UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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AÇÕES DOS HORMÔNIOS TIREOIDIANOS SOBRE O SISTEMA REPRODUTOR E O SISTEMA NERVOSO CENTRAL: VIAS DE SINALIZAÇÃO, MECANISMOS DE AÇÃO E MODULAÇÃO DO CITOESQUELETO

Ariane Zamoner

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Dedicatória

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"A mente que se abre a uma nova idéia

jamais voltará ao seu tamanho original"

Albert Einstein

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PARTE I

Resumo

Os hormônios da tireóide (HT) têm sido descritos agindo em sítios nucleares e extranucleares, induzindo respostas celulares através de diversos mecanismos. Também tem sido reportado que as proteínas do citoesqueleto são alvos para os HT durante o desenvolvimento do testículo e do sistema nervoso. Os efeitos dos HT durante o desenvolvimento são mediados principalmente por receptores nucleares modulando a expressão gênica. Entretanto, há diversas evidências de mecanismos não genômicos dos HT associados a vias de sinalização ativadas por Ca^{2+} e proteínas quinases. Nesse contexto, nosso estudo investigou as ações genômicas e não genômicas dos HT em células testiculares e neurais durante o desenvolvimento. Nós inicialmente demonstramos o envolvimento de mecanismos mediados por Ca^{2+} na fosforilação da vimentina induzida por T_3 em testículos de ratos imaturos através de mecanismos independentes da síntese de proteínas. Também observamos um acúmulo de vimentina fosforilada em testículos de ratos hipertireoideos, podendo ser conseqüência da ativação da ERK regulando o citoesqueleto. Nós ainda demonstramos um aumento no metabolismo basal através da medida do consumo de O_2 e dos níveis de TBARS. Além disso, as defesas antioxidantes enzimáticas e não enzimáticas modificaram-se aparentemente de acordo com o aumento no consumo de O_2. Esses resultados corroboram com a idéia de que um aumento na geração de ROS induz estresse oxidativo e pode ser responsável pelas alterações bioquímicas envolvidas no aumento da capacidade metabólica em testículos de ratos hipertireoideos. Além disso, o hipotireoidismo inibiu a atividade das NTPDases em culturas de células de Sertoli sem alterar a expressão das NTPDases 1, 2 e 3. Os HT modificaram a atividade NTPDásica, provavelmente através de mecanismos não genômicos e, consequentemente, podem influenciar a função reprodutiva durante o desenvolvimento. Considerando-se os efeitos dos HT em células neurais, nós primeiramente demonstramos que T_3 e T_4 estimularam a fosforilação dos filamentos intermediários (FI) através de mecanismos GABAérgicos via PKA e PKCaMII em córtex cerebral de ratos de 10 dias de idade. A dependência de PKA, PKC e canais de Ca^{2+} tipo-L e -T também foi evidenciada no efeito dos HT sobre a captação de $^{45}Ca^{2+}$. Surpreendentemente, em córtex cerebral de ratos de 15 dias de idade, apenas o T_4 estimulou a fosforilação dos FIs. Os mecanismos envolvidos na ação do T_4 sobre o citoesqueleto dependem da ativação de um receptor acoplado a proteínas Gi, além da participação da PLC, PKC, MAPK, PKCaMII e níveis intracelulares de Ca^{2+}. Estes dados demonstram que o T_4 tem importantes funções fisiológicas modulando o citoesqueleto de células neurais durante o desenvolvimento. Nós também demonstramos a reorganização e a fosforilação do citoesqueleto induzidas por HT em células de glioma C6 e astrócitos, e a participação da via de sinalização da RhoA mediando a ação hormonal. Concluindo, nossos resultados evidenciaram ações genômicas e não genômicas dos HT promovendo a fosforilação dos FI, reorganização do citoesqueleto, influxo de Ca^{2+} e modulação da atividade das NTPDases via mecanismos mediados por Ca^{2+} e proteínas quinases. Estes resultados podem contribuir para o melhor entendimento dos mecanismos envolvidos nas desordens observadas no hipo e hipertireoidismo durante o desenvolvimento e maturação do cérebro e do testículo.

Abstract

Thyroid hormones (TH) have been shown to act at nuclear and extra nuclear sites, inducing cell responses by several mechanisms. It has also been reported that cytoskeletal proteins are a target for TH during hormone-induced differentiation and development of nervous system and testicular cells. The developmental effects of TH are mainly mediated by nuclear receptors regulating gene expression. However, there are increasing evidences of nongenomic mechanisms of TH associated with kinase- and Ca^{2+}-activated signaling pathways. In this context, our study investigated the genomic and nongenomic actions of TH on testicular and neural cells from developing rats. We first described the involvement of Ca^{2+}-mediated mechanisms on vimentin phosphorylation induced by T_3 in immature rat testis. This effect was demonstrated to be independent of protein synthesis. Furthermore, we observed an accumulation of phosphorylated vimentin in hyperthyroid rat testes, which could be a consequence of the extracellular-regulated kinase (ERK) activation regulating the cytoskeleton. We also demonstrate increased basal metabolic rate, measured by tissue oxygen consumption, as well as, increased TBARS levels. Moreover, the enzymatic and non-enzymatic antioxidant defences were modified apparently to respond according to the augmented oxygen consumption. These results support the idea that an increase in mitochondrial ROS generation, underlying cellular oxidative damage, is a side effect of hyperthyroid-induced biochemical changes by which rat testis increase their metabolic capacity. In addition, hypothyroidism inhibited NTPDase activity in Sertoli cell cultures from young rats without alter the expression of NTPDase 1, 2 and 3. Our findings also demonstrate that TH modifies the NTPDase activities, probably via non-genomic mechanisms and consequently may influence the reproductive function throughout development. Concerning the effect of TH on neural cells, we first demonstrate that T_3 and T_4 stimulate the intermediate filament (IF) phosphorylation through PKA, PKCaMII and GABAergic mechanisms in cerebral cortex of 10 day-old rats. The dependence of PKA, PKC, L- and T-type Ca^{2+} channels were also evidenced on the effect of TH on $^{45}Ca^{2+}$ uptake. Surprisingly, in cerebral cortex of 15 day-old rats, only T_4 stimulate IF phosphorylation. The mechanisms underlying the action of T_4 involve the activation of a G_i-protein coupled receptor. Moreover, we showed the participation of PLC, PKC, MAPK, PKCaMII and intracellular Ca^{2+} levels mediating the effects of T_4 on the cytoskeleton. These findings demonstrate that T_4 have important physiological roles modulating the cytoskeleton of neural cells during development. We further demonstrate that TH induce cytoskeleton reorganization and phosphorylation in C6 glioma cells and astrocytes and the participation of RhoA signaling pathway mediating this action. In conclusion, our results evidence genomic and nongenomic actions of TH promoting IF phosphorylation, cytoskeletal reorganization, Ca^{2+} influx and modulation of NTPDase activity by Ca^{2+}- and kinase-mediated mechanisms. These results may contribute to a better knowledge of the mechanisms involved in the disorders observed in hyper and hypothyroidism during brain and testis maturation and development.

LISTA DE ABREVIATURAS

ADO	Adenosina
ADP	Adenosina 5'-difosfato
AMP	Adenosina 5'-monofosfato
AMPc	AMP cíclico
ATP	Adenosina 5'-trifosfato
ATPe	ATP extracelular
D1	Deiodinase tipo 1
D2	Deiodinase tipo 2
D3	Deiodinase tipo 3
FI	Filamento intermediário
FSH	Hormônio folículo estimulante
GABA	Ácido γ-aminobutírico
GFAP	Proteína glial fibrilar ácida
GnRH	Hormônio liberador de gonadotrofinas
GPCR	Receptor acoplado a proteínas G
GTP	Guanosina trifosfato
HT	Hormônios tireoidianos
MAPK	Proteína quinase ativada por mitógeno
MF	Microfilamento
MT	Microtúbulo
NF	Neurofilamento
NTPDase	Nucleosídeo trifosfato difosfohidrolase

5’-nuc	5’-nucleotidase
PLC	Fosfolipase C
PKA	Proteína quinase A
PKC	Proteína quinase C
PKCaM	Proteína quinase dependente de cálcio e calmodulina
RE	Retículo endoplasmático
RNA	Ácido ribonucléico
RNAm	RNA mensageiro
ROS	Espécies reativas oxigênio
SNC	Sistema nervoso central
SNP	Sistema nervoso periférico
TR	Receptor para hormônios tireoidianos
TRE	Elemento de resposta à tireóide
TRH	Hormônio liberador de tireotrofina
TSH	Hormônio estimulante da tireóide
T_3	Triiodotironina
T_3 r	T_3 reverso
T_4	Tiroxina

1. INTRODUÇÃO

1.1. HORMÔNIOS TIREOIDIANOS

Os hormônios tireoidianos (HT) são responsáveis por efeitos fisiológicos que causam alterações em virtualmente todas as vias metabólicas e órgãos. Eles modulam o consumo de oxigênio, o metabolismo de lipídios, carboidratos e proteínas. Estes hormônios também alteram a razão de síntese e degradação de uma grande variedade de fatores de crescimento e hormônios (Norman & Litwack, 1997; Boelaert & Franklyn, 2005). Sendo assim, em estado de hipertireoidismo, a atividade hipermetabólica dos HT pode ser responsável pela elevada produção de radicais livres de oxigênio e nitrogênio e, consequentemente, induzir danos oxidativos nos tecidos alvos (Venditti & DiMeo, 2006).

Os efeitos dos HT podem ser divididos em duas categorias de resposta biológica: (a) efeitos na diferenciação celular e desenvolvimento, e (b) efeitos nas vias metabólicas. Essas duas ações são interconectadas, sendo que as mudanças no crescimento e desenvolvimento são conseqüência da modulação hormonal do metabolismo (Norman & Litwack, 1997; Boelaert & Franklyn, 2005).

1.1.1. Mecanismos de produção e secreção dos hormônios tireoidianos

A regulação fisiológica da secreção dos HT é um sistema complexo que envolve a glândula tireóide, o hipotálamo, a pituitária e a atividade neural (Norman & Litwack, 1997). A produção e secreção dos HT é regulada pelo hormônio estimulante da tireóide (TSH), que se une ao receptor de membrana na tireóide e estimula a síntese e liberação hormonais a partir da tireoglobulina. O principal hormônio liberado pela tireóide é a tiroxina (T_4), e em menores concentrações a triiodotironina (T_3), o T_3 reverso (T_3r) e as tirosinas monoiodinadas. Além disso, o TSH regula a produção de T_3 nos tecidos periféricos, modulando a atividade das deiodinases

(Norman & Litwack, 1997; Fisher, 1996). A síntese e liberação de TSH pela pituitária anterior, ocorrem em resposta ao hormônio liberador de tireotrofina (TRH), que é secretado pelo hipotálamo (para revisão ver Boelaert & Franklyn, 2005). Os níveis de TRH e TSH são controlados por um sistema de retroalimentação negativa, no qual concentrações plasmáticas de T_3 e T_4 agem tanto no hipotálamo como na pituitária, inibindo a produção e liberação hormonais. O objetivo desse mecanismo é manter os níveis circulantes de HT dentro de uma faixa normal de concentração (Boelaert & Franklyn, 2005). A inibição é direcionada primariamente para a secreção, e posteriormente para a síntese destes hormônios (Fisher, 1996; Norman & Litwack, 1997; Van Doorn *et al.*, 1983). Os mecanismos de regulação da atividade da tireóide formam o eixo hipotálamo-hipófise-tireóide que está representado na figura 1.

Os hormônios circulam no plasma associados a proteínas de ligação. A mais importante dessas proteínas é a globulina de ligação de tiroxina, sendo que a transtirretina e a albumina desempenham um papel secundário no transporte do hormônio na circulação. O T_4 é distribuído nos tecidos periféricos, onde é convertido a T_3 por deiodinação, através da ação das deiodinases teciduais. A maior parte do T_3 circulante parece ser derivada da deiodinação hepática do T_4 pela deiodinase tipo 1 (D1) (Fisher, 1996; Boelaert & Franklyn, 2005).

As deiodinases teciduais apresentam três isoenzimas (D1, D2 e D3). Estas isoenzimas apresentam padrões de expressão distintos durante o desenvolvimento e específicos para cada tecido, funcionam com níveis de substrato ótimo individuais, e são diferentemente reguladas por hormônios, citocinas e fármacos (Van der Geyten *et al.*, 1997; Köhrle, 1999; Boelaert & Franklyn, 2005). A atividade da D2 cerebral é marcadamente elevada pela deficiência de HT, mantendo as concentrações de T_3 no tecido se o T_4 circulante diminuir. Os níveis hormonais também podem ser modulados pela atividade da D3 cerebral, que catalisa a inativação do T_3. A regulação coordenada das atividades de D2 e D3 cerebrais parece ser importante para manter a homeostasia das iodotironinas neste tecido (Darras *et al.*, 1999).

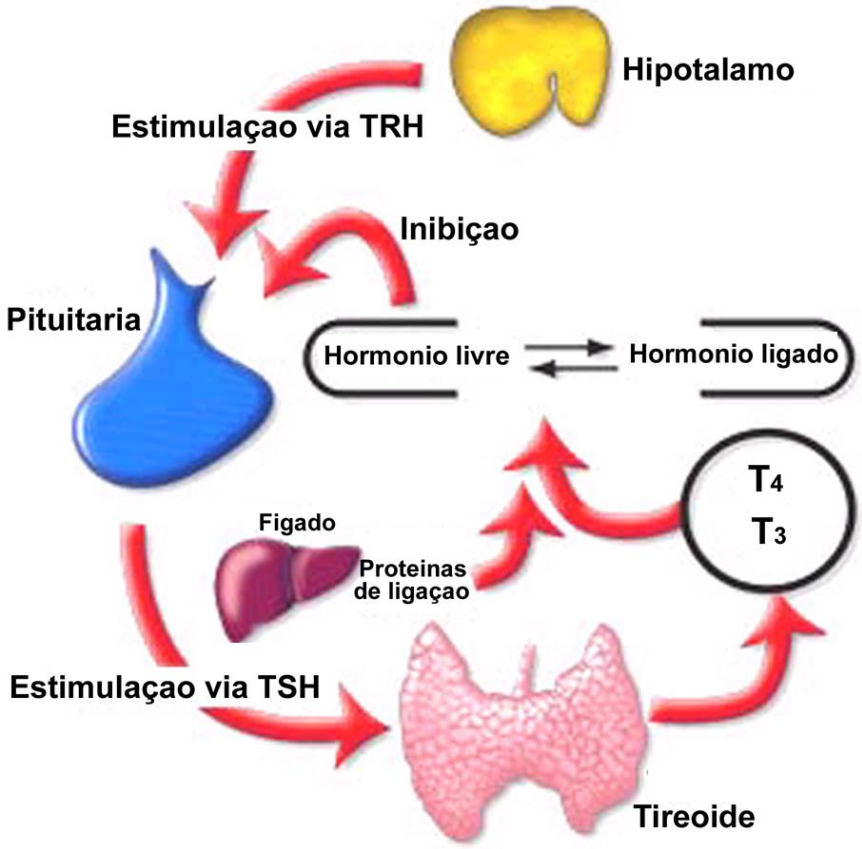

Figura 1. Representação esquemática do eixo hipotálamo-hipófise-tireóide. Disponível em: http://www.opt.pacificu.edu/ce/catalog/10644-PD/Lab_Tests.html

1.1.2. Mecanismos moleculares de ação hormonal

1.1.2.1. Mecanismo clássico, genômico, de ação dos hormônios da tireóide

Os HT são essenciais para o desenvolvimento, crescimento, maturação e metabolismo normais de diversos órgãos e tecidos (Boelaert & Franklyn, 2005). Esses efeitos são mediados principalmente pela ação do T_3, que age como ligante nos receptores nucleares para os HT. Estes receptores nucleares são codificados por dois protooncogenes altamente relacionados, c-*erb* Aα e c-*erb* Aβ, que codificam quatro espécies diferentes de RNA mensageiro, gerando distintas isoformas de receptores para os hormônios tireoidianos (TR): $TR\alpha_1$, $TR\alpha_2$, $TR\beta_1$, $TR\beta_2$ (Lazzar, 1993). A expressão e regulação para os TRs e para os genes responsivos aos HT é variável em diferentes tecidos, o que permite as múltiplas vias possíveis pelas quais estes hormônios podem exercer seus efeitos (Boelaert & Franklyn, 2005). $TR\alpha_1$ e $TR\beta_1$, por exemplo, são encontrados em praticamente todos os tecidos que respondem aos HT, todavia, outras isoformas apresentam

uma distribuição mais específica, como o TRβ_2, que é o receptor característico da pituitária (Anderson, 2001; Bernal *et al*., 2002).

O mecanismo clássico de ação do T_3 envolve a regulação positiva da expressão gênica através de sua ligação ao receptor para o HT, o qual se liga como homo ou heterodímero em sítios específicos do DNA, conhecidos como elementos de resposta à tireóide (TRE), localizados na região promotora dos genes alvos, onde interagem com co-repressores. Após a ligação com o ligante, os homodímeros dos receptores se dissociam em favor da formação de heterodímeros com o receptor retinóide-X (RXR), resultando na liberação dos co-repressores e recrutamento de coativadores da transcrição. Este complexo modula a transcrição dos genes alvos (Figura 2) (Boelaert & Franklyn, 2005; Moeller *et al*., 2006).

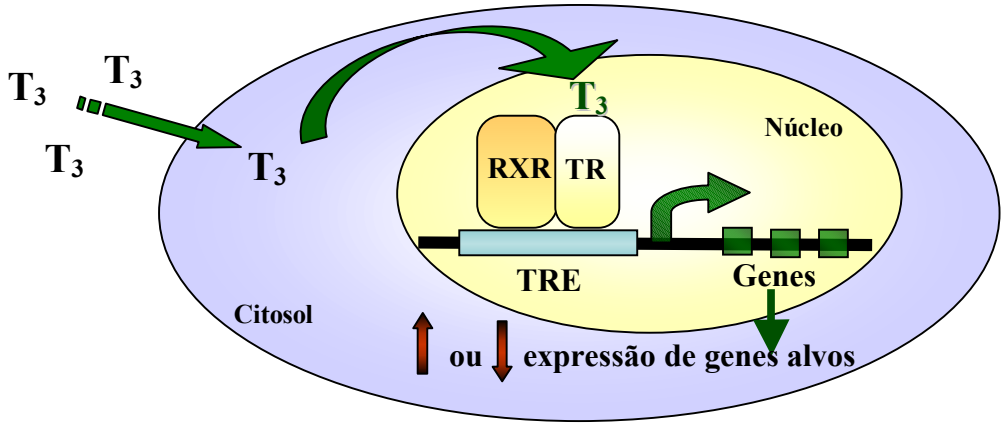

Figura 2. Mecanismo clássico de ação dos hormônios tireoidianos. Adaptado de Basset *et al*., 2003; Boelaert & Franklyn, 2005; Moeller *et al*., 2006.

1.1.2.2. Ações não genômicas dos hormônios tireoidianos

Os diferentes efeitos biológicos induzidos por T_3 podem provir de múltiplos sítios celulares. Além do modelo clássico, nuclear, de ação para os HT, diversos efeitos rápidos têm sido descritos como sendo ativados na membrana plasmática, no citosol, na mitocôndria, no envelope nuclear e no núcleo de diversos tipos celulares (Segal *et al*., 1989; Jannini *et al*., 1999; Arambepola *et al.,* 1998; Kobayashi & Horiuchi, 1995; Wrutniak *et al*., 1998; Lin *et al*., 1999; Silva *et al*., 2001; Wrutniak-Cabello *et al*., 2001; Menegaz *et al*., 2006).

A noção de ações não genômicas, independentes dos receptores nucleares, tem evoluído muito desde as hipóteses de trabalhos pioneiros sugerindo que os HT poderiam causar um estímulo rápido na captação de Ca^{2+}, o qual seria independente da síntese protéica (Segal & Ingbar, 1989; Segal *et al*., 1990). Atualmente, estas ações não genômicas têm sido descritas em diversos tipos celulares (Lin *et al*., 1999, Davis *et al.,* 2000; Silva *et al*., 2001; Kavok *et al*., 2001; D'Arezzo *et al*., 2004; Menegaz *et al*., 2006; Davis *et al*., 2005). Bergh e colaboradores (2005) descreveram um receptor de membrana para HT como um heterodímero de integrina αvβ3, que é acoplado à via de sinalização da proteína quinase ativada por mitógeno (MAPK) e por eventos intranucleares mediados por esta via de transdução de sinal, incluindo a modulação da transcrição gênica. Isso demonstra que pode haver uma inter-relação entre os eventos genômicos e não genômicos de ação para estes hormônios, envolvidos em suas funções biológicas. Além disso, foram descritos receptores acoplados a proteínas G (G protein coupled receptor - GPCR) para estes hormônios em células não nucleadas (Lin *et al*., 1999; Davis *et al*., 2000, 2002). A existência destes sítios de ligação na membrana plasmática já é conhecida há muitos anos em células vermelhas do sangue (Schwartz *et al*., 1967; Botta *et al*., 1983; Yoshida & Davis, 1981) e em sinaptossomas (Giguere *et al*., 1996). Entretanto, a identidade das proteínas envolvidas na ligação do hormônio à membrana plasmática não havia sido estabelecida nos estudos preliminares, e ainda há muita relutância em aplicar o termo "receptor" para estes sítios, apesar de sua relação aparente com certas funções locais na membrana, como o controle de bombas e canais iônicos, entre outras ações (Davis *et al*., 2005). As respostas celulares não genômicas para os HT têm sido frequentemente associadas a vias de transdução de sinal envolvendo a ativação de segundos mensageiros, incluindo as seguintes rotas de sinalização: fosfolipase C (PLC), proteína quinase C (PKC) e aumento na concentração intracelular de Ca^{++}; adenilato ciclase, proteína quinase A (PKA), AMP cíclico (AMPc), proteína de ligação ao elemento de resposta ao AMPc (CREB); Ras, Raf1 serina/treonina quinase, proteína quinase

quinase ativada por mitógeno (MEK) e MAPK (Lösel & Wehling, 2003). No SNC também têm sido relatados diversos sítios de ligação na membrana plasmática para HT, e há diversas hipóteses de que estes hormônios poderiam ter mecanismos de ação semelhantes a neurotransmissores, distintos de suas ações genômicas clássicas, regulando o desenvolvimento do sistema nervoso no início da vida fetal e pós-natal (Dratman, 1974; Mason *et al*., 1993; Dratman & Gordon, 1996; Martin *et al*., 1996; Sarkar, 2002). Estes efeitos incluem a inibição na captação de GABA (Mason *et al*., 1987), diminuição na atividade da $Na^{+}K^{+}$ATPase (Sarkar & Ray, 1993; 1998) e aumento pré-sináptico no influxo de Ca^{++} (Mason *et al*., 1990; Sarkar & Ray, 1993, 1998; Chakrabarti & Ray, 2000). Além disso, a ligação dos HT na membrana plasmática de diversos tipos celulares está envolvida em ações rápidas no fluxo iônico (Incerpi *et al*., 1999; Huang *et al*., 1999; Silva *et al*., 2001; D'Arezzo *et al*., 2004; Volpato *et al*., 2004; Menegaz *et al*., 2006), no transporte intracelular de proteínas (Safran *et al*., 1992; Zhu *et al*., 1998; Chen *et al*., 1999), na atividade de proteínas quinases (Lin *et al*., 1996, 1997, 1999; Kavok *et al*., 2001; Lösel & Wehling, 2003) e no citoesqueleto (Siegrist-Kaiser *et al*., 1990; Paul *et al*., 1999).

1.1.3. Hormônios tireoidianos e células de Sertoli

As células de Sertoli, pelo seu envolvimento na formação da barreira sangue-testículo, controlam o ambiente onde ocorre a espermatogênese. Essas células possuem sítios de ligação para os hormônios FSH (Griswold *et al*., 1993), testosterona (Mulder *et al*., 1976), T_3 (Palmero *et al*., 1995), retinol (Eskild *et al*., 1991) e 1α,25-diidroxi-vitamima D_3 (Majundar *et al*., 1994), substâncias estas que exercem efeitos moduladores importantes no processo espermatogênico.

Alterações na atividade da tireóide estão freqüentemente associadas a mudanças na função testicular (Jannini *et al*., 1995). Entretanto, o papel fisiológico dos hormônios tireoidianos na regulação da reprodução masculina ainda é pouco estudado. Os efeitos bioquímicos do T_3 *in vitro* e *in vitro* demonstraram que as células de Sertoli são o principal alvo para os HT no

testículo (Palmero *et al*., 1990; Ulisse *et al*., 1992, Jannini *et al*., 1999; Buzzard *et al*., 2000; Silva *et al*., 2001; Menegaz *et al*., 2006). O aumento da atividade metabólica dessa célula, causado por T_3, parece ser pré-requisito para a expansão da espermatogênese. Assim, o T_3, juntamente com o FSH, desempenha um papel central como regulador das primeiras fases do desenvolvimento tubular (Janinni *et al*., 1995, 1999).

Estudos da ligação hormônio-receptor e hibridização *in situ* indicaram que a célula de Sertoli é o alvo dos HT no testículo, com ação coincidente com a expressão dos receptores $TR\alpha_1$ (Jannini *et al*., 1999). Sítios de ligação para os TH foram encontrados em testículos de ratos durante toda a fase proliferativa (Buzzard *et al*., 2000). Entretanto, receptores funcionais para T_3 só foram detectados na fase imatura (Palmero *et al*., 1990; Ulisse *et al*., 1992; Jannini *et al*., 1999).

Muitas das funções das células de Sertoli estão sob o controle dos HT, como o estímulo no transporte de glicose, aumento a produção de gama-glutamil transpeptidase, proteína ligadora de andrógenos e fator de crescimento semelhante à insulina-I, estímulo na captação de aminoácidos, hiperpolarização celular (Silva *et al*., 2001; Volpato *et al*., 2004; Menegaz *et al*., 2006), inibição da atividade da aromatase e efeitos no metabolismo da testosterona (Silva *et al*., 2002). A diferenciação fetal das células de Sertoli e germinativas, e a subseqüente proliferação durante as fases fetal e pós-natal são eventos complexos que envolvem sinais desconhecidos para a sua inicialização e diferenciação. No rato, a proliferação máxima das células de Sertoli ocorre no final da gestação e no período perinatal (Orth, 1982), coincidindo com a função fetal da tireóide (Dubois & Dussault, 1977) e com a capacidade de ligação máxima do T_3 no testículo (Jannini *et al*., 1990, 1999).

Os HT desempenham um papel fundamental na regulação terminal das células de Sertoli. O T_3 exógeno, aplicado por um período transitório, aumenta o tamanho do testículo na fase imatura. No entanto, a administração prolongada de T_3 *in vitro* acelera o desenvolvimento

do testículo por reduzir o período proliferativo das células de Sertoli e dos gonócitos, diminuindo conseqüentemente, o número de células de Sertoli por testículo, antecipando a formação do lúmen tubular, e reduzindo o peso final do testículo no adulto. Entretanto, em testículos de animais adultos, o tratamento com HT não induz alterações morfológicas, confirmando o período crítico da efetividade desses hormônios na fase imatura (Janinni *et al*., 1995; 1999). Nesse contexto, alterações na atividade da tireóide são frequentemente associadas com mudanças na função reprodutiva, sendo que hipotireoidismo congênito, ou ocorrendo logo após o nascimento, está relacionado com um marcado atraso na maturação e desenvolvimento sexuais (Palmero *et al*., 1990; Longcope, 1991; Jannini *et al*, 1990, 1999). A Figura 3 ilustra os principais efeitos dos HT sobre o desenvolvimento testicular

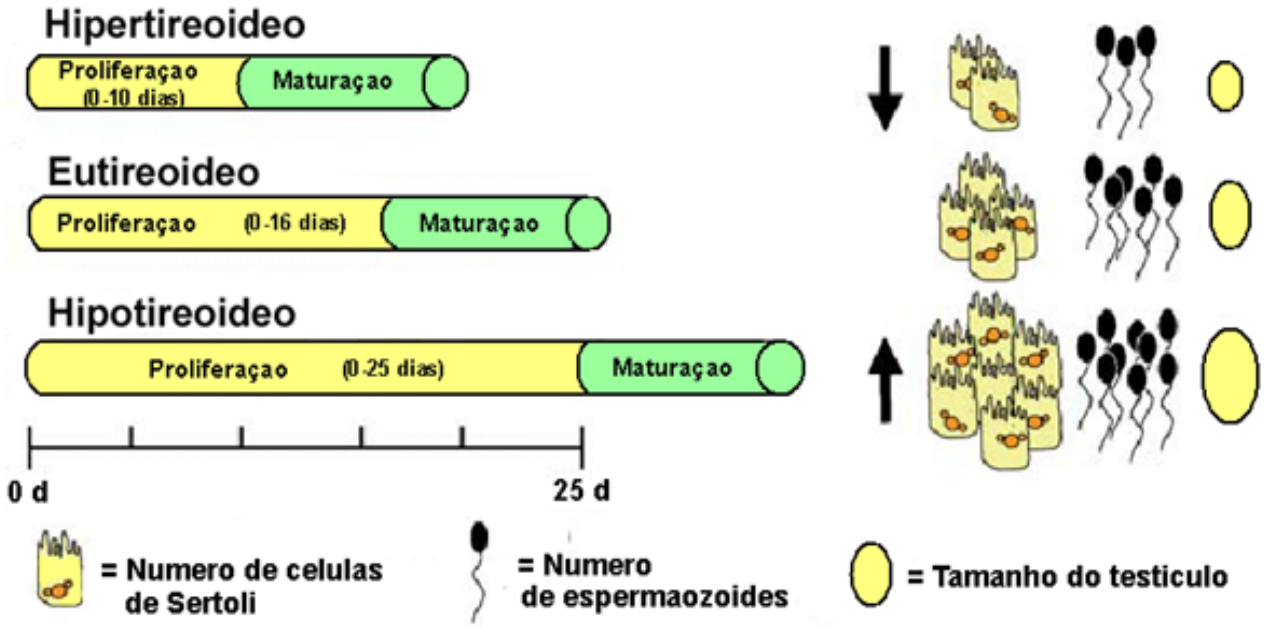

Figura 3. Ilustração dos efeitos dos HT sobre o desenvolvimento testicular. Em roedores eutireoideos, a célula de Sertoli prolifera até aproximadamente a segunda semana de vida pós-natal. O hipertireoidismo neonatal transitório resulta em cessamento precoce da proliferação das células de Sertoli, levando a uma menor população das mesmas no animal adulto, se comparada ao animal controle. Ao contrário, o hipotireoidismo neonatal transitório prolonga o período proliferativo e portanto, aumenta a população de células de Sertoli na fase adulta. Considerando que cada célula de Sertoli é capaz de suportar um número definido de células germinativas, o número de células de Sertoli é responsável pela capacidade reprodutiva e magnitude na produção de espermatozóides no adulto. Além disso, os efeitos decorrentes do hiper ou hipotireoidismo neonatal resultam em diminuição ou aumento no tamanho testicular, respectivamente, atribuídos a mudanças na população de células somáticas e germinativas (Holsberger & Cooke, 2005).

1.1.4. Hormônios tireoidianos e sistema nervoso central

Os HT são criticamente envolvidos no desenvolvimento e função do sistema nervoso central (SNC) (Bernal, 2002). O modelo melhor definido e estudado envolve o desenvolvimento hormônio-dependente do cérebro de ratos durante o período neonatal. Os HT desempenham um papel importante na morfogênese, regulação da expressão gênica, desenvolvimento, e aspectos envolvidos nos processos de aprendizado, memória e habilidades motoras (Schwartz *et al*., 1997). Em fases iniciais do desenvolvimento cerebral de mamíferos, os HT promovem proliferação e posteriormente estimulam a diferenciação celular (Oppenheimer & Schwartz, 1997). A ausência dos HT durante o período crítico do desenvolvimento neural, até seis meses de vida pós-natal em humanos, resulta em danos irreversíveis ao sistema nervoso, distúrbios no processo de migração neuronal, redução da sinaptogênese, defeitos na mielinização e diminuição da síntese e liberação de neurotransmissores, que são acompanhados de múltiplas alterações na morfologia cerebral (Dussault & Ruel, 1987; Geel *et al*., 1967; Oppenheimer & Schwartz, 1997, Anderson, 2001).

No sistema nervoso, os HT atuam principalmente sobre os mecanismos envolvidos com a neurotransmissão central e periférica, aumentando a síntese e sensibilidade a catecolaminas (Engstron *et al*., 1974). Estudos têm demonstrado uma redução da liberação de glutamato e da expressão dos receptores NMDA em cérebro de ratos após a indução de hipotireoidismo cirúrgico (Shuaib *et al*., 1994; Lee *et al*., 2003). Os mecanismos precisos pelos quais o hipotireoidismo induz alterações no sistema nervoso, como déficits de memória, ainda não estão completamente elucidados. Todavia, os distúrbios cognitivos, descritos em indivíduos hipotireoideos, são freqüentemente associados com a diminuída excitabilidade em SNC e periférico de indivíduos com hipotireoidismo não tratado (Pollard *et al*., 1982). Além disso, foram demonstrados efeitos regulatórios sobre a expressão de proteínas em astrócitos e gliomas C6 em cultura (Trentin & Alvarez-Silva, 1998; Trentin *et al*., 2001).

Os mecanismos moleculares precisos pelos quais os HT induzem seus efeitos no cérebro durante o desenvolvimento são pouco conhecidos e acredita-se que a maioria deles sejam mediados pela ativação de seus receptores nucleares, modulando a expressão gênica de proteínas específicas. As vias de sinalização dos TRs controlam muitos processos responsáveis pelo desenvolvimento neural (Anderson, 2001; Bernal *et al*., 2002). A versatilidade de suas ações é facilitada pela expressão regulada e diferenciada de TRs, que podem modular positiva ou negativamente a expressão gênica, além da ação das deiodinases, regulando os níveis de hormônio ativo no tecido cerebral. Isso determina as respostas tempo-específicas e relacionadas às vias de transdução de sinal evocadas (Forrest *et al*., 2002). A expressão dos TRs durante o desenvolvimento do SNC é altamente controlada, sendo que os TRα_1 são amplamente distribuídos durante o desenvolvimento neural, e a expressão dos TRβ é mais restrita (Bradley *et al*., 1994; Flamant & Samarut, 1998; Forrest & Vennström, 2000). Entretanto, embora distintos TRs facilitem determinadas funções específicas, apenas o amplo repertório de TRs não explica o grande espectro de ação e de efeitos para os HT (Forrest *et al*., 2002).

1.2. CITOESQUELETO

A habilidade das células eucarióticas em adotar uma variedade de formas e desenvolver movimentos coordenados e direcionados depende de uma rede complexa de filamentos protéicos que se estende por todo o citoplasma. Esta rede é denominada de citoesqueleto e é uma estrutura dinâmica que se reorganiza continuamente, sendo responsável pela mudança da forma, divisão e movimento celular (Elson, 1988; Ingber, 1993), e é provavelmente a única estrutura celular que liga diretamente a membrana plasmática ao núcleo (Janmey, 1998). Além disso, as proteínas do citoesqueleto formam trilhos para o transporte intracelular, promovendo e mantendo funções celulares diferenciadas (Kirkpatrick & Scott, 1999).

Alterações na dinâmica do citoesqueleto tornam a célula capaz de responder a modificações do meio intra e extracelular. Sendo assim, o comportamento e a função celulares são controlados pelas proteínas do citoesqueleto que consistem de três distintos, embora interconectados, sistemas de filamentos: os microfilaments (MFs), os filamentos intermediários (FIs) e os microtúbulos (MTs) (Alberts *et al*., 2002).

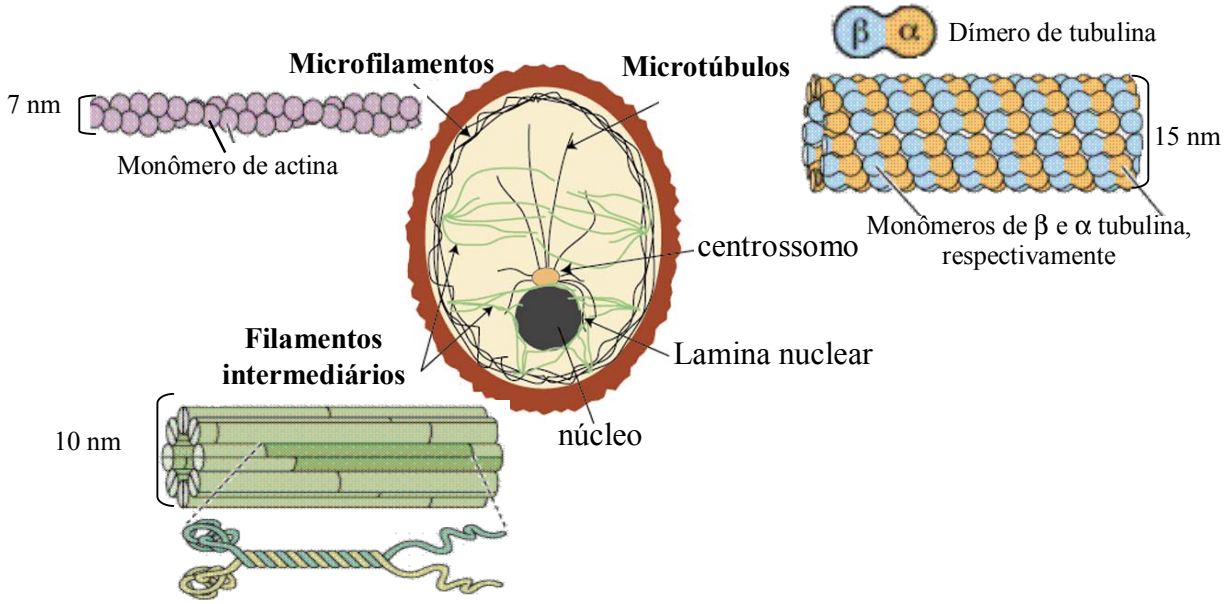

Figura 4. Organização dos microfilamentos, filamentos intermediários e microtúbulos no interior das células. Adaptado de fai.unne.edu.ar/biologia/celulamit/cytoskel.htm e www.sparknotes.com/

Os MFs e os MTs são polímeros de subunidades protéicas globulares de actina e tubulina, respectivamente, as quais são altamente conservadas nas células animais. Estas estruturas possuem a característica comum de terem sua dinâmica controlada pela hidrólise de nucleotídeos (Hermann & Aebi, 2000; Fuchs & Karakesisoglou, 2001). Por outro lado, os FIs são formados pela polimerização de uma família de subunidades protéicas fibrosas, específicas para os diferentes tipos celulares (Strelkov *et al*., 2003). Os MFs e os MTs são exclusivamente citoplasmáticos. Os MFs são pequenos filamentos flexíveis, situados na região cortical da célula e ancorados à membrana plasmática. Os MTs, no entanto, são estruturas tubulares e retilíneas que emanam do centrossomo, localizado próximo ao núcleo, estendendo-se em direção à

periferia celular (Omary *et al*., 2006). As Figuras 4 e 5 mostram a distribuição citoplasmática esquemática e por microscopia dos três tipos de filamentos do citoesqueleto numa linhagem de hepatócitos.

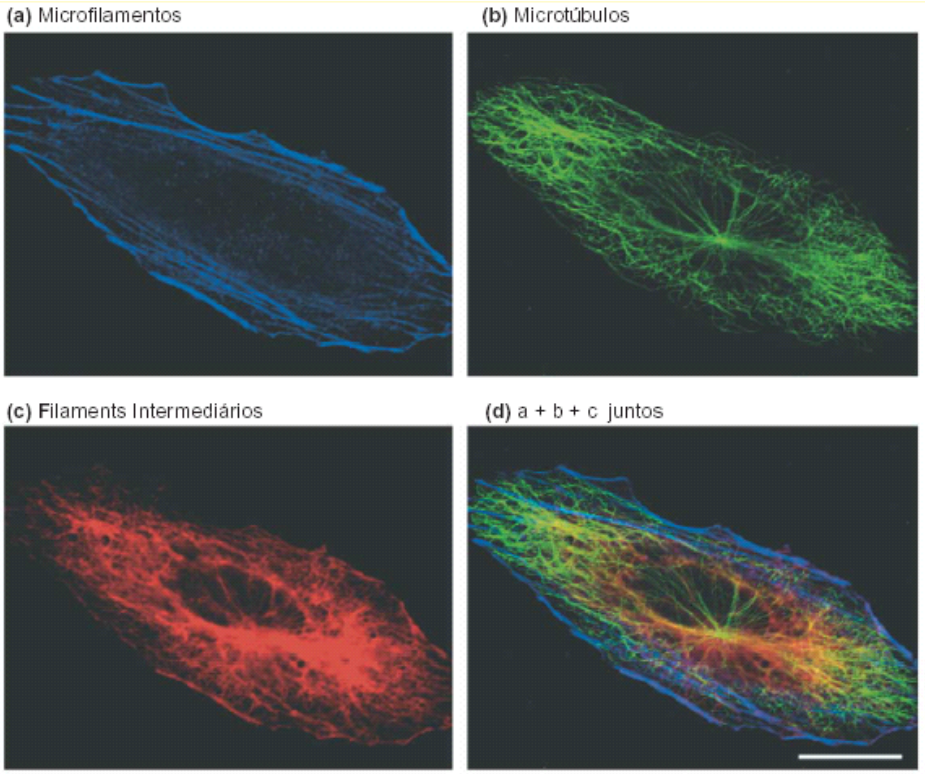

Figura 5. Microscopia de fluorescência demonstrando os três componentes do citoesqueleto. Culturas de linhagem de hepatócitos Huh7 com tripla marcação com (a) faloidina marcando microfilamentos (azul), (b) anti-tubulina para marcar microtúbulos (verde), e (c) anti-K8/K18 para visualizar FIs (vermelho). A sobreposição das imagens pode ser visualizada em (d). Observa-se a organização citoplasmática das três principais famílias de proteínas do citoesqueleto, que são altamente dinâmicas e podem variar em diferentes condições fisiológicas ou patológicas. Barra de escala =10 microns (Fonte: Omary *et al*., 2006).

1.2.1. Filamentos intermediários

Os FIs são codificados por uma família de mais de 65 genes, que codificam 5 diferentes categorias de FIs, 4 destas são proteínas citoplasmáticas e uma nuclear. O perfil de expressão dos FIs citoplasmáticos é célula- e tecido-específico, sendo que células mesenquimais expressam vimentina, as musculares desmina, os astrócitos proteína glial fibrilar ácida (GFAP), os neurônios neurofilamentos e as células epiteliais citoqueratina (Albert *et al*., 2002). As proteínas de FIs podem associar-se entre si, *in vitro*, e uma vez polimerizadas, tornam-se insolúveis em

condições onde tanto MTs quanto os MFs seriam rapidamente solubilizados (Zackroff & Goldman, 1979). Os FIs polimerizados possuem propriedades específicas que os tornam mais resistentes à deformação e à ruptura por tensão mecânica (Janmey *et al*., 1991; Janmey *et al*., 1998). Desta forma, as propriedades físico-químicas dos FIs conferem a estas proteínas, resistência à extração com tampões contendo detergentes não-iônicos e alta concentração iônica (Starger *et al*., 1978). Esta propriedade é utilizada até hoje para isolá-los de células e tecidos. Baseados nestas propriedades físicas e bioquímicas, os FIs têm sido vistos como um dos sistemas citoesqueléticos mais estáticos, proporcionando às células resistência ao estresse mecânico e à deformação (Coulombe *et al*., 2000). Entretanto, estudos recentes têm demonstrado que os FIs e seus precursores são altamente dinâmicos e exibem uma plasticidade complexa relacionada à sua associação e organização em células vivas (Helfand *et al*., 2004), sugerindo que os FIs podem ser tão dinâmicos quanto os MFs e os MTs (Yoon *et al*., 1998, 2001). Sendo assim, os FI funcionam para integrar mecanicamente as várias estruturas do espaço citoplasmático (Lazarides, 1980). Alterações na função dos FIs estão envolvidas em diversas patologias, onde as células tornam-se mais frágeis e não são capazes de suportar o estresse mecânico ou não-mecânico. Recentes estudos têm evidenciado um novo papel dos FIs influenciando também o crescimento e a morte celular através de interações dinâmicas com proteínas não-estruturais (Coulombe & Wong, 2004).

Os FIs citoplasmáticos estão envolvidos em processos fisiológicos como migração celular e crescimento tecidual, e também modulam respostas celulares ao estresse metabólico, levando à morte celular programada. Além disso, diversos trabalhos descrevem alterações dos FIs em resposta à injúria e nos processos de reparo tecidual, em câncer e outras doenças (DePianto & Coulombe, 2004). Entretanto, ainda é um grande desafio o melhor entendimento da função dos FIs citoplasmáticos. A Figura 6 ilustra as principais funções conhecidas dos FIs citoplasmáticos.

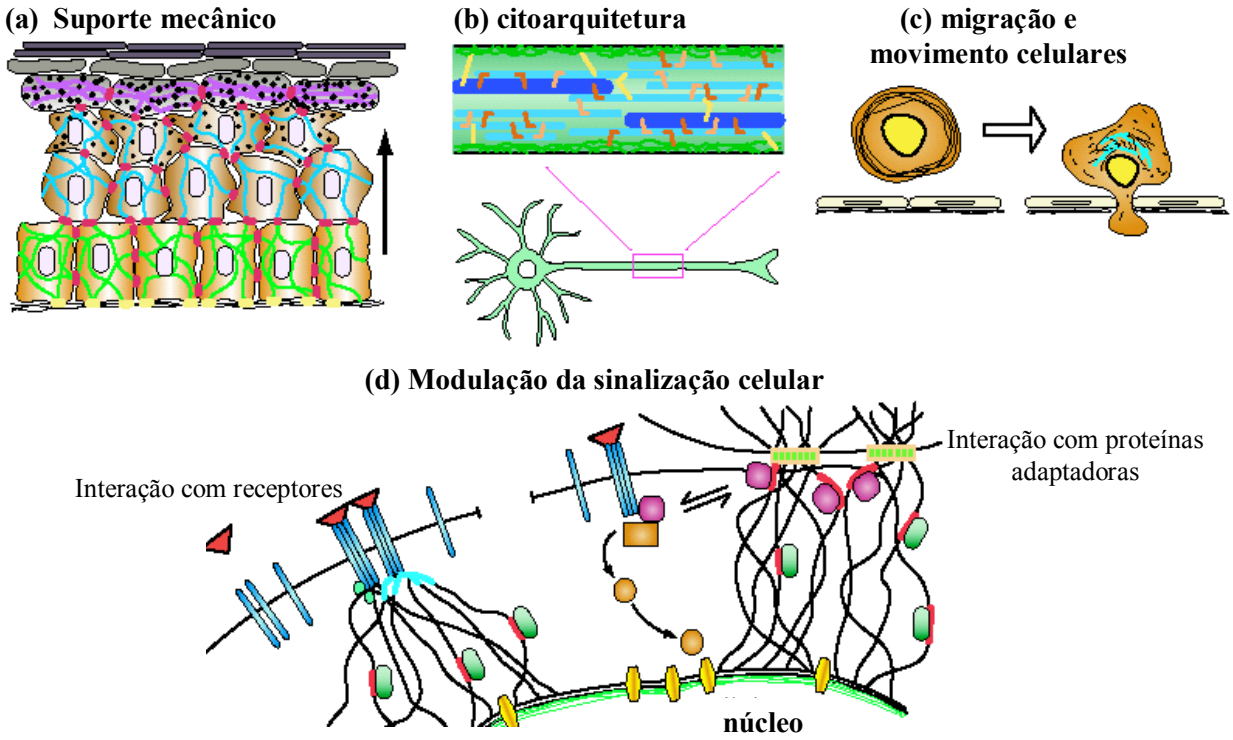

Figura 6. Principais funções dos filamentos intermediários citoplasmáticos. (a) suporte mecânico. Os FIs de queratina são abundantes nos queratinócitos e as mudanças demonstradas nas cores dos filamentos refletem sua expressão e composição diferencial nas diferentes camadas de células. Os filamentos se estendem por todo o citoplasma e são integrados entre as células por junções tipo desmossomos (pontos vermelhos) e entre as células basais e a lâmina basal por hemidesmossomos (pontos amarelos). Esta organização maximiza o suporte mecânico promovido pelos FIs. (**b**) Citoarquitetura. Em neurônios motores, o processo de crescimento axonal requer a interação com neurofilamentos (azul claro), subdivididos nas subunidades de baixo (NF-L), médio (NF-M) e alto (NF-H) peso molecular. Os domínios C-terminal das subunidades do NF-H (vermelho) e do NF-M (laranja) são hiperfosforilados e se projetam para fora do filamento, determinando o espaçamento interfilamento e consequentemente o calibre axonal. Os muitos NFs interagem com os MTs (azul escuro) e com os MFs (verde escuro) através de proteínas associadas ao citoesqueleto, (amarelo). (**c**) Migração celular. Na circulação, os linfócitos resistem ao estresse hemodinâmico e mecânico, devido principalmente à rede de FI de vimentina, que organiza a periferia citoplasmática (célula da esquerda). Após indução de resposta inflamatória, os FI de vimentina movem-se rapidamente para a região perinuclear. Isso ocorre devido a alterações na fosforilação da vimentina em sítios específicos (célula da direita com filamentos de cor diferenciada) modulando as propriedades viscoelásticas do citoplasma. (**d**) Modulação da sinalização celular. Os FIs podem se ligar e modular a atividade de proteínas de sinalização celular, influenciando o fluxo de sinais extracelulares. Dois mecanismos são ilustrados. Esquerda: interações de FIs com receptores de superfície modulando sua densidade e função; direita: interações entre FIs e proteínas adaptadoras (rosa), limitando a disponibilidade deste adaptador para se ligar ao receptor e transmitir o sinal para a célula. A regulação destas interações é demonstrada aqui por mudanças locais na cor do filamento (azul ou vermelha), que podem ser mediadas por modificações pós-traducionais dinâmicas, associação com outras proteínas ou diferenças na composição das subunidades de FIs Todos estes mecanismos participam na regulação das respostas celulares aos sinais pró-apoptóticos e outros eventos de sinalização. Adaptado de Coulombe & Wong (2004).

Os neurofilamentos (NFs) são FIs importantes na fisiologia neuronal, onde determinam o calibre axonal, auxiliam na manutenção da morfologia dos neurônios e são essenciais para o transporte axonal (Rao *et al*., 2003; Julien, 1999). Eles são sintetizados no corpo celular e transportados para o axônio através do transporte axonal, via mecanismos modulados por fosforilação. Além disso, o acúmulo de NFs tem sido descrito em várias doenças neurodegenerativas, como na esclerose amiotrófica lateral, na doença de Parkinson e na doença de Alzheimer. Ainda não está estabelecida a sua contribuição nos processos degenerativos característicos destas doenças, mas sugere-se que haja uma interrupção no transporte dessas proteínas nos neurônios afetados (Ackerley *et al*., 2000).

Outro FI importante é a proteina glial fibrilar ácida (GFAP), considerada uma proteína marcadora astrocitária, importante na modulação da motilidade e da forma celulares, fornecendo estabilidade estrutural aos astrócitos. Além disso, a GFAP participa de um sistema sofisticado de comunicação recíproca entre astrócitos e neurônios (McCall *et al*., 1996; Eliasson *et al*., 1999), regulando a liberação de neurotransmissores (Carmignoto, 2000), além de desempenhar um papel importante nos processos de gliose reativa (Pekny *et al*., 1999, Eng *et al*., 2000).

A vimentina é a proteína de FI expressa em células de origem mesenquimal, além de ser expressa transitoriamente em muitas células durante o desenvolvimento. Ela é encontrada também em astrócitos imaturos, em alguns astrócitos maduros (Alberts *et al*., 2002), e é a proteína de FI característica de células de Sertoli tanto na fase imatura quanto adulta do desenvolvimento sexual (Paranko *et al*., 1986).

Os FIs sofrem alterações dinâmicas na sua organização citoplasmática durante diferentes estágios do ciclo celular ou em resposta a sinais celulares (Steinert & Roop, 1988; Erickson *et al*., 1992; Helfand *et al*., 2004, 2005), sendo a fosforilação um mecanismo central na regulação da organização da rede citoplasmática de FIs (Eriksson & Goldman, 1993).

1.3. FOSFORILAÇÃO PROTÉICA

A fosforilação protéica compreende um mecanismo molecular dinâmico de fundamental importância na regulação biológica, onde diferentes sinais extracelulares produzem efeitos fisiológicos nas células-alvo através da regulação do estado de fosforilação de determinadas fosfoproteínas. Vários sinais extracelulares, chamados de primeiros mensageiros, tais como neurotransmissores, hormônios, fatores tróficos e drogas produzem a maior parte dos efeitos fisiológicos por regulação do estado de fosforilação de proteínas em células alvo (Walaas & Greengard, 1991; Grant & Pant, 2000; Helfand *et al*., 2004, 2005). Estes primeiros mensageiros desencadeiam um mecanismo em cascata alterando os níveis intracelulares de segundos mensageiros (AMPc, GMPc, Ca^{++}, fosfolipídeos, etc) que, por sua vez, agem sobre o sistema fosforilante, modificando o nível de fosforilação de substratos específicos, traduzindo-se na modulação de uma resposta fisiológica (Figura. 7) (Nestler & Greengard, 1994).

Os sistemas de fosforilação e desfosforilação consistem de uma proteína quinase, uma proteína fosfatase e um substrato protéico. O substrato protéico é convertido da forma desfosforilada à forma fosforilada por uma proteína quinase. Por sua vez, a forma fosforilada é convertida novamente à forma desfosforilada por uma proteína fosfatase. As proteínas quinases catalisam a transferência do grupo fosfato terminal do ATP ao grupamento hidroxila de um resíduo de aminoácido do substrato. As proteínas fosfatases catalisam a hidrólise da ligação éster, retornando a proteína novamente ao estado não fosforilado (Nestler & Greengard, 1994). Dessa forma, os aspectos moleculares envolvidos na transdução de sinal no ambiente intracelular baseiam-se principalmente na associação específica de proteínas e na modulação de sua fosforilação (ou desfosforilação). A fosforilação de alvos protéicos leva geralmente a mudanças imediatas em sua configuração e atividade. O balanço entre fosforilação e desfosforilação é determinante para a transdução de sinal intracelular (Walaas & Greengard, 1991; Nestler & Greengard, 1994).

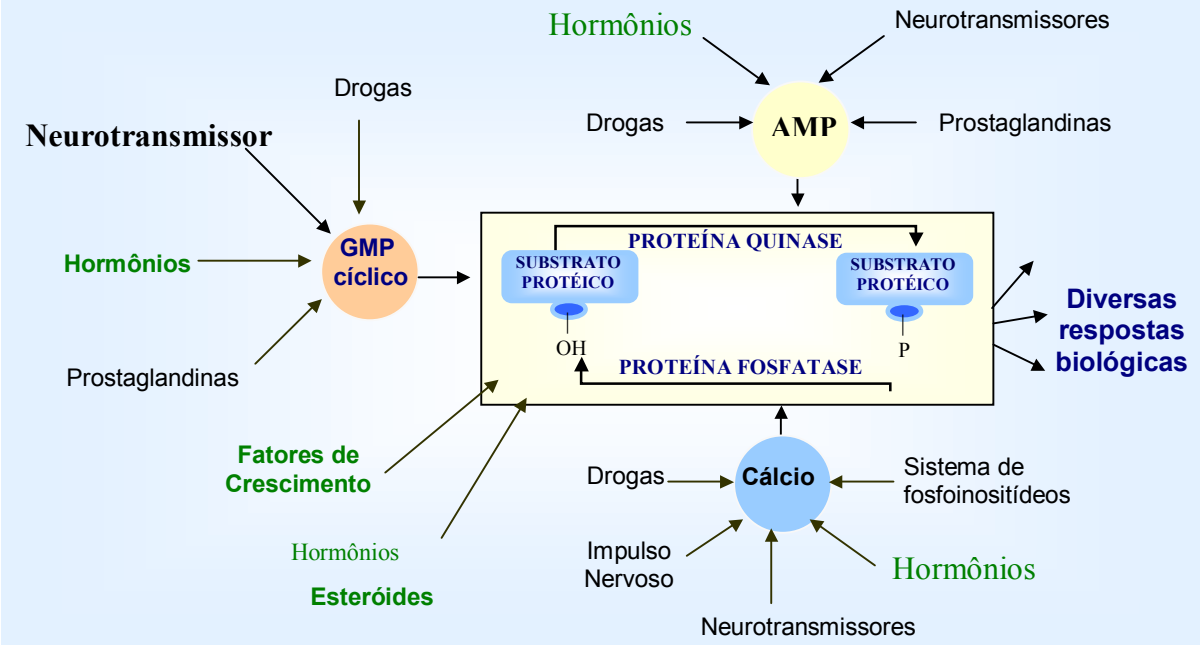

Figura 7. Representação esquemática do sistema de fosforilação de proteínas e alguns dos agentes regulatórios. Adaptado de Nestler & Greengard, 1994.

1.3.1. Fosforilação das proteínas do citoesqueleto

A constante reorganização do citoesqueleto através da fosforilação e desfosforilação de suas proteínas é regulada por diferentes rotas de sinalização, participando da resposta celular aos mecanismos regulatórios de transdução de sinal (Chang & Goldman, 2004; Helfand *et al*, 2005).

A fosforilação é um processo reversível, que desempenha um papel significativo no comportamento dinâmico de polimerização/despolimerização dos FIs e também regula as interações entre os FIs e outras proteínas, mediando as suas diversas funções nos diferentes tipos celulares (Sanhai *et al*., 1999). As subunidades de médio e alto peso molecular dos neurofilamentos (NF-M e NF-H) são altamente fosforiladas nos axônios e contribuem para as interações entre NF e outros elementos do citoesqueleto (Grant & Pant, 2000). Além disso, NF-M e NF-H localizam-se perifericamente nos NFs, sendo que as extremidades carboxi-terminal destas subunidades formam projeções laterais que se estendem perpendicularmente ao filamento central, e quando intensamente fosforiladas promovem repulsão entre os filamentos sendo

responsáveis pela manutenção do calibre axonal (Hirokawa & Takeda, 1998; Grant & Pant, 2000). A organização dos NFs no axoplasma está representada na Figura.8.

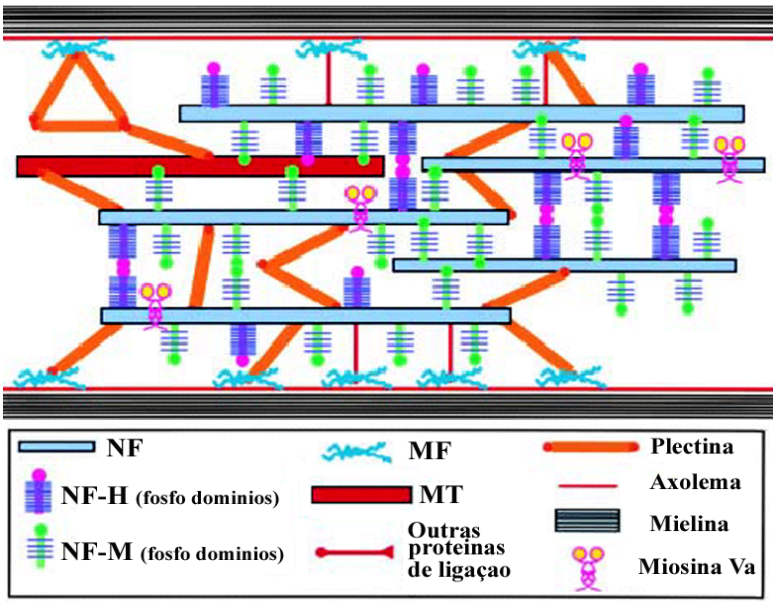

Figura 8. Organização dos neurofilamentos (NF) no axoplasma. Os NFs estão organizados tridimensionalmente no axoplasma através de interações com outros NF adjacentes, MF e MT através de proteínas de ligação como as plectinas, estabilizando assim o citoesqueleto neuronal. Os domínios carboxi-terminal de NF-M e NF-H são altamente fosforiláveis e formam pontes cruzadas entre os filamentos. A miosina Va age como proteína motora de ligação para os NFs. Na ausência de NF-M, NF-H ou outras proteínas de ligação, ocorre redução do calibre axonal e dificuldades em suportar e manter o volume do axônio (Adaptado de Rao et al., 2003).

Os FI são substratos para numerosas proteínas quinases, incluindo a proteína quinase A (PKA), proteína quinase C (PKC), quinase dependente de cálcio e calmodulina II (CaMKII), quinase cdc 42, quinase Jun amino-terminal (JNK) e p38, que alteram drasticamente a estrutura desses filamentos (Inagaki *et al*., 1987; Eriksson *et al*., 2004; Chang & Goldman, 2004).

As fosfatases também desempenham um papel importante na manutenção da integridade estrutural dos FIs (Goto *et al*., 1998; Inagaki *et al*., 1996). O balanço entre as atividades de proteínas quinases e fosfatases parece ser essencial não só na modulação da estrutura e plasticidade dos FIs, como também na adesão célula-substrato, motilidade celular e reorganização dos filamentos de actina durante a interfase. O estudo do mecanismo molecular da ação dessas proteínas é de grande importância na compreensão da dinâmica das proteínas do citoesqueleto e seu envolvimento no funcionamento da célula (Inada *et al*., 1999).

1.4. SINALIZAÇÃO CELULAR

A habilidade das células em receber e atuar através de sinais recebidos a partir da membrana celular é fundamental para a vida de todos os organismos. As várias rotas de sinalização intracelulares ativadas por receptores de superfície diferem em sua complexidade e modo pelo qual ativam sinais, desencadeando assim uma variedade de respostas biológicas. A ligação de moléculas sinalizadoras aos seus receptores específicos leva a alterações nos níveis de segundos mensageiros intracelulares, os quais desencadeiam uma série de reações, modificando a atividade de enzimas intermediárias de cascatas de sinalização celular. Este mecanismo é fundamental na propagação e amplificação do sinal iniciado pelo ligante extracelular (Cooper *et al*., 2001; Alberts *et al*., 2002). Várias proteínas chave podem ser utilizadas nas diversas vias de transdução de sinal, incluindo diferentes tipos de proteínas quinases, fosfatases, GTPases e moléculas adaptadoras sem atividade enzimática própria. Todas estas proteínas podem agir como intermediárias das cascatas de transdução de sinal celulares. Sabe-se também que o citoesqueleto celular é um importante alvo de diferentes estímulos extracelulares, através da modulação de diversas vias de sinalização (Coulombe & Wong, 2004; Helfand *et al*., 2005). Diversos trabalhos têm descrito que as proteínas de FIs e suas subunidades desempenham um papel crucial na transdução de sinais específicos da periferia celular para o núcleo em muitos tipos celulares. A via das MAPKs está envolvida em muitos destes efeitos (Sung *et al*., 2001; Helfand *et al*., 2005).

1.5. CÁLCIO

A sinalização celular modulada por Ca^{2+} compreende uma série de eventos conectando os estímulos externos com as respostas intracelulares através do aumento nos níveis citosólicos deste íon como transdutor de sinal. Os estímulos externos mais comuns são neurotransmissores, hormônios ou fatores de crescimento. Em células excitáveis, o estímulo químico inicial pode

envolver a excitação da membrana, ativando rotas de sinalização via Ca^{2+} (Bird & Putney, 2006).

O Ca^{2+} pode entrar nas células via canais de cálcio dependentes de voltagem (CCDV) ou modulados por ligante, assim como através de entrada capacitativa. Os CCDV são capazes de aumentar muito os níveis citosólicos de Ca^{2+} e são essenciais para uma grande variedade de funções celulares, tais como contração muscular, propagação de potenciais de ação, manutenção da atividade elétrica e regulação da liberação de neurotransmissores (Hui, 1991). Estes canais são designados de CCDV do tipo-L, -T, -P, -Q e –R, diferindo entre si em sua cinética de ativação, modo de regulação e inativação via Ca^{++} e sensibilidade a toxinas específicas (Dunlap *et al*., 1995). O influxo de Ca^{2+} pode estar envolvido em diversas funções celulares e pode também ser regulado por diversos mecanismos, incluindo atividade de proteínas quinases, neurotransmissores, hormônios, nucleotídeos e fatores de crescimento, sendo que este evento desempenha um papel central na transdução de sinal de diversos tipos celulares, incluindo o SNC e o trato reprodutivo (Gao *et al*., 1997; Ko *et al*., 2003; Ko *et al*., 2005; Bird & Putney, 2006).

1.6. PURINAS EXTRACELULARES NA SINALIZAÇÃO CELULAR

Os nucleotídeos de adenina formam uma classe ubíqua de moléculas intracelulares com capacidade de regular diversas funções no ambiente extracelular através de sua ação em receptores de superfície denominados purinoceptores (Ralevic & Burnstock, 1998). Os compostos purinérgicos possuem efeitos descritos sobre a função cardíaca, agregação plaquetária e tônus vascular (Huttemann *et al*, 1984; Olsson & Pearson, 1990; Burnstock, 1991; Collins & Hourani, 1993), e também podem atuar como fatores tróficos e reguladores endógenos de crescimento e diferenciação celulares, tanto durante o desenvolvimento, como na vida adulta (Abbracchio *et al,* 1994), na fertilização (Foresta *et al.,* 1992), na embriogênese (Knudsen &

Elmer, 1987) e na organogênese (Smuts, 1981). Além disso, em mamíferos, o ATP e a adenosina (Ado) comportam-se como neurotransmissores e neuromoduladores, tanto no SNC como no SNP (Craig & White, 1993; Sperlagh *et a.l*, 1995).

A comunicação celular mediada por nucleosídeos e/ou nucleotídeos pode ocorrer de duas maneiras: 1) pela ligação a receptores localizados na membrana plasmática, e 2) pelo transporte para o citoplasma, através de transportadores específicos (somente os nucleosídeos). Os purinoceptores são classificados em dois tipos, P1 e P2, que respondem a Ado e a ATP, respectivamente (Ralevic & Burnstock, 1998). Diferentes subtipos de purinoceptores foram descritos em células de Sertoli e germinativas, e sua ativação induz importantes mudanças funcionais nestas células. As células de Sertoli expressam receptores purinérgicos P2 associados com a sinalização via fosfatidil inositol e mobilização de Ca^{2+} intracelular induzindo diversas respostas biológicas (Filippini *et al*., 1994). A ativação de receptores para ATP nas células de Sertoli pode causar despolarização celular dependente do influxo de Na^{+}, com conseqüente abertura de canais de Ca^{++} dependentes de voltagem (Foresta *et al*., 1995). Além disso, o ATP extracelular (ATPe) desempenha um importante papel regulatório nos processos de diferenciação, transdução de sinal em resposta a estímulos externos e interações célula-célula (Burnstock, 1981; Gordon, 1986; Filippini *et al*., 1990). A Ado, gerada pela hidrólise de ATPe em células de Sertoli, pode agir em receptores P1, do tipo A1, recentemente demonstrados como essenciais para aquisição da capacidade fertilizante (Minelli *et al*., 2004). Estes receptores participam na modulação da atividade secretória das células de Sertoli (Conti *et al*., 1988).

Os eventos de sinalização induzidos pelos nucleotídeos de adenina extracelulares são controlados pela ação das ectonucleotidases, incluindo os membros das famílias das ecto-nucleotídeo pirofosfatase/fosfodiesterases (E-NPPs) e ecto-nucleosídeo trifosfato difosfohidrolases (E-NTPDases) assim como da ecto-5'-nucleotidase. Os membros da família das E-NTPDases constituem uma classe de ecto-enzimas caracterizadas por sua capacidade de

hidrolisar nucleotídeos di- e trifosfatados, mas não nucleotídeos monofosfatados (Plesner, 1995). A ecto-5'-nucleotidase catalisa o passo final de degradação dos nucletídeos extracelulares, a hidrólise do nucleosídeo 5'-monofosfato para seu respectivo nucleosídeo e fosfato inorgânico (Pi) (Zimmermann, 2000). Estas ecto-enzimas constituem uma cascata enzimática altamente organizada, responsável pela regulação das vias de sinalização mediadas pelos nucleotídeos e nucleosídeos, controlando desta forma, a razão, a quantidade e o tempo de degradação dos nucleotídeos e finalmente a formação dos nucleosídeos (Zimmermann, 2000; Bigonnesse *et al*., 2004). A atividade das ectonucleotidases foi descrita previamente em células de Sertoli em cultura (Casali *et al*., 2001) e pode estar envolvida na regulação das concentrações extracelulares dos nucleotídeos e nucleosídeos de adenina e, consequentemente, modulando seus efeitos locais (Filippini *et al*., 1994; Casali *et al*., 2001; Ko *et al*., 2003).

Muitas das respostas fisiológicas à estimulação hormonal dependem de uma integração contínua entre diferentes vias de sinalização. No sistema reprodutor masculino, além da regulação hormonal, diversas evidências sugerem uma participação importante na modulação da função das células de Sertoli por nucleotídeos de adenina extracelulares devido à presença de purinoceptores nestas células (Monaco *et al*., 1984; Stiles *et al*., 1986; Monaco *et al*., 1988; Conti *et al*., 1989; Filippini *et al*., 1994; Rivkees, 1994; Ko *et al*., 2003; Meroni *et al*., 1998). Considerando as importantes funções modulatórias dos HT sobre a proliferação e diferenciação das células de Sertoli e os efeitos dos nucleotídeos sobre estas células, a modulação das ecto-nucleotidases por estes hormônios pode desempenhar um papel relevante controlando os níveis destes nucleotídeos no trato reprodutor.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Investigar as ações dos hormônios tireoidianos, T_3 e T_4, sobre o sistema reprodutor e o sistema nervoso central. Ênfase especial será dada à modulação do citoesqueleto através da fosforilação e reorganização de filamentos intermediários, bem como às vias de sinalização envolvidas nesses efeitos. Estudaremos também a produção de estresse oxidativo em células testiculares, bem como os efeitos dos hormônios sobre as ectonucleotidases em células de Sertoli.

2.2. OBJETIVOS ESPECÍFICOS

Investigar os efeitos não genômicos do T_3 sobre o conteúdo e a fosforilação da vimentina presente na fração citoesquelética e o envolvimento do Ca^{2+} no mecanismo de ação do hormônio em testículos de ratos imaturos.

Estudar o efeito do hipertireoidismo sobre a expressão e a fosforilação da vimentina, o envolvimento da modulação da MAPK, bem como a indução de estresse oxidativo e atividade antioxidante em testículos de ratos jovens.

Investigar a modulação na atividade e na expressão das NTPDases em culturas de células de Sertoli de ratos jovens submetidos ao hipotireoidismo congênito, bem como o efeito dos HT sobre a atividade NTPDásica .

Estudar os efeitos não genômicos dos HTs sobre o sistema fosforilante associado ao citoesqueleto em córtex cerebral de ratos de 10 e 15 dias de idade, e identificar a dependência de mecanismos envolvendo receptores de membrana, e vias de sinalização ativando diferentes proteínas quinases.

Verificar o efeito dos HTs sobre a captação de $^{45}Ca^{2+}$ em fatias de córtex cerebral de ratos jovens e identificar os canais e/ou proteínas quinases que participam desta ação.

Verificar os efeitos dos HTs sobre a morfologia celular, a organização e a fosforilação do citoesqueleto em culturas de células C6 e de astrócitos primários, bem como identificar os mecanismos envolvidos no efeito hormonal.

PARTE II

Capítulo 1

INVOLVEMENT OF CALCIUM-DEPENDENT MECHANISMS IN T3-INDUCED PHOSPHORYLATION OF VIMENTIN OF IMMATURE RAT TESTIS

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Involvement of calcium-dependent mechanisms in T_3-induced phosphorylation of vimentin of immature rat testis

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Abstract

Thyroid hormones have been shown to act at extra nuclear sites, inducing target cell responses by several mechanisms, frequently involving intracellular calcium concentration. It has also been reported that cytoskeletal proteins are a target for thyroid and steroid hormones and cytoskeletal rearrangements are observed during hormone-induced differentiation and development of rat testes. However, little is known about the effect of 3,5,3′-triiodo-L-thyronine (T_3) on the intermediate filament (IF) vimentin in rat testes. In this study we investigated the immunocontent and in vitro phosphorylation of vimentin in the cytoskeletal fraction of immature rat testes after a short-term in vitro treatment with T_3. Gonads were incubated with or without T_3 and ^{32}P orthophosphate for 30 min and the intermediate filament-enriched cytoskeletal fraction was extracted in a high salt Triton-containing buffer. Vimentin immunoreactivity was analyzed by immunoblotting and the in vitro ^{32}P incorporation into this protein was measured. Results showed that 1 μM T_3 was able to increase the vimentin immunoreactivity and in vitro phosphorylation in the cytoskeletal fraction without altering total vimentin immunocontent in immature rat testes. Besides, these effects were independent of active protein synthesis. The involvement of Ca^{2+}-mediated mechanisms in vimentin phosphorylation was evident when specific channel blockers (verapamil and nifedipine) or chelating agents (EGTA and BAPTA) were added during pre-incubation and incubation of the testes with T_3. The effect of T_3 was prevented when Ca^{2+} influx was

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blocked or intracellular Ca^{2+} was chelated. These results demonstrate a rapid nongenomic Ca^{2+}-dependent action of T_3 in phosphorylating vimentin in immature rat testes.

Keywords: T_3; Vimentin; Testis; Cytoskeleton; Phosphorylation

Introduction

The cytoskeleton of eukaryotic cells comprises a network formed mainly of microfilaments, microtubules and intermediate filaments (IF). IFs have been proposed to be the major mechanical stabilizers and integrators of the cytoplasm, nonetheless several studies demonstrate that there is a rapid exchange of subunits between smaller oligomers and polymerized IF, suggesting a dynamic behavior of these cytoskeletal constituents (Chou et al., 2001). Other than structural scaffolding, cytoplasmic IFs also fulfil cell-type-specific functions, such as the contribution of vimentin IFs to sphingolipid synthesis (Gillard et al., 1998) and of K8–K18 keratin filaments in protecting the liver hepatocyte against drug-induced and apoptotic stress (Ku et al., 1998; Caulin et al., 2000). The IFs can undergo dynamic changes in their organization during different stages of the cell cycle or as a result of cell signaling (Spencer et al., 1998). Furthermore, it has been shown that IF proteins are altered following malnutrition, brain ischemia, exposure to toxic substances, as well as hormonal action (Paranko et al., 1986; De Mattos et al., 1994; Hall et al., 1995; De Morais et al., 1996; Spencer et al., 1998).

Vimentin is the major cytoskeletal constituent of a great variety of cells (Gyoeva and Gelfand, 1991; Shah et al., 1998), such as endothelial cells (Xu et al., 2004), Leydig cells (Ortega et al., 2004), and radial glia (Nakagawa et al., 2004). In addition, vimentin is often expressed transiently during development (Menet et al., 2001). Both vimentin and cytokeratins have been described in Sertoli cells during foetal and postnatal periods, but vimentin is the only IF described in immature and adult rat testes (Paranko et al., 1986; Romeo et al., 1995) where it plays important roles in the modifications of Sertoli cell morphology, junctional processes and cytoplasmic organization occurring during spermatogenesis (Russell and Peterson, 1985; Tanemura et al., 1994).

There is considerable evidence in the literature pointing to phosphorylation as the most important posttranslational modification modulating both reciprocal interactions of IF proteins with other cytoskeletal components and the continuous exchange of IF subunits between a soluble pool and polymerized intermediate filaments (Inada et al., 1999). The phosphorylation sites are located on N- and C-terminal domains of IF subunits. The N-terminal phosphorylation has been demonstrated to be essential for IF disassembly (Inagaki et al., 1987), while C-terminal phosphorylation has been implicated in interactions with other intracellular structures (Chou et al., 1996).

The classical mechanism of thyroid hormones has been established as a genomic action, including binding to intracellular hormone receptors that share characteristics of nuclear transcription factors. These effects are described to occur after a given time lag necessary to modify protein transcription (Davis et al., 2002; Silva et al., 2002). However, recently, a number of reports indicate that thyroid hormones exert several effects in cells lacking classical receptors (Silva et al., 2002). In addition, the effect of these hormones on the membrane transport system of chick embryo was reported to be very

rapid, occurring in minutes, a time lag non-compatible with the classical scheme of a nuclear receptor action (Incerpi et al., 1999a). These findings led to the identification of non-classical thyroid hormone binding elements in the plasma membrane (Silva et al., 2002). Through binding to these sites, thyroid hormones could exert short-term effects, including those on ion fluxes at the plasma membrane (Incerpi et al., 1999b; Huang et al., 1999), on intracellular protein trafficking (Chen et al., 1999; Zhu et al., 1998; Safran et al., 1992), on signal-transducing cytoplasmic kinase activities (Lin et al., 1996, 1999) and on the cytoskeleton (Siegrist-Kaiser et al., 1990).

Pioneer studies performed by Segal (1990) conclusively identified calcium as the first messenger for the rapid action of the thyroid hormone triiodothyronine (T_3) at the level of the plasma membrane, emphasizing calcium uptake as the first apparent event following the binding of the hormone to its membrane receptor. Furthermore, previous studies had shown a rapid effect of T_3 on different membrane processes, including amino acid accumulation and membrane potential in Sertoli cells of immature rat testis (Silva et al., 2001; Volpato et al., 2004).

Several evidences in the literature have pointed to the regulation of Sertoli cell IF cytoskeleton by steroid hormones (Show et al., 2003) and thyroid hormones (Paul et al., 1999) and to nongenomic Ca^{2+}-dependent mechanisms mediating the effects of thyroid hormones in Sertoli cells (Volpato et al., 2004). Taking into account these findings, we hypothesized that beyond the classical genomic mechanism of action, thyroid hormones elicit rapid nongenomic mechanisms, mediated by Ca^{2+}, leading to vimentin reorganization, underlying the cytoskeletal remodeling in immature rat testis. To test this hypothesis, we investigated a short-term effect of T_3 on the in vitro phosphorylation of vimentin associated with the cytoskeletal fraction of immature rat testes, emphasizing the involvement of Ca^{2+} in such effects.

Materials and methods

Chemicals

[^{32}P] Na_2HPO_4 was purchased from CNEN, São Paulo, Brazil. 3,5,3′-triiodo-L-thyronine (T_3), ethyleneglycol-*O*-*O*′z-bis(2-aminoethyl)-*N*′,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM), cycloheximide, nifedipine, verapamil, benzamidine, leupeptin, antipain, pepstatin, chymostatin, anti-vimentin antibody (clone vim 13.2), peroxidase conjugated rabbit anti-mouse IgG, acrylamide and bis-acrylamide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The L-[U-^{14}C]Leucine and chemiluminescence ECL kit were obtained from Amersham, (Oakville, Ontario, Canada).

Animals

Fifteen-day-old male Wistar rats were obtained from our breeding stock and maintained on a 12 h light/12 h dark cycle in a constant temperature (22 °C) colony room. On the day of birth the litter size was culled to eight pups. Litters smaller than eight pups were not included in the experiments. Water and a 20% (w/w) protein commercial chow were available ad libitum. All the animals were carefully monitored and maintained in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation.

Protein synthesis assays

Protein synthesis was measured by L-[U-^{14}C]leucine incorporation assays, as described below: rat testes were removed, weighed (47.41 ± 0.782 mg; $n = 56$), decapsulated, and pre-incubated for 30 min in a buffer (KRb) containing 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$. The buffer was bubbled with O_2/CO_2 (95:5;v/v) up to pH 7.4. Incubation was carried out in KRb buffer with or without 1 μM T_3, in the presence of L-[U-^{14}C]leucine (0.1 μCi/mL) for 30 min in a Dubnoff metabolic incubator at 34 °C and gassed with O_2/CO_2 (95%:5%; v/v). Incubation was stopped when cold trichloroacetic acid (TCA) was added to a final concentration of 7% and samples were processed as described by Silva et al. (1995). The total protein was measured according to the method of Lowry et al. (1951). The results of protein synthesis were expressed as cpm/μg protein.

Treatment of rat testes with thyroid hormone and/or drugs

Following cervical dislocation, testes from 15-day-old rats were rapidly removed and decapsulated onto Petri dishes placed on ice. One testis was initially pre-incubated at 30 °C for 20 min in the basic medium containing 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 25 mM Na-HEPES (pH 7.4), 12 mM glucose, 1 mM $CaCl_2$ and the following protease inhibitors: 1 mM benzamidine, 0.1 μM leupeptin, 0.7 μM pepstatin, 0.7 μM chymostatin and 0.7 μM antipain. When specified 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M T_3, 50 μM BAPTA-AM, 2 mM EGTA, 100 μM verapamil or 100 μM nifedipine was added to the pre-incubation and/or incubation medium. Incubation was carried out in 100 μL of the basic medium for 30 min at 30 °C (Silva et al., 2001; Volpato et al., 2004). In some experiments 0.35 mM cycloheximide was added to pre-incubation (1 h) and incubation medium and the experiment was carried out as described. In all experiments, one testis was used as control and the contralateral one as treated group.

In vitro ^{32}P incorporation into vimentin

The in vitro phosphorylation of vimentin was carried out as previously described by us (Funchal et al., 2003), with modifications. In brief, testes of 15-day-old rats were pre-incubated as described above. Incubation was carried out with 100 μL of the basic medium described above, containing 80 μCi [^{32}P] orthophosphate with or without the addition of different concentrations of T_3 and/or the drugs described above. The labeling reaction was allowed to proceed for 30 min at 30 °C and then stopped with 1 mL of cold stop buffer containing 150 mM NaF, 5 mM EDTA, 5 mM EGTA, 50 mM Tris–HCl, pH 6.5, and the protease inhibitors described above. Testes were then washed twice by decanting with the stop buffer to remove excess radioactivity. After the in vitro phosphorylation procedures, the IF enriched cytoskeletal fraction was extracted as described below.

Extraction of IF enriched cytoskeletal fraction from rat testes

IF enriched cytoskeletal fraction was prepared as described by Funchal et al. (2003). Briefly, tissue was homogenized in 600 μL of ice-cold buffer containing 5 mM KH_2PO_4, 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 1% Triton X-100 and the protease inhibitors described above. The

homogenate was centrifuged at 15,800×*g* for 10 min at 4 °C in an Eppendorf centrifuge. The insoluble material was resuspended in 600 μL of the same buffer and centrifuged as described. The pellet constituted the high salt Triton insoluble IF enriched cytoskeletal fraction. This pellet was then dissolved in 1% sodium dodecyl sulfate (SDS). Some experiments used total protein homogenate of the testis. For this, tissue was homogenized in 300 μL of a lysis solution (2 mM EDTA, 50 mM Tris–HCl, pH 6.8, 4% SDS). For SDS-PAGE analysis, samples were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8 and boiled for 3 min. Equal protein concentrations of the total protein homogenate or the IF-enriched cytoskeletal fraction were analyzed by 10% SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS). In the experiments on in vitro ^{32}P incorporation, the nitrocellulose membranes containing the IF-enriched cytoskeletal fraction were exposed to X-ray films (X-Omat XK1) at −70 °C with intensifying screens, and finally the autoradiograms were obtained and quantified as described below.

Immunoblotting analysis

The nitrocellulose membranes were washed for 10 min in Tris-buffered saline (TBS; 0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% defatted dried milk). After incubation, the blot was washed twice for 5 min with TBS plus 0.05% Tween-20 (T-TBS), and then incubated overnight at 4 °C in blocking solution containing the monoclonal anti-vimentin antibody (clone vim 13.2), diluted 1:400. The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in TBS containing peroxidase conjugated rabbit anti-mouse IgG diluted 1:4000. The blot was washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blot was then developed using a chemiluminescence ECL kit. Autoradiograms and immunoblots were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an OptiQuant version 02.00 software (Packard Instrument Company).

Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparison tests when the *F*-test was significant. All analyses were performed using the GraphPAD InStat Software version 1.12a.

Results

Fig. 1 shows a Coomassie blue stained SDS-PAGE of the IF-enriched cytoskeletal fraction extracted from immature rat testes, an autoradiograph of a nitrocellulose membrane and the corresponding immunoblot of the cytoskeletal fraction confirming that this fraction contains vimentin, as identified by the monoclonal antibody. It can be seen in the autoradiograph that vimentin is a good substrate for the endogenous phosphorylation system. The effect of T_3 at doses from 10^{-9} to 10^{-4} M was tested on the in vitro incorporation of ^{32}P into vimentin from immature rat testes incubated for 30 min. Significantly increased phosphorylation was observed at doses from 10^{-7} to 10^{-5} M (Fig. 2). Therefore, we have chosen 10^{-6} M T_3 for subsequent experiments on protein phosphorylation.

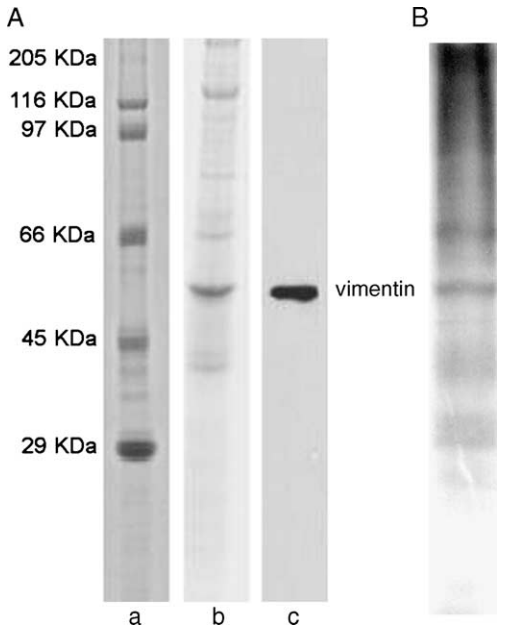

Fig. 1. Polyacrylamide gel electrophoresis (SDS-PAGE), autoradiograph and immunoblotting of the cytoskeletal fraction. (A) Lane a, molecular weight standards (KDa). Lane b, SDS-PAGE of the high salt Triton insoluble cytoskeletal fraction of 15-day-old rat testes. The gel was stained with Coomassie blue R-250. Lane c, immunoblotting of the cytoskeletal fraction using anti-vimentin monoclonal antibody. Development was carried out with an ECL kit. (B) Autoradiograph of a nitrocellulose membrane containing the cytoskeletal fraction obtained after in vitro incubation of the gonads with ^{32}P orthophosphate.

We first demonstrated that when immature rat testes were incubated for 30 min with 1 μM T_3, the L-[U-^{14}C]leucine incorporation into total proteins of testis was significantly increased, suggesting that 1 μM T_3 was able to increase protein synthesis after 30 min incubation (Fig. 3). Also, the presence of

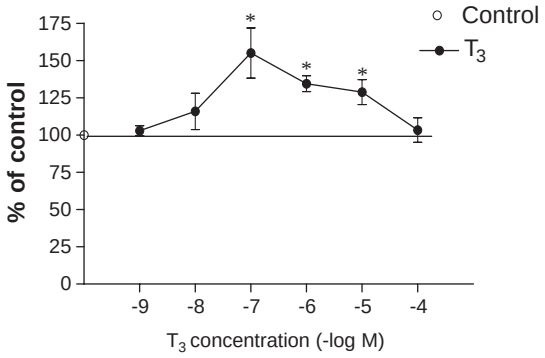

Fig. 2. Effect of different concentrations of T_3 on in vitro vimentin phosphorylation in immature rat testis. Fifteen-day-old rat testes were incubated with 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M T_3 in the presence of ^{32}P orthophosphate for 30 min. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into vimentin was measured as described in Materials and methods. Data are reported as means ± S.E.M. of six to eight animals expressed as percentage of controls. Statistically significant differences from controls, as determined by ANOVA followed by Tukey–Kramer multiple comparison test, are indicated: *$P < 0.01$.

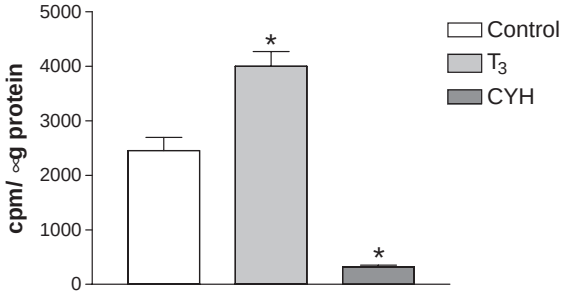

Fig. 3. Effect of T_3 and cycloheximide on in vitro incorporation of ^{14}C-leucine into proteins of immature rat testis. Immature rat testes were treated with 1 μM T_3 or 0.35 mM cycloheximide (CYH) in the presence of ^{14}C-leucine for 30 min as described in Materials and methods. Radioactivity incorporated into proteins was measured. Data are reported as means ± S.E.M. of six to eight animals. Statistically significant differences from controls, as determined by ANOVA followed by Tukey–Kramer multiple comparison test, are indicated: *$P < 0.001$.

cycloheximide (0.35 mM) in the incubation medium significantly inhibited (87%) the basal protein synthesis. However, when rat testes were incubated for 30 min with 1 μM T_3 we did not observe any alteration on total vimentin immunocontent in tissue homogenate, while the immunocontent of the high salt Triton insoluble vimentin in the cytoskeletal fraction was significantly increased. Otherwise, 0.35 mM cycloheximide, a protein synthesis inhibitor, did not prevent the increased vimentin immunocontent detected into the cytoskeletal fraction (Fig. 4), suggesting that the effect of T_3 on vimentin immunocontent was independent of protein synthesis.

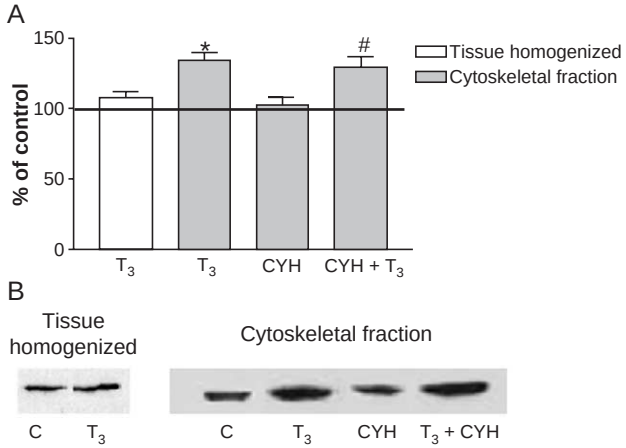

Fig. 4. Effect of T_3 and cycloheximide treatment on vimentin immunocontent of immature rat testis. (A) Immature rat testis was treated with 1 μM T_3 and/or 0.35 mM CYH for 30 min and the immunocontent of total vimentin from tissue homogenate or high salt Triton insoluble vimentin were measured. The immunoblotting was carried out with monoclonal anti-vimentin antibody diluted 1:400. Scans from six different animals were quantified as described in Materials and methods and for each animal the area of control samples was referred to as 100%. Results are expressed as mean ± S.E.M. The blots were developed using an ECL kit. Statistical analysis: one-way ANOVA followed by Tukey–Kramer multiple comparison test. *$P < 0.001$ compared with control; #$P < 0.05$ compared with CYH group. (B) A composite of nitrocellulose replicas of total and Triton-insoluble vimentin in the absence or presence of T_3 and/or cycloheximide. All lanes received an equivalent amount of protein.

We investigated the effect of T_3 on the in vitro phosphorylation of the Triton insoluble vimentin. Results showed that 1 μM T_3 treatment increased the in vitro phosphorylation of the cytoskeletal-associated vimentin (Fig. 5) suggesting a T_3-induced activation of the phosphorylating system associated to the cytoskeletal fraction. In addition, we measured the in vitro ^{32}P incorporation into Triton-insoluble vimentin in the presence of 1 μM T_3 plus 0.35 mM cycloheximide. Results showed that even in the presence of cycloheximide, the effect of T_3 on the in vitro phosphorylation of cytoskeletal vimentin was observed (Fig. 5).

Taking into account the importance of Ca^{2+} in a plethora of intracellular events that result in regulation of cell function, particularly the cytoskeletal roles (Mangoura et al., 1995; Stull, 2001), we examined the involvement of Ca^{2+} in the T_3-mediated increase of the in vitro ^{32}P incorporation into vimentin. For this, gonads were treated with 2 mM EGTA with or without 1 μM T_3. Results showed that when the extracellular calcium was chelated with EGTA the effect of T_3 on vimentin phosphorylation was prevented (Fig. 6). In order to verify the involvement of voltage-dependent Ca^{2+} channels (VDCC) on the effect of T_3, immature rat testes were co-incubated with 1 μM T_3 and the specific L-calcium channel (L-VDCC) blockers nifedipine (100 μM) or verapamil (100 μM), respectively. We first observed that the blockers themselves, at the concentrations used, were not able to significantly disturb the phosphorylating system (Fig. 6). In addition, co-incubation of the gonads with 1 μM T_3 plus 100 μM nifedipine or 1 μM T_3 plus 100 μM verapamil totally prevented the effect of T_3 on the phosphorylating system (Fig. 6), suggesting that Ca^{2+} influx via VDCC was involved in the ability of T_3 to alter the phosphorylating/dephosphorylating equilibrium of vimentin recovered in the cytoskeletal fraction. To further investigate the role of intracellular Ca^{2+} in this process we performed experiments using the membrane-permeable form of BAPTA, namely BAPTA-AM. Gonads were incubated with 1 μM T_3 plus 50 μM BAPTA-AM, a concentration that was shown to be unable to alter the phosphorylating system per se. Results showed that BAPTA prevented the stimulatory effect induced by T_3 in immature rat testes (Fig. 6), indicating that an increase in the intracellular Ca^{2+} concentration could be one of the

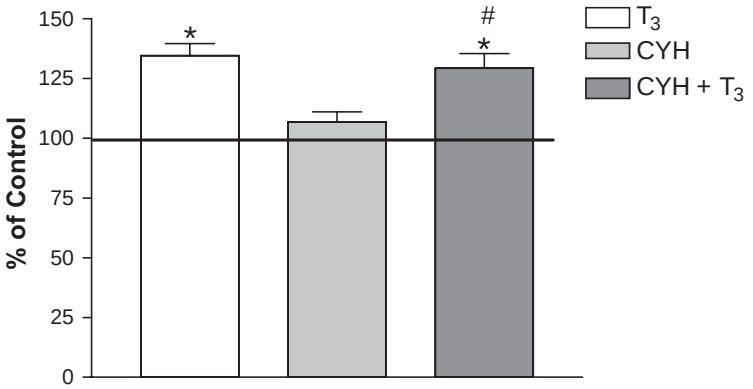

Fig. 5. Effect of T_3 and cycloheximide on in vitro vimentin phosphorylation of immature rat testis. Fifteen-day-old rat testes were incubated with 1 μM T_3, 0.35 mM CYH or 1 μM T_3 plus 0.35 mM CYH in the presence of ^{32}P orthophosphate. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into vimentin was measured as described in Materials and methods. Data are reported as means ± S.E.M. of six to eight animals expressed as percentage of controls. Statistical analysis: one-way ANOVA followed by Tukey–Kramer multiple comparison tests. *$P<0.001$ compared with control; #$P<0.05$ compared with CYH group.

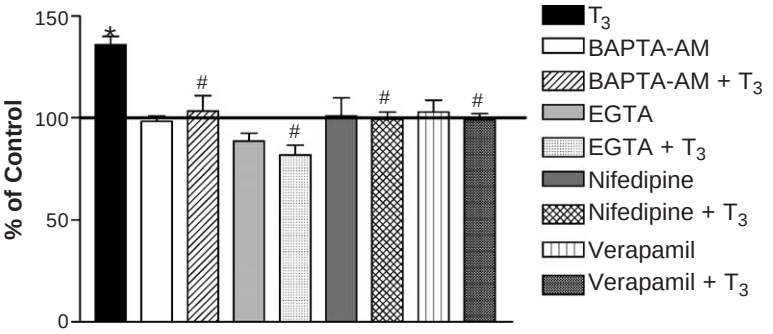

Fig. 6. Effect of L-VDCC inhibitors and Ca^{2+} chelators on T_3-induced alterations on IF-associated phosphorylating system. Gonads were pre-incubated and incubated for 30 min with 1 μM T_3 and ^{32}P-orthophosphate in the presence or absence of 50 μM BAPTA-AM (intracellular Ca^{2+} chelator), 2 mM EGTA (extracellular Ca^{2+} chelator), 100 μM nifedipine or 100 μM verapamil (L-VDCC inhibitors). The cytoskeletal fraction was extracted and the radioactivity incorporated into vimentin was measured as described in Materials and methods. Data are reported as means ± S.E.M. of six to eight animals in each group and expressed as percent of controls. Statistical analysis: one-way ANOVA followed by Tukey–Kramer multiple comparison test. *$P<0.001$ T_3 compared with control group; #$P<0.01$ "drug + T_3" compared with T_3 group.

mechanisms regulating the effects of this hormone on the phosphorylating system associated to the cytoskeleton.

Discussion

The classical genomic concept of thyroid hormones acting through nuclear receptors is well recognized (Lazar, 1993; Davis et al., 2000). However, nongenomic actions of thyroid hormones are now widely described and characterized by rapid responses, since they occur through plasma membrane transport systems (Incerpi et al., 1999b; Davis et al., 2000, 2002; Silva et al., 2002; Volpato et al., 2004). Although short-term effects of FSH and steroid hormones on vimentin have been described in Sertoli cells (Spruill et al., 1983; Sasaki et al., 1998; Show et al., 2003), little is known about short-term effects of thyroid hormones on cytoskeletal proteins in testicular cells. In the present study we have shown short-term effects of the thyroid hormone T_3 on the in vitro phosphorylation of cytoskeletal-associated vimentin in immature rat testis. We also demonstrated that such effect is independent of protein synthesis but dependent on intracellular Ca^{2+} levels and Ca^{2+} influx through VDCC.

In our experimental conditions T_3 was able to increase the in vitro ^{32}P incorporation into vimentin at concentrations ranging from 10^{-7} to 10^{-5} M, however we have chosen 10^{-6} M (1 μM) T_3 to undertake our experimental approach, since this concentration has been previously reported to elicit nongenomic effects in Sertoli cells, such as increased amino acid transport and changes in the membrane potential (Segal, 1989; Silva et al., 2001).

We demonstrated that incubation of immature rat testis with 1 μM T_3 for 30 min induced a significantly increased ^{14}C-leucine incorporation into proteins of the gonads, suggesting an increased protein expression, according to the classical genomic mechanism of action of T_3 (Zhang and Lazar, 2000). Besides, cycloheximide was able to block the basal protein synthesis on the testis. Despite these findings, T_3 failed to increase total vimentin immunocontent in tissue homogenate, suggesting no effect of the hormone on vimentin expression in this short period of incubation. However, the immunocontent

as well as the in vitro ^{32}P incorporation into the high salt Triton insoluble vimentin–representing aggregated or polymerized vimentin (Funchal et al., 2003)–were significantly increased following T_3 treatment. In addition, these effects were not prevented by 0.35 mM cycloheximide, a potent inhibitor of protein synthesis (Lin et al., 1997). These findings suggest that 30 min exposure of immature rat testis to 1 μM T_3 induced effects on the ability of vimentin to polymerize rather than on vimentin expression. Considering that it has been largely described that phosphorylation regulates the ability of IFs to polymerize (Shoeman and Traub, 1993), we could ascribe the activation of the phosphorylating system associated to cytoskeletal proteins as a possible mechanism inducing the altered equilibrium between soluble and polymerized vimentin. The increased cytoskeletal-associated vimentin would support a rapid mechanism of action for T_3 involving regulation of vimentin polymerization through phosphorylation/dephosphorylation equilibrium. Although several authors have described that phosphorylation can disassemble polymerized IFs into soluble forms (Inagaki et al., 1987, 1990; Huang et al., 1994; Chou et al., 1996), our results showed that upon short-term T_3 treatment we observed an increase of phosphorylated vimentin associated to the cytoskeletal fraction, suggesting that assembly of vimentin was triggered by phosphorylation. Our findings showing that phosphorylation can lead to increased assembly of vimentin into the cytoskeleton are in line with Mangoura et al. (1995), who described an increased assembly of phosphorylated vimentin into cytoskeletal polymers in chick embryonic cortical astrocytes treated with protein kinase C (PKC) activators. In this context, Cheng et al. (2003) described that vimentin phosphorylated by mitogen-activated protein kinase-activated protein kinase-2 (MAP-KAP-kinase-2) did not lose its capacity to organize into filaments.

There are several evidences in the literature pointing to cytoskeletal proteins acting as important framework for the modulation and control of essential cell processes, in particular, signal transduction events (Paramio and Jorcano, 2002). Moreover, it is described that the cytoskeletal dynamics can be regulated by hormones through genomic and nongenomic mechanisms. In this context, estradiol increases the pool of unstable microtubules and the expression of microtubule-associated proteins in hippocampal cells (Shah et al., 2003), while in epididymal cells, estradiol release is controlled by disorganization of microtubules and shortening of stress fibers (Marchlewicz et al., 2004). Testosterone regulates actin reorganization in LNCaP cells via a rapid, nongenomic, signaling pathway (Papakonstanti et al., 2003) and alters vimentin integrity in Sertoli cells by proteolytic degradation (Show et al., 2003). However, concerning the effects of thyroid hormones on cytoskeletal proteins, Paul et al. (1999) have demonstrated that these hormones induced the expression of phosphorylated acidic and basic variants of vimentin promoting the morphological maturation of cultured astrocytes. The authors also demonstrated that vimentin expression was significantly delayed or reduced in the thyroid hormone-deficient cells. However, concerning the effects of thyroid hormones on cytoskeletal proteins, Paul et al. (1999) have demonstrated that these hormones induced the expression of phosphorylated acidic and basic variants of vimentin promoting the morphological maturation of cultured astrocytes. The authors also demonstrated that vimentin expression was significantly delayed or reduced in the thyroid hormone-deficient cells. However, addition of thyroid hormones (T_3 and T_4) to these hypothyroid cultures led to increased expression of vimentin variants to normal levels.

Membrane binding sites for all steroid hormones have been detected in a number of cell types (Wehling, 1997; Grazzini et al., 1998; Gorczynska and Handelsman, 1995; Lyng et al., 2000) and Ca^{2+}-mediated mechanisms have been described to be involved in the activation of such sites. The rise in intracellular Ca^{2+} concentrations in response to a stimulus could be originated from a calcium influx

pathway, from release of calcium from an internal store, or through some combination of these (Peuchen et al., 1996). The major mechanism by which calcium is mobilized from the endoplasmic reticulum in most cell types is through activation of inositol triphosphate (IP_3) receptor. The IP_3-mediated calcium release is followed by a signal that results in extracellular calcium entry into the cell and refilling of the internal store (Irvine, 1992). Previous evidences showed the existence of an ionic mechanism related to extracellular Ca^{2+} and K^+ fluxes in the rapid, nongenomic action of T_3 in Sertoli cells (Volpato et al., 2004). In addition, Segal (1990) described that calcium is an important first messenger for the rapid effect of thyroid hormone at the level of the plasma membrane. Taking into account these findings we investigated the involvement of calcium on the in vitro phosphorylation of vimentin induced by T_3. To further investigate the participation of calcium influx in this effect, we proceeded to the blockage of the VDCC, known to be important mechanism of calcium influx (Davare and Hell, 2003). In our approach we used verapamil and nifedipine, two specific L-VDCC blockers (Volpato et al., 2004; Niikura et al., 2004; Jobling et al., 2004). Results showed that in the presence of each VDCC blocker, T_3 was not able to exert any effect on vimentin phosphorylation, suggesting the involvement of VDCC on the effect of this hormone in the cytoskeleton. We therefore investigated the role of intracellular Ca^{2+} mobilization in this process using the membrane-permeable form of BAPTA which is freely taken up into cells where it is hydrolyzed by cytosolic esterases and trapped intracellularly as the active Ca^{2+} chelator BAPTA. This reagent exchanges Ca^{2+} more than 100 times faster than other agents such as EGTA, because of the faster rates of association and dissociation. Therefore, BAPTA is an important tool to control fast changes in Ca^{2+} concentration (Mafra et al., 2002). Our results showed that when the intracellular calcium was chelated with BAPTA, the effect of T_3 on vimentin phosphorylation was totally prevented. Taken together, our data suggest that the stimulatory action of T_3 on the phosphorylating system associated with the cytoskeletal fraction is related to changes in Ca^{2+} influx and mobilization of intracellular Ca^{2+} pools. The importance of intracellular calcium concentration for the thyroid hormone action has been previously demonstrated by Lin et al. (1999), who described the activation of mitogen-activated protein kinase (MAPK) signaling pathway by iodothyronine (T_4). Activation of MAPK requires phospholipase C activity, producing inositol 1,4,5-triphosphate (IP_3). Production of IP_3 raises intracellular calcium concentration by releasing calcium from ER, activating PKC isoforms. Indeed, Davis et al. (2002) have demonstrated that the MAPK pathway could not be activated by T_4 in HeLa cells in viable BAPTA-treated cells, indicating that intracellular Ca^{2+} is required for the T_4 effect. A role for PKC in the signal transducing pathway and protein phosphorylation in short-term effects of thyroid hormones was also provided by Lawrence et al. (1989) and Incerpi et al. (1999b) who described that thyroid hormones activate PKC in rat erythroid cells and myoblasts, respectively. The involvement of PKC in vimentin phosphorylation was also demonstrated in chick embryonic cortical astrocytes (Mangoura et al., 1995).

In summary, in the light of the current knowledge, vimentin represents a novel target of action of thyroid hormones in immature rat testis. We demonstrated that T_3 altered the phosphorylation levels and the immunocontent of the cytoskeletal-associated vimentin through a rapid and Ca^{2+}-dependent mechanism. On the basis of our data this effect was independent on de novo protein synthesis, strongly supporting our hypothesis of nongenomic mechanism of action of thyroid hormones on vimentin. Consistent with these findings, the results of the present study suggest that Ca^{2+}-mediated changes in vimentin phosphorylation, elicited by T_3, could play important roles in the reorganization of the cytoskeleton, regulating cell physiology in immature rat testes. This novel aspect of the T_3 action opens an interesting area of research, where many questions are open to new research efforts.

Conclusion

Taking into account the evidences in the literature, we conclude that our study is in line with others, demonstrating that phosphorylation of vimentin can be modulated by short-term actions of thyroid hormones. T_3, at concentrations described to induce rapid nongenomic effects (Silva et al., 2001), markedly affect the phosphorylating system associated with cytoskeletal vimentin in immature rat testis, leading to increased vimentin polymerization. In addition, we demonstrated that Ca^{2+} influx by VDCC and mobilization of intracellular Ca^{2+} stores are involved in such effects, probably activating a signaling cascade leading to vimentin phosphorylation and cytoskeletal reorganization.

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Capítulo 2

HYPERTHYROIDISM IN THE DEVELOPING RAT TESTIS IS ASSOCIATED WITH OXIDATIVE STRESS AND HYPERPHOSPHORYLATED VIMENTIN ACCUMULATION

Artigo submetido para Cellular and Molecular Endocrinology

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HYPERTHYROIDISM IN THE DEVELOPING RAT TESTIS IS ASSOCIATED WITH OXIDATIVE STRESS AND HYPERPHOSPHORYLATED VIMENTIN ACCUMULATION

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Running title: Hyperthyroidism and rat testis cytoskeleton

Abstract

Hyperthyroidism was induced in rats and somatic indices and metabolic parameters were analyzed in testis. In addition, the morphological analysis evidenced testes maturation and intense protein synthesis and processing, supporting the enhancement in vimentin synthesis in hyperthyroid testis. Furthermore, vimentin phosphorylation was increased, indicating an accumulation of phosphorylated vimentin associated to the cytoskeleton, which could be a consequence of the extracellular-regulated kinase (ERK) activation regulating the cytoskeleton. Biomarkers of oxidative stress demonstrated an increased basal metabolic rate measured by tissue oxygen consumption, as well as, increased TBARS levels. In addition, the enzymatic and non-enzymatic antioxidant defences appeared to respond according to the augmented oxygen consumption. We observed decreased total glutathione levels, with enhancement of reduced glutathione, whereas most of the antioxidant enzyme activities were induced. Otherwise, superoxide dismutase activity was inhibited. These results support the idea that an increase in mitochondrial ROS generation, underlying cellular oxidative damage, is a side effect of hyperthyroid-induced biochemical changes by which rat testis increase their metabolic capacity.

Keywords

Hyperthyroidism; oxidative stress; vimentin; phosphorylation; ERK MAPK.

Introduction

Thyroid hormones (TH) are essential for normal postnatal growth and development, and are known to play fundamental roles in the regulation of the energy metabolism of almost all mammalian tissues. It is well described that TH is not a major regulator of the adult testis (Barker and Klitgaard 1952; Oppenheimer et al. 1974), nevertheless, T_3 receptors are present in high quantities in neonatal Sertoli cells, indicating that the developing Sertoli cell and testis may be an important TH target (Palmero et al. 1988, 1989; Jannini et al. 1990; Jannini et al., 1999). In addition, alterations in thyroid activity are frequently associated with changes in male reproductive functions, since hypothyroidism induced or occurring soon after birth is associated with a marked delay in sexual maturation and development (Longcope, 1991).

Vimentin is the major cytoskeletal constituent of a great variety of cells (Gyoeva and Gelfand, 1991; Shah et al., 1998), and is often expressed transiently during development (Menet et al., 2001). Both vimentin and cytokeratins are intermediate filament (IF) proteins and have been described in Sertoli cells during foetal and postnatal periods, but vimentin is the only IF described in immature and adult rat testes (Paranko et al., 1986; Romeo et al., 1995), where it plays important roles in the modifications of Sertoli cell morphology, junctional processes and cytoplasmic organization occurring during spermatogenesis (Russell and Peterson, 1985; Tanemura et al., 1994). In addition, there are several evidences in the literature pointing to phosphorylation as the most important posttranslational modification modulating both reciprocal interactions of IF proteins with other cytoskeletal components and the continuous exchange of IF subunits between a soluble pool and polymerized IF (Inada et al., 1999; Zamoner et al., 2005, 2006; Inagaki et al.,1987; Chou et al., 1996). In this context, we have recently described that TH are able to modulate IF phosphorylation in testis and cerebral cortex of rats through nongenomic mechanisms (Zamoner et al., 2005, 2006).

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases that transmit signals from extracellular stimuli to multiple substrates involved in cell growth, differentiation, and apoptosis. Three major subfamilies of MAPKs, extracellular regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, have been identified. The outcome of MAPK activation depends on the mode of activation, cell type, and threshold of activity. The ERK MAPKs generally regulate cell growth and differentiation and there is now increasing evidence that activation of the ERK MAPKs can be induced by a variety of stimuli, including FSH and TH (Cowley et al., 1994; Papkoff et al, 1994; Cobb, 1999; Lin et al., 1999; Rama et al., 2000; Ng and Bogoyevitch, 2000; Chang and Karin, 2001; Jin et al., 2005; Yu et al., 2005). In addition, the ERK-dependent signaling is involved in the proliferation and differentiation of the Sertoli cells in a stage-specific manner modulated by FSH (Crepieux et al., 2001). However, little is known about how the ERK MAPK kinase pathway is affected in Sertoli cells after hyperthyroidism.

On the other hand, it has been largely described that hyperthyroidism is associated with hypermetabolic state and increased oxygen consumption in tissues. In this process, oxygen can undergo univalent reduction by one-electron transfer, which allows the formation of oxygen radicals and other reactive species (ROS), such as the superoxide anion radical ($^{\bullet}O_2^-$), the hydrogen peroxide (H_2O_2) and the hydroxil radical ($^{\bullet}OH$) (Venditti and Di Meo, 2006). When ROS generation exceeds the antioxidant capacity of cells, oxidative stress develops (Sies, 1991). In this context, it is described that hyperthyroidism enhances ROS generation and induces changes in antioxidant defenses in different tissues, including liver (Fernádez et al., 1985; Huh et al., 1998), heart (Venditti et al., 1997; Asayama et al., 1987; Civelek et al., 2001; Shinohara et al., 2000), brain (Adamo et al., 1989; Das and Chainy, 2004), testis (Choudhury

et al., 2003), blood (Videla et al., 1988; Bianchi et al., 1999; Bednarek et al., 2004) and muscle (Asayama et al., 1987; Shinohara et al., 2000; Zaiton et al., 1993).

In the present report we investigate some metabolic parameters of induced hyperthyroidism in rat testis. We first focused on somatic indices, morphological alterations, vimentin expression and phosphorylation and MAPK signaling activation. We also investigate some aspects of oxidative stress (oxygen consumption, lipid peroxidation measured through thiobarbituric acid reactive substances - TBARS, oxidized glutathione - GSSG), enzymatic (glutathione reductase - GR, glutathione-S-transferase - GST, glutathione peroxidase - GPx, superoxide dismutase - SOD and catalase - CAT) and non-enzymatic antioxidant defences (total - TG and reduced glutathione levels - GSH) in immature hyperthyroid rat testis.

Materials and Methods

Chemicals

[^{32}P] Na_2HPO_4 was purchased from CNEN, São Paulo, Brazil. 3,5,3'-triiodo-L-thyronine (T_3), benzamidine, leupeptin, antipain, pepstatin, chymostatin, anti-vimentin antibody (clone vim 13.2), peroxidase conjugated rabbit anti mouse IgG, acrylamide and bis-acrylamide were obtained from Sigma Chemical Co. (St Louis, MO, USA). The antibodies p44/42 MAP Kinase (anti ERK1/2) and Phospho-p44/42 MAP Kinase (Thr202/Tyr204) were obtained from Cell Signaling Technology (Boston, MA, USA). The chemiluminescence ECL kit and peroxidase conjugated anti rabbit IgG were obtained from Amersham, (Oakville, Ontario, Canada). TRIzol Reagent, SuperScript-II RT from Invitrogen (Carlsbad, CA, USA) and Taq DNA Polymerase from CENBIOT (Porto Alegre, RS, Brazil).

Animals

Fifteen-day-old male Wistar rats were obtained from our breeding stock and maintained on a 12 h light/12 h dark cycle in a constant temperature (22 °C) colony room. On the day of birth the litter size was culled to eight pups. Litters smaller than eight pups were not included in the experiments. Water and a 20% (w/ w) protein commercial chow were available ad libitum. All the animals were carefully monitored and maintained in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation.

Induction of hyperthyroidism

All males of the same litter were divided in T_3-treated and control groups. For hyperthyroidism induction, pups (8-day-old) received daily intraperitoneal injections of T_3, 80 µg/ kg body weight, for 7 days. T_3 was dissolved using 0.0025 M NaOH and the final solution was prepared with 0.9% saline solution. Control animals received intraperitoneal injections of 0.9% saline solution during the same period. Animals were killed by decapitation 24 hours after the last injection. In all animal groups the testis weight was measured on the day of the experiment.

Radioimmunoassay

Blood was collected, separated by centrifugation (500 x g, 10 min) and the serum was stored at -20ºC. Twelve to fifteen samples of control and treated groups were used to measure the serum hormone levels. Serum levels of T_3 and T_4, total and free, and TSH (ultra-sensitivity method) were determined by radioimmunoassay. The h-TSH assay sensitivity was 0.003 µ U/ml. The assay procedures were run according to the manufacturer's specifications (CRIESP – São Paulo, Brazil).

Light and transmission electron microscopy

For light microscopy, the testis were removed, fixed in Bouin's solution overnight, and subsequently transferred to 70% ethanol for storage. The specimens were then dehydrated in a graded series of ethanol and embedded in paraplast plus. They were sectioned in 5 μm slices, stained with hematoxylin and eosin and examined by light microscopy.

For transmission electron microscopy, the contralateral testis were fixed with 3% glutaraldehyde, buffered with 0.2 M phosphate buffer (PB), pH 7.4 and post-fixed with 1% osmium tetroxide in the same buffer. They were dehydrated in a crescent concentration series of ethanol and passage through propylene oxide, the specimens were embedded with Spurr. Thin sections were obtained with glass knives in a Porter Blum MT-2 ultramicrotome, stained with uranyl acetate and lead citrate (Watson, 1958; Reynolds, 1963) and observed with a JEM-1200Ex transmission electron microscope.

In vitro ^{32}P incorporation into vimentin

The in vitro phosphorylation of vimentin was carried out as previously described by us (Funchal et al., 2003; Zamoner et al., 2005). In brief, testes of 15 day-old rats were pre-incubated at 30°C for 20 min in a Krebs-Hepes medium containing 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 25 mM Na-HEPES (pH 7.4), 12 mM glucose, 1 mM $CaCl_2$, and the following protease inhibitors: 1 mM benzamidine, 0.1 μM leupeptin, 0.7 μM antipain, 0.7 μM pepstatin and 0.7 μM chymostatin. Incubation was carried out with 100 μl of the basic medium described above, containing 80 μCi [^{32}P] orthophosphate. The labeling reaction was allowed to proceed for 30 min at 30 °C and then stopped with 1 mL of cold stop buffer containing 150 mM NaF, 5 mM EDTA, 5 mM EGTA, 50 mM Tris-HCl, pH 6.5, and the

protease inhibitors described above. Testes were then washed twice by decanting with the stop buffer to remove excess radioactivity. After the in vitro phosphorylation procedures, the IF-enriched cytoskeletal fraction was extracted as described below.

Extraction of IF enriched cytoskeletal fraction

The extraction of IF-enriched cytoskeletal fraction was proceeded as described by Funchal et al. (2003). Briefly, testis was homogenized in 600 µl of ice-cold buffer containing 5 mM KH_2PO_4, 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 1 % Triton X-100 and the protease inhibitors described above. The homogenate was centrifuged at 15,800 x g for 10 min at 4 °C in an Eppendorf centrifuge. The insoluble material was resuspended in 600 µl of the same buffer and centrifuged as described. The pellet constituted the high salt Triton insoluble IF enriched cytoskeletal fraction. This pellet was then dissolved in 1% sodium dodecyl sulfate (SDS). Some experiments used total protein homogenate of the testis. For this, tissue was homogenized in 300 µl of a lysis solution (2 mM EDTA, 50 mM Tris–HCl, pH 6.8, 4% SDS). The protein content was measured by the method of Lowry et al (1951). For SDS-PAGE analysis, samples were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8 and boiled for 3 min. Equal protein concentrations of the total protein homogenate or the IF-enriched cytoskeletal fraction were analyzed by 10% SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS). In the experiments on in vitro ^{32}P incorporation, the nitrocellulose membranes containing the IF-enriched cytoskeletal fraction were exposed to X-ray films (X-Omat XK1) at –70 °C with intensifying screens, and finally the autoradiograms were obtained and quantified as described below.

Immunoblotting analysis

The nitrocellulose membranes were washed for 10 min in Tris-buffered saline (TBS; 0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% defatted dried milk – MTBS). After incubation, the blot was washed twice for 5 min with TBS plus 0.05% Tween-20 (T-TBS), and then incubated overnight at 4°C in blocking solution containing the monoclonal anti-vimentin antibody (clone vim 13.2), diluted 1:400, and p44/42 MAP Kinase antibody (anti ERK1/2) and Phospho-p44/42 MAP Kinase (Thr202/Tyr204) antibody diluted 1:1000 for ERK/phosphoERK detection. The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in MTBS containing peroxidase conjugated rabbit anti-mouse IgG diluted 1:4000 (for anti vimentin) or anti-rabbit IgG 1:1000 (for ERK and phosphoERK). The blot was washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blot was then developed using a chemiluminescence ECL kit. Autoradiograms and immunoblots were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an OptiQuant version 02.00 software (Packard Instrument Company).

Reverse transcription polymerase chain reaction – RT-PCR

Total RNA from testes cells was isolated using TRIzol® reagent. CDNA was synthesized from 1 μg of total RNA. RNA was primed with 0.5 μg of oligo $(dT)_{12-18}$ primer (reaction volume: 20 μL). After the RNA was denatured (10 min at 70 °C) and cooled on ice, the following reagents were mixed and then added: 10 mM of each deoxynucleoside triphosphate (dNTP), 6 μL 5 x reverse transcrition (RT) buffer, 0.1 mM DTT, and 200 U Superscript ™ RT (reaction volume 30 μL). Reaction was performed by incubation for 1 h at 42 °C.

Amplification reactions consisted of 0.2 μM primer (described in table 1), 10 mM of each dNTP, polymerase chain reaction (PCR) buffer, 5 μL of the cDNA reaction, 0.5 U of Taq DNA polymerase. Amplification was carried out during 30 PCR cycles, each cycle consisting of a denaturation step at 94 °C for 1 min, an annealing step at 60 °C for 2 min, and an extension step at 74 °C for 3 min. after the last cycle, incubation for another 7 min at 74 °C was performed. The PCR products (5 μL) were analysed on agarose gels containing 0.5 μ g/mL ethidium bromide (Guma et al. 2001).

Oligonucleotides of 5' primers and 3' primers of target genes:

mRNA specie	5'primer sequence (5'to 3')	3'primer sequence (5'to 3')	Size of PCR product (bp)
Vimentin	GCCTATGTGACCCGGT CCTCGGCAGTGCGCCT	AGACGTGCCAGAGAAGCATTGTCAA	478
β-Actin	GTGGGCCGCTCTAGGCACCAA	CTCTTTGATGTCAACGCACGATTTC	540

Oxygen consumption measurements

Testis from controls, and T_3-treated pups were carefully excised, surface-dried with filter paper, weighted, and exhaustively washed in ice-cold isotonic Krebs Ringer-bicarbonate (KRb) solution containing 15 mM glucose, pH 7.4. Small slices (50-80 mg) from these tissues were resuspended in 2 ml of the KRb buffer settled in a Tucker chamber containing a Clark electrode, and rates of oxygen consumption were recorded on an oxygraphe for two minutes at a controlled temperature (25 ± 1ºC). The oxygen consumption was expressed as μmol O_2/min/g and measured in triplicate (Estabrook, 1984).

Antioxidant enzyme assays

Tissue was homogenized (approximately 500 mg of tissue in 9 volumes of buffer) in a buffer containing 0.1% Triton X-100, 0.12 M NaCl, 30 mM NaH_2PO_4, pH 7.4, and also containing

freshly prepared protease inhibitors (0.3 mM PMSF and 0.05 mM trypsin inhibitor). The use of Triton is essential for the measurement of total antioxidant enzymatic capacity of the tissue. Homogenization was carried out at 4°C, using 15 strokes in a Potter-Elvehjem homogeneizer, followed by centrifugation at 1000 x g for 5 min at 4°C. The supernatants were used for enzymatic evaluations and TBARS contents. Aliquots of the extracts were stored in liquid nitrogen (-170 °C) and examined separately for each enzyme. Superoxide dismutase (SOD) activity was measured according to the method of cytochrome *c* reduction (Flohé and Gunzler, 1984). Catalase (CAT) activity was determined by the decrease in hydrogen peroxide (10 mM solution) concentration at 240 nm (Aebi, 1984). Glutathione peroxidase (GPx) was measured through the system glutathione/NADPH/glutathione reductase, by the dismutation of *tert*-butylhydroperoxide (Flohé and Gunzler, 1984). Glutathione reductase (GR) was measured through the oxidation rate of NADPH, in a reaction medium containing 0.1 M NaH_2PO_4 buffer, pH 7.0 containing 0.1% DPTA and 1.0 mM GSSG (Carlberg and Mannervik, 1985). The enzyme glutathione S-transferase (GST) was evaluated according to Habig et al (1974), using CDNB as substrate.

Glutathione assay

GSH was measured according to Beutler (1975), using Elmann's reagent (DTNB). Tissue acid extracts were obtained by the addition of 12% trichloroacetic acid (1:4 v/v), and then centrifuged. Supernatants from the acid extracts were added to 0.25 mM DTNB in 0.1 M NaH_2PO_4, pH 8.0, and the formation of thiolate anion was determined at 412 nm. TG was measured according to the method of Tietze (1969), and GSSG was calculated in equivalents of GSH (1 GSSG=2 GSH).

Lipid oxidation (TBARS)

Determination of thiobarbituric acid-reactive substances (TBARS) was used to assay endogenous lipid oxidation according to Ohkawa (1979) and Bird and Draper (1984). Fresh homogenates were added to 0.2 mM butylhydroxytoluene (BHT) to avoid further artifactual lipid oxidation.Tissue acid extracts were obtained by the addition of the homogenate to trichloroacetic acid 12% (1:4 v/v), and then centrifuged. Supernatants were centrifuged at 5000 x g for 3 min, added to 0.67% (w/v) 2-thiobarbituric acids, maintained in boiling water for 60 min, cooled at 5°C for 30 min, and then measured spectrophotometrically at 535 nm. Absorbances were expressed as nmol TBARS/g tissue (E_{535} = 153 mM^{-1} cm^{-1}). Frozen samples were not used because even when reacted with BHT they showed artifactual lipid autoxidation and, therefore, enhanced TBARS levels (Wilhelm Filho et al., 2001).

Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison tests when the F-test was significant or by Student *t* test. All analyses were performed using the GraphPAD InStat Software version 1.12a.

Results

In vivo treatment with T_3 (80 μg/kg for 7 days) induced hyperthyroidism, resulting in increased serum concentrations of free (FT_3) and total T_3 (T_3), associated with significantly reduced serum concentrations of free (FT_4) and total T_4 (T_4). In addition, the TSH levels were below the detection limit of the method (Table 1). Moreover, hyperthyroid animals presented increased testicular and decreased body weights (Table 2). Light micrographs of testis sections from control and hyperthyroid groups are shown in Figure 1 (A and B),

demonstrating lumen formation and similar diameter of seminiferous tubules in control and hyperthyroid testis without altering neither cell integrity nor cell population . Transmission electron microscopy of Sertoli cells from hyperthyroid rats showed developed Golgi apparatus presenting swollen vesicles, as well as abundant secretion vesicles (Figure 1C and 1D). Moreover, hyperthyroid Sertoli cells (Figure 1F) showed more abundant polyribosomes than control cells (Figure 1E). It was also observed more lipid droplets dispersed inside and outside the cells in hyperthyroid than in control seminiferous tubules (Figure 1 G and H).

We then investigated the effect of hyperthyroidism on the in vitro phosphorylation of the Triton insoluble cytoskeletal-associated vimentin. Results showed that the hormonal treatment increased the in vitro phosphorylation of this protein (Figure 2). In addition, we analyzed vimentin immunocontent in total homogenate and in the cytoskeletal fraction of hyperthyroid rat testis. Results showed that hyperthyroidism increased vimentin immunocontent both in tissue homogenate and in the cytoskeletal fraction from rat testis (Figure 3). Furthermore, RT-PCR results suggested that not only vimentin immunocontent was increased in hyperthyroid testis but also its mRNA expression (Figure 4). We next investigated the effect of hyperthyroidism on MAPK pathway. Results showed that the expression of total ERK1/2 was diminished in hyperthyroid testis, while phospho ERK1/2 was unaltered, indicating an increased ratio phosphoERK/totalERK, which suggests an increased MAPK activation in treated group compared with control animals (Figure 5).

Considering the hypermetabolic activity described for hyperthyroidism in several tissues (Venditi and Di Meo, 2006), we tested various parameters of oxidative metabolism, such as oxygen consumption, lipid peroxidation and GSSG measurement, as well as the enzymatic and non-enzymatic antioxidant defences in T_3-treated rats testis. Hyperthyroid testis presented increased oxygen consumption, as compared to control group, compatible with the higher

metabolic activity (Figure 6A) and increased TBARS levels (Figure 6B), indicating high lipid peroxidation and evidencing that hyperthyroid status is associated with oxidative stress in testes cells. Moreover, hyperthyroidism showed decreased TG levels, despite of increased GSH and unaltered GSSG levels (Figure 7A). In adittion, GR, GST and GPx activities were significantly higher in hyperthyroid than in control group (Figure 7B). Furthermore, hyperthyroid rat testis presented higher CAT activity (Figure 8A), while SOD activity (Figure 8B) was decreased as compared to control animals.

Discussion

The biochemical effects of TH demonstrate that the Sertoli cell is the main direct target in the testis for TH, and that the prepuberal period is the temporal frame for its action. The increased metabolic activity of the Sertoli cell caused by T_3 appears to be a prerequisite for the expansion of spermatogenesis. Thus, T_3 shares with FSH the role of pivotal regulator of the early phases of tubular development (Jannini et al 1995).

The effectiveness of T_3 administration (80 μg/kg body weight) in inducing hyperthyroidism was confirmed by the increase in FT_3 and T_3 associated with decrease in TSH circulating levels, as well as, the consequent improvement of testicular growth. In addition, we observed low circulanting levels of FT_4 and T_4, which are in agreement with Cohen et al. (1989), who described that supraphysiological administration of L-T_3 in children promotes increase in T_3 with decrease in T_4 levels. Furthermore, Fisher (1996) described that excessive iodine intake can block thyroid hormone biosynthesis resulting in reduced T_4 secretion. In this context, our experimental model produced alterations in TH circulanting levels according to an hyperthyroid status induced by administration of exogenous T_3. This is further supported by the decreased body weight we observed, which is consistent with the increased catabolic

activity described in hyperthyroidism (Norman and Litwack 1997). Moreover, the increased testis weight is supported by previous evidences that TH treatment is able to increase testicular size (Jannini et al 1993, Van Haaster et al 1993), probably associated with the accelerated testis maturation induced by this hormone (Jannini et al 1995).

Several laboratories have shown that T_3 can decrease proliferation and stimulate maturation of Sertoli cells (Francavilla et al., 1991; Cooke et al. 1994; Jannini et al. 1995; Palmero et al. 1995; Arambepola et al. 1998). In the present study, optical microscopy showed an apparent cellular integrity and organization of the seminiferous tubules, indicating that our treatment did not provoke significant structural alterations in the germinative epithelium. The presence of lipid droplets (Ramos and Dym 1979) and the appearence of a lumen in the seminiferous tubules observed in hyperthyroid rats mark the maturation of the Sertoli cells (Tindall et al., 1975; Russell et al., 1989). In addition, the swollen Golgi apparatus and the increased polyribosomes indicate an intense protein synthesis and processing in hyperthyroid testis.

Although we have recently described that in vitro administration of T_3 was able to stimulate the phosphorylating system associated with the IF proteins in rat testis and cerebral cortex of young rats (Zamoner et al 2005; 2006), the effect of induced hyperthyroidism on the cytoskeleton of testicular cells needs investigation. In the present study we observed an increased in vitro ^{32}P incorporation into the cytoskeletal associated vimentin from hyperthyroid rat testis. This effect was accompanied by increased vimentin immunoreactivity both in tissue homogenate and in the cytoskeletal fraction. In this context, increased vimentin expression was suggested by RT-PCR results. These findings indicate that induced hyperthyroidism increases the expression of phosphorylated vimentin in testicular cells, as well as, the recovering of the phosphorylated vimentin in the cytoskeletal fraction, reflecting

the assembled or polymerized form of this IF protein (Funchal et al 2003). It has been described that phosphorylation can disassemble polymerized IFs into soluble forms (Inagaki et al., 1987). However, our results suggest that assembly of vimentin was triggered by phosphorylation. The present findings agree with previous evidence of the ability of T_3 to increase the level of cytoskeletal-associated vimentin through phosphorylation (Zamoner et al 2005), and are consistent with cAMP-dependent protein kinase activity modulating vimentin polymerization in fibroblasts (Lamb et al 1989). Furthermore, phosphorylation of vimentin by MAPK-activated protein kinase-2 has no effect on its assembly ability (Cheng et al 2003). There is also evidence that the IF assembly/disassembly equilibrium in a variety of cell types is engaged in different physiological activities (Helfand et al, 2004). This conversion is regulated, at least in part, by phosphorylation mediated by protein kinases involved in various signaling processes (Inagaki et al 1996). In addition, treatments that activate signal-transduction pathways frequently alter the distribution of cytoskeletal networks throughout the cell (Chen et al 2006; Liu et al 2006).

Thyroid hormones have been described to induce phosphorylation and nuclear translocation (activation) of MAPK mediating both genomic and non genomic events, including protein synthesis and cytoskeletal remodeling in different cell types (Lin et al 1999; Ghosh et al., 2005). The extracellular signal-regulated kinases (ERKs) are constituent of MAPKs family (Chang and Karin 2001) and have been described to induce survival actions of TH in different cell types (Ghosh et al 2005; Lin et al 2005). We then investigated a possible role of ERK activation modulating the cytoskeletal-associated vimentin in this experimental model of hyperthyroidism. Surprisingly, the results showed unaltered phosphorylated ERK1/2 levels in contrast to decreased total immunocontent of these proteins in hyperthyroid rat testis. In this context, this result might indirectly means a disruption of the

phosphorylation/dephosphorylation balance towards the phosphorylated (activated) forms. It is known that ERK activation causes important downstream effects modulating the cytoskeleton (Chen et al 2006; Liu et al 2006) in response to different stimuli. In this regard, Perlson et al (2005) demonstrated a role for the complex vimentin and phosphoERKs in response to neuronal injury. Taken together the present observations, we could propose that the increased vimentin synthesis and phosphorylation observed in hyperthyroid testis could be tentatively ascribed to ERK-mediated mechanisms.

Considering that it has been reported that MAPK signaling cascades seem to be activated by ROS (Baas and Berk, 1995), we investigated the oxidative capacity in hyperthyroid rat testis. Hypermetabolic state in hyperthyroidism is associated with the enhanced level of electron carriers, by which hyperthyroid tissues increase their metabolic capacity (Venditti and Di Meo, 2006). Consistent with the induction of the hyperthyroid state, the oxygen consumption was significantly increased in the testis of treated animals. As a consequence, hyperthyroid tissues exhibit an increased ROS and RNS production (Halliwell and Gutteridge 1999). ROS can attack polyunsaturated fatty acids in the biomembrane, proteins, enzymes and nucleic acids damaging cell function (Halliwell and Gutteridge 1999; Kehrer 1993). Lipid peroxidation has been frequently used as an index of oxidative stress in hyperthyroid tissues and altered lipid peroxidation in rat liver, estimated by TBARS levels, has been associated with T_3-induced hyperthyroidism (Fernández et al 1985; Venditti et al 1997; Tapia et al 1999). Accordingly, the present results show that T_3 treatment increase TBARS levels in the hyperthyroid rat testes, supporting a disturbance of the normal cell balance between production of ROS and the capacity to neutralize their action. Thus, in order to understand the oxidative stress in hyperthyroid rat testis, it is worthwhile examining the changes in tissue antioxidant capacity to neutralize free radicals. We first examined the levels of the water-

soluble antioxidant glutathione. Under normal steady-state conditions, the GSH concentration in liver is maintained by continuous synthesis and use, the latter being carried out by processes such as oxidation to GSSG (Kaplowitz et al 1985). Otherwise, the interconversion GSH/GSSG/GSH is maintained by the concerted action of glutathione peroxidase (GPx) and glutathione reductase (GR), respectively (Venditti and Di Meo 2006). We found that in hyperthyroid rat testis the GSH levels were increased although GSSG was unaltered. These findings are consistent with differently increased activities of GPx and GR which is compelled to replenish GSH levels. Nonetheless, despite the increased recovering of GSH levels, consistent with an increased antioxidant defence, total TG levels are decreased, highlighting a not well defined relationship between hyperthyroidism and this antioxidant response in rat testes. In addition, the increase in GST activity suggest that it was induced either by a direct action as an antioxidant enzyme or via the second phase reactions through the conjugation/excretion process of hydroperoxides (Halliwell and Gutteridge, 1999). However, decreased TG could be ascribed to decreased SOD activity we observed, probably leading to decreased H_2O_2 production by the mitochondria. In addition, we could suppose that decreased SOD activity, despite the high electron transport velocity, would contribute to exceed mitochondrial antioxidant defences. In this context, concerning SOD activity, contradictory results are found in the literature. Total SOD was found to decrease in liver (Fernández et al 1988) and to increase in heart (Mano et al 1995; Shinohara et al 2000) in young hyperthyroid rats. Furthermore, CAT activity reduces hydrogen peroxide to water and oxygen and it is found mainly in peroxisomes, and to a lesser extent in the cytosol and microsomal fraction of the cell. Our results showed that hyperthyroidism increased CAT activity. Interestingly, CAT and GPx activities in the testis increased concomitantly in hyperthyroid rats. This combined response suggests that the generation of endogenous hydrogen peroxide and other

hydroperoxides was increased in the testis under the stimulation of oxygen consumption, probably as a consequence of an overall enhancement of ROS generation. Considering the overall depletion of TG, the increase in GSH contents and the stimulation verified in the antioxidant enzymatic activities after T_3 treatment, it is evident that this hormone provoked a high oxidative insult to the testis.

Taking together these results, the enhancement of oxygen consumption found after T_3 treatment probably elicited an increase in ROS generation, which was accompanied by enhanced TBARS levels, and there was a tendency to deplete the cellular pool of antioxidants such as total glutathione, while most of the antioxidant enzymatic activities were induced.

In summary, it is possible to conclude that hyperthyroidism was induced by T_3 injection and that this experimental condition was able to promote testes maturation associated to an increased metabolic activity and several changes in oxidative metabolism. Morphological results are in agreement with the stimulated synthesis of vimentin observed in hyperthyroid testis. In addition, the hyperthyroid state promoted oxidative stress in the testis which could be associated with phosphorylated vimentin accumulation and MAPK cascade activation. While oxidative stress in the developing testis appears to be responsible for the accumulation of phosphorylated vimentin and MAPK signaling, the mechanistic details of these events need further investigation. However, since multiples parameters of oxidative stress and antioxidant activities are altered during the process of phosphorylated vimentin accumulation in the developing rat testis, an understanding of their interrelationship may provide insight into the mechanism of this process during reproductive development.

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Figure 1. Optical and transmission electron microscopy of control and hyperthyroid rat testis. Hematoxylin-eosin stained sections of seminiferous tubules from control (A) and hyperthyroid (B) rat testis showing lumen formation (Lu) and tubular integrity. (C,D,E and F), transmission electron microscopy of Sertoli cells. (D) shows strongly developed Golgi apparatus (Go) and abundant mitochondria (Mi) and increased polyribosomes (Ri) in Sertoli cell of hyperthyroid rat testis (F), compared to controls (C and E respectively). (H) shows abundant intra and extracellular lipid droplets (Li) in hyperthyroid seminiferous tubules as compared to control (G). Nu, nucleous.

Figure 2. Effect of hyperthyroidism on in vitro vimentin phosphorylation in immature rat testis. Fifteen-day-old rat testes were incubated in the presence of ^{32}P orthophosphate for 30 min. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into vimentin was measured as described in Materials and Methods. Data are reported as means ± S.E.M. of 10 animals and expressed as percentage of control. Statistically significant difference from controls, as determined by Student's *t* test is indicated: *P<0.001.

Figure 3. Effect of induced hyperthyroidism on vimentin immunocontent of immature rat testis. (A) The immunocontent of total (tissue homogenate) and high salt Triton-insoluble vimentin were measured. The immunoblotting was carried out with monoclonal anti-vimentin antibody diluted 1:400. The blots were developed using an ECL kit. Scans from ten different animals were quantified as described in Materials and Methods. Results are expressed as mean ± S.E.M. Statistical analysis: one-way ANOVA followed by Tukey–Kramer multiple comparison test. *P < 0.001. (B) A composite of nitrocellulose replicas of total and Triton-insoluble vimentin in the control and hyperthyroid groups.

Figure 4. RT-PCR analysis of vimentin expression in controls and hyperthyroid rat testis. (1) DNA ladder, the larger band correspond to 600 base pairs; (2) β-actin; (3) vimentin control; (4) vimentin hyperthyroid.

Figure 5. Effect of induced hyperthyroidism on ERK1/2 immunocontent from immature rat testis. Immunocontent of total (A) and phosphorylated (B) ERK1/2 in testis homogenate. The blots were developed using an ECL kit. Scans from ten different animals were quantified as described in Materials and Methods. Results are expressed as mean ± S.E.M. Statistical analysis: one-way ANOVA followed by Tukey–Kramer multiple comparison test. *P < 0.01. (C) Representative nitrocellulose replicas of total and phosphorylated ERK1/2 in control and hyperthyroid groups. ERK1 = p44 ; ERK2 = p42.

Figure 6 . Effect of hyperthyroidism on oxygen consumption and lipid peroxidation in immature rat testis. (A) Oxygen consumption. (B) Thiobarbituric acid reactive substances (TBARS) measurement of lipid peroxidation. Data are reported as means ± S.E.M. of 8 animals in each group. Statistically significant differences from controls, as determined by Student's *t* test followed is indicated: *P<0.01; **P<0.0001.

Figure 7. Effect of hyperthyroidism on glutathione levels and glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities in immature rat testis. (A) Total (TG), reduced (GSH) and oxidized (GSSG) glutathione levels. (B) Enzymatic activities of GR, GST and GPx. Data are reported as means ± S.E.M. of 8 animals in each

group. Statistical analysis: one-way ANOVA followed by Tukey–Kramer multiple comparison test. *P < 0.01; **P< 0.001.

Figure 8. Effect of hyperthyroidism on catalase (CAT) and superoxide dismutase (SOD) activities in immature rat testis. (A) CAT activity; (B) SOD activity. Data are reported as means ± S.E.M. of 8 animals in each group. Statistically significant differences from controls, as determined by Student's *t* test followed is indicated: *P<0.01.

Table 1. Effect of induced hyperthyroidism on serum levels of thyroid hormones and TSH in rats.

	Control	Hyperthyroid
FT_3 (ng/ml)	3.26 ± 0.01	5.66 ± 0.07*
FT_4 (ng/dl)	2.20 ± 0.003	0.92 ± 0.50*
TSH (μU/ml)	0.03 ± 0.001	< 0.003*
T_3 (ng/ml)	9.40 ± 0.01	16.31 ± 1.21*
T_4 (μg/dl)	6.10 ± 0.001	0.30 ± 0.01*

Serum levels of free and total T_3 (FT_3 and T_3 respectively), free and total T_4 (FT_4 and T_4 respectively) and TSH. Data are reported as means ± S.E.M; n= 8 for controls and n= 8 for hyperthyroid group. Statistically significant differences from controls, as determined by Student's *t* test are indicated: *P<0.05.

Table 2. Effect of induced hyperthyroidism on body and testicular weight.

Treatment	Body weight (g)	Testis weight (mg)
Control	28.0 ± 1.4	109.0 ± 9,0
Hyperthyroid	20.0 ± 2.3*	142.0 ± 11.0*

Data are reported as means ± S.E.M; n= 11 for control and hyperthyroid groups. Statistically significant differences from controls, as determined by Student's *t* test are indicated: *P< 0.0001.

Figure 1
Click here to download high resolution image

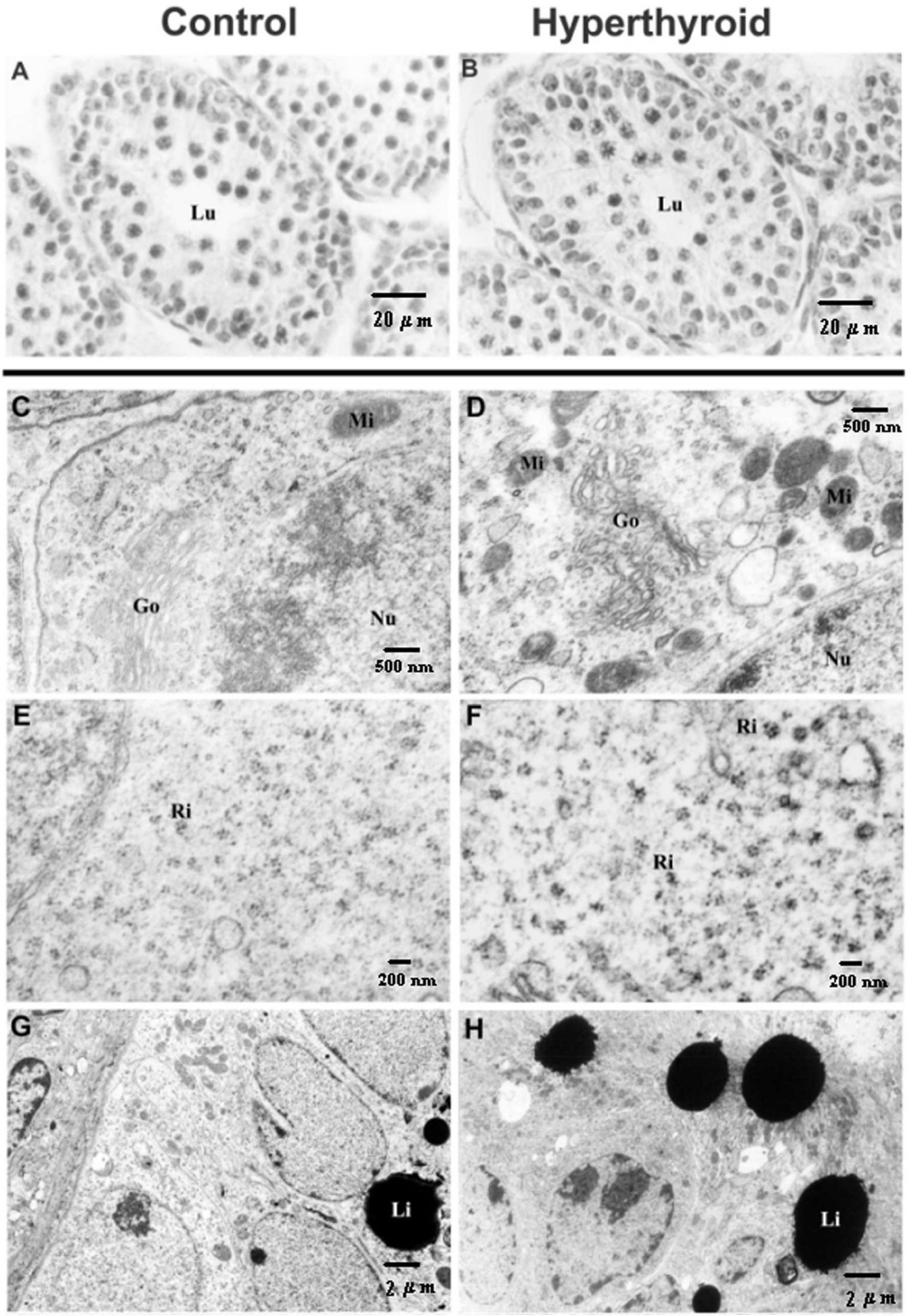


Figure 2

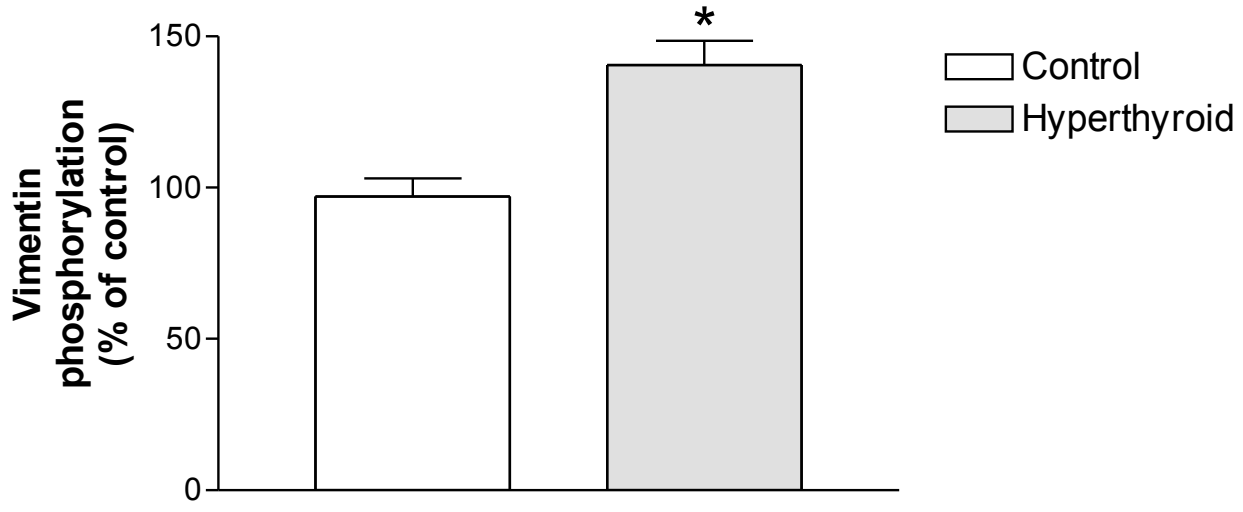

Vimentin phosphorylation (% of control)
150
100
50
0
*
Control
Hyperthyroid

Figure 3

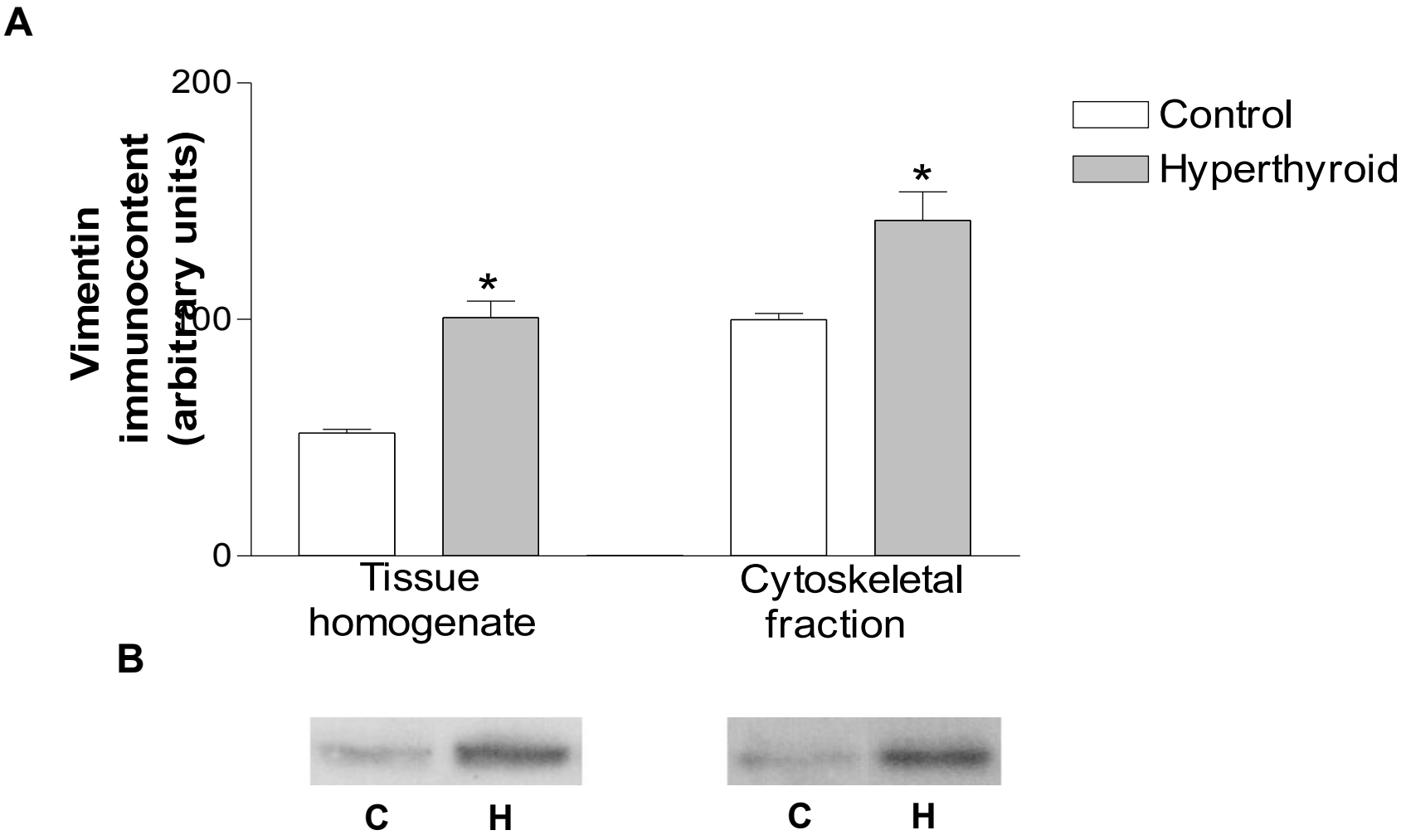

A
Vimentin immunocontent (arbitrary units)
200
100
0
*
*
Control
Hyperthyroid
Tissue homogenate
Cytoskeletal fraction
B
C
H
C
H

Figure 4

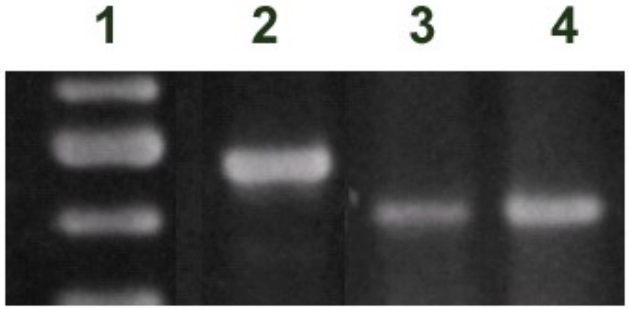


Figure 5

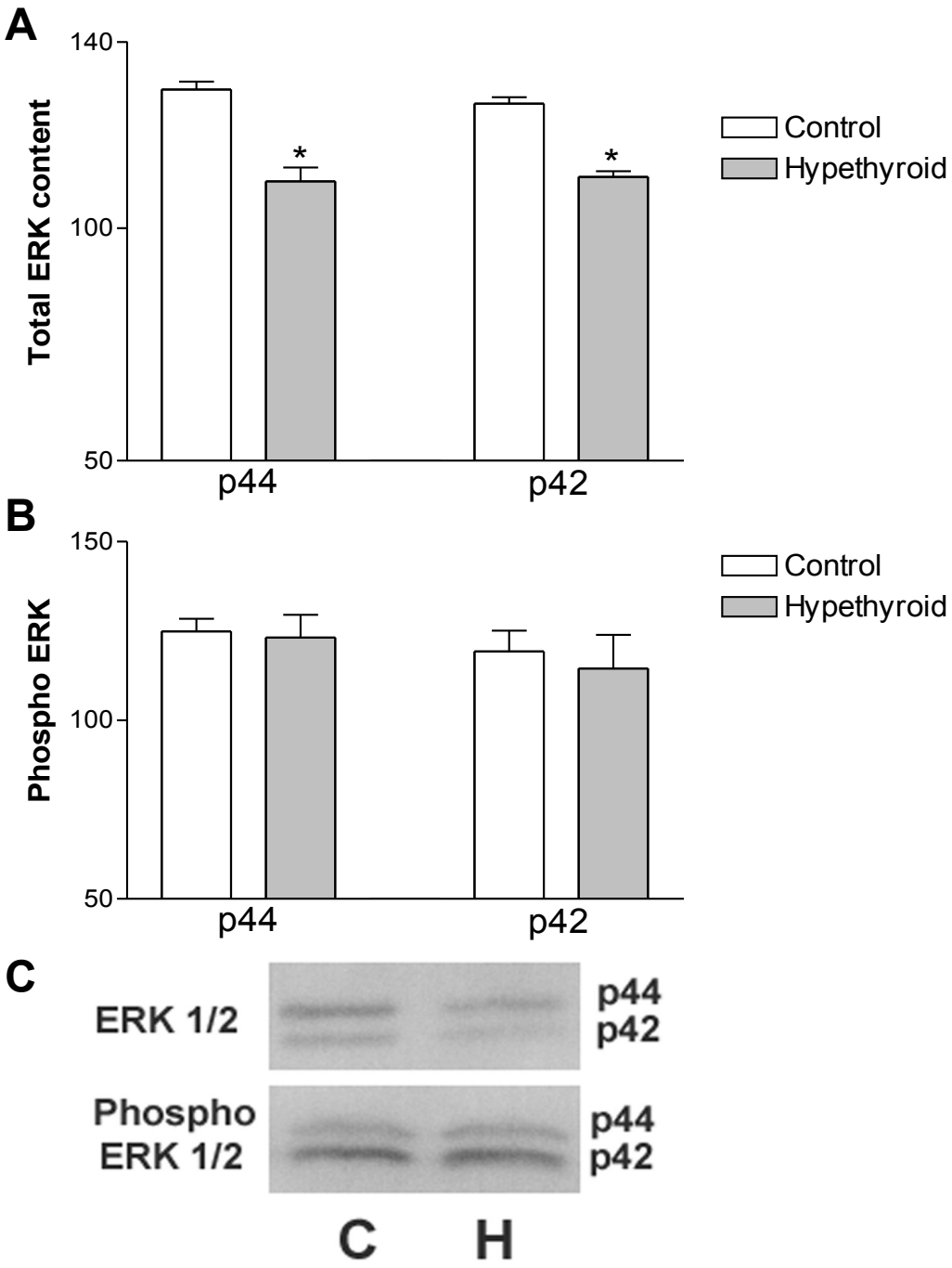

A
140
100
50
Total ERK content
*
*
p44
p42
Control
Hypethyroid
B
150
100
50
Phospho ERK
p44
p42
Control
Hypethyroid
C
ERK 1/2
p44
p42
Phospho
ERK 1/2
p44
p42
C
H

Figure 6

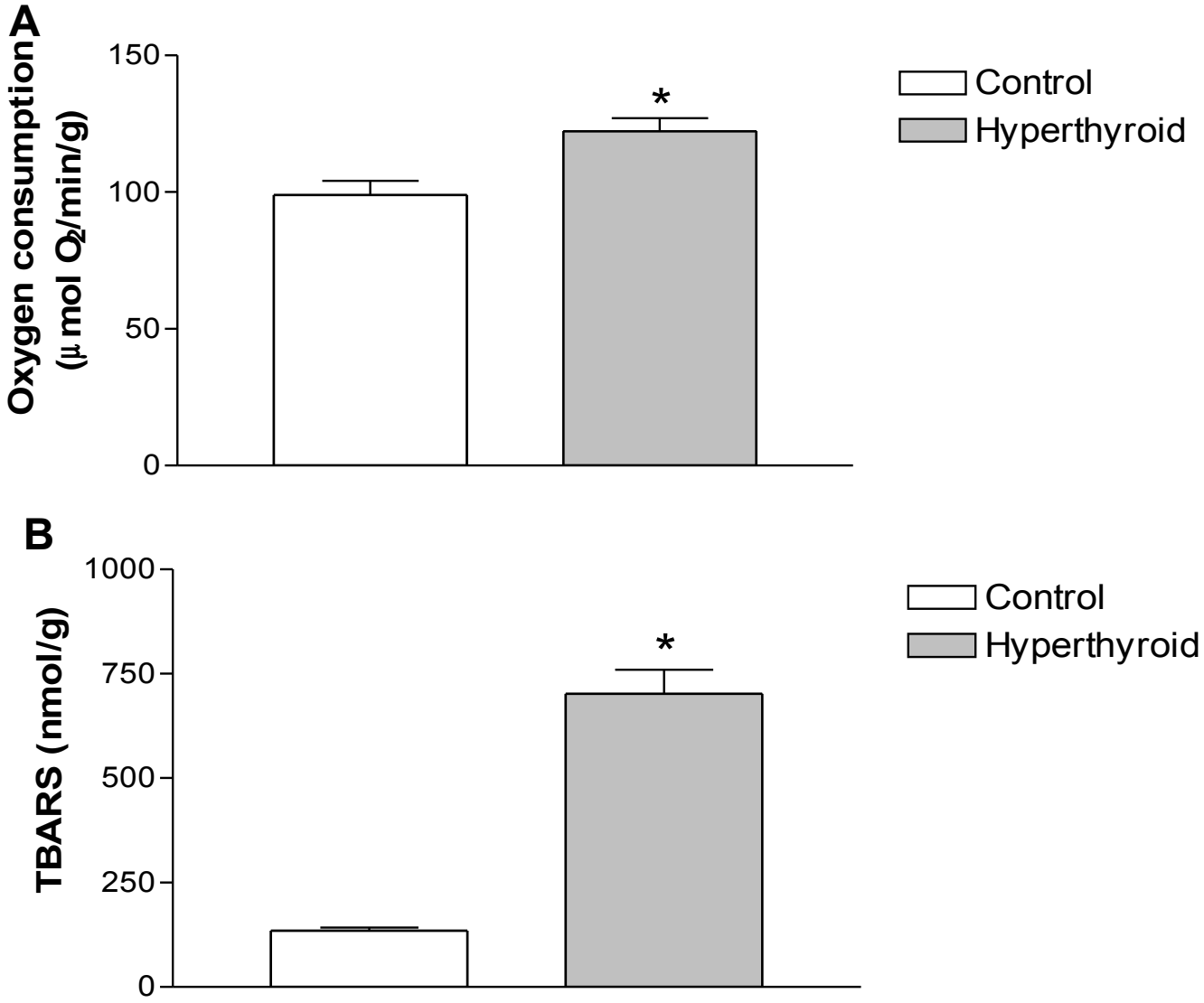

A
Oxygen consumption
(μ mol O2/min/g)
150
100
50
0
*
Control
Hyperthyroid
B
TBARS (nmol/g)
1000
750
500
250
0
*
Control
Hyperthyroid

Figure 7

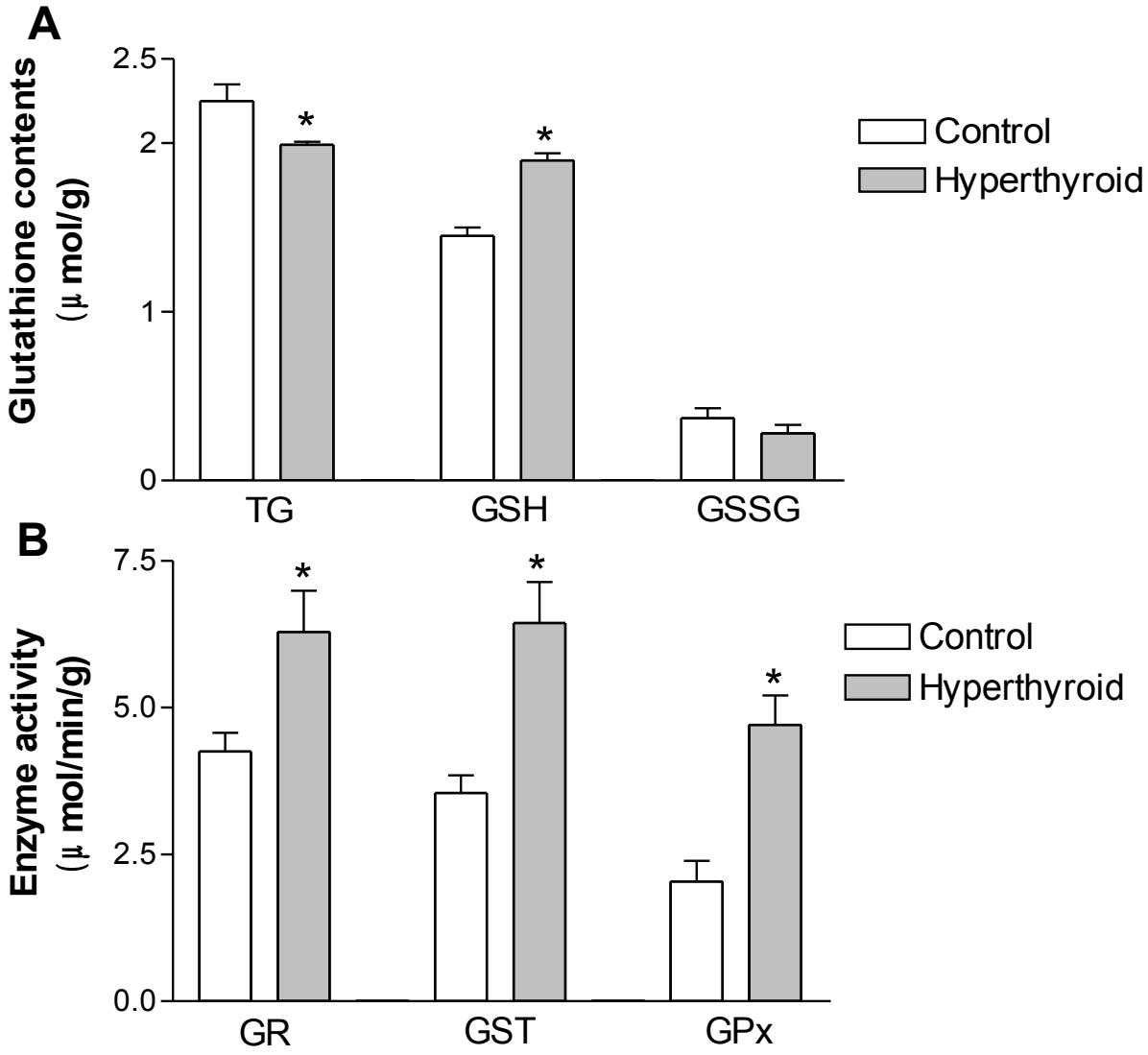
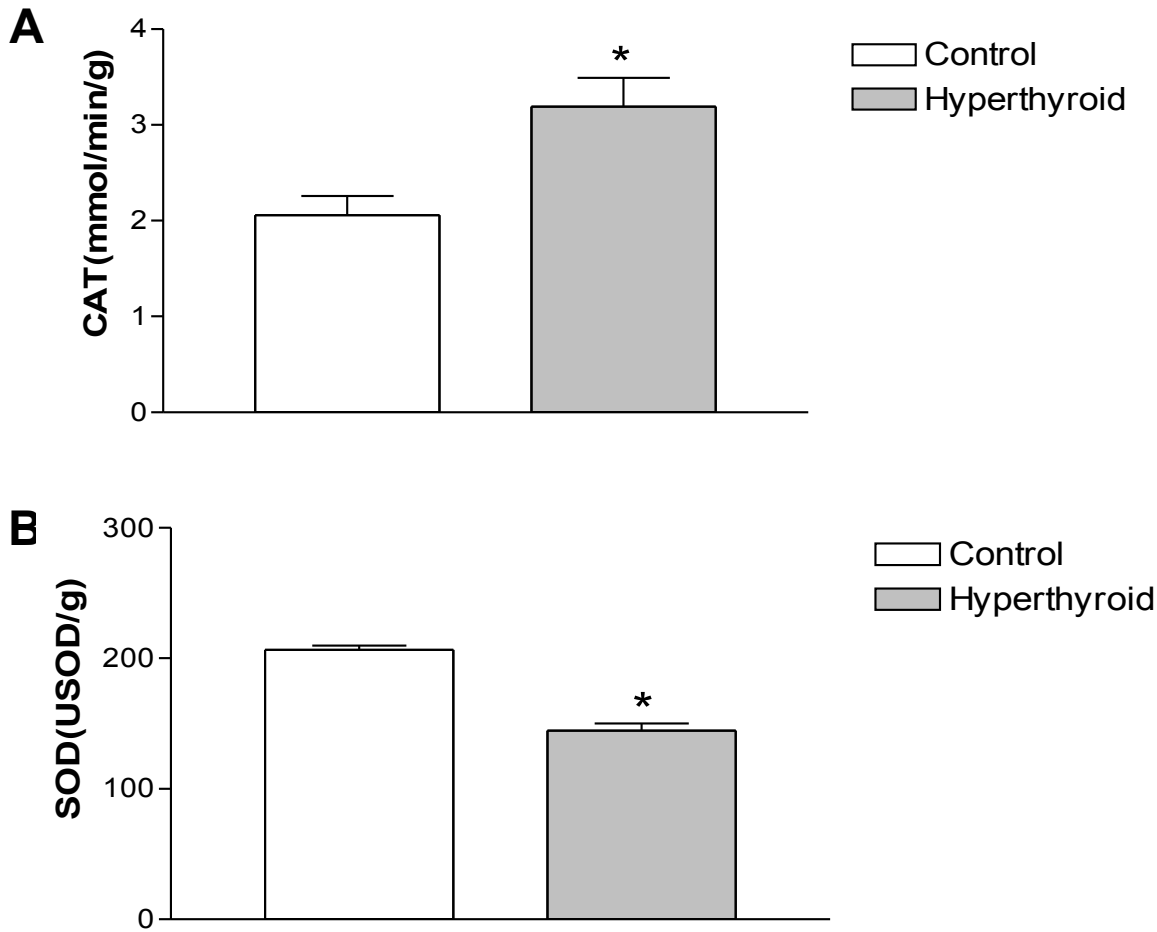

A
Glutathione contents
(μ mol/g)
2.5
2
1
0
*
*
TG
GSH
GSSG
Control
Hyperthyroid
B
Enzyme activity
(μ mol/min/g)
7.5
5.0
2.5
0.0
*
*
*
GR
GST
GPx
Control
Hyperthyroid

Figure 8


A
CAT(mmol/min/g)
0
1
2
3
4
*
Control
Hyperthyroid
B
SOD(USOD/g)
0
100
200
300
*
Control
Hyperthyroid

Capítulo 3

GENOMIC-INDEPENDENT ACTION OF THYROID HORMONES ON NTPDASE ACTIVITIES IN SERTOLI CELL CULTURES FROM CONGENITAL HYPOTHYROID RATS

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Genomic-independent action of thyroid hormones on NTPDase activities in Sertoli cell cultures from congenital hypothyroid rats

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Abstract

The Sertoli cells play an essential role in the maintenance and control of spermatogenesis. The ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) and 5′-nucleotidase activities can modulate the extracellular adenine nucleotide levels, controlling nucleotide-mediated signaling events in Sertoli cells. Since thyroid hormones (TH) and adenine nucleotides and nucleosides play important modulatory roles in Sertoli cell proliferation and differentiation, the aim of our study was to investigate the effect of hypothyroidism upon the NTPDase and 5′-nucleotidase activities in Sertoli cell cultures, as well as to verify whether these effects may be reversed by short and long-term supplementation with TH. Congenital hypothyroidism was induced by adding 0.02% methimazole in the drinking water from day 9 of gestation and continually until 18 days of age. Hypothyroidism significantly decreased the extracellular ATP and ADP hydrolysis and this effect was significantly reversed when cell cultures were supplemented with 1 μM T_3 or 0.1 μM T_4 for 30 min. In contrast, AMP hydrolysis was not altered by hypothyroidism, but was increased by T_4 supplementation for 24 h. The presence of the enzymes NTPDase 1, 2 and 3 was detected by RT-PCR in Sertoli cell cultures, however, hypothyroidism was not able to alter the expression of these enzymes. These findings demonstrate that TH modify NTPDase activities in hypothyroid Sertoli cells, probably via nongenomic mechanisms and, consequently, may influence the reproductive function throughout development.

Keywords: NTPDases; 5′-nucleotidase; Hypothyroidism; Sertoli cells; Thyroid hormones; Nongenomic action

Introduction

The Sertoli cells present in the seminiferous tubules provide physical support to germ cells, form the blood testis barrier and secrete protein products which are thought to be essential to the maintenance and control of spermatogenesis (Maran et al., 1999). The hormonal factors controlling the duration of Sertoli cell proliferation are critical determinants of fertility (Jannini et al., 1995). Thyroid hormones (TH) are essential for normal postnatal growth and development, and are known to play a fundamental role in the regulation of the energy metabolism of almost all mammalian tissues. Alterations in thyroid activity are frequently associated with changes in male reproductive functions, since hypothyroidism, induced or occurring soon after birth, is associated with a marked delay in sexual maturation and development (Longcope, 1991).

It is well known that the physiological responses of the cells to hormones are dependent on continuous cross-talking of different signal transduction pathways (Filippini et al., 1994). In addition to the hormonal modulation of Sertoli cell functions, there are

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several reports evidencing that extracellular adenine nucleotides can modulate responses through the purinoceptors present in these cells (Monaco et al., 1984, 1988; Stiles et al., 1986; Conti et al., 1989; Filippini et al., 1994; Rivkees, 1994; Ko et al., 1998; Meroni et al., 1998). Purinoceptors are classified into two types, P1 and P2, responsive to adenosine and ATP, respectively (Ralevic and Burnstock, 1998). Different subtypes of purinoceptors were detected on Sertoli and germinative cells, and their activation can induce important functional changes in these cells. Sertoli cells express P_2-purinergic receptors associated with phosphatidyl inositol turnover and $[Ca^{++}]_i$ mobilization inducing a number of biological effects (Filippini et al., 1994). ATP receptor activation in Sertoli cells can cause Na^+ influx-dependent membrane depolarization, with consequent opening of voltage-gated Ca^{2+} channels (Foresta et al., 1995). Therefore, extracellular ATP (ATPe) has been shown to play important regulatory roles in differentiation, transduction of external stimuli, and cell–cell interaction (Burnstock, 1981; Gordon, 1986; Filippini et al., 1990). Adenosine generated by ATPe hydrolysis in Sertoli cells can act on the A1 purinoceptors, recently demonstrated to be essential for acquisition of fertilizing capacity (Minelli et al., 2004). These receptors are coupled to a G-inhibitory protein when activated by adenosine, leading to inhibition of adenylyl cyclase (Monaco and Conti, 1986; Monaco et al., 1988) and participating in the modulation of secretory activity in Sertoli cells (Conti et al., 1988).

Signaling events induced by extracellular adenine nucleotides are controlled by the action of ecto-nucleotidases, including members of ecto-nucleotide pyrophosphatases/phosphodiesterases (E-NPPs) and ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) families as well as the ecto-5′-nucleotidase. Members of the E-NTPDase family constitute a class of ecto-enzymes characterized by their capacity to hydrolyze nucleoside tri- and diphosphates (Plesner, 1995). The ecto-5′-nucleotidase catalyzes the final step of extracellular nucleotide degradation, the hydrolysis of nucleoside 5′-monophosphates to the respective nucleosides and Pi (Zimmermann, 2000). These ecto-enzymes constitute a highly organized enzymatic cascade for the regulation of nucleotide-mediated signaling, controlling the rate, amount and timing of nucleotide degradation and ultimately nucleoside formation.

Ecto-nucleotidase activities, previously described in Sertoli cell cultures, are able to promote the hydrolysis of the extracellular nucleotides ATP, ADP and AMP to adenosine (Casali et al., 2001). These enzymatic activities can regulate the extracellular concentration of adenine nucleotides and nucleosides, modulating their local effects (Filippini et al., 1994; Casali et al., 2001; Ko et al., 2003).

Since TH play important modulatory roles in Sertoli cell proliferation and differentiation, and also taking into account our previous evidence of short-term effects of TH in rat testis (Zamoner et al., 2005), the aim of this study was to investigate the effect of hypothyroidism upon the extracellular hydrolysis of ATP and ADP by NTPDase and AMP by 5′-nucleotidase activities in Sertoli cell cultures, as well as to verify the reversibility of such effects after short- and long-term supplementation with T_3 or T_4 in the culture medium.

Materials and methods

Materials

L-thyroxine (T_4), 3,5,3′-triiodo-L-thyronine (T_3), methimazole, nucleotides (ATP, ADP, AMP), Malachite Green Base, soybean trypsin inhibitor I–S, DNase I, collagenase I, hyaluronidase I–S and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Culture medium (DMEM/F-12) and soybean trypsin were purchased from Grand Island Biological (Grand Island, NY, USA). Twenty four-well plates were from Costar (Cambridge, MA, USA). All other reagents were of the highest analytical grade.

Animals

18-day-old male pups used in the study were originated from litters in our Wistar rat colony. They were maintained on a 12 h light/12 h dark cycle in a constant temperature (22 °C) colony room. On the day of birth, the litter size was culled to eight pups. Litters smaller than eight pups were not included in the experiments. Water and a 20% (w/w) protein commercial chow were available ad libitum. All the animals were carefully monitored and maintained in accordance with the ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation.

Induction of hypothyroidism

Wistar rats were mated and the day of appearance of the vaginal plug was considered day 0 of fetal age. Congenital hypothyroidism was induced by adding 0.02% methimazole in the drinking water from day 9 of gestation, and continually until the day of the experiment (Pipaón et al., 1992). Euthyroid rats, receiving only water during the same period, were used as controls. Control and hypothyroid animals were used at 18 days of age. Since methimazole readily passes the placental barrier and is transmitted to the suckling pups in the mother's milk, the fetus and neonates also became hypothyroid (Oppenheimer and Schwartz, 1997). This protocol ensures a decreased growth rate and low levels of T_3 and T_4 (Pipaón et al., 1992).

Serum hormone levels

Blood was collected, separated by centrifugation (500 ×*g* for 10 min) and the serum was stored at −20 °C. Five to six samples of control and hypothyroid groups were used to measure the serum hormone levels. Serum levels of T_3, T_4 and TSH were determined by chemiluminescence using the Bayer-Architect kit. The assay procedures were run according to the manufacturer's specifications.

Sertoli cell cultures

Sertoli cells were isolated from the testis of 18-day-old Wistar rats by sequential enzymatic digestion, as described by Casali et al. (2001). The testes were sequentially digested with

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0.25% trypsin and DNase (10 μg/ml) for 30 min at 37 °C to remove the interstitial tissue. The seminiferous tubules obtained were dissociated with collagenase (1 mg/ml) and hyaluronidase (1 mg/ml) to separate the Sertoli cells from myoid cells and germ cells by centrifugation at 40 ×*g* for 10 min. The cultures were grown at confluence on 24 multiwell plates (0.6×10^6 cells/well and the final protein concentration was approximately 100 μg/well) at 34 °C in a water-saturated atmosphere with 95% air and 5% CO_2 in DMEM/F-12 (1:1) for 24 h. On the second day of culture, the monolayer was washed with Hank's Buffer Saline Solution (HBSS) pH 7.4 and maintained for 3 more days in serum free DMEM/F-12 (1:1). On the fourth day of culture, Sertoli cell monolayers were used for the assays.

Hormone treatment

The Sertoli cells were treated with DMEM/F-12 supplemented with 1 μM T_3 or 0.1 μM T_4 for 30 min or 24 h. All assays were performed on the fourth day of culture.

Ecto-nucleotidase assays

The Sertoli cell monolayers were incubated as previously described by Casali et al. (2001). Briefly, the Sertoli cell monolayers were washed 3 times with the reaction medium containing 135 mM NaCl, 5 mM KCl, 10 mM glucose and 10 mM HEPES, 2 mM $CaCl_2$ pH 7.4. The reaction was started by adding ATP, ADP or AMP to the reaction medium to a final concentration of 1.0 mM and incubated for 10 min. The final volume was 0.2 ml and incubation was carried out at 34 °C. After incubation, a sample of the supernatant was taken and mixed with cold trichloroacetic acid (TCA) to a final concentration of 5%. This mixture was centrifuged for 10 min at 16,000 ×*g* at 4 °C and aliquots were taken for the assay of released inorganic phosphate (Pi), according to the procedure of Chan et al. (1986). Controls to correct for non-enzymatic hydrolysis of nucleotides were performed by measuring the Pi released into the same reaction medium incubated without cells. Possible contamination by Pi released from cultures was discharged by incubating the monolayers in reaction medium without nucleotides. All assays were performed in quadruplicate. All enzyme conditions of this assay were previously described (Casali et al., 2001) in order to ensure the linearity of reaction.

RT-PCR

Total RNA from Sertoli cell culture was isolated with Trizol™ reagent (Life Technologies) in accordance with the manufacturer's instructions. The cDNA species were synthesized with SuperScript II (Life Technologies) from 2 μg of total RNA in a total volume of 20 μl with an oligo (dT) primer in accordance with the manufacturer's instructions. cDNA reactions were performed for 1 h at 42 °C and stopped by boiling for 5 min. Two microliters of cDNA were used as a template for PCR with primers specific for E-NTPDases 1, 2 and 3. As a control for cDNA synthesis, β-actin-PCR was performed. Two microliters of the cDNA were used for PCR in a total volume of 25 μl using a concentration of 0.5 μM of each primer indicated below, 50 μM of dNTPs and 1 U Taq polymerase (Life Technologies) in the supplied reaction buffer.

The PCR cycling conditions were as follows: for β-actin, an initial 2 min denaturation step at 94 °C, 1 min at 94 °C, 1 min at 59.8 °C, 1 min at 72 °C for 34 cycles (210 bp amplification product); for NTPDase 1, an initial 3 min denaturation step at 92 °C, 30 s at 92 °C, 1 min 30 s at 67 °C, 1 min at 72 °C for 27 cycles (543 bp amplification product); for NTPDase 2, an initial 3 min denaturation step at 95 °C, 1 min at 95 °C, 1 min at 64 °C for 40 cycles (331 bp amplification product); for NTPDase 3, an initial 3 min denaturation step at 94 °C, 1 min at 94 °C, 1 min at 60.9 °C for 26 cycles (267 bp amplification product). All PCRs included a final 10 min extension at 72 °C. Ten microliters of the PCR reaction were analyzed on a 1.5% agarose gel. The following set of primers were used: for β-actin: 5′TAT GCC AAC ACA GTG CTG TCT GG3′ and 5′TAC TCC TGC TTC CTG ATC CAC AT3′ (from Invitrogen); for NTPDase 1: 5′GAT CAT CAC TGG GCA GGA GGA AGG3′ and 5′AAG ACA CCG TTG AAG GCA CAC TGG3′; for NTPDase 2: 5′GCT GGG TGG GCC GGT GGA TAC G3′ and 5′ATT GAA GGC CCG GGG ACG CTG AC3′ (from Invitrogen) and for NTPDase 3: 5′CGG GAT CCT TGC TGT GCG TGG CAT TTC TT3′ and 5′TCT AGA GGT GCT CTG GCA GGA ATC AGT3′ (from Imprint).

Cellular protein determination

After the assays, the Sertoli cell monolayers for each well were dried and digested with 0.5 N NaOH and total protein was measured by the method of Lowry et al. (1951).

Statistical analysis

Data from five independent experiments developed in quadruplicate were statistically analyzed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparison tests when the *F*-test was significant. All analyses were performed using the GraphPAD InStat Software version 1.12a.

Results

Rat pups exposed to the congenital hypothyroidism model (methimazole in drinking water during gestational and suckling

Table 1
Effect of congenital hypothyroidism on serum levels of thyroid hormones and TSH

	Control	Hypothyroid
T_3 (ng/mL)	1.3±0.12	0.56±0.01*
T_4 (μg/dL)	4.2±0.8	0.7±0.01*
TSH (ng/mL)	2.4±0.14	3.6±0.14*

Data are reported as means±S.E.M; $n=8$ for controls and $n=8$ for hypothyroid group. Statistically significant differences from controls, as determined by Student's *t*-test are indicated: *$P<0.05$.

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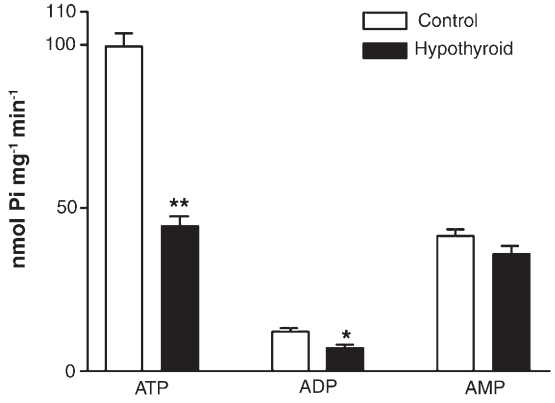

Fig. 1. Effect of hypothyroidism on ATP, ADP and AMP hydrolysis in Sertoli cell cultures. Data are reported as means±S.E.M. of five to six independent experiments performed in quadruplicate. Statistically significant differences from controls, as determined by one-way ANOVA followed by Tukey–Kramer multiple comparison test are indicated: *$P<0.001$.

periods), showed decreases in body (control: 22.1±0.4 g; hypothyroid: 13.9±0.3 g) and testicular (control: 50±0.8 mg; hypothyroid: 21±0.4 mg) weights compared to controls, as determined by Student's t-test (*$P<0.0001$; $n=15$). In addition, serum levels of T_3 and T_4 in methimazole-exposed pups were significantly lower than in control pups, while serum levels of TSH were increased. These results support the effectiveness of methimazole in producing typical evidence of severe TH deficiency (Table 1).

The effects of hypothyroidism on the ATPe and ADPe hydrolyzing activities of NTPDase as well as on the AMPe hydrolyzing activity of ecto-5′-nucleotidase in Sertoli cell cultures are shown in Fig. 1. When ATPe and ADPe hydrolysis were measured, we observed a significant decrease of approximately 67% and 62%, respectively, in hypothyroid compared to control cells. It is important to note that the intensities of the effects on both substrates were similar. In contrast, the AMPe hydrolysis was not changed under the same experimental conditions.

Fig. 2 shows the expression of NTPDases 1, 2 and 3 in Sertoli cells. Our results suggest that hypothyroidism does not alter the expression of the enzymes tested in these cells, suggesting that the effects observed are not due to a decreased synthesis of these proteins. Interestingly, the decrease in the ATPe and ADPe hydrolysis was reversed when hypothyroid cultures were treated with 1 μM T_3 or 0.1 μM T_4 after 30 min

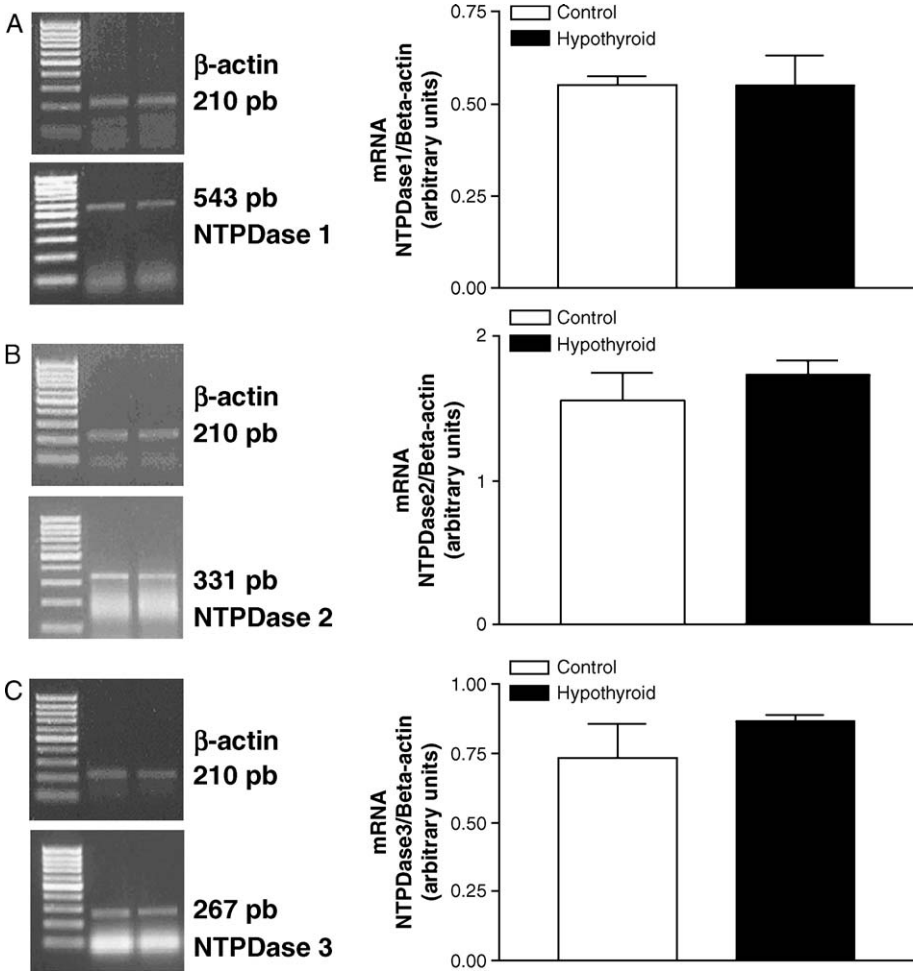

Fig. 2. Semi-quantitative RT-PCR mRNA for NTPDases 1, 2 and 3 from Sertoli cell cultures from controls and hypothyroid rats. The expression was evaluated by NTPDase to β-actin ratio. Bars represent arbitrary units of densitometry and are relative to mean±S.E.M. of NTPDase 1 (A), 2 (B), 3 (C) mRNA/β-actin mRNA ratio of at least three independent cell cultures for each group.

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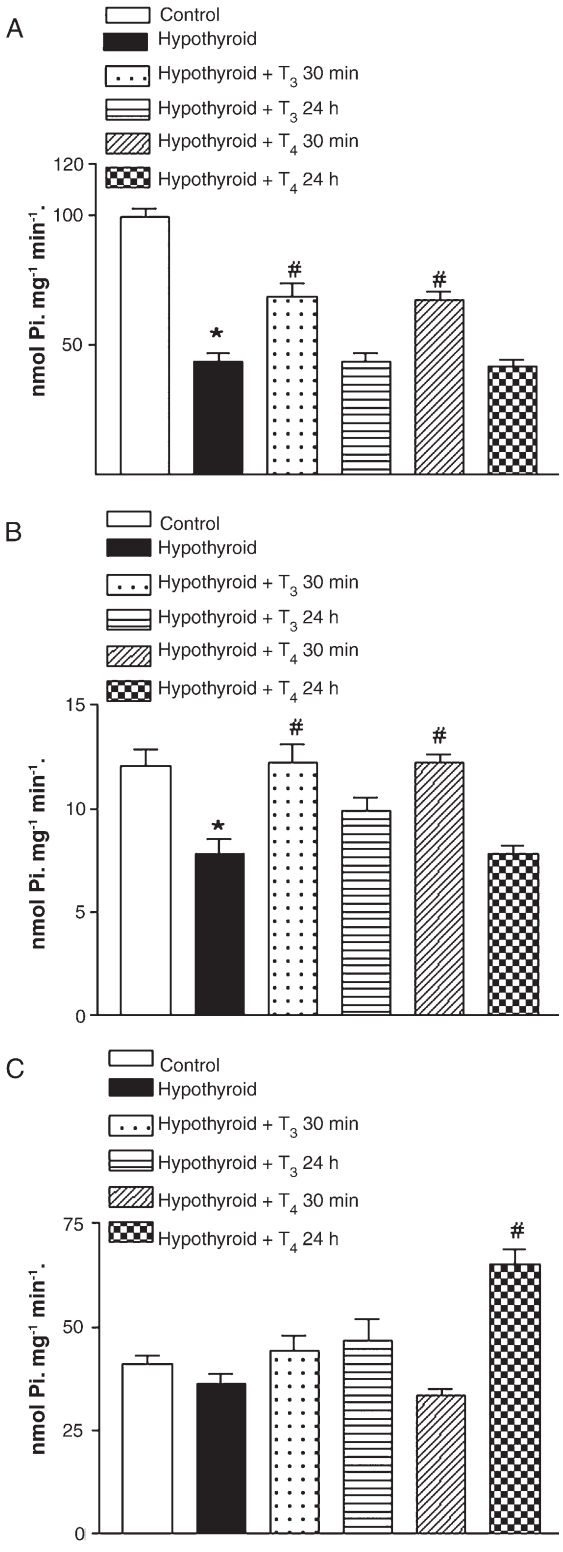

Fig. 3. Effect of short- (30 min) and long-term (24 h) supplementation with thyroid hormones (TH) on ATP, ADP and AMP hydrolysis in hypothyroid Sertoli cell cultures. Data are reported as means ± S.E.M. of five to six independent experiments performed in quadruplicates. The statistical analysis was determined by one-way ANOVA followed by Tukey–Kramer multiple comparison test. $^{*}P<0.001$ hypothyroid compared with control group; $^{\#}P<0.01$ "hypothyroid + TH" compared with hypothyroid group.

but not after 24 h (Fig. 3A and B), emphasizing a transient effect of the hormones. Moreover, we did not observe significant differences in the AMPe hydrolysis in Sertoli cells from hypothyroid rats when the TH were added in the culture medium for 30 min. In contrast, when cells were treated with 0.1 μM T_4 for 24 h the AMPe hydrolysis was stimulated (Fig. 3C).

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Discussion

Sertoli cells play a pivotal role in regulation and maintenance of spermatogenesis. The physiological role of TH in testis is still enigmatic, even if alterations in thyroid activity are frequently associated with changes in testicular function (Longcope, 1991). The presence of specific nuclear TH receptors, described in prepubertal Sertoli cells, implies the existence of an early and critical influence of TH on testis development (Jannini et al., 1999).

Our present investigation, showing that congenital hypothyroidism affects the body and testis weight after methimazole exposure, is supported by previous data reported by Francavilla et al. (1991), van Haaster et al. (1993), and Mendis-Handagama et al. (1998). In addition, we observed a reduction in the serum levels of TH with a marked increase in TSH secretion after methimazole treatment, confirming the induction of hypothyroidism (Maran et al., 1999). We also demonstrated that TH deficiency was able to decrease the E-NTPDase activities responsible for the hydrolysis of ATPe and ADPe, without altering AMPe hydrolysis by Sertoli cell cultures, known to be mediated by the ecto-5′-nucleotidase enzyme. Interestingly, the expressions of the three NTPDases tested (NTPDases 1, 2 and 3) were not altered, as demonstrated by RT-PCR. Although we can not exclude eventual translational mechanisms, our results suggest a modulatory effect on the catalytic activity of NTPDases, emphasizing a genomic-independent action on these enzymes both in hypothyroid and hormone-treated cells. This was further supported by the evidence that ATPe and ADPe hydrolysis was reversed when hypothyroid cultures were supplemented with TH for a short-term period (30 min), but not after a long-term period (24 h) with the hormones, suggesting a transient effect, probably mediated by nongenomic signaling mechanisms.

Although the mechanism of TH has been classically established as a genomic action, a number of reports indicate that these hormones exert several effects in cells lacking nuclear receptors (Davis et al., 2002; Silva et al., 2002). These findings led to the identification of non-classical TH binding elements in the plasma membrane (Silva et al., 2002). Through binding to these sites, TH could exert short-term effects, including those on ion fluxes at the plasma membrane (Incerpi et al., 1999; Huang et al., 1999), on intracellular protein trafficking (Safran et al., 1992; Zhu et al., 1998; Chen et al., 1999), on signal transducing cytoplasmic kinase activities (Lin et al., 1996, 1999) and on the cytoskeleton (Zamoner et al., 2005). In this context, our results showing the short-term effect of TH on ATPe and ADPe hydrolysis could be ascribed to nongenomic instead of the classical genomic actions, suggesting that more than one mechanism could contribute to the modulatory effects of TH on extracellular nucleotide levels in Sertoli cells.

Evidence in the literature shows that extracellular purines, such as ATP and adenosine, can modulate cellular processes in the male reproductive system through triggering of membrane purinergic receptors (Monaco et al., 1984, 1988; Stiles et al., 1986; Conti et al., 1989; Filippini et al., 1994; Rivkees, 1994; Ko et al., 1998, 2003 Meroni et al., 1998; Minelli et al., 2004). Sertoli cells express A1 purinoceptors, recently demonstrated to be essential for acquisition of fertilizing capacity (Minelli et al., 2004) and participating in the modulation of inhibin secretion (Conti et al., 1988). Indeed, these cells also express P_2Y and different subtypes of P_2X receptors, according to the stage of the seminiferous epithelium. The role of nucleotides as important modulators of different cell functions is well established in the testis, where there is a complex network of cell–cell interactions both in testicular somatic and germinal cells (Gordon, 1986; Filippini et al., 1990, 1994).

The inhibition of ATPe and ADPe hydrolysis observed in hypothyroid Sertoli cell cultures could be a consequence of the inhibition of NTPDase activities and might prolong the effects of these nucleotides at their respective receptors. In this context, it is important to emphasize that we are now providing the first evidence for the expression of the enzymes NTPDase 1, 2 and 3 in Sertoli cells, suggesting that at least these three isoforms could be involved in ATPe and ADPe hydrolysis in these cells. Although another plasma membrane bound NTPDase, named NTPDase 8, has been cloned and characterized, its expression in rodent testis is very low (Bigonnesse et al., 2004), nonetheless we cannot exclude the participation of this enzyme in the effects observed in Sertoli cells.

Extracellular adenosine plays important roles in regulating the metabolic function of germ cells and, consequently, in their maturation processes. This nucleoside could be produced from AMPe hydrolysis through the action of ecto-5′-nucleotidase that can participate together with NTPDases in the control of the extracellular adenosine levels (Casali et al., 2001). Results show that, in contrast to the inhibition of NTPDase, the ecto-5′-nucleotidase activity was not affected in hypothyroid cells, as evidenced by unaltered AMPe hydrolysis. Moreover, ecto-5′-nucleotidase activity was not modified after the short-term exposure (30 min) to the hormones. Surprisingly, long-term (24 h) exposure of cell cultures to T_4 stimulated this activity, emphasizing a T_3-independent mechanism of action for T_4 on ecto-5′-nucleotidase in Sertoli cells. The mechanisms underlying this effect are not clear.

Although the ecto-5′-nucleotidase activity was unaltered, we propose that the inhibited NTPDase activities could lead to an accumulation of ATPe, as well as a depletion of adenosine in extracellular medium. Considering the physiological role of ATPe in regulating Sertoli cell functions, such as modulating calcium influx, it may be suggested that increased ATPe concentrations may induce cytotoxic effects and be involved in the alterations observed during maturation processes of these cells. In addition, these events could be related to the reproductive dysfunctions described in hypothyroidism.

In summary, our findings indicate that hypothyroidism may influence the NTPDase activities from Sertoli cells through a direct effect that does not involve conventional genomic actions. Moreover, the reversion of the ATPe and ADPe hydrolysis when hypothyroid cultures were treated with T_3 or T_4 for 30 min, also reinforces the idea of nongenomic mechanisms underlying TH actions in these cells.

In conclusion, this study shows that TH, which are important regulators of the male reproductive system, modulate the extracellular adenine nucleotide levels in hypothyroid cultured

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Sertoli cells. Although TH- and hypothyroidism-induced changes in extracellular nucleotide metabolism may have some implications in purinoceptor signaling, the consequences of these modifications remain to be clarified. Thus, the effect of congenital hypothyroidism and TH supplementation on NTPDase activities in Sertoli cells can influence the actions mediated by the adenine nucleotides and nucleosides on reproductive functions throughout development.

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Capítulo 4

SHORT-TERM EFFECTS OF THYROID HORMONES ON CYTOSKELETAL PROTEINS ARE MEDIATED BY GABAERGIC MECHANISMS IN SLICES OF CEREBRAL CORTEX FROM YOUNG RATS

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Short-Term Effects of Thyroid Hormones on Cytoskeletal Proteins Are Mediated by GABAergic Mechanisms in Slices of Cerebral Cortex from Young Rats

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SUMMARY

Thyroid hormones play important roles in brain function. However, few information is available about the effect of 3,5,3′-triiodo-L-thyronine (T_3) or thyroxine (T_4) on the *in vitro* phosphorylation of intermediate filament (IF) proteins from cerebral cortex of rats. In this study we investigated the involvement of GABAergic mechanisms mediating the effects of T_3 and T_4 on the *in vitro* incorporation of ^{32}P into IF proteins from cerebral cortex of 10-day-old male rats. Tissue slices were incubated with or without T_3, T_4, γ-aminobutiric acid (GABA), kinase inhibitors or specific GABA antagonists and ^{32}P-orthophosphate for 30 min. The IF-enriched cytoskeletal fraction was extracted in a high salt Triton-containing buffer and the *in vitro* ^{32}P incorporation into IF proteins was measured. We first observed that 1 μM T_3 and 0.1 μM T_4 significantly increased the *in vitro* incorporation of ^{32}P into the IF proteins studied through the PKA and PKCaMII activities. A similar effect on IF phosphorylation was achieved by incubating cortical slices with GABA. Furthermore, by using specific GABA antagonists, we verified that T_3 induced a stimulatory effect on IF phosphorylation through noncompetitive mechanisms involving $GABA_A$, beyond $GABA_B$ receptors. In contrast, T_4 effects were mediated mainly by $GABA_B$ mechanisms. In conclusion, our results demonstrate a rapid nongenomic action of T_3 and T_4 on the phosphorylating system associated to the IF proteins in slices of cerebral cortex of 10 day-old male rats and point to GABAergic mechanisms mediating such effects.

KEY WORDS: phosphorylation; thyroid hormones; intermediate filaments; PKA; PKCaM II; GABA.

INTRODUCTION

Intermediate filaments (IFs) are major components of the cytoskeleton and nuclear envelope in most types of eukaryotic cells. They are expressed in cell-type-specific

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patterns and play an important structural or tension-bearing role in the cell. Evidence is now emerging that IF also acts as an important framework for the modulation and control of essential cell processes, in particular, signal transduction events (Paramio and Jorcano, 2002). The neuronal cytoskeleton comprises a protein network formed mainly by microtubules (MT) and neurofilaments (NF), the IFs of neurons. Neurofilaments are composed of three different polypeptides whose approximate molecular weight are 200, 160, and 68 KDa, and are commonly referred to as heavy (NF-H), medium (NF-M), and light (NF-L) neurofilament subunits (Ackerley *et al.*, 2000). Glial fibrillary acidic protein (GFAP) is the intermediate filament of mature astrocytes (Eng *et al.*, 2000) and vimentin is the IF of cells of mesenchimal origin (Alberts *et al.*, 2002).

The amino and the carboxy-terminal tail domains of NF subunits are potential phosphorylation sites (Nixon and Sihag, 1991). The phosphorylation sites located on the amino terminal domain of the neurofilament subunits are phosphorylated by second messenger-dependent protein kinases including protein kinase C, cyclic AMP- (PKA), and Ca^{2+}/calmodulin-dependent (PKCaMII) protein kinases (Sihag and Nixon, 1990). The functional role of neurofilament phosphorylation is to date not completely clear. However, the regulation of IF polymerization by phosphorylation is well described in the literature. Vimentin filaments reconstituted *in vitro* undergo complete disassembly when phosphorylated by purified protein kinase A or protein kinase C (Inagaki *et al.*, 1987). A similar *in vitro* disassembly induced by phosphorylation has been noted for almost all major IF proteins, such as glial fibrillary acidic protein (GFAP) (Inagaki *et al.*, 1990), desmin (Inada *et al.*, 1998), keratin (Ku and Omary, 1997), α-internexin (Tanaka *et al.*, 1993), NF-L (Hashimoto *et al.*, 1998), and lamin (Peter *et al.*, 1992). On the other hand, the carboxy terminal side arm domains of NF-H and NF-M subunits are extensively phosphorylated by several protein kinases, such as glycogen synthetase kinase (GSK) 3, extracellular signal-regulated kinase (ERK), stress activated protein kinase, protein kinase K, protein kinase C, and Cdk-5 (Hisanaga *et al.*, 1993; Shetty *et al.*, 1993; Guidato *et al.*, 1996; Sun *et al.*, 1996). It has been demonstrated that *in vitro* phosphorylation of COOH-terminal domains of NF-H and NF-M straightens individual neurofilaments and promotes their alignment into bundles (Leterrier *et al.*, 1996), whereas *in vivo* phosphorylation of these proteins is associated with an increased interneurofilament spacing (Hsieh *et al.*, 1994). As a consequence, NF-H and NF-M COOH-terminal side arms extend and form crossbridges among neurofilaments and other cytoskeletal elements (Gotow *et al.*, 1994).

Thyroid hormones (TH) are critically involved in development and function of the central nervous system. Expression and cell distribution of cytoskeletal components have been for long time considered to be important effects of thyroid hormone during brain development, and responsible for the effects of the hormone on axonal growth, and dendritic architecture (Bernal, 2002). In this context, Paul *et al.* (1999) have reported that TH induced the expression of phosphorylated forms of vimentin in cultured astrocytes.

The classical mechanism of TH has been established as a genomic action, including binding to intracellular hormone receptors that share characteristics of nuclear transcription factors. These effects are described to occur after a given time lag

necessary to modify protein transcription (Davis *et al.*, 2002; Silva *et al.*, 2002). However, recently, a number of reports have indicated that TH exerts several effects in cells lacking classical receptors (Lin *et al.*, 1997). In addition, the effect of these hormones on the membrane transport system of chick embryo was reported to be very rapid, occurring in minutes, a time lag noncompatible with the classical scheme of a nuclear receptor action (Incerpi *et al.*, 1999a). These findings led to the identification of nonclassical TH binding elements in the plasma membrane. Through binding to these sites, TH could exert short-term effects, including those on ion fluxes at the plasma membrane (Huang *et al.*, 1999; Incerpi *et al.*, 1999b; Silva *et al.*, 2001; Volpato *et al.*, 2004), on intracellular protein trafficking (Safran *et al.*, 1992; Zhu *et al.*, 1998; Chen *et al.*, 1999), on signal-transducing cytoplasmic kinase activities (Lin *et al.*, 1996, 1999) and on the cytoskeleton (Siegrist-Kaiser *et al.*, 1990). Also, we reported a rapid nongenomic and Ca^{2+}-dependent action of T_3 on the phosphorylating system associated with vimentin in immature rat testes (Zamoner *et al.*, 2005).

In the present investigation we studied the short-term effects of thyroid hormones, T_3 and T_4, on the *in vitro* incorporation of ^{32}P into IF proteins in slices from cerebral cortex of 10-day-old rats. Additionally, the role of protein kinases on the effects exerted by T_3 and T_4 on cytoskeletal protein phosphorylation was examined. We also evaluated the influence of GABAergic system on the effects elicited by the TH, since growing evidence in the literature suggest a role for altered GABAergic function mediated by TH (Mason *et al.*, 1987; Hashimoto *et al.*, 1991; Sandrini *et al.*, 1991; Narihara *et al.*, 1994). Despite these evidences, little is known about the effects of GABA on the phosphorylating system associated to the cytoskeleton.

METHODS

Radiochemical and Compounds

[^{32}P]Na_2HPO_4 was purchased from CNEN, São Paulo, Brazil. 3,5,3′triiodo-L-thyronine, thyroxine, gamma-aminobutyric acid (GABA), bicuculline methiodide, benzamidine, leupeptin, antipain, pepstatin, chymostatin, antibodies, acrylamide, and bis-acrylamide were obtained from Sigma (St. Louis, MO, USA). KN-93 and H-89 were obtained from Calbiochem (La Jolla, CA, USA). Phaclofen and picrotoxin were purchased from Tocris Neuramin (Bristol, UK). ECL kit was from Amersham (Oakville, Ontario).

Animals

Ten-day-old male Wistar rats were obtained from our breeding stock. Rats were maintained on a 12-h light/12-h dark cycle in a constant temperature (22°C) colony room. On the day of birth the litter size was culled to eight pups. Litters smaller than eight pups were not included in the experiments. Water and a 20% (w/w) protein commercial chow were provided *ad libitum*. The experimental protocol followed

the "Principles of Laboratory Animal Care" (NIH publication 85-23, revised 1985) and was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul.

Preparation and Labelling of Slices

Rats were killed by decapitation, the cerebral cortex were dissected onto Petri dishes placed on ice and cut into 400 μm thick slices with a McIlwain chopper. In all experiments, slices from the same cerebral cortex were used both for control and treated groups.

Preincubation

Tissue slices were initially preincubated at 30°C for 20 min in a Krebs-Hepes medium containing 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 25 mM Na-HEPES (pH 7.4), 12 mM glucose, 1 mM $CaCl_2$, and the following protease inhibitors: 1 mM benzamidine, 0.1 μM leupeptin, 0.7 μM antipain, 0.7 μM pepstatin, and 0.7 μM chymostatin in the presence or absence of 1 mM GABA, 50 μM bicuculline, 50 μM picrotoxine, 50 μM phaclofen, 10 μM KN-93, 10 μM H-89, 1.0 μM T_3, and 0.1 μM T_4 when indicated.

Incubation

After preincubation, the medium was changed and incubation was carried out at 30°C with 100 μl of the basic medium containing 80 μCi of [^{32}P] orthophosphate with or without addition of 1 mM GABA, 50 μM bicuculline, 50 μM picrotoxine, 50 μM phaclofen, 10 μM KN-93, 10 μM H-89, 1.0 μM T_3, and 0.1 μM T_4 when indicated. The labeling reaction was normally allowed to proceed for 30 min at 30°C and stopped with 1 mL of cold stop buffer (150 mM NaF, 5 mM, EDTA, 5 mM EGTA, Tris-HCl 50 mM, pH 6.5, and the protease inhibitors described above). Slices were then washed twice with stop buffer to remove excess radioactivity.

Preparation of the High Salt-Triton Insoluble Cytoskeletal Fraction From Tissue Slices

After treatment, preparations of IF-enriched cytoskeletal fractions were obtained from cerebral cortex of 10-day-old rats as described by Funchal *et al.* (2003). Briefly, after the labelling reaction, slices were homogenized in 400 μl of ice-cold high salt buffer containing 5 mM KH_2PO_4, (pH 7.1), 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 1% Triton X-100, and the protease inhibitors described above. The homogenate was centrifuged at 15800 $\times$ *g* for 10 min at 4°C, in an Eppendorf centrifuge, the supernatant discarded, and the pellet homogenized with the same volume of the high salt medium. The resuspended homogenate was centrifuged as described and the supernatant was discarded. The Triton-insoluble IF-enriched

pellet, containing neurofilament subunits, vimentin, and glial fibrillary acidic protein (GFAP), was dissolved in 1% SDS and protein concentration was determined.

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The cytoskeletal fraction was prepared as described above. Equal protein concentrations were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli (1970). After drying, the gels were exposed to X-ray films (X-Omat XK1) at -70°C with intensifying screens and finally the autoradiograph was obtained. Cytoskeletal proteins were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an Optiquant version 02.00 software (Packard Instrument Company). Density values were obtained for the studied proteins.

Immunoblotting Analysis

Cytoskeletal fractions (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Trans-blot SD semi-dry transfer cell, BioRad) for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS). The nitrocellulose membranes were washed for 10 min in Tris-buffered saline (TBS; 0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% defatted dried milk). After incubation, the blot was washed twice for 5 min with TBS plus 0.05% Tween-20 (T-TBS), and then incubated overnight at 4°C in blocking solution containing the following monoclonal antibodies: anti NF-150 (clone NN-18) diluted 1:100, anti NF-68 (clone NR-4) diluted 1:300, anti vimentin (clone vim 13.3) diluted 1:400, and antiglial fibrillary acidic protein (GFAP) (clone G-A-5) diluted 1:400. The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in TBS containing peroxidase-conjugated rabbit antimouse IgG diluted 1:4000. The blot was washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blot was then developed using a chemiluminescence ECL kit.

Protein Determination

The protein concentration was determined by the method of Lowry *et al.* (1951) using serum bovine albumin as the standard.

Statistical Analysis

Data were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Tukey test when the *F*-test was significant. All analyses were performed using the SPSS software program on an IBM-PC compatible computer.

RESULTS

Figure 1 shows the immunoblotting (A) and the autoradiograph (B) of the IF-enriched cytosketeton fraction from cerebral cortex of 10- day-old male rats. Lane a displays the molecular weight standards. The pattern of the Triton-insoluble IF proteins from cerebral cortex of rats is demonstrated in lane b, where the bands of NF-M, NF-L, vimentin, and GFAP can be seen. Lanes c–f show the correspondent immunoblotting where the bands of 150 and 68 kDa are immunoreactive with NF-M and NF-L antibodies, respectively (lanes c and d)., while the 54 kDa band is identified as vimentin (lane e) and the 50 kDa band as GFAP (lane f). It can be seen in the autoradiograph that NF-M, NF-L, vimentin, and GFAP are good substrates for the endogenous phosphorylation system (Fig. 1B). When tissue slices of cerebral cortex were incubated for 30 min with 1 μM T_3 or 0.1 μM T_4 we observed an increased ^{32}P incorporation pattern into the cytoskeletal proteins studied (NF-M, NF-L, vimentin, and GFAP) (Fig. 2). Then, we investigated whether PKA and PKCaMII were involved in the activating effect of TH on the phosphorylating system associated to IF proteins extracted in high-salt Triton containing buffer from cerebral cortex of 10-day-old male rats. In this series of experiments tissue slices were preincubated with the specific kinase inhibitors before treatment with the hormones. We added H-89 and KN-93, the specific protein kinase inhibitors of PKA and PKCaMII, respectively, to the incubation system in the presence of T_3 or T_4. Results showed that H-89 prevented the hyperphosphorylation induced by the TH, conversely KN-93

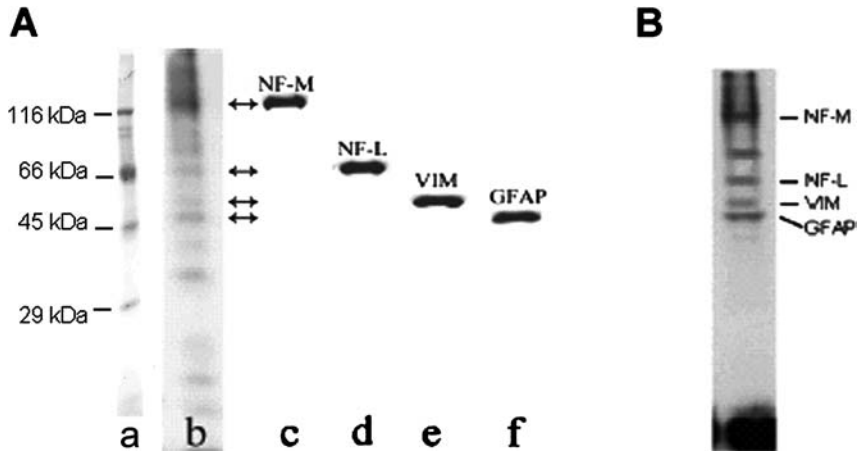

Fig. 1. Polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, and autoradiograph of the cytoskeletal fraction. (A) *Lane* a, molecular weight standards (KDa), from top to bottom: β-galactosidase (116), bovine albumin (66), egg albumin (45), carbonic anidrase (29). *Lane* b, nitrocellulose membrane of the high salt Triton insoluble cytoskeletal fraction stained with Ponceau. (c–f) Immunoblotting of the IF-enriched cytoskeletal fraction with anti IF monoclonal antibodies: (c) NF-M, middle molecular weight neurofilament subunit; (d) NF-L, low molecular weight neurofilament subunit; (e) Vim, vimentin; (f) GFAP, glial fibrillary acidic protein. B) Autoradiograph of the Triton-insoluble IF-enriched cytoskeletal fraction from a 10-day-old rat.

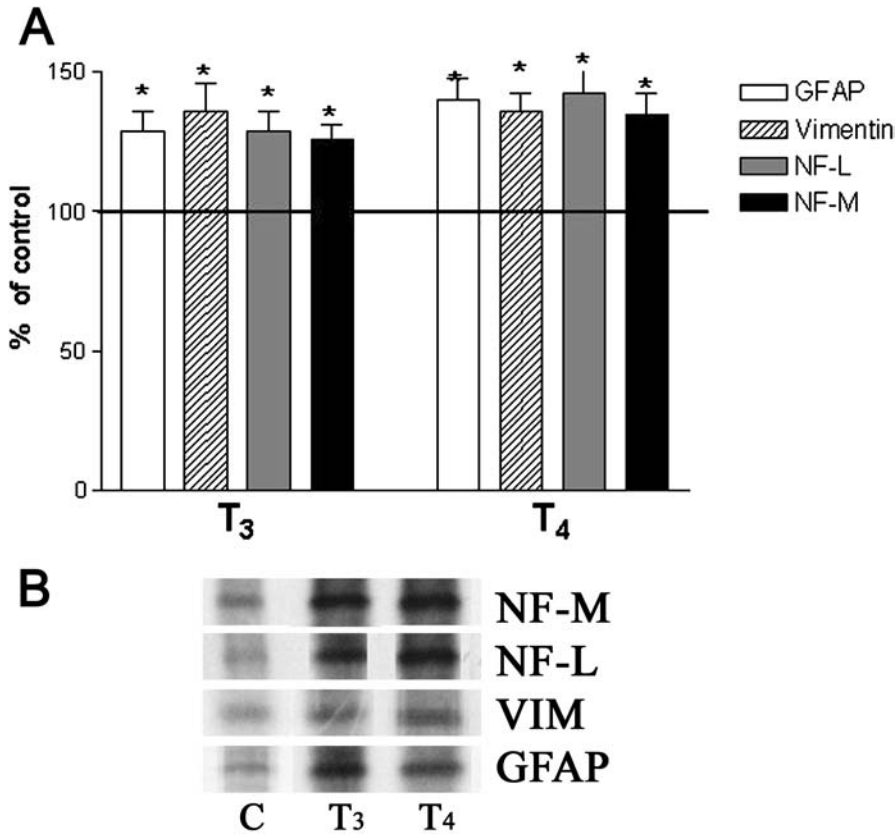

Fig. 2. Effect of thyroid hormones, T_3 and T_4, on the phosphorylation of intermediate filament subunits from cerebral cortex of rats. (A) Slices of cerebral cortex of 10-day-old male rats were incubated for 30 min with 1 μM T_3 or 0.1 μM T_4 in the presence of ^{32}P-orthophosphate. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into GFAP, vimentin (VIM), NF-L, and NF-M was measured from the autoradiographs of the SDS-PAGE of the phosphorylated enriched cytoskeletal fraction, as described in Material and Methods. (B) Representative autoradiographs of the proteins studied. Data are reported as means $\pm$ SEM expressed as percentage of controls from five independent experiments using six to eight animals in each experiment. Statistically significant differences from controls, as determined by ANOVA followed by Tukey test are indicated: $^*P < 0.001$.

totally prevented the effect of T_3 but partially blocked the action of T_4 (Figs. 3(A) and (B)). These results suggest that TH activating effect on the phosphorylation of the cytoskeletal proteins from cerebral cortex of 10-day-old male rats is mediated by PKA and PKCaMII.

In order to verify whether the stimulatory effect of T_3 and T_4 on the phosphorylation of IF subunits was mediated by GABA receptors, tissue slices were incubated with ^{32}P-orthophosphate in the presence of 1.0 mM GABA. We verified that GABA was able to mimic the effect of TH. Moreover, we did not observe an additional stimulatory effect when slices were incubated with the hormones in the presence of

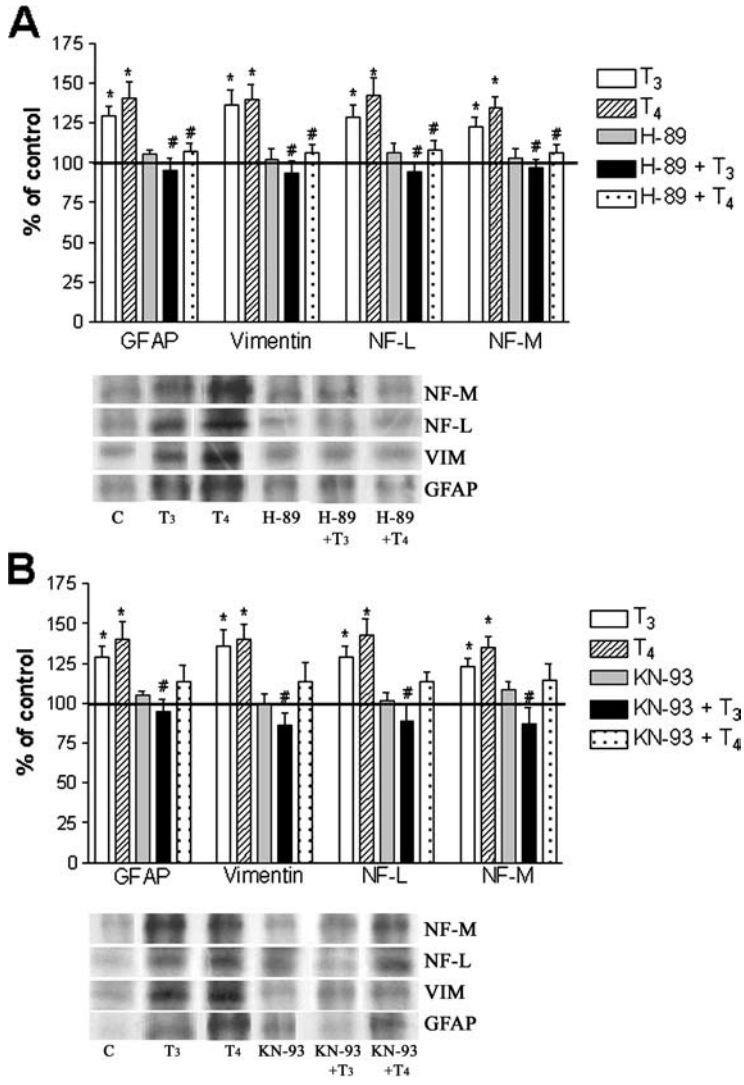

Fig. 3. Effect of treatment of tissue slices with T_3 or T_4 on cAMP- or Ca^{2+}/calmodulin-dependent *in vitro* incorporation of ^{32}P into IFs in the cytoskeletal fraction of cerebral cortex of 10 day-old male rats. Slices of cerebral cortex were preincubated and incubated with 1 μM T_3 or 0.1 μM T_4 and ^{32}P-orthophosphate, in the presence or absence of 10 μM H- 89 (A) or 10 μM KN-93 (B), as described in Material and Methods. The cytoskeletal fraction was extracted and the radioactivity incorporated into glial fibrillary acidic protein (GFAP), vimentin (VIM), low molecular weight neurofilament subunit (NF-L), and middle molecular weight neurofilament subunit (NF-M) was measured from the autoradiographs of the SDS-PAGE of the phosphorylated enriched cytoskeletal fraction. Representative autoradiographs of the proteins studied are shown in the insets. Data are reported as means ± SEM expressed as percent of controls from five independent experiments using six to eight animals for each experiment. Statistical analysis: one-way ANOVA followed by Tukey–Kramer multiple comparison test. $^{*}P < 0.001$ hormones compared with control group; $^{\#}P < 0.01$ "drug + hormone" compared with respective hormone group.

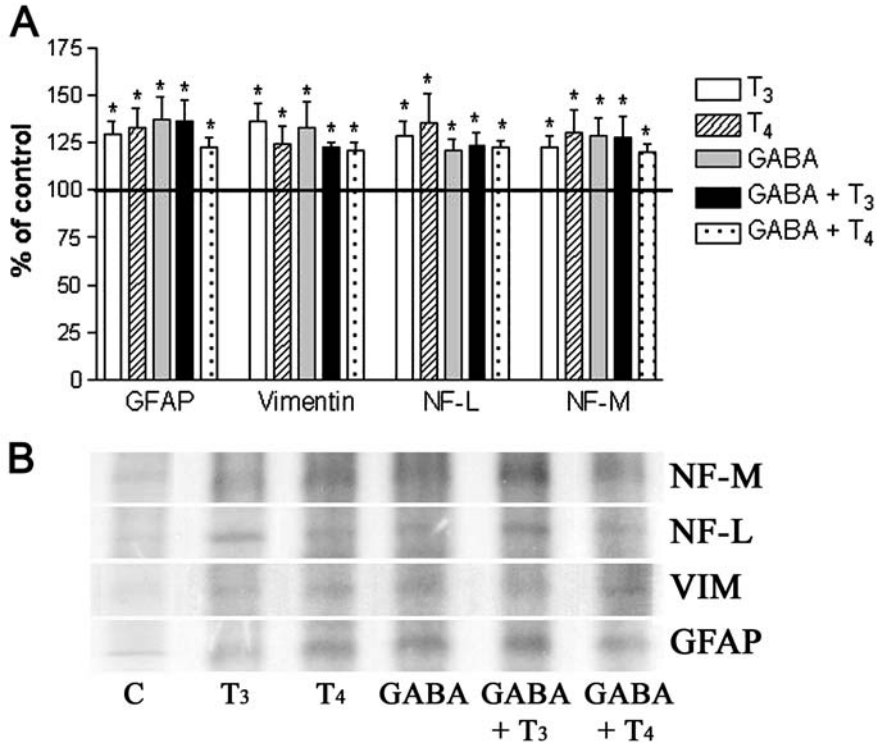

Fig. 4. Effect of GABA on T_3 and T_4-induced phosphorylation of intermediate filament subunits of cerebral cortex of rats. (A) Slices of cerebral cortex of 10-day-old male rats were incubated with 1 μM T_3 or 0.1 μM T_4 and/or 1 mM GABA in the presence of ^{32}P-orthophosphate. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into glial fibrillary acidic protein (GFAP), vimentin (VIM), low molecular weight neurofilament subunit (NF-L), and middle molecular weight neurofilament subunit (NF-M), was measured from the autoradiographs of the SDS-PAGE of the phosphorylated-enriched cytoskeletal fraction. (B) Representative autoradiographs of the proteins studied. Data are reported as means ± SEM expressed as percentage of controls from five independent experiments using six to eight animals for each experiment. Statistically significant differences from controls, as determined by ANOVA followed by Tukey–Kramer multiple comparison tests are indicated: $^*P < 0.001$.

GABA, suggesting that GABA and the hormones act through the same mechanism (Fig. 4). Next we tested the effect of 1.0 μM T_3 or 0.1 μM T_4 in the presence of GABAergic receptor antagonists in preincubation and incubation medium. Incubation of tissue slices with T_3 or T_4 in the presence of the competitive $GABA_A$ antagonist bicuculline showed that this antagonist was not able to prevent the effect of the hormones on the phosphorylating system associated to the cytoskeletal proteins (Fig. 5(A)). However, picrotoxin, a noncompetitive $GABA_A$ inhibitor, was able to prevent hyperphosphorylation induced by T_3 treatment but it was ineffective in preventing T_4 effects (Fig. 5(B)). Finally, when tissue slices were incubated with T_3 following preincubation with the specific $GABA_B$ antagonist phaclofen, the stimulatory effect of the hormone on the *in vitro* phosphorylation of the various

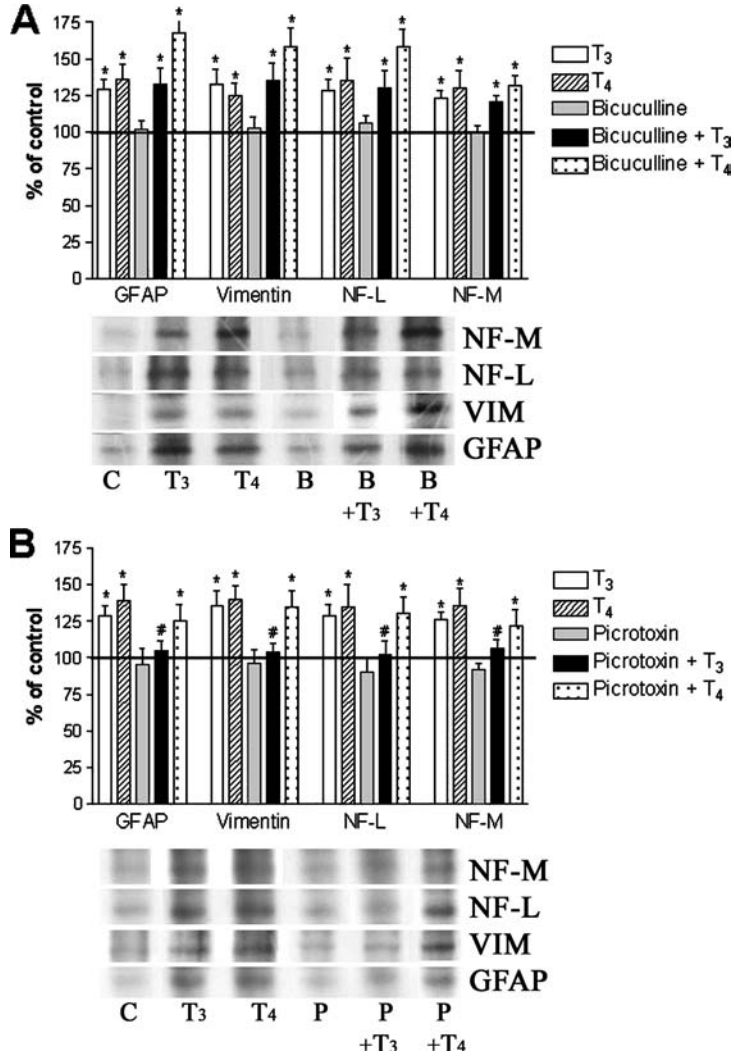

Fig. 5. Effect of the $GABA_A$ and $GABA_B$ antagonists on T_3 and T_4-induced phosphorylation of intermediate filament subunits of cerebral cortex of rats. Slices of cerebral cortex of 10-day-old male rats were incubated with 1 μM T_3 or 0.1 μM T_4 and/or 50 μM bicuculline (A), 50 μM picrotoxin (B), 50 μM Phaclofen (C) in the presence of ^{32}P-orthophosphate. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into glial fibrillary acidic protein (GFAP), vimentin (VIM), low molecular weight neurofilament subunit (NF-L), and middle molecular weight neurofilament subunit (NF-M), was measured from the autoradiographs of the SDS-PAGE of the phosphorylated enriched cytoskeletal fraction. Representative autoradiographs of the proteins studied are shown in the insets. Data are reported as means ± SEM expressed as percentage of controls from five independent experiments using six to eight animals for each experiment. Statistical analysis: one-way ANOVA followed by Tukey–Kramer multiple comparison test. $^*P < 0.001$ hormones compared with control group; $^\#P < 0.01$ "drug + hormone" compared with respective hormone group.

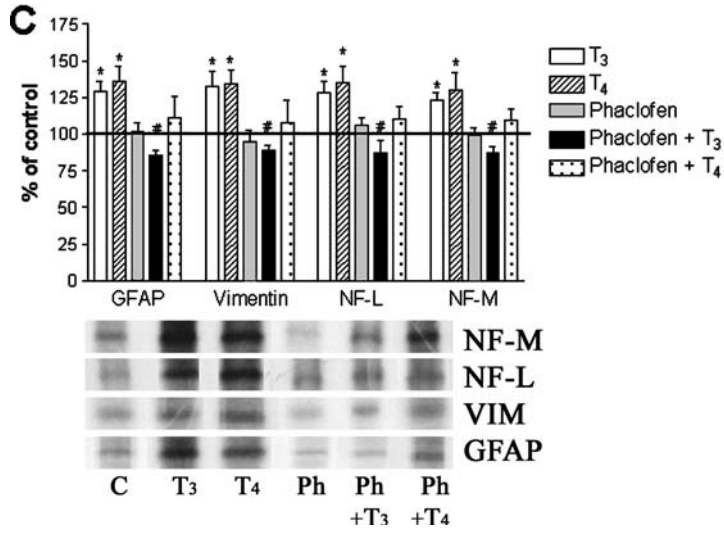

Fig. 5. Continued.

cytoskeletal proteins was prevented. Nonetheless, we observed that phaclofen only partially prevented the effect of T_4 (Fig. 5C). Taken together, these results suggest that T_3 increased the *in vitro* phosphorylation of IF subunits through mechanisms mediated by $GABA_B$ and noncompetitive $GABA_A$ receptors, while T_4 effects are mediated only partially by $GABA_B$ receptors (Fig. 5(C)).

DISCUSSION

The classical genomic concept of TH acting through nuclear receptors is well recognized (Lazar 1993; Davis *et al.*, 2000). However, nongenomic actions of TH are now widely described and characterized by rapid responses, since they occur through plasma membrane transport systems (Incerpi *et al.*, 1999b; Davis *et al.*, 2000; Silva *et al.*, 2001; Davis *et al.*, 2002; Silva *et al.*, 2002; Volpato *et al.*, 2004). Although short-term effects of T_4 on actin polymerization have been described in cultured astrocytes (Siegrist-Kaiser *et al.*, 1990) little is known about short-term effects of TH on the activity of the phosphorylating system associated to the IF proteins in the central nervous system of young rats. In the present report we investigated some mechanisms underlying the short-term effect of thyroid hormones T_3 and T_4, on the *in vitro* phosphorylation of IF proteins from cerebral cortex slices of 10-day-old male rats. We have chosen 1 μM T_3 and 0.1 μM T_4 to undertake our experimental approach since these concentrations have been reported to elicit nongenomic effects. One μM T_3 increased the *in vitro* ^{32}P incorporation into vimentin in 15-day-old rat testis (Zamoner *et al.*, 2005), and elicited membrane hyperpolarization in Sertoli cells (Silva *et al.*, 2001). Otherwise 1 μM T_3 and 0.1 μM T_4 increased amino acid transport and induced changes in the membrane potential in Sertoli cells (Segal 1989; Silva *et al.*, 2001; Volpato *et al.*, 2004).

We observed that T_3 and T_4 were able to increase the *in vitro* ^{32}P incorporation into neurofilament subunits as well as into vimentin and GFAP after 30 min incubation of tissue slices and these effects were mediated by the protein kinases PKA and PKCaMII. Our conclusions are based on the experiments using the protein kinase inhibitors H-89, a cell-permeable, selective, and potent inhibitor of PKA (Chijiwa *et al.*, 1990) and KN-93, a specific PKCaMII inhibitor (Tokumitsu *et al.*, 1990), in the presence of T_3 or T_4. Using this approach, we have previously identified that these kinases are associated to the cytoskeletal fraction from cerebral cortex of young rats (de Freitas *et al.*, 1995). However, considering T_4 action, the partial prevention of hyperphosphorylation observed in tissue slices coincubated with T_4 and KN-93 suggests the involvement of other protein kinases in such effect.

In the present report we also showed that the action of the hormones was mimicked by GABA. In order to verify and better understand the involvement of the GABA receptors in such effect, we used the $GABA_A$ antagonists bicuculline and picrotoxin and the $GABA_B$ antagonist phaclofen in the next set of experiments. The convulsant bicuculline is the classical $GABA_A$-receptor antagonist, which, by competing with GABA for binding to one or both sites on the $GABA_A$ receptor (Olsen and DeLorey, 1999) reduces ionic current by decreasing the opening frequency and mean open time of the channel (MacDonald and Olsen, 1994; Johnston, 1996). On the other hand, the convulsant compound picrotoxin is an ionic channel blocker, which causes a decrease in mean channel open time. Picrotoxin works by preferentially shifting opening channels to the briefest open state (1 ms) (Olsen and DeLorey, 1999). We showed that bicuculline was not able to prevent hyperphosphorylation elicited either by T_3 or T_4, while picrotoxin was effective in preventing only T_3-induced hyperphosphorylation of the IF proteins studied, strongly suggesting the participation of noncompetitive $GABA_A$ receptors in T_3 action. We also observed that the potent selective $GABA_B$ receptor antagonist phaclofen totally prevented the stimulatory effect of T_3 on the *in vitro* phosphorylation of IF proteins. Nonetheless, the effect of T_4 was only partially prevented by this antagonist. Taken together, it can be concluded that T_3 induced a short-term stimulation of the *in vitro* phosphorylation of the proteins studied through noncompetitive $GABA_A$, in addition to $GABA_B$ receptors, while T_4 acted through $GABA_B$ receptors in slices from cerebral cortex of 10-day-old male rats. Moreover, on the basis on our data showing a partial involvement of $GABA_B$ receptors in the T_4 action, we could suppose a more complex signaling mechanism involved in the short-term effect of this hormone on the cytoskeletal proteins. At this point, it is important to emphasize that our findings further support the action of T_4 at the cell membrane as an enhancer of signal transduction. Moreover, the differential mechanisms of action of T_3 and T_4 support that besides the important actions via nuclear receptors, TH can nongenomically alter distinct signal-transducting pathways targeting the cytoskeleton.

Our results showing short-term effects of TH mediated by GABAergic mechanisms are supported by previous reports. In this context, Martin *et al.* (1996) have demonstrated that TH could modulate $GABA_A$ receptors. Moreover, the short-term effects of the TH mediated by the activity of GABA receptors that we are evidencing in the present report are in line with Chapell *et al.* (1998), who described that micromolar concentrations of T_3 can directly activate $GABA_A$ receptor chloride channels, and these responses were inhibited by picrotoxin but not

bicuculline. Our results indicated that T_3 activated the $GABA_A$ receptor in a noncompetitive manner consistent with a channel-opening mechanism of action. The inability of the competitive $GABA_A$ receptor antagonist bicuculline to block T_3-induced hyperphosphorylation suggests that T_3 does not act at the GABA-binding site on the $GABA_A$ receptor, but acts at an unidentified site on the receptor complex.

Otherwise, $GABA_B$ receptors are known to be present in both presynaptic terminals and postsynaptic cells (Harrison, 1990) as well as in astrocytes (Gaiarsa *et al.*, 2002). Komatsu (1996) has described that postsynaptic $GABA_B$ receptors are involved in the induction of LTP of inhibitory synaptic transmission and that this effect was mediated at least in part by facilitation of the monoamine-induced IP_3 formation, which then causes Ca^{2+} release from the internal stores in postsynaptic cells. The increased intracellular Ca^{2+} levels could be on the basis of PKA and PKCaMII activation induced by T_3 and T_4 that we have observed in this report

Moreover, our results showing that the short-term TH treatment increased the astrocytic IF protein (GFAP) phosphorylation, via GABAergic mechanisms is supported by previous reports demonstrating that astrocytes also express a large variety of receptors for neurotransmitters and neuropeptides (Porter and McCarthy, 1997). Furthermore, Runquist and Alonso (2003) suggested that GFAP expression and the morphology of adult astrocytes are affected by GABAergic signaling, reinforcing the phisiological role of GABA pathway modulating the cytoskeleton. In addition, our present results showing that the stimulatory effects of TH on GFAP phosphorylation were prevented by GABA antagonists are in line with these findings indicating that astrocytes express high levels of GABA receptors (Rosewater and Sontheimer, 1994).

However, the exact mechanisms underlying the involvement of GABA receptors mediating the altered phosphorylation of cytoskeletal proteins in short-term effects of TH are still unknown. Growing evidence in the literature demonstrate an interaction between $GABA_A$ receptors and the cytoskeleton, which is presumably needed for receptor trafficking, anchoring, and/or synaptic clustering (Wang and Olsen, 2000). On the other hand, it has been described that microtubules participate in the maintenance of normal subcellular distribution of $GABA_A$ receptors in neurons and that the organization of microfilaments may play a role in modulating the gene expression of $GABA_A$ receptor subunits (Ho *et al.*, 2001).

In summary, our results provide the first evidence of a rapid response mechanism on the phosphorylation level of IF proteins mediated by TH both in neuronal and glial cells through GABAergic pathways. Thus, the complexity of the processes underlying the differential mechanism of action to T_3 and T_4 suggest the existence of multiple sites of regulation to the TH. However, further experiments will be necessary to clarify the physiological implications of the GABAergic mechanisms involved in the rapid action of TH on the phosphorylating system associated with the cytoskeletal proteins of rat brain.

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Capítulo 5

IONIC INVOLVEMENT AND KINASE ACTIVITY ON THE MECHANISM OF ACTION OF THYROID HORMONES ON $^{45}CA^{2+}$ UPTAKE IN CEREBRAL CORTEX FROM YOUNG RATS

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Ionic involvement and kinase activity on the mechanism of nongenomic action of thyroid hormones on $^{45}Ca^{2+}$ uptake in cerebral cortex from young rats

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Abstract

Thyroid hormones (TH) play important roles in brain development. Although most of the nongenomic actions of TH are known to be calcium-dependent, the effects of 3,5,3′-triiodo-L-thyronine (T_3) or thyroxine (T_4) on calcium influx in cerebral cortex of rats are not clear. In this study we investigate some mechanisms involved in the effect of T_3 and T_4 on Ca^{2+} uptake in slices of cerebral cortex from 10-day-old male rats. Results indicated 10^{-6} M T_3 or 10^{-7} M T_4 was able to increase $^{45}Ca^{2+}$ uptake after 30 s of hormone exposure. The involvement of L- and T-type voltage-dependent Ca^{2+} channels (VDCC) on the effect of TH on $^{45}Ca^{2+}$ uptake was evidenced by using nifedipine and flunarizine, L- and T-type channel blockers, respectively. Otherwise, chloride currents were not involved in the hormone actions, as demonstrated by using 9-anthracene carboxylic acid, a Cl^--channel blocker. In addition, results demonstrated a PKC-dependent mechanism for both T_3 and T_4, as evidenced by stearoylcarnitine chloride, a specific PKC inhibitor. Furthermore, we verified that the T_3 action was also mediated by PKA activity, as demonstrated coincubating T_3 and KT 5720 (PKA inhibitor), and reinforced by using theophylline, a phosphodiesterase inhibitor. In contrast, concerning the effect of T_4, results suggest a partial involvement of PKA activity, and demonstrated that high cAMP levels were not able to support the effect of T_4, suggesting the participation of G inhibitory protein-coupled receptor in the action of this hormone on $^{45}Ca^{2+}$ uptake. In conclusion, our results evidence a nongenomic action of TH promoting Ca^{2+} influx by ionic channels involving mechanisms dependent on kinase activities. It is possible that the modulation of Ca^{2+} channels by kinase activities represent an important membrane action of TH signaling mechanism in the central nervous system during development.

Keywords: Thyroid hormones; Calcium influx; Cerebral cortex; Calcium channels; PKC; PKA

1. Introduction

The thyroid hormones (3,5,3′-triiodo-L-thyronine, T_3; L-thyroxine, T_4; TH) play crucial roles in the growth and differentiation of many organs, including the central nervous system (CNS) (Oppenheimer and Schwartz, 1997). Late brain development is characterized by maturation of the organ. The process of axonal and dendritic growth, synapse formation, myelinatin, cell migration, and proliferation of specific population of cells, such as the glial cells and certain late arising neurons, all occur late in brain development and are regulated by TH (Anderson, 2001). Deficiency of TH during the perinatal period results in severe mental and physical retardation, known as cretinism in humans (Koibuchi and Chin, 2000). Furthermore, adverse consequences of thyroid hormone insufficiency depend both on severity and developmental timing, indicating that TH deficiency may produce different effects during development (Zoeller and Crofton, 2005).

TH exert their action primarily at the nuclear level by regulating the transcription of thyroid-hormone-responsive

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genes, however there are numerous physiological effects that can not be mediated by action of the genome, like transcription and translation, as judged from their insensitivity toward appropriate inhibitors and/or the short-time frame in which the response occurs (Davis et al., 2005). These nongenomic responses are frequently associated with secondary messenger signaling pathways including the phospholipase C (PLC), inositol triphosphate (IP3), diacyl glycerol (DAG), protein kinase C (PKC) and $[Ca^{2+}]_i$ pathway, the adenylyl cyclase, protein kinase A (PKA) and the cyclic AMP-response element binding protein (CREB) pathway and the Ras, Raf1 serine/threonine kinase, mitogen activated protein kinase (MEK) and the mitogen activated protein kinase (MAPK) pathway (Lösel and Wehling, 2003). Moreover, we have demonstrated a rapid nongenomic action of T_3 and T_4 on the phosphorylation of cytoskeletal proteins in slices of cerebral cortex of rats through GABAergic mechanisms (Zamoner et al., 2006). Membrane binding sites for TH were identified many years ago in erythrocytes and hepatocytes (Pliam and Goldfine, 1977; Botta et al., 1983; Segal, 1989), but the linkage between binding sites and hormone actions is not well established. It has been suggested that nongenomic effects for TH are initiated at the αVβ3 integrin membrane receptor (Berg et al., 2005) and are connected via one or more signal transduction pathways to nuclear events and to local changes in cell membrane function (Davis et al., 2005). The mechanisms by which TH non-genomically affect the activity of plasma membrane ion channels and ion pumps are not well understood. In this context, we have recently reported a rapid nongenomic action of T_3 dependent on extracellular Ca^{2+} currents on the phosphorylating system associated with vimentin in immature rat testes (Zamoner et al., 2005). It is possible that the actions of TH on Ca^{2+} influx could represent a mechanism of hormone signaling involving protein kinase cascades via nongenomic mechanisms. This is supported by pioneer studies performed by Segal (1990) conclusively identifying Ca^{2+} as the first messenger for the action of T_3 at the level of the plasma membrane, which emphasize Ca^{2+} uptake as the first apparent event following the binding of the hormone to its membrane receptor.

Ca^{2+} influx can be regulated through three major mechanisms: voltage-dependent Ca^{2+} channels (VDCC), second messenger-mediated channels and receptor-mediated channels (Rink and Hallam, 1989). VDCC are found in a variety of excitable cell types, including neurons. By definition, VDCC can be activated by membrane depolarization. There are different VDCC types believed to be involved in several cellular functions, such as neurotransmitter release (Bird and Putney, 2006). Ca^{2+} influx through VDCC could be regulated by complex mechanisms, including PKC and PKA activities, which play central roles in signal transduction in many cell types (Divecha and Irvine, 1995; Nishizuka, 1995; Gao et al., 1997; Ko et al., 2005).

Considering previous evidences that nongenomic actions of TH involve modifications of intracellular Ca^{2+} levels activating Ca^{2+}-sensitive signaling pathways, in the present investigation we studied the short-term effects of T_3 and T_4, on the $^{45}Ca^{2+}$ uptake in slices from cerebral cortex of 10-day-old male rats. Additionally, we investigated the involvement of VDCC and protein kinase activities on such effects.

2. Materials and methods

2.1. Chemicals

3,5,3′-Tri-iodo-L-thyronine (T_3), L-thyroxine (T_4), 9-anthracene carboxylic acid (9-AC), stearoylcarnitine chloride (ST); KT 5720, theophylline, nifedipine and flunarizine were obtained from the Sigma Chemical Company, St. Louis, MO, USA; [^{45}Ca] $CaCl_2$ (sp.act. 321 KBq/mg Ca^{2+}) was purchased from IPEN, São Paulo, Brazil. Optiphase Hisafe III biodegradable liquid scintillation were obtained from Perkin-Elmer (Boston, USA). All other chemicals were of analytical grade.

2.2. Animals

Wistar rats bred in our animal house and maintained in an air-conditioned room (about 21 °C) with controlled lighting (12 h/12 h light/dark cycle) were used in this study. The suckling rats were kept with their mothers until sacrifice by decapitation. Pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil) and tap water were available *ad libitum*. All the animals were carefully monitored and maintained in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation.

2.3. Experimental procedures

Animals were killed by decapitation, the cerebral cortex was dissected onto Petri dishes placed on ice, cut into thick slices and weighted. For Ca^{2+} influx measurements, cerebral cortex slices from 10-day-old male rats were pre-incubated in Krebs Ringer-bicarbonate (KRb) buffer (122 mM NaCl; 3 mM KCl; 1.2 mM $MgSO_4$; 1.3 mM $CaCl_2$; 0.4 mM KH_2PO_4; 25 mM $NaHCO_3$) for 15 min in a Dubnoff metabolic incubator at 32 °C, pH 7.4 and gassed with O_2:CO_2 (95:5; v/v). After that, the medium was changed by fresh KRb with 0.2 μCi/mL $^{45}Ca^{2+}$ during 60 min. When specified tissue slices were incubated for a further 30, 60 or 300 s in the absence or the presence of T_3 or T_4 (10^{-5}, 10^{-6}, 10^{-7}, 10^{-9} and 10^{-11} M). In some experiments channel blockers or kinase inhibitors were added during the last 20 min before the hormone addition and maintained during all the incubation period (see figures). 9-AC and flunarizine were diluted at final concentration of 10 μM; nifedipine at 100 μM; KT 5720 and ST at 1 μM in KRb buffer.

Extracellular $^{45}Ca^{2+}$ from the cortical slices was thoroughly washed off in 127.5 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 10 mM HEPES, 11 mM glucose, 10 mM $LaCl_3$, pH 7.3 (30 min in washing solution). The presence of La^{3+} during the washing stage was found to be essential to prevent release of the intracellular $^{45}Ca^{2+}$. After washing, tissue slices were transferred to screw cap tubes containing 1 mL of distilled water. They were frozen at −20 °C in a freezer and afterwards boiled for 10 min; 100 μL aliquots of tissue medium were placed in scintillation fluid and counted in a LKB rack beta liquid scintillation spectrometer (model 1215; EG & G-Wallac, Turku, Finland). The results were expressed as pmol $^{45}Ca^{2+}$/mg tissue or percent of control as specified in figures (Batra and Sjögren, 1983, with minor modifications).

2.4. Statistical analysis

The results are means ± S.E.M. expressed as the pmol $^{45}Ca^{2+}$/mg tissue or % of control. When multiple comparisons were performed, evaluation was done using one-way ANOVA followed by Tukey–Kramer multiple comparison test. Differences were considered to be significant when $P < 0.05$.

3. Results

We have recently described that 10^{-6} M T_3 and 10^{-7} M T_4 were able to increase cytoskeletal protein phosphorylation in

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slices from cerebral cortex of 10-day-old rats (Zamoner et al., 2006). Taking into account these findings, we initiated our present study testing the time-course effect of 10^{-6} M T_3 and 10^{-7} M T_4 on $^{45}Ca^{2+}$ uptake in cerebral cortex slices of 10-day-old male rats. Fig. 1 shows that TH increase $^{45}Ca^{2+}$ uptake after 30 s and after 5 min (300 s) exposure, without altering this parameter in 60 s treatment. Taking into account these results, we used the shorter effective time of exposure to the hormones (30 s) to carry out our study.

In addition, Fig. 2 shows the dose–response curve of T_3 and T_4 at doses ranging from 10^{-11} to 10^{-5} M on $^{45}Ca^{2+}$ uptake during 30 s in the presence of the respective hormones. Significantly increased uptake was observed at doses of 10^{-6} M T_3 and 10^{-7} M T_4. Therefore, we have chosen these hormone concentrations and 30 s exposure time for subsequent experiments on $^{45}Ca^{2+}$ uptake by tissue slices.

Taking into account the importance of Ca^{2+} in a plethora of intracellular events regulating cell function (Bird and Putney, 2006), we studied the involvement of two Ca^{2+} channel subtypes in the stimulatory action of TH on $^{45}Ca^{2+}$ uptake. We first demonstrated that when tissue slices were incubated for 30 s with the hormones and 100 μM nifedipine or 10 μM flunarizine, L- and T-voltage-dependent Ca^{2+} channel (VDCCs) blockers, respectively, the effects of the hormones were totally prevented (Fig. 3A).

Considering that $GABA_A$ receptors are involved in the effect of TH on cytoskeletal protein phosphorylation (Zamoner et al., 2006) and that voltage-dependent Ca^{2+} conductance can also be modulated by changing Cl^- currents

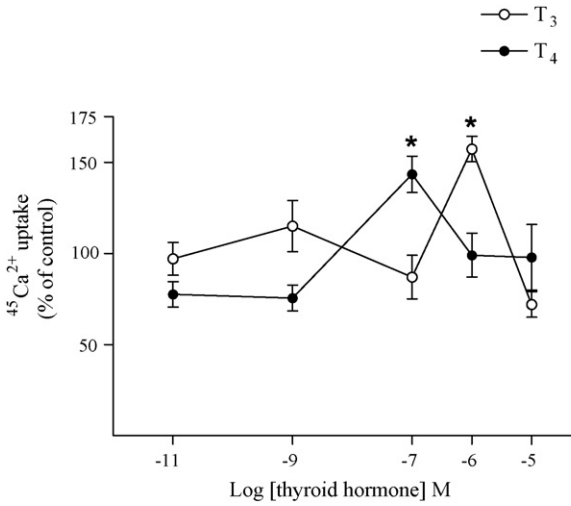

Fig. 2. Dose–response curve of thyroid hormones on $^{45}Ca^{2+}$ uptake on cerebral cortex. Slices of cerebral cortex were pre-incubated for 60 min in the presence of 0.2 μCi/mL of $^{45}Ca^{2+}$. The slices were incubated with or without T_3 or T_4 at different concentrations (10^{-11}, 10^{-9}, 10^{-7}, 10^{-6} and 10^{-5} M) for 30 s. Values are means ± S.E.M. for three independent experiments carried out in quadruplicate, expressed as percent of control. Statistically significant differences from controls, as determined by ANOVA followed by Tukey–Kramer multiple comparison test, are indicated $^{*}P \leq 0.001$.

(Garcia et al., 1997) we investigated the involvement of Ca^{2+}-activated chloride currents on the effects of TH on $^{45}Ca^{2+}$ uptake by using 10 μM 9-AC, a chloride channel blocker. Fig. 3B shows that this blocker was unable to alter the effect of the hormones.

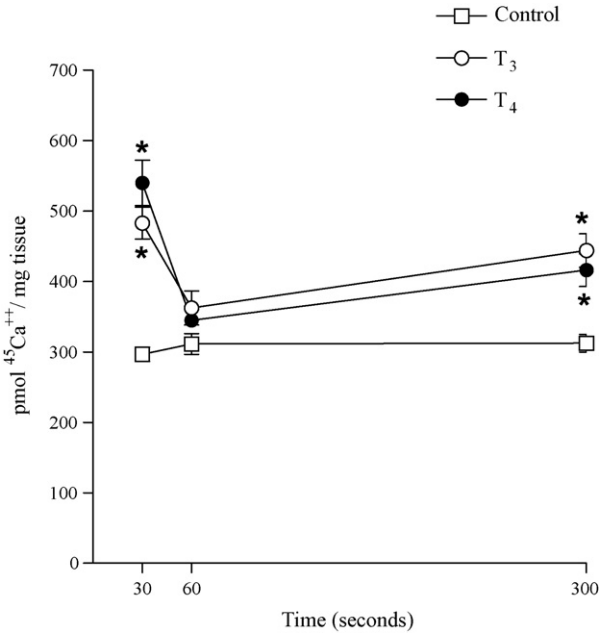

Fig. 1. Time-course effect of thyroid hormones on $^{45}Ca^{2+}$ uptake in cerebral cortex of rats. Slices of cerebral cortex were pre-incubated for 60 min in the presence of 0.2 μCi/mL of $^{45}Ca^{2+}$. After that the slices were incubated with or without 10^{-6} M T_3 or 10^{-7} M T_4 for 30, 60 and 300 s. Values are means ± S.E.M. for three independent experiments carried out in quadruplicate, expressed as pmol $^{45}Ca^{2+}$/mg tissue. Statistically significant differences from controls, as determined by ANOVA followed by Tukey–Kramer multiple comparison test, are indicated $^{*}P \leq 0.001$.

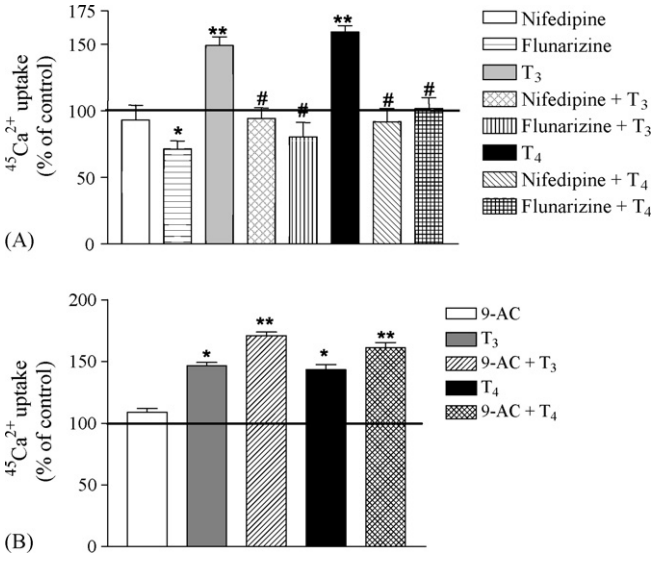

Fig. 3. Involvement of voltage-dependent calcium channels and chloride currents on $^{45}Ca^{2+}$ uptake in cerebral cortex of rats. Slices of cerebral cortex were pre-incubated for 60 min with 0.2 μCi/mL of $^{45}Ca^{2+}$. In the last 20 min preincubation 100 μM nifedipine or 10 μM flunarizine (A), or 10 μM 9-AC (B) was added to the reaction medium. After that, the slices were treated with or without 10^{-6} M T_3 or 10^{-7} M T_4 for 30 s (incubation). Values are means ± S.E.M. for three independent experiments carried out in quadruplicate, expressed as percentage of controls. Statistically significant differences as determined by ANOVA followed by Tukey–Kramer multiple comparison test, are indicated: $^{**}P \leq 0.001$ and $^{*}P \leq 0.01$ compared with control group; $^{\#}P \leq 0.01$ compared with respective hormone group.

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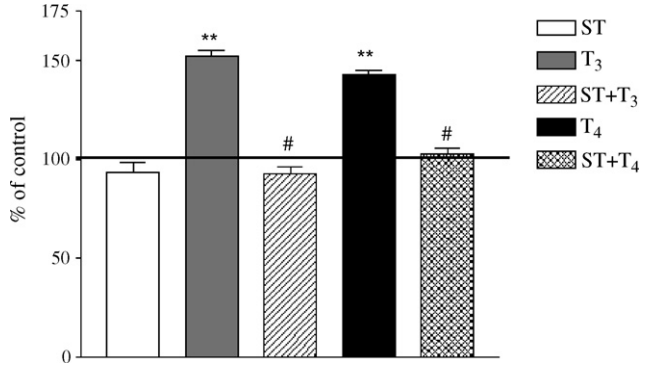

Fig. 4. Involvement of protein kinase C activity on the stimulatory action of thyroid hormones on $^{45}Ca^{2+}$ uptake in cerebral cortex. Slices of cerebral cortex were pre-incubated for 60 min in the presence of 0.2 μCi/mL of $^{45}Ca^{2+}$ and at the last 20 min 1 μM ST was added to the reaction medium. After that the slices were treated with or without 10^{-6} M T_3 or 10^{-7} M T_4 for 30 s. Values are means ± S.E.M. for three independent experiments carried out in quadruplicate expressed as percentage of controls. Statistically significant differences as determined by ANOVA followed by Tukey–Kramer multiple comparison test, are indicated $^{**}P \leq 0.001$ compared with control group; $^{\#}P \leq 0.01$ compared with respective hormone group.

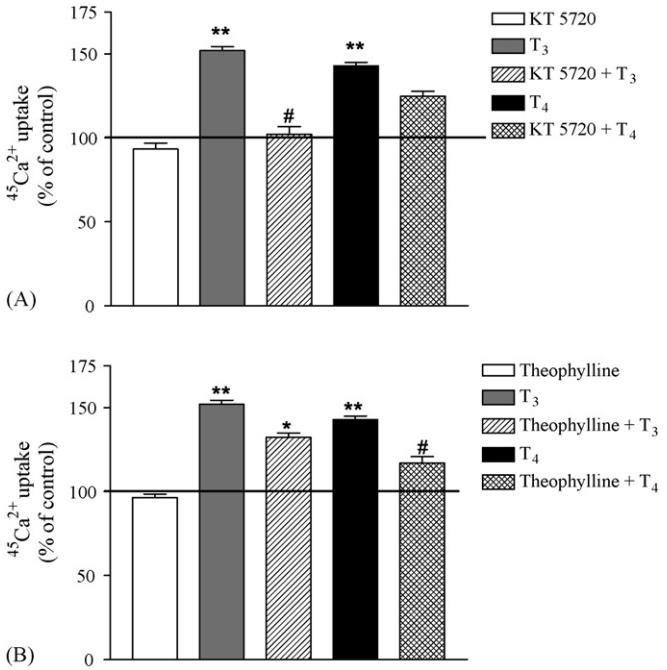

Fig. 5. Involvement of protein kinase A activity and cyclic AMP levels on the stimulatory action of thyroid hormones on $^{45}Ca^{2+}$ uptake in cerebral cortex. Slices of cerebral cortex were preincubated for 60 min in the presence of 0.2 μCi/mL of $^{45}Ca^{2+}$ and at the last 20 min 1 μM KT 5720 (A), or 100 μM theophylline (B) was added to the reaction medium. After that the slices were treated with or without 10^{-6} M T_3 or 10^{-7} M T_4 for 30 s. Values are means ± S.E.M. for three independent experiments carried out in quadruplicate expressed as percentage of controls. Statistically significant differences, as determined by ANOVA followed by Tukey–Kramer multiple comparison test, are indicated $^{**}P \leq 0.001$ compared with control group; $^{*}P \leq 0.05$ compared with respective inhibitor; $^{\#}P \leq 0.01$ compared with respective hormone group.

We also investigated whether PKC and PKA were involved in the activating effect of TH on $^{45}Ca^{2+}$ uptake. We added the specific protein kinase inhibitors ST and KT5720, PKC and PKA inhibitors, respectively, to the incubation system in the presence of the hormones. Results showed that 1 μM ST totally prevented the effect of both T_3 and T_4 (Fig. 4), while 1 μM KT5720 was able to totally prevent the effect of T_3 but only partially prevent T_4 action (Fig. 5A). Otherwise, an increased cAMP concentration was not able to significantly alter the effect of T_3 on $^{45}Ca^{2+}$ uptake, as evidenced by treating tissue slices with T_3 in the presence of 100 μM theophylline, a cyclic AMP phosphodiesterase inhibitor. However, slices incubated with T_4 plus theophylline showed a partial involvement of cyclic AMP on the effect of the hormone on $^{45}Ca^{2+}$ uptake (Fig. 5B).

4. Discussion

The present study was conducted to investigate some aspects of the mechanisms underlying the action of TH on Ca^{2+} uptake in slices of cerebral cortex of young rats. The present study demonstrates that incubation of cerebral cortex slices of 10 day-old rats with 10^{-6} M T_3 and 10^{-7} M T_4 for 30 or 300 s increased $^{45}Ca^{2+}$ uptake, leading to temporal intracellular Ca^{2+} oscillations. The increased short-term $^{45}Ca^{2+}$ influx detected after 30 s exposure to the hormones might elicit a rapid Ca^{2+} uptake by endoplasmic and/or cell membrane pumps providing cytoplasmic Ca^{2+} buffering, as evidenced after 60 s. Otherwise, the depleted intracellular Ca^{2+} pool would provoke the capacitative Ca^{2+} entry, as observed after 300 s, to provide for sustained Ca^{2+} signaling. Considering these findings we have chosen 30 s to undertake our study, since this was the shorter incubation time we have tested, moreover, at this short incubation time we try to minimize the influence of compensatory mechanisms modulating the intracellular Ca^{2+} levels. We have also tested the effect of different concentrations of the hormones on $^{45}Ca^{2+}$ uptake, ranging from 10^{-11} to 10^{-5} M. Results showed a stimulatory action of T_4 (10^{-7} M) on $^{45}Ca^{2+}$ uptake that is 10 times more potent than that of T_3 (10^{-6} M). Furthermore, it is important to emphasize that T_4 elicited this effect at a physiological concentration, while T_3 produced a similar effect at a supraphysiological concentration. Supporting our results, it has been demonstrated that T_4 had a more potent action compared with T_3 on the antiviral action of IFN-γ in HeLa cells (Lin et al., 1997) and on the amino acid accumulation in rat testis (Menegaz et al., 2006).

Since Ca^{2+} enters cells via voltage- or ligand-dependent channels or by means of capacitative entry (Bird and Putney, 2006), we concentrated our efforts investigating the role of VDCC on Ca^{2+} entry elicited by TH. VDCC are designated L-, T-, P-, Q- and R-type differing in their gating kinetics, modes of Ca^{2+}-inactivation and Ca^{2+}-regulation, and sensitivity to specific toxins (Dunlap et al., 1995).

Our results showed that the effects of both T_3 and T_4 on $^{45}Ca^{2+}$ uptake were totally prevented by nifedipine, a specific L-type Ca^{2+} channel blocker (Bird and Putney, 2006) and by flunarizine, a nonspecific T-type channel blocker (Perrier et al., 1992), suggesting the involvement of L- and T-type Ca^{2+} channels in the $^{45}Ca^{2+}$ uptake mechanism mediated by TH. Moreover, it is important to consider the involvement of Cl^- ions in signal

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transduction pathways. In this context, 9-AC, an aromatic compound has been widely used as an anion probe to study various chloride channels (Clemo et al., 1998; Estevez et al., 2003; Qu et al., 2003; Zhou et al., 1997). However, our results showed that coincubation of both T_3 and T_4 with 9-AC did not prevent the stimulatory effect of the hormones on $^{45}Ca^{2+}$ uptake.

Otherwise, increase of intracellular Ca^{2+} via VDCC has been described for TH (Watanabe et al., 2005), strengthening therefore, the view that TH activate these channels. Moreover, rapid highly localized Ca^{2+} spikes regulate fast responses to a variety of extracellular signals. Cells respond to such oscillations using sophisticated regulatory mechanisms of $[Ca^{2+}]_i$, including phosphorylation of cytoplasmic domains of VDCC in different tissues. In this context, we provide evidence that coincubating tissue slices with 10^{-6} M T_3 or 10^{-7} M T_4 and ST, a PKC inhibitor, totally prevented the effect of the hormones on $^{45}Ca^{2+}$ uptake, suggesting a modulation of Ca^{2+} channels by PKC activity. This is in agreement with Shistik et al. (1998) who propose the PKC-mediated up-regulation of L-type Ca^{2+} channels in cardiac and smooth muscle cells. The underlying mechanisms involving the action of PKC on TH-induced Ca^{2+} influx are not clear, but we could suppose that the hormones activate the inositol phospholipid signaling pathway producing diacylglycerol and IP_3 which results in PKC activation as well as in the opening of IP_3-gated Ca^{2+}-release channels in the ER membrane. It has been shown that the two branches of this pathway often collaborate in producing a full cellular response. This hypothesis is supported by previous evidences that TH activates the inositol phospholipid pathway (Kavok et al., 2001; D'Arezzo et al., 2004).

Nonetheless, KT5720, a specific PKA inhibitor (Kam et al., 2005), was able to totally prevent the stimulatory effect of T_3 but only partially prevented the effect of T_4 on $^{45}Ca^{2+}$ uptake. In this context, Hall et al. (2006) described the upregulation of L-type Ca^{2+} channel by PKA in the brain and heart. The importance of PKA activity on T_3-induced $^{45}Ca^{2+}$ uptake was confirmed by coincubating cortex slices with T_3 and theophylline, a phosphodiesterase inhibitor, since in the presence of high cAMP levels the effect of the hormone persisted, supporting previous evidences that L-type Ca^{2+} current was increased by TH in rat ventricular myocytes by the activation of the adenylate cyclase cascade (Watanabe et al., 2005). Otherwise, concerning the effect of T_4, we demonstrated that high cAMP levels and PKA activity are not directly involved in Ca^{2+} influx, as evidenced coincubating tissue slices with T_4 and KT5720 or theophylline. The PKA inhibitor only partially prevented the effect of T_4, whereas the high cAMP levels induced by theophylline were unable to support the effect of the hormone on $^{45}Ca^{2+}$ uptake, leading to the prevention of such effect. These findings are compatible with a mechanism of action mediated by G inhibitory protein (G_i). In this context, Lin et al. (1999) suggest that T_4 binds to a G_i protein-coupled receptor (GPCR). It is known that G_i family can inhibit adenylyl cyclase or activate phosphodiesterase activity, leading to inhibition of VDCC (Nestler and Duman, 2006).

Taken together, we propose that T_3 stimulates Ca^{2+} uptake through L- and T-type VDCC in a PKC- and PKA-dependent manner and such effect is supported by high cAMP levels, suggesting the participation of inositol phospholipid pathway and G_s protein mechanisms. The stimulatory action of T_4 on VDCC is also mediated by PKC, probably through inositol phospholipid pathway; however the involvement of PKA is compatible with G_i-mediated mechanisms (Lin et al., 1999). Taken together, it can be concluded that T_4 have a more complex signaling mechanism involved in the modulation of Ca^{2+} influx. Consistent with these findings, the results of our previous report (Zamoner et al., 2006) also evidenced differential mechanisms of action for T_3 and T_4 targeting the cytoskeleton in cerebral cortex from 10 day-old rats, supporting that T_3 and T_4 can nongenomically alter distinct signal-transducting pathways.

In conclusion, our findings evidence that T_3 and T_4 have membrane-initiated actions modulating Ca^{2+} channels which could not be necessarily mediated by similar downstream events, suggesting the presence of multiple sites of hormonal regulation and supporting a role for TH as modulators of signal transduction pathways in the CNS.

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Capítulo 6

G_i PROTEIN-COUPLED RECEPTOR- AND CALCIUM-MEDIATED NONGENOMIC MECHANISMS OF ACTION OF THYROXINE ON THE CYTOSKELETON IN CEREBRAL CORTEX OF YOUNG RATS

Artigo submetido para European Jounal of Neuroscience

Capítulo 7

THYROID HORMONES REORGANIZE THE CYTOSKELETON OF GLIAL CELLS THROUGH GFAP PHOSPHORYLATION AND RHOA-DEPENDENT MECHANISMS

Artigo aceito para publicação na Cellular and Molecular Neurobiology

View Letter

Date: May 04, 2006

To: "Regina Pessoa-Pureur" rpureur@ufrgs.br

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Dear Dr Regina Pessoa-Pureur:

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THYROID HORMONES REORGANIZE THE CYTOSKELETON OF GLIAL CELLS THROUGH GFAP PHOSPHORYLATION AND RHOA- DEPENDENT MECHANISMS

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RUNNING READ: Thyroid hormones and glial cytoskeleton

ABSTRACT

Thyroid hormones (3,5,3'-triiodo-L-thyronine, T_3; 3,5,3',5'-L-tetraiodothyronine, T_4; TH) play crucial roles in the growth and differentiation of the central nervous system. In this study we investigated the actions of TH on proliferation, viability, cell morphology, in vitro phosphorylation of glial fibrillary acidic protein (GFAP) and actin reorganization in C6 glioma cells. We first observe that long-term exposure to TH stimulates cell proliferation without induce cell death. We also demonstrate that after 3, 6, 12, 18 and 24 h treatment with TH, C6 cells and cortical astrocytes show a process-bearing shape. Furthermore, immunocytochemistry with anti-actin and anti-GFAP antibodies reveals that TH induce reorganization of actin and GFAP cytoskeleton. We also observe an increased in vitro ^{32}P incorporation into GFAP recovered into the high salt Triton insoluble cytoskeletal fraction after 3 and 24 h exposure to 5×10^{-8} and 10^{-6} M T_3, and only after 24 h exposure to 10^{-9} M T_4. These results evidence a T_3 action on the phosphorylating system associated to GFAP and suggest a T_3- independent effect of T_4 on this cytoskeletal protein. In addition, C6 cells and astrocytes treated with lysophosphatidic acid, an upstream activator of the RhoA GTPase pathway, totally prevented the morphological alterations induced by TH, indicating that this effect could be mediated by the RhoA signaling pathway. Considering that IF network can be regulated by phosphorylation leading to reorganization of IF filamentous structure and that alterations of the microfilament organization may have important implications in glial functions, the effects of TH on glial cell cytoskeleton could be implicated in essential neural events such as brain development.

KEYWORDS: thyroid hormones, C6 glioma cells, astrocytes, cytoskeleton, GFAP phosphorylation, RhoA signaling pathway

INTRODUCTION

Astrocytes, the most abundant glial cell type in the brain, play a vital role in the homeostatic regulation of the central nervous system (CNS). These cells are involved in neurotransmitter uptake, neuronal metabolic support, pH regulation, and protection against toxic episodes affecting cell viability (Blanc et al 1998; Takuma et al 2004). C6 rat glioma cell line which are immortalized glial cells with properties of both astrocytes and oligodendrocytes (Volpe et al. 1975; Mangoura et al 1989) present several similarities with primary glial cells in culture. For this reason, C6 cells have provided a useful model to study glial cell properties, glial growth factors and sensitivity of glial cells to various substances and conditions (Vernadakis et al 1991; Vernadakis et al 1992).

Glial fibrillary acidic protein (GFAP) is the major intermediate filament (IF) protein in mature astrocytes. The high abundance of GFAP in astrocytes and its strong conservation among vertebrates suggests that it plays a critical function in the CNS. Although the exact functions of GFAP in astrocytes remain to be elucidated, the onset of GFAP expression is observed during astrocyte differentiation in rats (Dahl 1981). GFAP is also dramatically up regulated in gliosis along with astrocyte hypertrophy (Eng and Lee 1995). These features support the hypothesis that GFAP determines to a large extent the complex astrocyte morphology that includes multiple processes that contact blood vessel walls, ensheath neuronal synapses, abut nodes of Ranvier, and interdigitate with one another at the pial surface to form the glial limitans (Messing and Brenner 2003). The amino and the carboxy-terminal tail domains of GFAP subunits are potential phosphorylation sites. Thereafter, it has become increasingly evident that site-specific phosphorylation of IF proteins alters filament structure as well as organization of IF networks. Then, phosphorylation and dephosphorylation of specific amino acid residues in the amino-terminal tail of GFAP are

involved in the regulation of GFAP assembly (Inagaki et al 1994), while phosphorylation of carboxy-terminal domains are involved in the interactions between IFs or with other cell structures (Hsieh et al 1994; Gotow et al 1994; Leterrier et al 1996).

The actin cytoskeleton is the major determinant of cell morphology, being involved in cell motility, phagocytosis, migration, adhesion, cytokinesis, and endocytosis (Zigmond 1996). Actin also plays active roles in astrocytes. In this context, there is considerable evidence that toxins such as cytochalasin D, which prevent actin polymerization, are able to alter various astrocytic functions, including calcium uptake, release, and signaling, as well as, Cl^- conductance, glutamate uptake, and hormone modulation of cell growth (Cotrina et al 1998; Lascola et al 1998; Duan et al 1999; Sergeeva et al 2000). In contrast, the rearrangement of actin cytoskeleton represents an important biological response to several extracellular stimuli, which are mediated by the Rho family of small GTPases.

Rho GTPases in the GTP-bound active state can interact with a number of effectors to transduce signals leading to diverse biological responses, including actin cytoskeletal rearrangements, cell cycle regulation, control of apoptosis, and membrane trafficking (Van Aelst and D'Souza-Schorey 1997; Hall 1998; Bishop and Hall 2000). When the bound GTP is hydrolyzed to GDP, Rho proteins return to the inactive basal state. The Rho GTPases include many distinct family members in mammalian cells (Rac 1-3, Cdc42, TC10, RhoA-E, G, H, and Rnd1 and 2), but the best characterized are the Cdc42, Rac1 and RhoA. In fibroblasts, Cdc42 triggers the formation of filopodia, whereas Rac1 regulates the formation of lamellipodia and RhoA triggers the assembly of focal contacts and stress fibers (Aspenstrom 1999). Furthermore, growth factors and lysophosphatidic acid (LPA) are upstream regulators of RhoA. Moreover, it has been shown that LPA and RhoA are

involved in the regulation of astrocyte morphology (Suidan et al 1997; Manning et al 1998).

Thyroid hormones (3,5,3'-triiodo-L-thyronine, T_3; 3,5,3',5'-L-tetraiodothyronine, T_4; TH) play crucial roles in the growth and differentiation of many organs, including the CNS (Porterfield and Hendrich 1993; Koibuchi and Chin 2000; Anderson 2001), regulating the axonal and dendritic growth, synapse formation, myelination, cell migration and proliferation of specific glial and neuronal populations (Porterfield and Hendrich 1993; Rodriguez-Pena 1999; Anderson 2001; Gomes et al 2001). Even though the regulatory role of T_3 in brain development is well established, the cellular and molecular mechanisms by which this hormone produces its biological effects remain elusive. It has been demonstrated that T_3 regulates protein expression in rat astrocytes and C6 cells (Trentin and Alvarez-Silva 1998; Trentin et al 2001). Despite this findings, astroglial cells are heterogeneous with respect to their ability to respond to T_3, including their capacity to secrete different growth factors or related molecules. Furthermore, it has been demonstrated the T_4-dependent ability to regulate the extracellular matrix on the astrocyte surface, suggesting a mechanism by which this hormone can influence neuronal migration in brain (Farwell and Dubord-Tomasetti 1999).

It is well established that, during rat brain development, astrocytes are target cells for TH (Gould 1990). Moreover, we have recently reported that TH alter phosphorylation of glial and neuronal IF proteins in cerebral cortex of rats (Zamoner et al 2005). Therefore, taking into account that phosphorylation is an important mechanism regulating cytoskeletal dynamics and that cytoskeleton is essential for normal cell morphology, in the present study we investigated the effects of exposing cultured C6 glioma cells or cortical astrocytes to different concentrations of TH on cell morphology, viability, proliferation and GFAP

phosphorylation. We also studied whether the RhoA signaling pathway was involved in the effects elicited by the hormones.

METHODS

Radiochemicals and compounds- [^{32}P]orthophosphate was purchased from CNEN, São Paulo, Brazil. 3,5,3'triiodo-L-thyronine, thyroxine, benzamidine, leupeptin, antipain, pepstatin, chymostatin, acrylamide, bis-acrylamide, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), anti-actin (clone 1A4), trypsin and material for cell culture were purchased from Sigma (St. Louis MO, USA). Polyclonal anti-GFAP (glial fibrillary acidic protein) was purchased from DAKO. Dulbecco´s modified Eagle´s medium (DMEM) was from Gibco BRL (Carlsbad, CA, USA). Fetal calf serum (FCS) was purchased from Cultilab (Campinas, SP, Brazil). ECL kit was from Amersham, Oakville, Ontario and Trans-blot SD semi-dry transfer cell was from BioRad.

Maintenance of cell line: The C6 rat glioma cell line was obtained from American Type Culture Collection (Rockville, Maryland, USA). We used C6-glioma cells at passage 90, expressing GFAP. The cells were grown and maintained in DMEM (pH 7.4) containing 2.5 mg/ml Fungizone® and 100 U/l gentamicin, and supplemented with 5 % FCS. Cells were kept at a temperature of 37^{o} C, a minimum relative humidity of 95 %, and an atmosphere of 5 % CO_2 in air.

Astrocyte Cultures: Primary cerebral cortex astrocyte cultures were prepared as described previously (Gottfried et al 1999). Briefly, cerebral cortex of newborn Wistar rats (P1-2)

was dissected and placed in a Ca^{2+} - and Mg^{2+} -free balanced salt solution (CMF-BSS) pH 7.4 containing (mM): NaCl,137; KCl, 5.36; Na_2HP_4, 0.27; KH_2PO_4, 1.1; glucose, 6.1. The tissue was then cleaned of meninges and dissociated by sequential passage through a Pasteur pipette. The cell suspension was allowed to settle and the supernatant transferred to another tube. The sedimented cells were resuspended in CMF-BSS and dissociated again. The two supernatants were combined and the resulting cell suspension centrifuged at 150 *g* for 5 min. The supernatant was discarded the pellet resuspended in culture medium (DMEM, pH 7.6), supplemented with 8.39 mM HEPES, 23.8 mM $NaHCO_3$, 0.1% fungisone; 0.032% garamicine, and 10% FCS. The cells were plated at a density of 5 x 10^4 cells/well onto 96-well plates pre-treated with poly-L-lysine. Cultures were maintained in 5% CO2 /95% air at 37 ºC and allowed to grow to near consequence (2 weeks). Medium was changed every 3-4 days. Immediately before medium changes, plates were shaken manually to remove non-adherent cells. Western blotting with an antibody to neuron specific β tubulin III excluded contamination with neurons.

Cell Counting: Glioma cells were seeded at 5 X 10^3 cells per well in DMEM/ 5 % FCS in 24-well plates, grown for 24 h in 5 % FCS and quiescence was induced by reducing the concentration of FCS to 0.5 % for 24 h. The medium was changed 2 h prior to treatment after which cells were treated for 24 h at 37^{o}C in an atmosphere of 5% CO_2 / 95% air in DMEM (pH 7.4) containing 0% FCS in the absence (controls) or presence of TH in different concentrations. At the end of this period, the medium was removed, cells were washed with PBS and 200 µl of 0.25 % trypsin/EDTA solution was added to detach the cells, which were immediately counted in a hemocytometer under a optic microscope.

Coomassie brilliant blue assay: Glioma cells were seeded at 5 X 10^3 cells per well in DMEM 5 % FCS in 24-well plates, grown for 24 h in 5 % FCS and quiescence was induced

by reducing the concentration of FCS to 0.5 % for 24 h. The medium was changed 2 h prior to treatment after which cells were treated for 24 h at 37°C in an atmosphere of 5% CO_2 / 95% air in DMEM (pH 7.4) containing 0% FCS in the absence (controls) or presence of TH in different concentrations. At the end of this period, the medium was removed, cells were washed with PBS and fixed for 20 min with 4% paraformaldehyde in PBS. The cells were incubated for 15 min with Coomassie brilliant blue and than washed exhaustively to remove Coomassie brilliant blue excess. Finally, the cells were homogenized in 1 mL SDS 1 % and protein measurement was obtained by spectrophotometry at 600 nm. A linear correlation was used between absorbance and cell number along a large range of cell densities and used to measure cell proliferation (Margis and Borojevic 1989).

Lactate Dehydrogenase Assay (LDH Assay): The viability was assessed by measuring the release of the cytosolic enzyme lactate dehydrogenase into the medium. C6 cells were incubated in the presence or absence of TH for 24 hours. LDH measurement was carried out in 25 ml aliquots using the LDH kit from Doles reagents.

Propidium iodide: Cellular damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake. Cells were treated during 24 h with TH and 7.5 mM PI, at 37°C in an atmosphere of 5% CO_2 / 95% air in DMEM + 0 % FCS, after which they were analyzed and photographed with a Nikon inverted microscope using a TE-FM Epi-Fluorescence accessory. Optical density was determined with the Optiquant version 02.00 software (Packard Instrument Company).

Morphological studies: After cells reached confluence, the culture medium was removed by suction and the cells were incubated for 3, 6, 12, 18 and 24 h at 37°C in an atmosphere of 5% CO_2 / 95% air in DMEM (pH 7.4) containing 0% FCS in the presence or absence

(controls) of the TH at different concentrations. Morphological studies were performed using phase contrast optics.

Immunocytochemistry: C6 cells were cultured, treated with TH for 24 h and fixed for 20 minutes with 4% paraformaldehyde in phosphate buffer saline (PBS) (in mM): KH_2PO4, 2.9; $Na_2HPO_47H_2O$, 38; NaCl, 130; KCl, 1.2, rinsed with PBS and permeabilized for 10 min in PBS containing 0.2 % Triton X-100. To reduce nonspecific background staining the fixed cells were then blocked for 60 min with PBS containing 0.5% bovine serum albumin and incubated overnight with polyclonal anti-GFAP (1:200) or monoclonal anti-actin (1:200), followed by peroxidase-conjugated IgG (1:1000) for 2 h. Finally the cells were treated with 0.05% diaminobenzidine containing 0.01% hydrogen peroxide for 10 min.

In vitro phosphorylation- C6 cells were preincubated for 3 and 24 h in the presence or absence of TH, then the medium was changed and incubation was carried out at 30 ^oC with 1000 µl of a medium containing (in mM) NaCl, 124; KCl, 4; $MgSO_4$, 1.2; Na-HEPES 25; (pH 7.4), glucose, 12; $CaCl_2$,1 and the following protease inhibitors: 1mM benzamidine, 0.1 µM leupeptin, 0.7 µM antipain, 0.7 µM pepstatin, 0.7 µM chymostatin, and 10 µCi of [^{32}P] orthophosphate with or without addition of the TH. The labeling reaction was normally allowed to proceed for 1 h at 30 ^oC and stopped with 1 ml of cold stop buffer (150 mM NaF, 5 mM, EDTA, 5 mM EGTA, Tris-HCl 50 mM, pH 6.5, and the protease inhibitors described above). Cells were then washed twice by decantation with stop buffer to remove excess radioactivity.

Preparation of the high-salt Triton insoluble cytoskeletal fraction from C6 cells- After treatment, preparations of total IF were obtained from C6 cells as described by Funchal et al (2003). Briefly, after the labeling reaction, cells were homogenized in 200 µl of ice-cold

high salt buffer containing 5 mM KH_2PO_4, (pH 7.1), 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 1 % Triton X-100 and the protease inhibitors described above. The homogenate was centrifuged at 15800 x g for 10 min at 4 °C, in an Eppendorf centrifuge, the supernatant discarded and the pellet homogenized with the same volume of the high-salt medium. The resuspended homogenate was centrifuged as described and the supernatant was discarded. The Triton-insoluble intermediate filament-enriched pellet, containing GFAP, was dissolved in 1% SDS and protein concentration was determined by the method of Lowry et al (1951).

Polyacrylamide gel electrophoresis (SDS-PAGE) - The cytoskeletal fraction and the total protein homogenate were prepared as described above. Equal protein concentrations were loaded onto 10 % polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli (1970). After drying, the gels were exposed to X-ray films (X-Omat XK1) at -70 ^{o}C with intensifying screens and finally the autoradiograph was obtained. Proteins were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an Optiquant version 02.00 software (Packard Instrument Company). Density values were obtained for the studied proteins.

Immunoblotting Analysis- Cytoskeletal fractions were separated by 10% SDS-PAGE (40 µg/lane of total protein) and transferred in a Trans-blot SD semi-dry transfer cell to nitrocellulose membranes for 1 hr at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS). The blot was then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% defatted dry milk). After incubation, the blot was washed twice for 5 min with blocking solution plus 0.05% Tween-20 (T-TBS) and then incubated

overnight at 4°C in blocking solution containing the monoclonal antibody anti glial fibrillary acidic protein - GFAP (clone G-A-5) diluted 1:400 (Sigma). The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated rabbit anti mouse IgG diluted 1:2000. The blot was again washed twice for 5 min with T-TBS and twice for 5 min with TBS. The blot was developed using a chemiluminescence ECL kit.

Statistical analysis- Data were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Tukey test when the F-test was significant. All analyses were performed using the SPSS software program on an IBM-PC compatible computer.

RESULTS

In our control culture conditions, C6 glioma cells presented a rounded and flat morphology with abundant cytoplasm, as observed in phase-contrast microscopy in Figure 1. In order to evaluate the effect of TH on the morphology of C6 glioma cells in culture, cells were treated with different concentrations of TH (5 x 10^{-8}, 10^{-7}, and 10^{-6} M T_3 or 10^{-9}, 10^{-8} and 10^{-7} M T_4) and observed after different exposure times (3, 6, 12, 18 and 24 h). Our results show that TH in the concentrations tested induced morphological alterations at 3-h exposure, comprising fusiform and process-bearing cells up to 24 h exposure (Figure 1).

Cell viability was evaluated by the propidium iodide (PI) method (Pringle et al 1996), which measures the incorporation of PI by the C6 cells and by lactate deshydrogenase activity (LDH), measuring the release of the cytosolic enzyme lactate dehydrogenase into the culture medium by damaged cells. Cells were incubated in the presence of 10^{-6} M T_3 or 10^{-7} M T_4, for 24h and the assays were proceeded. Figure 2 A and B shows that even the

long-term exposure of cells to supra physiological concentrations of TH was not able to induce cell death. Indeed, we observed increased cell proliferation after 24 h treatment of C6 cultures with different concentrations of T_3 (5 x 10^{-8} and 10^{-6} M) or T_4 (10^{-9} and 10^{-7}M), by cell counting and Coomassie brilliant blue assays (Figure 3).

Immunocytochemical analysis using anti-actin or anti-GFAP antibodies was also carried out to test the involvement of cytoskeletal actin and IF reorganization in the cell morphology alterations induced by TH. Under basal conditions, cells presented intense cytoplasmic immunolabeling to actin (Figure 4) and GFAP (Figure 5). In addition, nearly all C6 cultured cells (>98%) stained positively to GFAP, attesting to their astrocyte phenotype. In addition, actin and GFAP stained filaments formed a meshwork extending across the cytoplasm. When cells were exposed to T_3 (5 x 10^{-8} or 10^{-6} M) or T_4 (10^{-9} or 10^{-7} M) for 24 h, we observed a rearrangement of actin and GFAP filament networks that were more distributed along the cytoplasm and processes. Thus we verified whether exposure of C6 cells to TH could alter the in vitro phosphorylation of GFAP recovered into the high-salt Triton-insoluble cytoskeletal fraction, since it is largely described in the literature that phosphorylation regulates the assembly/disassembly ability of IF proteins (Inada et al 2000). Results showed that Triton-insoluble GFAP from C6 cells treated with T_3 (5 x 10^{-8} or 10^{-6} M) for 3 or 24 h presented hyperphosphorylation as compared with controls (Figure 6). Nevertheless, in cells treated with T_4 (10^{-9} or 10^{-7} M) for 3h GFAP in vitro phosphorylation was not altered. However, after 24 h treatment with 10^{-9} M T_4, we observed GFAP hyperphosphorylation, while in 10^{-7} M T_4 was not able to alter the phosphorylating system associated to GFAP (Figure 6).

Considering our findings showing that TH alter cell morphology through cytoskeleton reorganization in C6 glioma cells, we investigated the morphological alterations induced by

TH in cortical astrocytes. Figure 7 demonstrates that T_3 and T_4 induced morphological alterations in astrocytes after different time and concentration exposures to the hormones. We observed a transformation from polygonal to process-bearing shape of these cells.

To evaluate whether the effects of TH on the rearrangement of actin filaments in C6 cells was mediated by a Rho signaling pathway, cultured C6 cells were treated with 2 μM LPA, which is a specific upstream regulator of Rho A, in the absence (controls) or presence of 10^{-6} M T_3 or 10^{-7} M T_4 for 24 h (Figure 8). It should be emphasized that the organization and dynamic of actin cytoskeleton are regulated by small Rho GTPases, linking the extracellular stimuli to the organization of the actin cytoskeleton (Guasch et al 2003). Initially, we verified that cells treated with LPA presented a similar morphology observed in control cultures (Figure 8). However, the most interesting finding was that the presence of LPA ensured a normal C6 morphology in cells treated with both T_3 (10^{-6} M) or T_4 (10^{-7} M) for 24 h. Similar results were obtained with cultured astrocytes treated with T_3 and T_4 for 24 h (Figure 9), which presented process-bearing cells after 24 h exposure to 10^{-6} M T_3 or 10^{-7} M T_4 (Figure 9c and e). In this case, we also observed that LPA- treated cells were protected against TH-induced altered cell morphology (Figure 9d and f). Taken together our results indicate that the morphological alterations induced by TH in C6 cells are mediated by altering phosphorylation/dephosphorylation ability of GFAP subunits and modulated via RhoA pathway both in C6 glioma cells and in astrocytes in culture.

DISCUSSION

We have previously described that TH increased the in vitro incorporation of ^{32}P into the IF proteins (GFAP, vimentin and neurofilament subunits) through PKA and PKCaMII

activities from cerebral cortex of young rats (Zamoner et al 2006). Because aberrant cytoskeletal phosphorylation/dephosphorylation may have serious consequences for cellular function and structure and Rho proteins are well known for their effects on the actin cytoskeleton (Ridley 2001), in the present study we investigated the involvement of GFAP phosphorylation and RhoA mechanisms underlying TH effects on cytoskeletal organization in cultured C6 glioma cells and cortical astrocytes.

We demonstrated that T_3 and T_4 provoked marked morphological alterations in C6 glioma cells at doses as low as 10^{-9} M for T_4 and 5 x 10^{-8} M for T_3. Nonetheless, long time exposure (24 h) of C6 cells to 10^{-6} M T_3 or 10^{-7} M T_4 was not able to provoke cell death, instead induced cell proliferation. The proliferative effects of T_3 we are evidencing in our study are in line with Trentin et al (2001) who described that 5 x 10^{-8} M T_3 stimulates cerebellar astrocyte and C6 glioma cell proliferation through secretion of mitogenic growth factors. However, it is important to emphasize that, in our experimental conditions, T_4 also induced C6 cell proliferation, suggesting a novel potential mitogenic action for this hormone.

Immunocytochemistry showed that the alterations in cell shape, elicited by TH, involved rearrangement of actin and GFAP filaments. Therefore, exposure of C6 glioma cells to different TH concentrations altered actin filament organization in these cells. We observed by immunocytochemical staining with actin antibody that 24 h after addition of T_3 (5 x 10^{-8} or 10^{-6} M), or T_4 (10^{-9} or 10^{-7} M), actin filaments reorganize leading to a process-bearing cellular shape. By using GFAP antibody, we also evidenced an altered organization of GFAP filaments supporting therefore the reorganization of actin cytoskeleton. Our data indicate similar alterations in cell morphology in cultures supplemented by both T_3 or T_4. The present results show that TH induce morphological

changes in C6 cells accompanied by a reorganization of the GFAP and actin networks. Taking into account the increasing evidence that the structural organization of the IF network is regulated spatially and temporally by phosphorylation (Inagaki et al 1996; Funchal et al 2005) and since the high-salt Triton insoluble cytoskeletal fraction is enriched in polymerized or aggregated forms of IF proteins (Funchal et al 2003), we studied the effect of different concentrations of TH on GFAP phosphorylation after 3 and 24 h exposure to the hormones. Results indicated that the in vitro phosphorylation of GFAP recovered in the cytoskeletal fraction was increased after T_3 treatments. On the other hand, the effect of T_4 on GFAP phosphorylation was slightly different, highlighting a T_4-dependent differential pattern of action on the endogenous phosphorylating system associated to the IF cytoskeleton in C6 cells. In fact, T_4 did not alter GFAP phosphorylation after 3 h exposure to the hormone, otherwise, after 24 h of treatment only the low concentration used (10^{-9} M) induced GFAP hyperphosphorylation, demonstrating no dose-response relationship for both hormones on the cytoskeletal-associated phosphorylating system. On the basis of our data we could propose that the reorganization of GFAP filaments observed after 3 h exposure to T_3 could be related to the altered activity of the GFAP-associated phosphorylating system. Nonetheless, concerning the effect of T_4, it appears that despite the ability of the hormone in altering cell shape, we could not ascribe this effect to the phosphorylation levels of GFAP, since they were not affected. However, in order to explain the reasons by which the highest concentration of T_4 used in our experimental conditions did not alter GFAP phosphorylation we must also consider a possible intracellular T_4 to T_3 conversion by the action of type II 5´deiodinase (Pallud et al., 1997) which is inhibited by high concentrations of T_4 (Larsen and Berry 1995; Larsen et al 1998; Anderson, 2001). Nonetheless, it is important to emphasize that although T_4 did not

alter GFAP phosphorylation it was effective in altering cell shape and cause actin and GFAP reorganization with all the concentrations used as demonstrated in figures 4 and 5 respectively, supporting a T_4-triggered mechanism on cytoskeleton rearrangement that probably is independent on GFAP phosphorylation. Interestingly, despite previous evidences of the presence of high concentrations of specific deiodinases converting T_4 into T_3 in glial cells (Pallud et al 1997), our results suggest a T_3-independent action for T_4 regulating cytoskeletal dynamics, although the use of an inhibitor of deiodinase could provide a more conclusive response. In addition, our results demonstrating the action of T_4 on the cytoskeleton are in agreement with previous evidences describing that T_4 dynamically alters microfilament organization in cultured astrocytes by regulating actin polymerization via an extranuclear mechanism (Siegrist-Kaiser et al 1990; Farwell and Leonard 1997). T_4 can also modulate the ability of integrins to cluster into focal contacts upon binding to laminin (Farwell et al., 1995). In this context, Bergh et al (2005) has described a plasma membrane receptor site for T_4 on integrin $\alpha V\beta 3$. Moreover, our results represent a further demonstration of T_4 ability to modulate glial cytosleleton.

In addition, we demonstrated that besides the alterations in cell shape induced by T_3 and T_4 at different concentrations on C6 glioma cells, these hormones also provoked marked morphological alterations in cultured astrocytes. This was evidenced after different time and concentration exposures to the hormones, leading to contraction of cell body and a process-bearing morphology of these cells.

We also verified that LPA, a specific RhoA activator, prevented the effects of the TH on C6 and astrocyte morphology, implying that Rho GTPase signaling pathway probably mediated these effects. In this context, it has been established that RhoA is the best-characterized member of the Rho family of low molecular weight GTPases, which are

mediators of cell growth and actin cytoskeletal rearrangement in mammalian cells. The role of RhoA in actomyosin-based contractility and in stress fiber formation has already been demonstrated in several cell lines, including S3T3 fibroblasts (Ridley and Hall 1992) and astrocytes (Guasch et al 2003). It has been shown that RhoA action is dependent on LPA, one of the factors present in serum and known to act through a G protein coupled receptor (GPCR), eliciting stress fiber formation and focal adhesion complex assembly in a RhoA-dependent manner (Fukushima et al 1998).

Our results are in line with previous studies showing that RhoA and LPA are involved in astrocyte morphology (Guasch et al 2003; Sah et al 2000). Furthermore, we have recently described that branched chain keto acids altered cultured astrocyte morphology through actin and GFAP reorganization, and these effects were prevented by LPA, suggesting the involvement of RhoA signaling pathway (Funchal et al 2004). The exact explanation for our results showing the involvement of the RhoA signaling pathway in the morphological alterations provoked by TH is unknown. However, we could presume that these hormones may somehow interfere with RhoA signaling mechanisms, representing a further demonstration of a mechanism of action for TH that could be related with the hormone effects in brain. As regards the consequences of alterations of the actin cytoskeleton network, it may have important implications on various critical astrocyte functions, such calcium signaling and uptake (Cotrina et al 1998; Sergeeva et al 2000), glutamate transport (Duan et al 1999), Cl^- conductance (Lascola et al 1998), endo and exocytosis of vesicles and protein trafficking (Ridley 2001).

The small G proteins are mainly involved in the control of the cytoskeleton architecture and cell movement (Ridley 2001), but they also participate in several other important cellular activities, including the control of cell proliferation (Sah et al 2000).

Although LPA prevented the morphological alterations induced by TH, supporting the involvement of RhoA mechanisms underlying the hormone action on cytoskeletal reorganization, the participation of this signaling mechanism on GFAP phosphorylation and cell proliferation needs to be futher investigated.

Although Friedman et al 1977 have described the inhibition of thyroid adenylate cyclase by TH as a possible negative feedback mechanism, several evidences in the literature point to the ability of TH to increase cyclicAMP (cAMP) levels in other cell types (Leitman et al., 1996; Sarkar et al 1999; Zamoner et al., 2006). In this context, our group demonstrated that TH modulate PKA- mediated IF phosphorylation in cerebral cortex (Zamoner et al 2006), supporting the hypothesis that the increased GFAP phosphorylation we have observed could be through PKA-dependent mechanisms. Although our data do not exclude the involvement of other signaling pathway, further experiments will be necessary to clarify this point. Otherwise, it has been demonstrated that increased cAMP levels lead to an inhibition of RhoA activity in several cell types including thyroid, melanocytes, endothelial, lymphoid and neuronal cells (Fortemaison et al 2005; Scott et al 2003; Lang et al 1996; Dong et al 1998). In addition, inactivation of Rac, a small GTPase, by PKA has also been described in endothelial cells (Bakre et al 2002). Furthermore, TH effects on the cytoskeleton dynamic we are evidencing involve an inhibition of RhoA activity, as demonstrated by LPA supplementation, strongly suggesting the PKA involvement on TH actions on IF and microfilament organization in C6 glioma cells and cortical astrocytes.

C6 cells are described as glial cells with characteristics of both astrocytes and oligodendrocytes (Volpe et al 1975). The C6 glioma cell line we used in the present work is enriched in GFAP filaments indicating the astrocytic phenotype of these cells, allowing us to extrapolate the results obtained for TH on C6 cell cytoskeleton to cortical astrocytes.

This is emphasized by the evidence that LPA prevented the morphological alterations induced by T_3 and T_4 both in C6 cells and cortical astrocytes.

In conclusion, our results demonstrate that TH at different concentrations alter the phosphorylating levels leading to reorganization of GFAP, an important brain protein related to cellular structure and function. In addition, these hormones also reorganize actin cytoskeleton probably via RhoA signaling pathway. Since actin cytoskeleton participates in major astrocytic functions, actin reorganization induced by TH might have important consequences, including alterations in protein trafficking, uptake and release of neurotransmitters, and calcium control. Taking into account our present results, we propose that T_3- and T_4- dependent regulation of cytoskeleton dynamic in astrocytes could provide a mechanism by which these morphogenic hormones can influence neuronal migration and brain development.

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Figure 1. Effect of thyroid hormones (T_3 and T_4) on C6 glioma cell morphology. Cells were cultured to confluence in DMEM + 5 % fetal calf serum (FCS). The medium was then changed to DMEM + 0 % FCS and cells incubated for 3, 6, 12, 18 and 24 h in the presence or absence of the thyroid hormones at different concentrations (5 x 10^{-8}, 10^{-7} or 10^{-6} M T_3 and 10^{-9}, 10^{-8} and 10^{-7} M T_4). After incubation, phase-contrast images were recorded as described in Material and Methods. Original images were adjusted by increasing contrast. Scale bar = 50 μm.

Figure 2. Cell viability in C6 glioma cells treated with thyroid hormones (T_3 and T_4). C6 cells were cultured to confluence in DMEM + 5 % fetal calf serum (FCS). A) Cells were transferred to DMEM + 0% FCS containing 7.5 μM propidium iodide in the absence or presence of 10^{-6} M T_3 or 10^{-7} M T_4 and the incubation continued for 24 h. Representative images of three separate experiments with propidium iodide method. Scale bar = 10 μm. B) Cell viability was assessed by measuring the release of the cytosolic enzyme lactate dehydrogenase into the medium. C6 cells were incubated in DMEM + 0% FCS containing 10^{-6} M T_3 or 10^{-7} M T_4 for 24 hours. Data are reported as means ± S.E.M. of three different experiments carried out in triplicates expressed as percentage of controls and analyzed by one-way ANOVA.

Figure 3. Effect of thyroid hormones (T_3 and T_4) on C6 cell proliferation. Cells were cultured in DMEM + 5% fetal calf serum (FCS). The medium was then changed to DMEM + 0% FCS in the presence or absence of the thyroid hormones (5 x10^{-8} and 10^{-6}M T_3, or 10^{-9} or 10^{-7}M T_4) for 24. Cell proliferation was measured by Coomassie brilliant blue assay (A) and by cell counting in a hemocytometer under a optic microscope (B) as described in

Material and Methods. Data are reported as cell number x 10^3 from three independent cultures. Statistically significant differences from controls as determined by one-way ANOVA followed by Tukey test are indicated: *P <0.01.

Figure 4. Actin-immunostaining of C6 glioma cells exposed to thyroid hormones (T_3 and T_4). Cells were cultured to confluence in DMEM + 5% fetal calf serum (FCS). The medium was then changed to DMEM + 0% FCS in the presence or absence of the thyroid hormones (5 x10^{-8} and 10^{-6}M T_3 or 10^{-9} or 10^{-7}M T_4) for 24 hours, after which they were fixed and immunostained with anti-actin as described in Material and Methods. Original images were adjusted by increasing contrast . Scale bar = 50 μM.

Figure 5. GFAP-immunostaining of C6 glioma cells exposed to thyroid hormones (T_3 and T_4). Cells were cultured to confluence in DMEM + 5% fetal calf serum (FCS). The medium was then changed to DMEM + 0% FCS in the presence or absence of the thyroid hormones (5 x10^{-8} and 10^{-6}M T_3, or 10^{-9} or 10^{-7}M T_4) for 24 hours, after which they were fixed and immunostained with anti-GFAP as described in Material and Methods. Original images were adjusted by increasing contrast . Scale bar = 50 μM.

Figure 6. Effect of thyroid hormones (T_3 and T_4) on the in vitro phosphorylation of GFAP in C6 glioma cells. A) Cell cultures were incubated for 3 and 24 hours with different concentrations of T_3 and T_4 with the addition of ^{32}P orthophosphate during the last hour. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into GFAP was measured as described in Material and Methods. Data are

reported as means ± S.E.M. of three different experiments carried out in quadruplicates expressed as percentage of controls. Statistically significant differences from controls as determined by ANOVA followed by Tukey test are indicated: *P <0.01; **P < 0.001. B) Representative ^{32}P-labelled GFAP bands. C) Immunoblotting of the cytoskeletal fraction using anti GFAP monoclonal antibody.

Figure 7. Effect of thyroid hormones (T_3 and T_4) on the morphology of cortical astrocytes in culture. Cells were cultured to confluence in DMEM + 10% fetal calf serum (FCS). The medium was then changed to DMEM without FCS in the presence or absence of the thyroid hormones (5 x 10^{-8} and 10^{-6} M T_3, or 10^{-9} or 10^{-7} M T_4) for 3, 12 and 24 hours. After incubation, phase-contrast images were recorded as described in Material and Methods. Original images were adjusted by increasing contrast. Scale bar = 50 μm.

Figure 8. Effect of lysophosphatidic acid (LPA) on thyroid hormones (T_3 and T_4)-induced alterations in C6 glioma cells morphology. Cells were cultured to confluence in DMEM + 5% fetal calf serum (FCS). The medium was then changed to DMEM + 0% FCS with the addition of 2μM LPA in the presence or absence of the thyroid hormones (10^{-6} M T_3 or 10^{-7} M T_4) for 24 hours. After incubation, cells were fixed and phase-contrast images were recorded as described in Material and Methods. Original images were adjusted by increasing contrast. Scale bar = 50 μm.

Figure 9. Effect of lysophosphatidic acid (LPA) on thyroid hormones (T_3 and T_4)-induced alterations in cortical astrocyte morphology. Cells were cultured to confluence in DMEM + 10% fetal calf serum (FCS). The medium was then changed to DMEM + 0% FCS with the

addition of 2 μM LPA in the presence or absence of the thyroid hormones (10^{-6} M T_3 or 10^{-7} M T_4) for 24 hours. After incubation, cells were fixed and phase-contrast images were recorded as described in Material and Methods. Original images were adjusted by increasing contrast. Scale bar = 50 μm.

Figure 1

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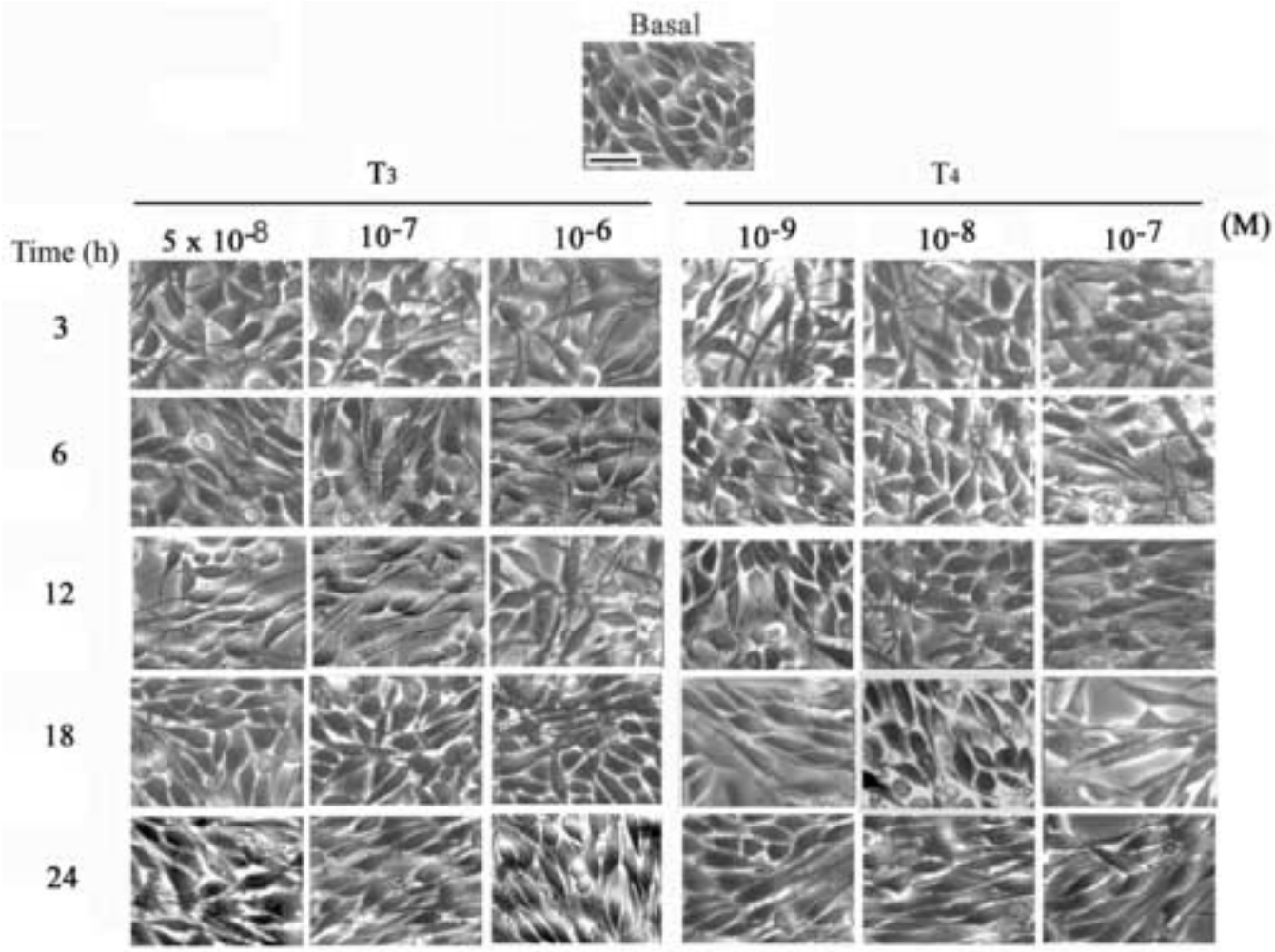

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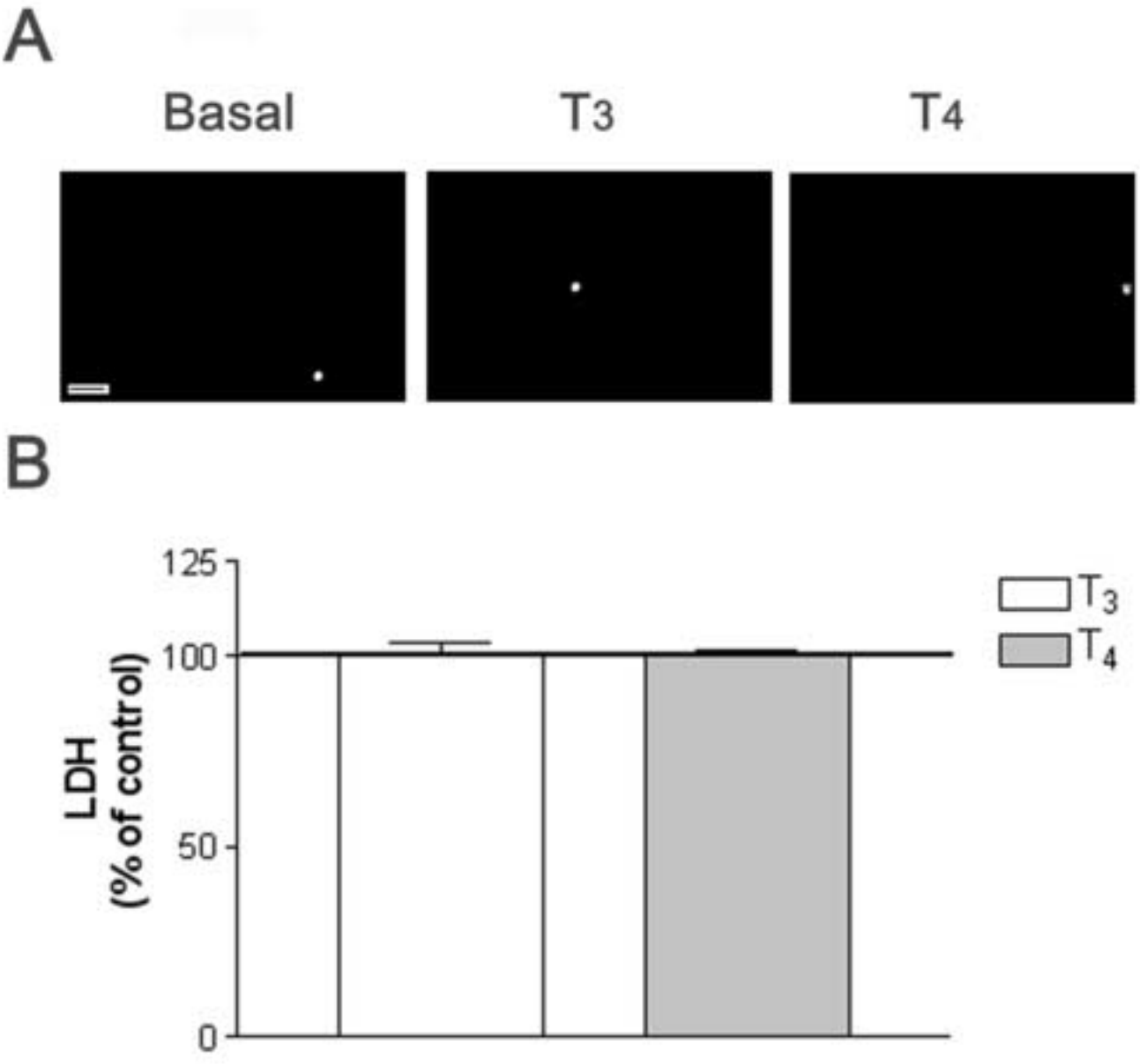

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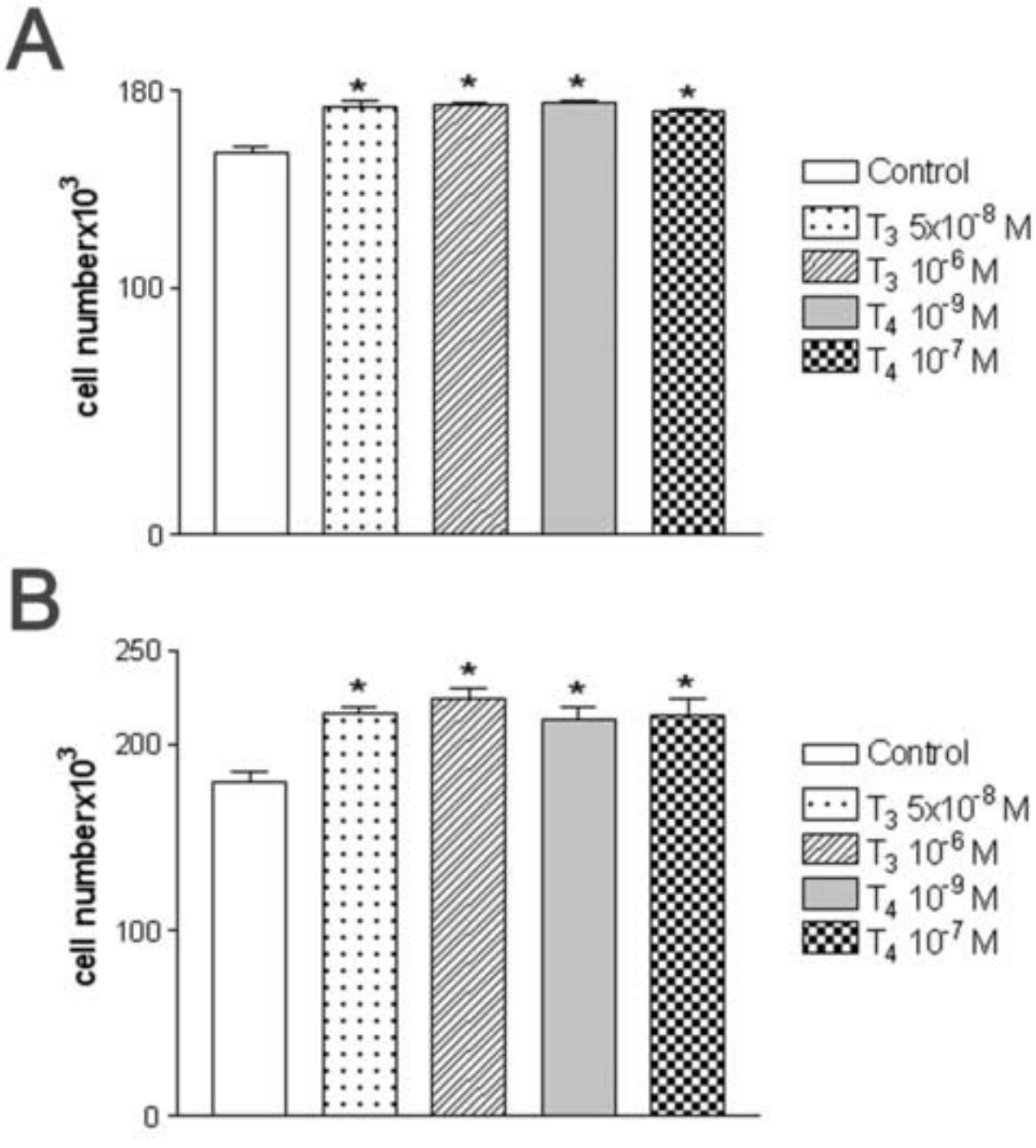

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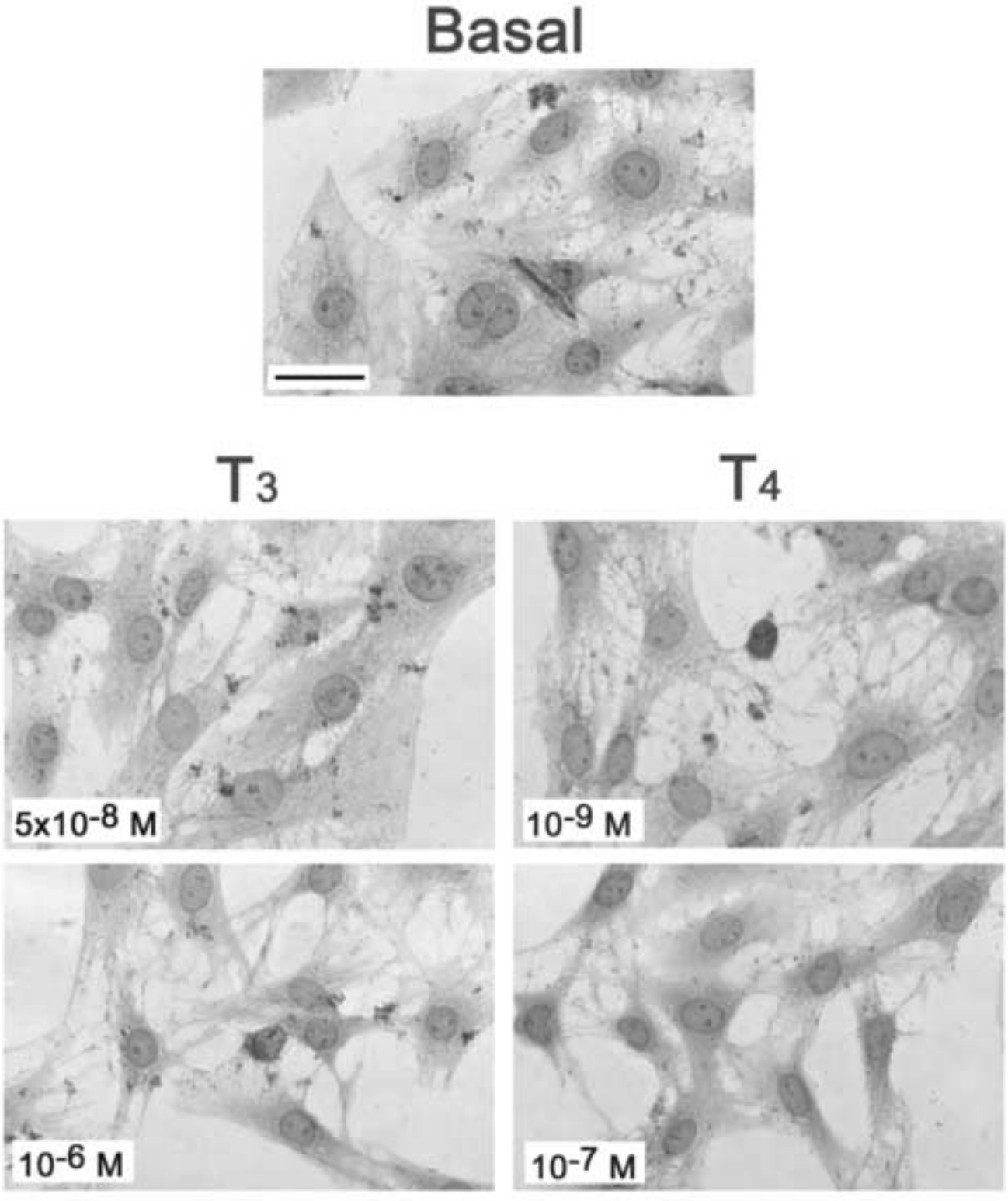

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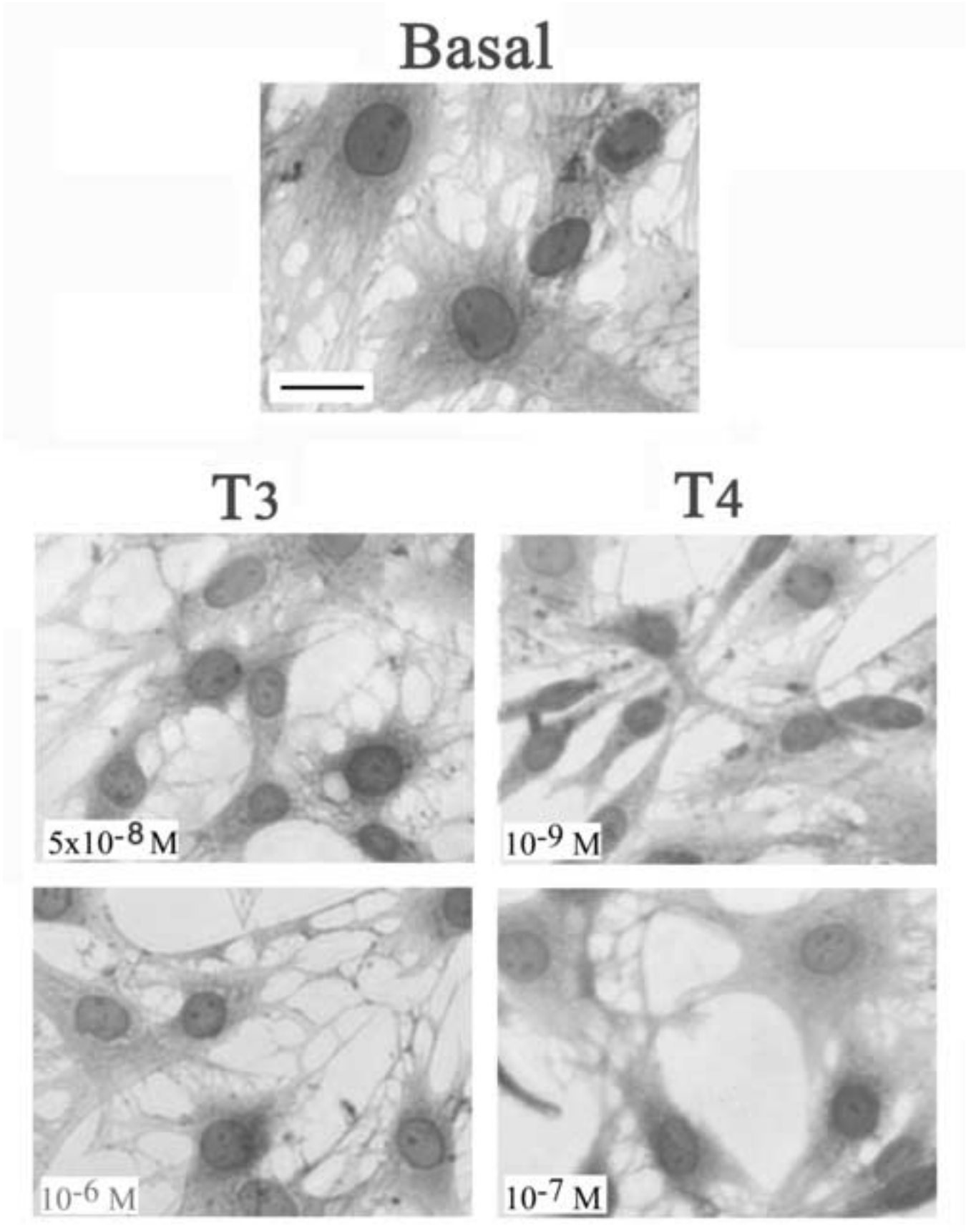

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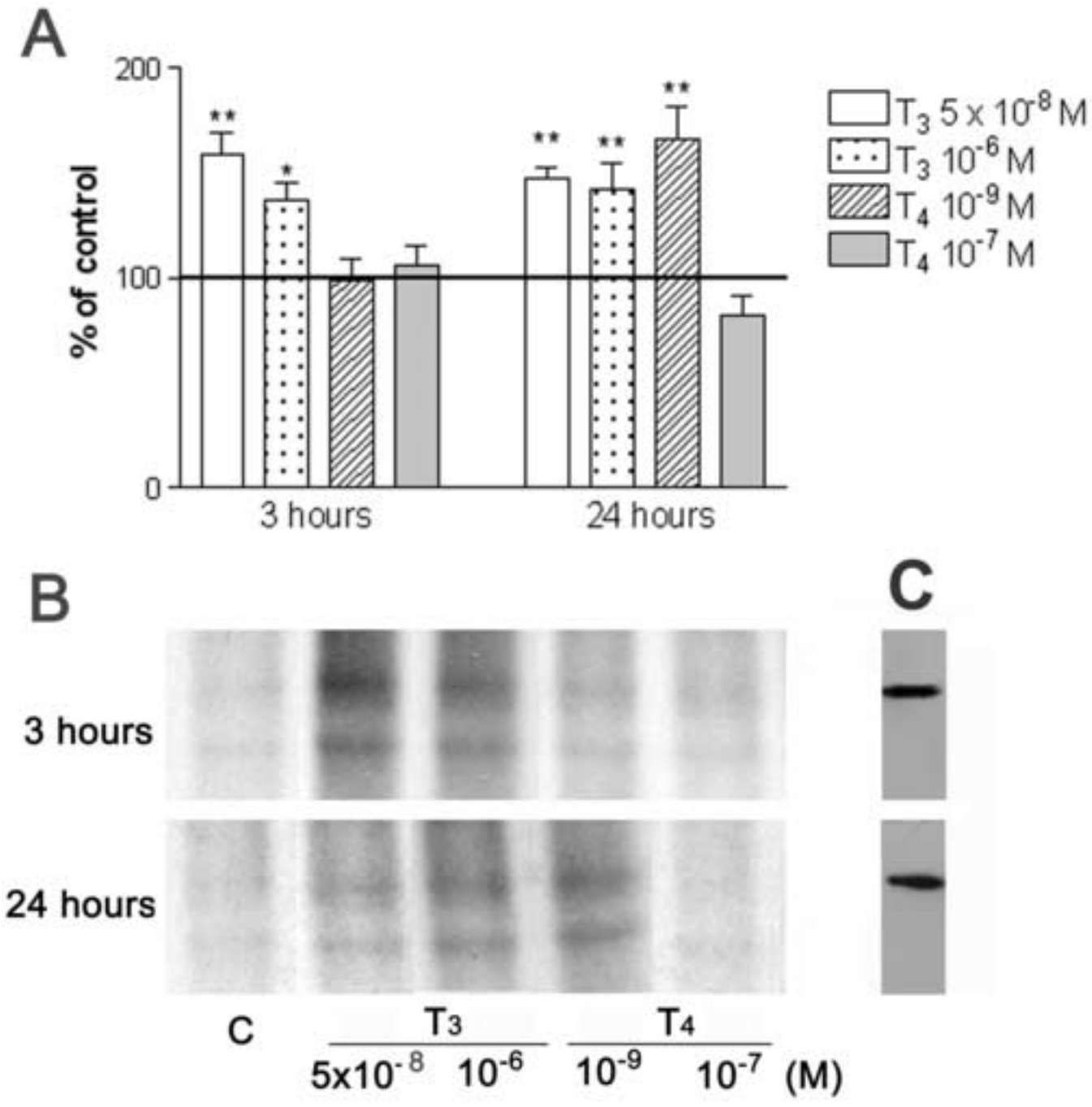

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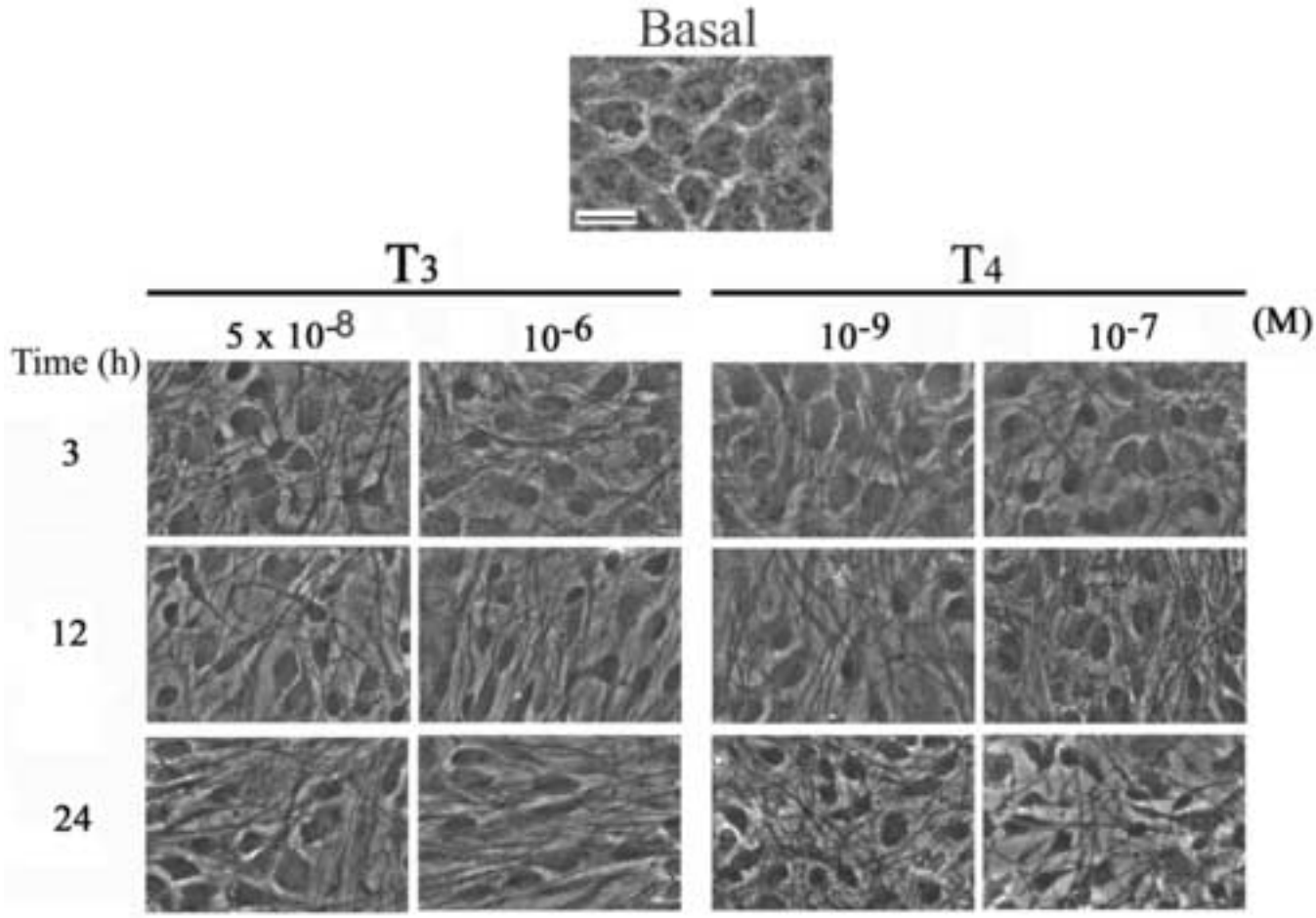

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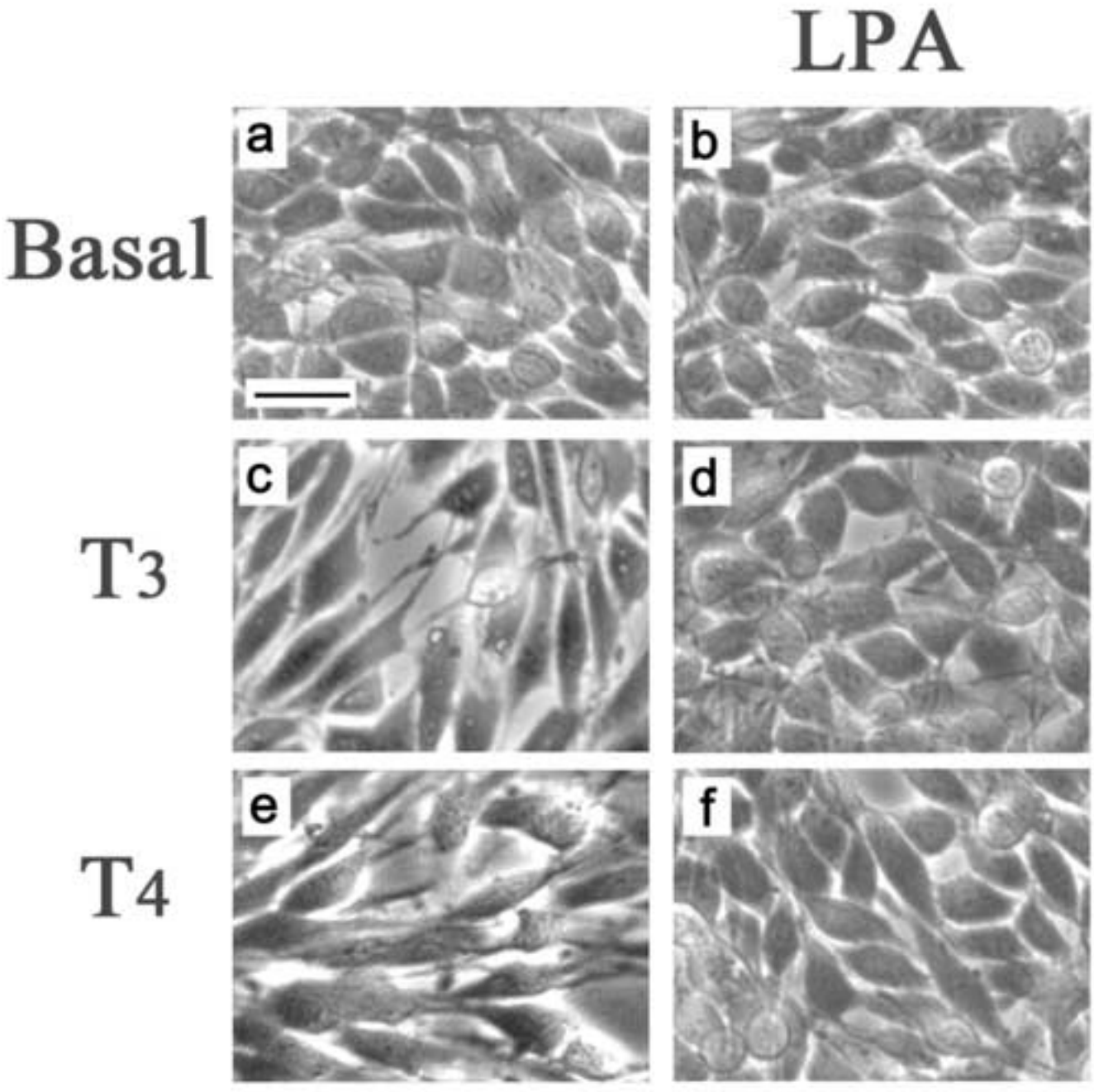

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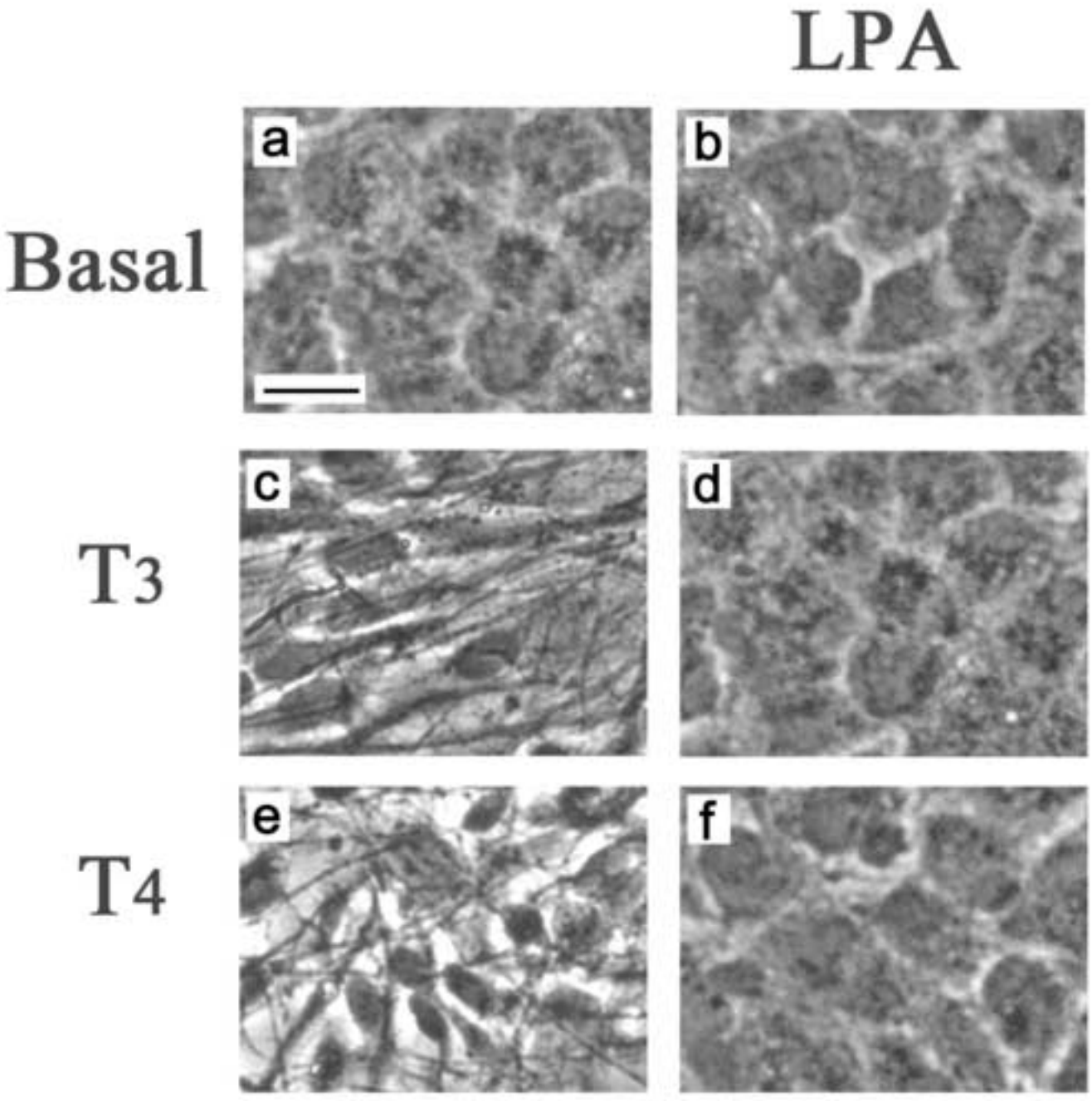

PARTE III

1. DISCUSSÃO

Os HT são essenciais para o crescimento e desenvolvimento normais, e são conhecidos pelo seu papel fundamental na regulação metabólica de muitos órgãos e tecidos (Norman & Litwack, 1997). O T_3 é responsável pelas principais ações desses hormônios, conhecidas como sendo mediadas pela interação do hormônio com receptores específicos localizados no núcleo celular e pela regulação da expressão gênica (Yen, 2001). Estes efeitos genômicos requerem um período de tempo para que ocorra a síntese protéica e a conseqüente manifestação das respostas biológicas (Wagner *et al*., 1995; Darimont *et al*., 1998). Entretanto, os HT também podem agir através de mecanismos não genômicos, extranucleares, os quais ocorrem em um curso de tempo de segundos a minutos, provavelmente mediados por receptores específicos na membrana plasmática de diferentes tipos celulares (Pliam & Goldfine, 1977; Segal *et al*., 1989; Botta *et al*., 1983; Silva *et al*., 2001). Os efeitos não genômicos dos HT são muito conhecidos e foram demonstrados na membrana plasmática, no citoesqueleto, no citoplasma e na mitocôndria de células de mamíferos (Davis *et al*., 2002). No entanto, os mecanismos envolvidos em tais efeitos são difíceis de estabelecer. Sabe-se, porém, que envolvem modulação de Ca^{2+}-ATPase, captação de glicose e polimerização de actina (Davis & Davis, 2002). Os efeitos não genômicos dos HT foram relacionados também com a atividade do trocador Na^+/H^+ na captação de aminoácidos neutros e na manutenção do potencial de membrana (Silva *et al*., 2001; Volpato *et al*., 2004; Menegaz *et al*., 2006). Os mecanismos de transdução de sinal envolvidos parecem ter a participação da rota do fosfatidilinositol, PKC, modulação da MAPK e de canais iônicos (Lin *et al*., 1999; Davis *et al*., 2000; Volpato *et al*., 2004), e alguns desses efeitos têm sido demonstrados em células não somente de mamíferos, mas de outros animais (Incerpi *et al*., 2002). Os efeitos genômicos e não genômicos para os HT são descritos como essenciais para o desenvolvimento sexual e do SNC (Bernal, 2002; Silva *et al*., 2002; Incerpi *et al*., 2005). Além disso, os HT

também estão envolvidos na diferenciação sexual do cérebro (Lechan & Fekete, 2005), porém os mecanismos moleculares envolvidos nas suas ações não são totalmente esclarecidos. Nós avaliamos algumas ações desses hormônios em células testiculares e neurais, tendo o citoesqueleto como um dos principais alvos para investigar as vias genômicas e não genômicas de sinalização hormonal.

1.1. Efeitos dos HT *sobre as células testiculares*

As células de Sertoli expressam receptores funcionais para os HT, influenciando tanto a esteroidogênese quanto a espermatogênese, e agindo principalmente durante a fase imatura do desenvolvimento animal. É bem descrito que esses hormônios afetam a proliferação das células de Sertoli e a formação tubular, aumentam a síntese protéica, estimulam a captação de aminoácidos e promovem mudanças no potencial de membrana das células de Sertoli (Silva *et al*., 2002; Volpato *et al*., 2004; Menegaz *et al*., 2006).

Considerando a importância do T_3 na fase imatura de desenvolvimento sexual, inicialmente demonstramos que este hormônio estimula a fosforilação e o aumenta o conteúdo da vimentina presente na fração citoesquelética em testículos de ratos de 15 dias de idade. Este efeito ocorreu de maneira independente da síntese protéica e dependente dos níveis intra e extracelulares de cálcio. Além disso, este efeito foi modulado diferentemente durante o desenvolvimento, sendo que evidenciamos que o T_3 estimula a fosforilação da vimentina também em ratos adultos, porém, não possui efeito em testículos de ratos púberes (dados não mostrados). Isso confirma que o T_3 desempenha um papel importante na dinâmica do citoesqueleto das células testiculares, sendo sua ação regulada durante o desenvolvimento.

Muitos dos efeitos não genômicos dos HT são mediados por aumento nos níveis intracelulares de Ca^{2+} em resposta a um estímulo no influxo deste íon, liberação dos estoques intracelulares ou devido a uma combinação destes eventos (Peuchen *et al*., 1996). Nossos

resultados evidenciaram a participação do influxo de cálcio através de L-CCDV e dos níveis intracelulares deste íon no mecanismo de ação do T_3 sobre a fosforilação da vimentina em células testiculares. Isso demonstra que flutuações nos níveis intracelulares de Ca^{2+} podem desempenhar um importante papel na reorganização do citoesqueleto, regulando, desta maneira, a fisiologia testicular.

É descrito que a dinâmica do citoesqueleto está diretamente implicada na sobrevivência, diferenciação e proliferação celular. Sinais extracelulares, hormônios e drogas são capazes de induzir as principais alterações na estrutura do citoesqueleto, regulando o comportamento celular. Há também muitas evidências indicando a fosforilação de proteínas do citoesqueleto como um importante mediador de transdução intracelular de sinais (Inada *et al.,* 1999; Helfand *et al*., 2005). Além dos efeitos demonstrados após tratamento *in vitro* com T_3, investigamos o efeito *in vivo* deste hormônio, através da indução do hipertireoidismo, sobre o citoesqueleto testicular e observamos uma hiperfosforilação da vimentina associada a um aumento na expressão desta proteína, como demonstrado pela análise de RT-PCR e imunoconteúdo total da proteína no tecido. Nós também observamos um aumento na relação entre a forma fosforilada e total da ERK1/2. Sendo assim, o aumento na expressão e/ ou fosforilação da vimentina pode estar associado à ativação da via da MAPK. A atividade da ERK modulando o citoesqueleto já foi descrita anteriormente em resposta a diversos estímulos (Chen *et al*., 2006; Liu *et al*., 2006). Além disso, Perlson e colaboradores (2005) descreveram o envolvimento de um complexo entre vimentina e fosfo-ERK em resposta à injúria neuronal. Esses resultados indicam que o tratamento *in vitro* com o hormônio interfere com os mecanismos que regulam a expressão da proteína e formação do filamento de vimentina. Isso também enfatiza a alta sensibilidade dos constituintes do citoesqueleto às alterações hormonais provocadas pelo tratamento *in vivo* com T_3. Dessa forma, o T_3 pode agir genômica ou não genômicamente em células testiculares e o citoesqueleto é um importante alvo de ação hormonal.

Os resultados, demonstrando as alterações nos FI em testículos de ratos, sugerem os HT como importantes moduladores da expressão e/ou dinâmica desses filamentos no período imaturo do desenvolvimento sexual.

As funções da fosforilação das proteínas de FI incluem desestabilização e reorganização do filamento, solubilidade, localização em domínios celulares específicos, associação com outras proteínas citoplasmáticas ou associadas à membrana, proteção contra estresse fisiológico e mediação de funções celulares especificas (Inagaki *et al*., 1996; Paul *et al*., 1999). Consistente com estes dados, os nossos resultados *in vivo* e *in vitro* sugerem que os HT são fatores regulatórios da dinâmica da vimentina de células testículares de ratos e que a fosforilação desempenha um papel fundamental na organização da estrutura do citoesqueleto, podendo ser essencial no processo de maturação da célula de Sertoli, assim como ser importante nos processos reprodutivos.

A célula de Sertoli prolifera principalmente na fase fetal e no início da vida pós-natal. Muitos estudos têm demonstrado que os HT estão envolvidos e podem ser os principais fatores que regulam o período de proliferação e a maturação dessas células (Majdic *et al*., 1998; Cooke & Meisami, 1991; Hess *et al*., 1993). Além disso, os receptores para HT são encontrados em grande quantidade na fase imatura do desenvolvimento sexual (Jannini *et al*., 1990; Jannini *et al*., 1994; Jannini *et al*., 1999). Isso sugere uma possível ação dos HT regulando a morfologia testicular durante as primeiras semanas de vida.

A análise histológica testicular nos animais controles e hipertireoideos na fase imatura do desenvolvimento sexual apresentou túbulos seminíferos com o lúmen tubular formado em ambos os grupos estudados, o que pode refletir a secreção do fluido testicular e a maturação das células de Sertoli (Russel *et al*., 1989; Van Haaster *et al.*, 1993). Nos estudos com microscopia eletrônica, observou-se que animais hipertireoideos apresentaram o aparelho de Golgi das células de Sertoli bastante desenvolvido, com vesículas dilatadas, além de vesículas de secreção,

refletindo um processamento de proteínas altamente ativo. Foi observada também a presença de polirribossomos em quantidades maiores que nos animais controles. Esses resultados indicam que as células estavam em intensa atividade de síntese e processamento de proteínas, corroborando em parte, com os resultados referentes ao aumento de expressão de vimentina nos animais hipertireoideos, além de indicar que a dose hormonal e o tempo de tratamento não causaram degeneração celular, nem toxicidade ao tecido. Segundo Francavilla *et al*. (1991) e Russel *et al*. (1989), a presença de gotas de lipídeos e o aparecimento do lúmen nos túbulos seminíferos são marcadores da maturação das células de Sertoli em ratos.

As ações dos HT sobre a regulação metabólica são bem estabelecidas e o hipertireoidismo está associado a danos oxidativos em diversos tecidos (Venditti & DiMeo, 2006). Dessa forma, verificamos que o hipertireoidismo aumenta o consumo de oxigênio e os níveis de TBARS, refletindo um aumento na peroxidação lipídica em testículos de ratos imaturos hipertireoideos. A peroxidação lipídica é um processo que envolve a redução da integridade da membrana, podendo alterar diversas funções biológicas e contribuindo significativamente para o desenvolvimento das disfunções sexuais observadas durante alterações na atividade da tireóide. É importante salientar que a peroxidação lipídica não é alterada em testículos de ratos hipertireoideos adultos (Choudhury *et al*., 2003), reafirmando a fase imatura do desenvolvimento sexual como importante alvo de estudo. Nós também avaliamos as concentrações de glutationa total, reduzida e oxidada (GT, GSH e GSSG, respectivamente) em testículos de ratos hipertireoideos e verificamos um aumento nos níveis de GSH, com diminuição de GT, sem alterar os níveis de GSSG. Considerando que a interconversão GSH/GSSG/GSH é mantida pela atividade da glutationa peroxidase (GPx) e glutationa redutase (GR), respectivamente (Venditti & DiMeo, 2006), a indução observada na atividade destas enzimas está de acordo com o aumento na concentração de GSH observado. Além disso, observamos indução na atividade da glutationa-S-transferase (GST) e da catalase, com diminuição na atividade da superóxido

dismutase. Estas respostas combinadas sugerem a geração de estresse oxidativo nos testículos de ratos hipertireoideos, provavelmente como conseqüência de um aumento na geração de radicais livres de oxigênio (ROS). Sendo assim, o aumento no consumo de oxigênio provavelmente desencadeia a geração de ROS, que é acompanhada pelo aumento nos níveis de TBARS, e isso tende a consumir o "pool" celular de antioxidantes, como a GT, enquanto observa-se indução na maioria das defesas antioxidantes enzimáticas.

É importante salientar que, além do controle hormonal, o sistema reprodutivo também pode ser modulado pela ação das purinas extracelulares, como ATP e adenosina, agindo sobre os purinoceptores (Mônaco *et al*., 1984; Ko *et al*., 2003; Mineli *et al*., 2004). O papel dos nucleotídeos extracelulares, como importantes moduladores de diferentes funções celulares, é bem estabelecido em testículos, onde há uma rede complexa de interações célula-célula entre as células de Sertoli e germinativas (Gordon, 1986; Filippini *et al*., 1994). Os níveis dos nucleotídeos de adenina extracelulares são controlados pela ação das E-NTPDases assim como da ecto-5'-nucleotidase, que regulam a hidrólise de ATP até adenosina (Plesner, 1995; Zimmermann, 2000). Nossos resultados evidenciaram uma inibição na atividade das NTPDases em culturas de células de Sertoli de ratos com hipotireoidismo congênito. A inibição na hidrólise de ATP e ADP, observada nas células de animais hipotireoideos, pode acarretar em acúmulo de ATP extracelular (ATPe). Considerando o papel fisiológico do ATPe regulando as funções das células de Sertoli, como a modulação do influxo de Ca^{2+}, podemos atribuir que níveis elevados de ATP podem induzir efeitos citotóxicos ou podem estar envolvidos nas alterações observadas durante os processos de proliferação e maturação destas células. Estes eventos podem estar relacionados com as disfunções reprodutivas descritas durante o hipotireoidismo (Longcope, 1991). Além disso, não foram observadas alterações na expressão das NTPDases 1, 2 e 3. O tratamento das células de Sertoli de ratos hipotireoideos com T_3 ou T_4 por períodos curtos, mas não por períodos longos de exposição hormonal, promoveu indução da atividade das NTPDases.

Estes resultados indicam que o hipotireoidismo influencia a atividade das NTPDases em células de Sertoli através de mecanismos que não envolvem as ações genômicas clássicas mediadas por alterações da tireóide. Além disso, a reversão dos efeitos do hipotireoidismo após o tratamento *in vitro* com HT reforça a idéia de mecanismos não genômicos desses hormônios sobre as células de Sertoli envolvendo a modulação da atividade das NTPDases. Dessa forma, propomos que os mecanismos envolvidos na regulação das células de Sertoli pelos HT podem estar relacionados também com a ativação de receptores purinérgicos presentes nestas células. O ATPe pode desempenhar um papel fundamental nestes efeitos, visto que se observa uma modulação dos níveis extracelulares deste nucleotídeo tanto no hipotireoidismo quanto após a suplementação com T_3 ou T_4.

A Figura 9 apresenta um modelo proposto para ação dos HT sobre as células testiculares.

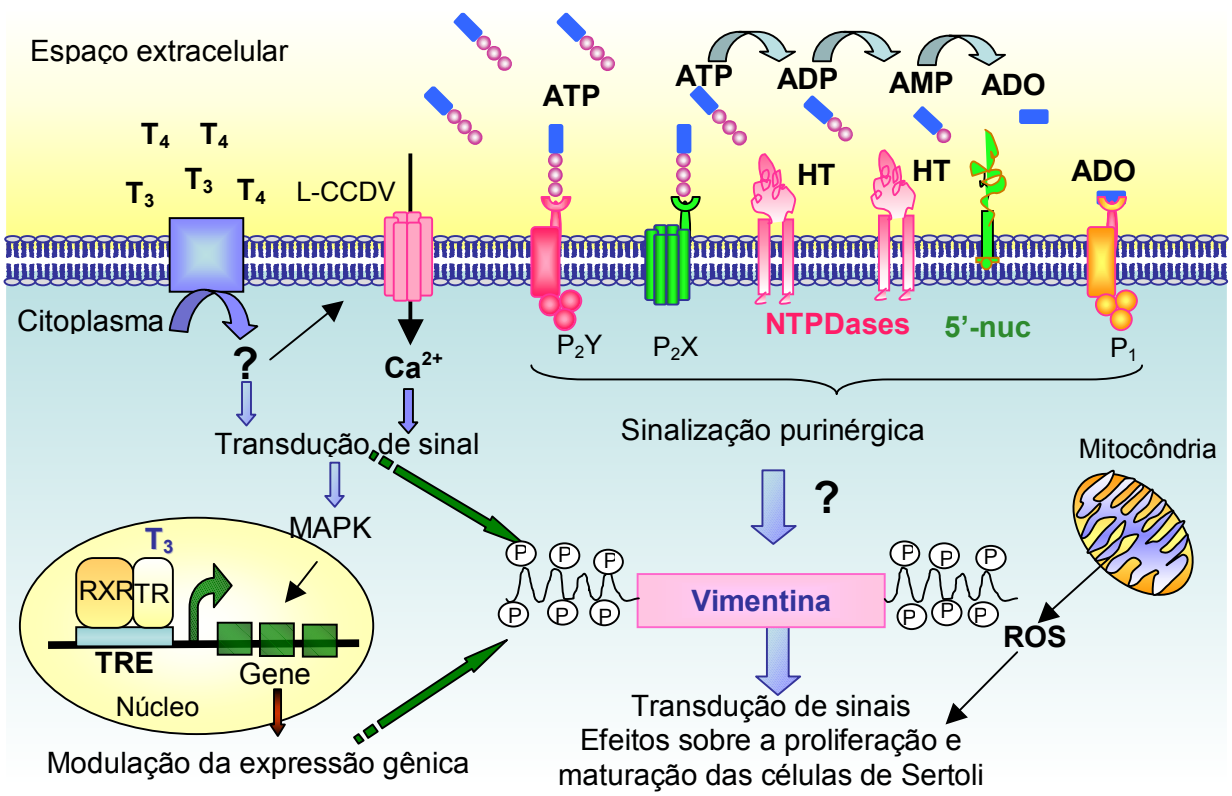

Figura 9. Mecanismo proposto para ação dos HT sobre as células testiculares. As células de Sertoli possuem receptores nucleares ativos para T_3 na fase imatura do desenvolvimento, e os

efeitos dos HT podem envolver a modulação da transcrição de diversos genes, inclusive da vimentina. Considerando as ações não-genômicas descritas para o T_3 modulando a fosforilação da vimentina através de mecanismos envolvendo o aumento nos níveis intracelulares de Ca^{2+}, propomos que estes hormônios podem agir tanto na membrana quanto no núcleo, ativando diferentes rotas de sinalização, além da possibilidade de uma inter-relação entre os efeitos genômicos e não-genômicos para estes hormônios, como evidenciado pela atividade da MAPK. Além disso, a indução de estresse oxidativo está relacionada com ação dos HT sobre as células testiculares devido à elevada atividade metabólica, bastante conhecida para estes hormônios em outros tecidos. Também salientamos que os HT podem regular a atividade das NTPDases e da 5'-nucleotidase (5'nuc), modulando desta forma os níveis extracelulares dos nucleotídeos de adenina e, conseqüentemente, a ação destes sobre os purinoceptores (P2X, P2Y e P1). Sendo assim, a ação dos nucleotídeos sobre seus receptores também poderia desempenhar um papel importante na modulação da dinâmica da vimentina, assim como na ativação cascatas de sinalização importantes para a atividade reprodutiva através de diversas rotas metabólicas, desencadeando uma resposta biológica.

Em resumo, o conjunto dos resultados observados em células testiculares demonstra uma resposta do citoesqueleto, em particular da vimentina, à ação hormonal do T_3 durante a maturação sexual, enfatizando o complexo envolvimento das flutuações nos níveis de Ca^{2+} intracelular e dos mecanismos de fosforilação neste processo. Além disso, o estudo do envolvimento do sistema purinérgico sobre a dinâmica citoesqueleto pode ser de extrema importância para o melhor entendimento dos efeitos dos HT sobre o desenvolvimento sexual. Entretanto, pesquisas mais aprofundadas são necessárias para esclarecer os mecanismos deste evento. Presume-se que interferências neste sistema poderiam provavelmente auxiliar num melhor entendimento das ações do T_3 nas células testiculares normais, e poderiam contribuir, ao menos em parte, para o esclarecimento das disfunções celulares que ocorrem em pacientes com hipo ou hipertireoidismo.

1.2. Efeitos dos HT *sobre as células neurais*

O papel dos HT na diferenciação do SNC é intensamente documentado devido a suas funções criticas em vários aspectos durante a morfogênese, regulação da expressão gênica, desenvolvimento de habilidades motoras e ações sobre a atividade cognitiva (Schwartz *et al.*,

1997; Bernal *et al*., 2002). A expressão e distribuição dos componentes do citoesqueleto têm sido consideradas como sendo importantes alvos dos HT durante o desenvolvimento do SNC, e são responsáveis pelos efeitos do T_3 sobre o crescimento axonal e a arquitetura dendrítica. Estas ações são conhecidas como sendo mediadas por mecanismos genômicos do T_3 sobre o desenvolvimento de células neurais (Anderson, 2001). Considerando-se que as propriedades dinâmicas da rede de FIs são reguladas pela atividade coordenada de proteínas quinases e fosfatases (Izawa & Inagaki, 2006), e que diversas ações não genômicas dos HT envolvem a modulação de cascatas de sinalização envolvendo quinases e fosfatases, nós utilizamos modelos experimentais de tratamento hormonal com o intuito de verificar efeitos não genômicos dos HT sobre o influxo de Ca^{2+} e a fosforilação do citoesqueleto de células neurais em ratos imaturos.

Inicialmente demonstramos que os HT estimularam a fosforilação das proteínas de FIs estudadas (GFAP, vimentina, NF-L e NF-M) através de mecanismos não genômicos dependentes da atividade da PKA e da PKCaMII em fatias de córtex cerebral de ratos de 10 dias de idade. O estudo dos mecanismos de sinalização dos HT nesta idade é de fundamental importância, visto que a atividade da tireóide em ratos de 10 dias de idade, equivale ao humano recém-nascido (Bernal, 2002). A união de neurotransmissores a seus receptores específicos pode causar alterações na concentração intracelular de Ca^{2+} e AMPc, promovendo a ativação de várias enzimas, entre elas proteínas quinases e fosfatases, provocando respostas celulares ao estímulo externo (Cooper *et al*., 2001). Considerando-se que trabalhos anteriores do nosso laboratório descreveram aumento na fosforilação de FIs de ratos jovens através da modulação do sistema GABAérgico (Funchal *et al*., 2004a) e que o T_3 tem sido descrito como importante modulador dos receptores $GABA_A$ (Chapell *et al*, 1998; Martin *et al*., 1996), nós investigamos o envolvimento desse sistema no mecanismo de ação dos HT sobre o sistema fosforilante associado ao citoesqueleto. Nossos resultados demonstraram que a hiperfosforilação induzida por T_3 envolve a ativação de receptores $GABA_A$ através de mecanismos não-competitivos, além

da ação via receptores $GABA_B$, como demonstrado pela utilização dos antagonistas picrotoxina e faclofen, respectivamente. Entretanto, o efeito do T_4 não envolve a participação de receptores $GABA_A$, e envolve apenas parcialmente a modulação de receptores $GABA_B$, sugerindo um mecanismo de sinalização mais complexo na ação deste hormônio sobre a modulação do citoesqueleto. Nesse contexto, é importante enfatizar que o T_4 é um importante ativador da transdução de sinais na membrana plasmática. Os mecanismos de ação diferenciais para T_3 e T_4 corroboram com a idéia de que além das importantes ações dos HT via receptores nucleares, os HT podem alterar, não genomicamente, distintas vias de sinalização celular, tendo o citoesqueleto como alvo.

Os efeitos dos HT sobre o citoesqueleto através do sistema GABAérgico estão de acordo com trabalhos anteriores descrevendo interações entre receptores $GABA_A$ e citoesqueleto, sendo responsáveis pelo transporte, ancoramento e atividade sináptica destes receptores (Wang & Olsen, 2000). Os microtúbulos participam da manutenção da distribuição normal desses receptores nos neurônios e os microfilamentos modulam a expressão gênica das subunidades dos receptores $GABA_A$ (Ho *et al*., 2001). Além disso, Runquist & Alonso (2003) descreveram que a sinalização GABAérgica modula a expressão de GFAP e a morfologia de astrócitos adultos, reforçando a importância deste sistema na modulação do citoesqueleto de células neurais. Entretanto, nosso trabalho demonstra a primeira evidência de modulação não genômica da dinâmica dos FIs modulada por HT tanto em células neuronais como gliais através de mecanismos GABAérgicos. A complexidade dos processos envolvidos nos mecanismos de ação distintos para T_3 e T_4, sugere que existem múltiplos sítios de regulação para os HT e que o T_4 tem um efeito independente de sua conversão a T_3 via deiodinases.

Diversos sinais extracelulares, como neurotransmissores, hormônios e fatores de crescimento, modulam a sinalização celular através de eventos moleculares e biofísicos, ativando respostas intracelulares através do aumento dos níveis citoplasmáticos de Ca^{2+} (Bird & Putney,

2006). Muitos dos mecanismos não genômicos dos HT desencadeados na membrana plasmática envolvem a modulação de bombas e canais iônicos (Lösel & Wehling, 2003). Considerando-se que o influxo de Ca^{2+} mediado por estes hormônios tem sido descrito há muitos anos (Segal *et al*., 1989), nós investigamos o efeito dos HT sobre a captação de $^{45}Ca^{2+}$ em fatias de córtex cerebral de ratos 10 dias de idade, visto que, nesta idade, observamos aumento na fosforilação dos FIs por mecanismos envolvendo a ativação da PKCaMII, que possui sua atividade modulada pelos níveis intracelulares deste íon. Nossos resultados demonstraram que tanto o T_3 quanto o T_4 estimulam a captação de $^{45}Ca^{2+}$ após 30 segundos (s) e 5 minutos de incubação com o hormônio. Curiosamente, 60 s de exposição hormonal não foi capaz de alterar o influxo deste íon. Isso indica uma janela temporal de ação altamente regulada por estes hormônios, demonstrando um efeito hormonal bifásico. O perfil diferencial de efeito observado na curva de tempo com os hormônios (30, 60 e 300 s) evidencia que há uma combinação complexa de mecanismos que podem ser modulados por voltagem ou por níveis intracelulares de cálcio, envolvendo a ativação de Ca^{2+}-ATPases específicas (Bird & Putney, 2006; Evans & Zamponi, 2006). Além disso, a ação estimulatória do T_4 na concentração de 10^{-7} M sobre a captação de Ca^{2+} demonstra que este hormônio é 10 vezes mais potente que o T_3, que apresentou efeito apenas em 10^{-6} M. Também é importante salientar que o T_4 desencadeia este efeito em concentrações fisiológicas (Davis *et al*., 2002), enquanto o T_3 apenas possui efeito em concentração supra-fisiológica. Estes resultados estão de acordo com trabalhos anteriores demonstrando que o T_4 é mais potente que o T_3 na atividade antiviral (Lin *et al* 1997; Davis *et al*., 2000) e na captação de aminoácidos (Menegaz *et al* 2006).

Os efeitos fisiológicos envolvendo a modulação dos níveis intracelulares de Ca^{2+} são dependentes da ativação dos diferentes subtipos de canais de Ca^{2+}, das várias proteínas regulatórias e das vias de sinalização ativadas em grupos específicos de células (Evans & Zamponi, 2006). Assim, nós verificamos a participação de L- e T-CCDVs no mecanismo de ação

dos HT sobre a captação de $^{45}Ca^{2+}$ em fatias de córtex cerebral de ratos, utilizando para isso bloqueadores específicos destes canais (nifedipina e flunarizina, respectivamente) (Bird & Butney, 2006; Perier *et al*., 1992). Apesar de diversos trabalhos descreverem a participação dos íons Cl^- dirigindo o influxo de Ca^{2+} (Kerschbaum *et al*., 1997) e participando da modulação da transdução de sinal mediada por Ca^{2+} (Lai *et al*., 2003), nossos resultados demonstraram que os canais de Cl^- não estão envolvidos no mecanismo de ação dos HT sobre a captação de $^{45}Ca^{2+}$ em fatias de córtex cerebral de ratos.

O aumento no influxo de Ca^{2+} via CCDV tem sido descrito para HT (Watanabe *et al*., 2005). O influxo rápido de Ca^{2+} ocorre em resposta a diversos sinais extracelulares e as células respondem a estas oscilações através de mecanismos regulatórios sofisticados, incluindo a fosforilação dos domínios citoplasmáticos dos CCDV. Neste contexto, Shistik e colaboradores (1998) descreveram uma supra-regulação dos L-CCDV mediada por PKC em células musculares lisas e cardíacas. Além disso, nossos resultados, demonstrando a participação da atividade da PKC na captação de $^{45}Ca^{2+}$ ativada por T_3 e T_4, estão de acordo com trabalhos anteriores sugerindo a modulação da atividade da PKC induzida por HT (Kavoc *et al*., 2001; Lösel & Wehling, 2003; D'Arezzo *et al*., 2004; Davis *et al*., 2005). A modulação dos CCDV via PKA também tem sido demonstrada (Hall *et al*., 2006), inclusive como sendo modulada por HT (Watanabe *et al*., 2005) em cérebro e coração. A importância da atividade da PKA na captação de $^{45}Ca^{2+}$ induzida por T_3 foi confirmada utilizando-se o KT5720 como inibidor específico desta quinase. Entretanto, o mecanismo de ação do T_4 parece não envolver a ativação da PKA, indicando que outros mecanismos podem estar envolvidos e confirmando os dados encontrados sobre o sistema fosforilante associado ao citoesqueleto observado em nosso estudo anterior que descreve mecanismos de ação diferenciais para T_3 e T_4. Dessa forma, é possível afirmar que T_3 e T_4 possuem mecanismos de ação desencadeados na membrana plasmática que são ativados em tempos muito curtos de incubação (30 s), modulando vias de transdução de sinal que não são

necessariamente mediadas pelos mesmos mecanismos, sugerindo múltiplos sítios de regulação hormonal.

Considerando-se que demonstramos que os HT estimulam não genomicamente a fosforilação dos FIs via mecanismos GABAérgicos em córtex cerebral de ratos de 10 dias de idade, investigamos o efeito destes hormônios sobre o sistema fosforilante associado ao citoesqueleto em 15 dias de idade, período que coincide com o pico de sinaptogênese em ratos. Nossos resultados demonstraram que nessa idade apenas o T_4 estimulou a fosforilação das proteínas de FI estudadas. Entretanto, o tratamento das fatias de tecido com GABA, glutamato, ATP ou epinefrina não promoveu alterações neste parâmetro. A evidência de que nem T_3 nem GABA alteraram a fosforilação em córtex cerebral de ratos de 15 dias de idade, apesar dos resultados anteriores demonstrando o envolvimento do GABA na fosforilação e no mecanismo de ação hormonal em córtex cerebral de ratos de 10 dias, sugere que os HT possuem mecanismos não genômicos rápidos e regulados diferentemente durante o desenvolvimento do SNC. Nossas observações demonstram uma nova e interessante contribuição dos HT sobre a função do citoesqueleto no cérebro em desenvolvimento. É importante salientar que Farwell e colaboradores (2005) descreveram ações não genômicas do T_4 sobre o citoesqueleto de actina. Além disso, eles demonstraram que é o T_4 e não o T_3, o HT modulador crítico do citoesqueleto neuronal, atuando não genomicamente.

A ação do T_4 sobre a fosforilação dos FIs é dependente de proteínas G inibitórias - Gi (sensíveis à toxina pertussis), sendo que as vias de transdução de sinal envolvidas no mecanismo de ação hormonal são a rota da PLC, PKC, MAPK, PKCaMII e Ca^{2+}. Nós sugerimos que o mecanismo de ação do T_4 seja iniciado na membrana celular através de um receptor acoplado a proteínas Gi (GPCR), modulando o citoesqueleto. Estes dados são evidenciados pela inibição da ação do T_4 por toxina pertussis, pelo fato de que a inibição da atividade da PKA não impede o efeito hormonal e devido à diminuição nos níveis de AMPc após exposição ao T_4 por um curto

período de tempo. Todos estes dados, em conjunto, evidenciam a participação de proteínas Gi no mecanismo não genômico de ação do T_4, a qual já foi previamente descrita em outros tecidos (Sundquist *et al*., 1992; Lin *et al*., 1997, 1999; Davis *et al*., 1995, 2000). É importante salientar, também, que embora o T_3 não altere a fosforilação do citoesqueleto em ratos de 15 dias de idade, ele também diminui os níveis intracelulares de AMPc, apesar deste efeito ser menor que o produzido por T_4. Dessa forma, propomos que podem ocorrer outros eventos não genômicos, além da fosforilação, sendo desencadeados por T_3 através da inibição da adenilato ciclase (AC). Essas observações enfatizam ainda mais os mecanismos diferenciais e a especificidade de ação destes hormônios durante o desenvolvimento do SNC.

A atividade da PKC modulada por HT já foi demonstrada previamente (Lawrence *et al*., 1989; Sundquist *et al*., 1992; Lin *et al*., 1999), e tem sido implicada no antiporter Na^+/H^+ (Incerpi *et al*., 1999), no aumento das correntes de Na^+ (Huang *et al*., 1999) e na atividade de Ca^{2+}-ATPases (Smallwood *et al*., 1988; Warnick *et al*., 1993; Davis *et al*., 1995). Por outro lado, Kavok e colaboradores (2001) descreveram o mecanismo de ação não genômico do T_4 como sendo dependente da ativação da PLC. Então, investigamos o envolvimento da via da PLC no mecanismo de ação do T_4 sobre a fosforilação dos FI, utilizando o U73122 como inibidor desta enzima. O envolvimento da PLC na sinalização mediada por T_4 sugere que a ativação da PKC seja dependente de diacilglicerol (DAG). Considerando que a PLC produz a hidrólise do fosfatidilinositol em tri-fosfato de inositol (IP_3) e DAG, o IP_3 produzido pode estar aumentando os níveis de Ca^{2+} intracelulares através de liberação dos estoques do retículo endoplasmático (RE) (Nestler & Dumas, 2006). Esta hipótese pode ser comprovada pela co-incubação do tecido com BAPTA-AM, um quelante de Ca^{2+} intracelular e T_4, o que impede a ação do hormônio. Além disso, nós também evidenciamos a participação do influxo de Ca^{2+} através da captação de $^{45}Ca^{2+}$. O aumento na concentração de Ca^{2+} no ambiente intracelular pode ser o responsável pela ativação da PKCaMII que está parcialmente envolvida no mecanismo de ação do T_4. Estes dados

são consistentes com trabalhos anteriores de nosso grupo demonstrando que esta quinase está associada ao citoesqueleto (Funchal *et al*., 2005).

As ações não genômicas dos HT também têm sido constantemente associadas à ativação da rota da MAPK, sendo esta via de transdução de sinal um importante integrador entre as vias genômicas e não genômicas de sinalização hormonal, comunicando a membrana com o núcleo celular (Lin *et al*., 1999; Davis *et al*., 2000; Kosawa *et al*., 2001; Shih *et al*., 2001; Lösel & Wehling, 2003; Davis *et al*., 2005). Além disso, sugere-se que a via de sinalização da MAPK pode ser ativada por PKC (Kolch *et al*., 1993; Marais *et al*., 1998; Lin *et al*., 1999; Davis *et al*., 2000). Sendo assim, os resultados demonstrados em nosso estudo corroboram os estudos de Lin e colaboradores (1999) que descrevem a ativação de proteínas Gi, PKC e MAPK em células não nucleadas. Estes resultados evidenciam que o T_4 atua através de mecanismos complexos e reforçam que ele não é um pró-hormônio, como tem sido considerado, mas tem ações fisiológicas importantes na dinâmica das células neurais durante o desenvolvimento, podendo ativar diversas vias de sinalização.

Considerando-se os nossos resultados demonstrando a ação dos HT sobre os FIs neuronais e gliais em fatias de tecido, investigamos também a ação destes hormônios sobre o citoesqueleto de células gliais em cultura, para avaliar o efeito hormonal direto sobre a organização do citoesqueleto. Neste contexto, demonstramos que os HT alteram a morfologia de células de glioma C6 e em cultura primária de astrócitos primários em diferentes concentrações e tempos de exposição hormonal. Através de análise imunocitoquímica, observamos que as alterações na forma das células C6 envolvem a reorganização dos filamentos de actina e de GFAP. Diversas evidências na literatura sugerem que a organização estrutural da rede de FIs é regulada espacialmente e temporalmente por fosforilação (Inagaki *et al*., 1996; Ackerley *et al*., 2000; Izawa & Inagaki, 2006). Sendo assim, avaliamos o efeito dos HT sobre a fosforilação da GFAP na fração citoesquelética enriquecida em FI polimerizados e/ou agregados (Funchal *et al*.,

2003) e observamos que o T_3 estimula a fosforilação da GFAP, enquanto o T_4, possui efeito estimulatório apenas com a concentração mais baixa utilizada e após 24 h de incubação. Entretanto, é importante salientar, que apesar de o T_4 não alterar a fosforilação, ele altera a morfologia e induz a reorganização do citoesqueleto de GFAP e de actina. Isso sugere, novamente, um mecanismo de ação diferencial para T_3 e T_4, sendo que o T_4 possui um efeito próprio e independente de sua conversão em T_3 via deiodinases.

Os resultados observados para T_4 sobre a reorganização dos filamentos de actina corroboram trabalhos anteriores que descrevem que este hormônio altera a dinâmica da organização dos MFs, regulando a polimerização de actina através de mecanismos extranucleares (Siegrist-Kaiser, 1990; Farwell & Leonard, 1997; Farwell *et al*., 2005). Assim, esses resultados constituem uma nova demonstração da habilidade do T_4 em modular o citoesqueleto.

Os FIs estão envolvidos na transdução de sinal em sítios da membrana plasmática que regulam a atividade de GTPases, condutividade iônica, liberação intracelular de cálcio, assim como fosforilação de proteínas (Janmey, 1998). Identificar as proteínas quinases envolvidas na fosforilação dos FIs e na sinalização celular também é de grande importância para se compreender como a reorganização celular dos FI é regulada. Neste contexto, nós também demonstramos que os efeitos dos HT sobre a morfologia de gliomas C6 e astrócitos em cultura primária foram desencadeados pela inibição da atividade da RhoA, uma pequena GTPase monomérica. Este mecanismo foi comprovado pela utilização do ácido lisofosfatídico (LPA), um ativador específico da RhoA, que previniu as alterações morfológicas induzidas pelos HT em ambos os tipos celulares, implicando que a inibição da via de sinalização desta GTPase provavelmente está mediando os efeitos hormonais. Nossos resultados confirmam estudos anteriores demonstrando que as atividades do LPA e da RhoA estão envolvidas na morfologia de astrócitos (Sah *et al*., 2000; Guasch *et al*., 2003; Funchal *et al*., 2004b). O mecanismo envolvido

na inibição da atividade da RhoA pelos HT não é conhecido. Entretanto, resultados anteriores demonstraram que níveis elevados de AMPc levam à inibição da RhoA em diversos tecidos, incluindo a tireóide (Lang *et al.,* 1996; Dong *et al.*, 1998; Scott & Leopardi, 2003; Fortemaison *et al.*, 2005), e que a PKA inibe a atividade de pequenas GTPases em células epiteliais (Bakre *et al.*, 2002). Além disso, nós descrevemos o envolvimento da PKA na fosforilação dos FIs mediada pelos HT em córtex cerebral de ratos de 10 dias de idade. Sendo assim, os efeitos dos HT sobre a dinâmica do citoesqueleto, evidenciados neste trabalho, envolvem a inibição da atividade da RhoA, e sugerimos o envolvimento da ativação da PKA nas ações hormonais sobre a organização dos FIs e MF de células C6 e astrócitos.

Os mecanismos de sinalização e as diferentes rotas de transdução de sinal para os HT nas células neurais estão propostos na Figura 10.

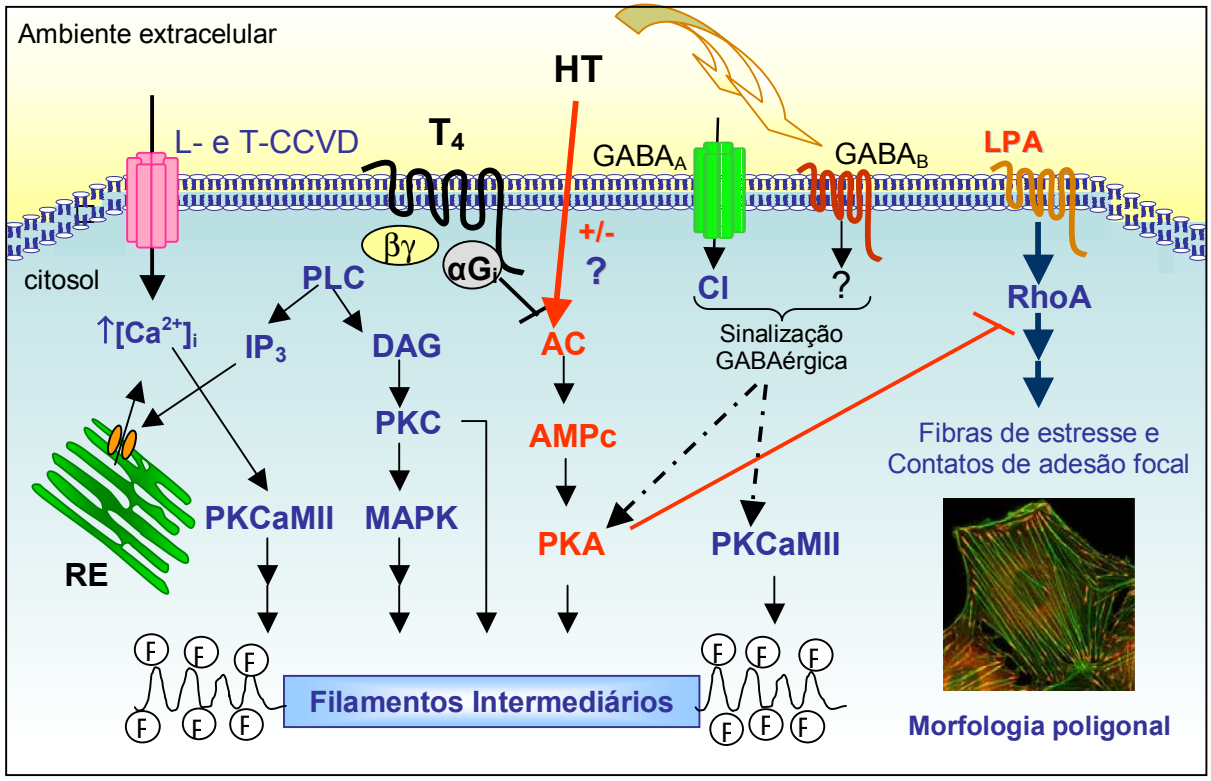

Figura 10. Mecanismos de sinalização propostos para as ações não genômicas dos HT sobre células neurais. O mecanismo de ação para o T_4 sobre o sistema fosforilante é postulado como iniciando pela interação do hormônio com o receptor acoplado à proteína Gi (GPCR). Dessa forma, observa-se uma inibição da atividade da AC, com conseqüente diminuição dos níveis de AMPc, e não ativação da PKA. A subunidade βγ da proteína G regula a atividade da PLC, formando IP_3 e DAG. O IP_3 formado libera Ca^{2+} de RE, e a ação conjunta do DAG e dos níveis

elevados de Ca^{2+} ativa a PKC, que pode estar ativando a MAPK. Evidenciamos também um aumento no influxo de Ca^{2+}, que pode estar sendo ativado por PKA e/ou PKC, contribuindo desta forma para o aumento dos níveis intracelulares de Ca^{2+}, e este pode estar ativando a PKCaMII. Dependendo do estágio do desenvolvimento, observa-se a ativação da via da AC levando à ativação da PKA. As atividades coordenadas da PKA, PKC, MAPK e PKCaMII estimulam a fosforilação das proteínas de FI por mecanismos desencadeados pelos HT. A ativação do sistema GABAérgico ou de GPCR é regulada diferentemente durante o desenvolvimento. Além disso, os HT são hábeis em induzir alterações morfológicas e reorganização do citoesqueleto de células gliais através de mecanismos dependentes da inibição da RhoA. A PKA possui uma modulação altamente regulada durante o desenvolvimento e pode ser responsável pela inibição da RhoA GTPase.

A reorganização do citoesqueleto é de extrema importância na regulação da estrutura e função celulares. Esta reorganização pode ter conseqüências importantes, incluindo alterações no transporte de proteínas, na captação e liberação de neurotransmissores e no controle dos níveis intracelulares de Ca^{2+}. Analisando nossos resultados em conjunto, propomos que os hormônios T_3 e T_4 são importantes moduladores da dinâmica do citoesqueleto de células neurais e que seus efeitos sobre a organização do citoesqueleto podem estar envolvidos nos seus efeitos morfogênicos sobre a migração neuronal, a sinaptogênese e o desenvolvimento do SNC.

2. CONCLUSÕES

2.1. Conclusão geral

Células neurais do córtex cerebral e células testiculares de ratos jovens são alvo de ações não genômicas, além das clássicas ações genômicas dos HT, sugerindo que a importância fisiológica destes hormônios durante o desenvolvimento dos sistemas nervoso e reprodutor também é devida a eventos rápidos que independem de mecanismos nucleares de longa duração. Experimentos *in vitro* demonstraram que o citoesqueleto de neurônios, astrócitos, bem como de células de Sertoli é modulado por ações não genômicas do T_3 e do T_4, através da regulação do sistema fosforilante endógeno associado aos filamentos intermediários. Além disso, os HT alteram a morfologia e a organização do citoesqueleto em células gliais em cultura.

As ações dos HT sobre o citoesqueleto são dependentes do desenvolvimento e acontecem através de mecanismos rápidos de sinalização celular, envolvendo a atividade de canais de Ca^{2+}, ativação de cascatas de proteínas quinases e de GTPases monoméricas. Além disso, evidenciamos que o T_4 desencadeia ações próprias sobre o citoesqueleto, diferentes daquelas induzidas pelo T_3, fortemente sugerindo que o T_4 participa de importantes ações na maturação dos tecidos alvos.

O hipertireoidismo induz estresse oxidativo e modula a expressão gênica, causando alterações histológicas compatíveis com o aumento da síntese protéica em testículos de ratos jovens. Por outro lado, o hipotireoidismo está associado à inibição da atividade das NTPDases em células testiculares através de mecanismos não genômicos.

2.2. Conclusões Específicas

O T_3 aumenta o conteúdo e a fosforilação da vimentina presente na fração citoesquelética através de mecanismos dependentes de cálcio em testículos de ratos imaturos.

O hipertireoidismo estimula a expressão e fosforilação da vimentina, associada ao aumento na forma fosforilada da MAPK em testículos de ratos imaturos.

O hipertireoidismo induz estresse oxidativo e atividade antioxidante em testículos de ratos de 15 dias de idade.

O hipotireoidismo congênito está associado à inibição da atividade das NTPDases, sem alterar a expressão das NTPDases 1,2 e 3, em cultura de células de Sertoli de ratos jovens.

Os HT estimulam, através de mecanismos não genômicos, a atividade NTPDásica em cultura de células de Sertoli de ratos hipotireoideos.

Os HT estimulam a fosforilação das proteínas de FI de córtex cerebral de ratos de 10 dias de idade via mecanismos GABAérgicos não genômicos dependentes da PKA e PKCaMII.

Os hormônio T3 estimula a captação de $^{45}Ca^{++}$ em fatias de córtex cerebral de ratos de 10 dias de idade via L- e T-CCDV e ativação de PKA e PKC, enquanto o mecanismo do T4 não depende da atividade de PKA.

Em córtex cerebral de ratos de 15 dias de idade, os HT estimulam não genomicamente a fosforilação de FI através de mecanismos dependentes de proteínas G inibitórias e ativação de cascatas de sinalização via cálcio, PLC, PKC, PKCaMII e MAPK.

Os HT induzem a reorganização do citoesqueleto em cultura de células C6 e astrócitos através de mecanismos dependentes de RhoA.

3. PERSPECTIVAS

Os resultados obtidos neste trabalho vislumbram novas possibilidades de estudos com a finalidade de elucidar as diferentes rotas de sinalização envolvidas no mecanismo de ação dos HT e na fisiopatologia do hipo- e hipertireoidismo. Dessa forma, nossas perspectivas são:

- ✓ Verificar o efeito do T_4 sobre as proteínas do citoesqueleto em testículos de ratos imaturos.
- ✓ Estudar a participação do sistema purinérgico nos efeitos dos HT sobre o sistema nervoso e o sistema reprodutor.
- ✓ Estudar os efeitos do hipo- e hipertireoidismo sobre a expressão e a dinâmica das proteínas do citoesqueleto em cérebro de ratos.
- ✓ Dosar os níveis de S-100 B em sangue e líquor de ratos com hipo- e hipertireoideos.
- ✓ Avaliar as alterações nos níveis de peptídeo β-amilóide em cérebro de ratos com hipotireoidismo congênito.
- ✓ Estudar o efeito do hipotireoidismo congênito sobre a fosforilação da proteína *tau.*
- ✓ Verificar os efeitos *in vivo* e *in vitro* dos HT sobre a captação e liberação de glutamato.

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5. ANEXOS

5.1. LISTA DE FIGURAS