UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS – BIOQUÍMICA

EFEITOS DO SULFITO E DO TIOSSULFATO SOBRE A HOMEOSTASE ENERGÉTICA E REDOX E FUNÇÃO MITOCONDRIAL EM CÉREBRO DE RATOS

MATEUS GRINGS

ORIENTADOR: Prof. Dr. GUILHIAN LEIPNITZ

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PARTE I

Introdução e Objetivos

RESUMO

O sulfito e o tiossulfato estão acumulados em tecidos e líquidos biológicos de pacientes afetados pela deficiência da sulfito oxidase (SO), uma enzima mitocondrial que catalisa a oxidação de sulfito derivado do metabolismo de aminoácidos sulfurados. A deficiência da SO é causada pela deficiência isolada da enzima SO ou por uma deficiência na rota de biossíntese de seu cofator molibdênio. Os indivíduos afetados por esta desordem apresentam disfunção neurológica progressiva, convulsões neonatais severas, subluxação do cristalino, hipotonia axial, hipertonicidade periférica e atraso no desenvolvimento, resultando geralmente em morte prematura. Considerando que a fisiopatologia do dano neurológico encontrado em pacientes deficientes para a SO ainda não está esclarecida, o objetivo do presente trabalho foi investigar os efeitos *in vitro* do sulfito e do tiossulfato sobre parâmetros de metabolismo energético e homeostase redox e mitocondrial em cérebro de ratos jovens. Inicialmente, verificamos que o sulfito inibe a atividade do complexo IV da cadeia respiratória, indicando que este composto prejudica o fluxo de elétrons, enquanto que o tiossulfato não afetou a atividade de nenhum dos complexos da cadeia respiratória em sobrenadantes de córtex cerebral. Também foi verificado que o sulfito e o tiossulfato diminuem a atividade da creatina quinase total (tCK) e de suas isoformas mitocondrial e citosólica, sugerindo que estes compostos prejudicam o tamponamento e a transferência de energia celular no cérebro. Além disso, melatonina, trolox (análogo solúvel do α-tocoferol), glutationa e o inibidor da óxido nítrico sintase N^{ω}-nitro-L-arginina metil éster atenuaram ou preveniram totalmente a inibição da tCK induzida por sulfito e tiossulfato, sugerindo o envolvimento de espécies reativas de oxigênio e nitrogênio nestes efeitos. O sulfito e o tiossulfato também aumentaram a oxidação da 2',7'-diclorofluorescina e inibiram a atividade da aconitase, enquanto que somente o sulfito aumentou a produção de peróxido de hidrogênio, reforçando o envolvimento de dano oxidativo nos efeitos provocados por estes metabólitos. Contudo, a atividade da enzima Na^+,K^+-ATPase sináptica não foi alterada pelo sulfito e tiossulfato. Em seguida, observamos que o sulfito dissipa o potencial de membrana mitocondrial na presença de Ca^{2+}, de forma dose-dependente de sulfito e Ca^{2+} em preparações mitocondriais de cérebro de ratos. O sulfito também induziu inchamento e diminuiu a capacidade de retenção de Ca^{2+}, os níveis de NAD(P)H na matriz e o imunoconteúdo de citocromo c em mitocôndrias quando Ca^{2+} estava presente no meio. Além disso, as alterações provocadas pelo sulfito foram prevenidas por rutênio vermelho, ciclosporina A e ADP, sugerindo que o sulfito induz transição da permeabilidade mitocondrial (MPT). Também foi verificado que dentre vários inibidores da MPT, incluindo antioxidantes, inibidores da fosfolipase A2 e o regente redutor ditiotreitol, apenas o agente alquilante de tióis N-etilmaleimida foi capaz de prevenir o inchamento mitocondrial causado por sulfito. O sulfito também diminuiu o conteúdo de grupamentos tiol de proteínas de membrana em preparações mitocondriais de cérebro, indicando que este composto age diretamente sobre grupamentos tiol contidos no poro de MPT. Assim, pode-se presumir que o prejuízo no metabolismo energético e na homeostase redox causados pelo sulfito e pelo tiossulfato e que a indução de MPT pelo sulfito podem estar envolvidos na disfunção neurológica observada nos portadores da deficiência da SO.

ABSTRACT

Sulfite and thiosulfate accumulate in tissues and biological fluids of patients affected by the deficiency of sulfite oxidase (SO), which is a mitochondrial enzyme that catalyzes the oxidation of sulfite derived from the metabolism of sulfur amino acids. SO deficiency is caused by the isolated deficiency of the enzyme SO itself or by a deficiency in the biosynthetic pathway of its molybdenum cofactor. Individuals affected by this disorder present progressive neurological dysfunction, severe neonatal seizures, lens subluxation, axial hypotonia, limb hypertonicity and failure to thrive, resulting often in early childhood death. Considering that the pathophysiology of the neurological damage found in SO deficient patients has not been totally established, the aim of the present work was to investigate the *in vitro* effect of sulfite and thiosulfate on parameters of energy metabolism, as well as redox and mitochondrial homeostasis in rat brain. First, we verified that sulfite inhibited the activity of complex IV of the respiratory chain in cerebral cortex supernatants, indicating that this compound impairs the electron transfer flow, whereas thiosulfate did not affect any of the activities of the respiratory chain complexes. It was also found that sulfite and thiosulfate markedly decreased the activity of total creatine kinase (tCK) and its mitochondrial and cytosolic isoforms, suggesting that these compounds impair brain cellular energy buffering and transfer. Moreover, melatonin, trolox (soluble analogue of α-tocopherol), glutathione and the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester attenuated or fully prevented the inhibition of tCK induced by sulfite and thiosulfate, suggesting the involvement of reactive oxygen and nitrogen species in these effects. Sulfite and thiosulfate also increased 2',7'-dichlorofluorescin oxidation and inhibited the activity of aconitase, whereas only sulfite increased hydrogen peroxide production, reinforcing the involvement of oxidative damage in the effects elicited by these metabolites. In contrast, synaptic Na^+,K^+-ATPase activity was not altered by sulfite and thiosulfate. Next, we observed that sulfite dissipates mitochondria membrane potential in the presence of Ca^{2+}, in a sulfite and Ca^{2+} dose-dependent manner. Sulfite also induced swelling and decreased Ca^{2+} retention capacity, matrix NAD(P)H pool and cytochrome *c* immunocontent in mitochondria when Ca^{2+} was present in the medium. Furthermore, the alterations elicited by sulfite were prevented by ruthenium red, cyclosporine A and ADP, supporting the involvement of mitochondrial permeability transition (MPT) in these effects. It was also verified that among various MPT inhibitors, including antioxidants, phospholipase A2 inhibitors and the reductant reagent dithiothreitol, only the thiol alkylating agent N-ethylmaleimide was able to prevent the sulfite-elicited mitochondrial swelling. Moreover, sulfite decreased membrane protein thiol group content in brain mitochondria, indicating that this compound acts directly on MPT pore containing thiol groups. Taken together, it may be presumed that the mitochondrial energy and redox homeostasis impairment caused by sulfite and thiosulfate and MPT induced by sulfite may be involved in the neurological dysfunction observed in patients affected by SO deficiency.

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LISTA DE ABREVIATURAS

Alam – Alameticina

ANT – translocador de nucleotídeos adenina

BSA – albumina bovina padrão

CAT – catalase

CCCP – carbonilcianeto m-clorofenil-hidrazona

cCK – creatina quinase citosólica

CK – creatina quinase

CoQ – coenzima Q

CP – clorpromazina

CsA – ciclosporina A

CTE – cadeia transportadora de elétrons

DCF – diclorofluoresceína

DCF-DA – 2',7'-diclorofluoresceína diacetato

DCFH – 2',7'-diclorofluorescina

DCIP – 2,6-dicloroindofenol

DTT – ditiotreitol

EIM – erros inatos do metabolismo

ERO – espécies reativas de oxigênio

ERN – espécies reativas de nitrogênio

FAU – unidade arbitrária de fluorescência

FCCP – carbonilcianeto-4-(trifluorometoxi) fenilhidrazona

GDH – glutamato desidrogenase

GM – glutamato + malato

GSH – gluationa reduzida

L-NAME - N^{ω}-nitro-L-arginina metil éster

mCK – creatina quinase mitocondrial

MDH – malato desidrogenase

MEL – melatonina

MMI – membrana mitocondrial interna

MPT – transição da permeabilidade mitocondrial

MPTP – poro de transição da permeabilidade mitocondrial

NAC – N-acetilcisteína

NEM – N-etilmaleimida

NMDA – N-metil-D-aspartato

Pi – fosfato inorgânico

PM – piruvato + malato

QUIN – quinacrina

RCR – razão de controle respiratório

RR – rutênio vermelho

SO – sulfito oxidase

SUC – succinato

tCK – creatina quinase total

TFZ – trifluoperazina

TNB – ácido 5-tio-2-nitrobenzoico

TRO – trolox

αKG – α-cetoglutarato

αKGDH – α-cetoglutarato desidrogenase

ΔΨm – potencial de membrana mitocondrial

I.1. INTRODUÇÃO

I.1.1. Erros Inatos do Metabolismo

Os erros inatos do metabolismo (EIM) são um grupo de doenças genética e fenotipicamente heterogêneas causadas por defeitos genéticos que levam a diminuição ou perda total da atividade de uma enzima específica de uma determinada rota metabólica. Como consequência deste bloqueio metabólico há o acúmulo de intermediários potencialmente tóxicos nos tecidos e líquidos biológicos dos pacientes e a deficiência de produtos essenciais ao organismo (Bickel, 1987; Mak et al., 2013).

O termo EIM foi utilizado pela primeira vez em 1908 por Sir Archibald E. Garrod quando estudava pacientes acometidos pela alcaptonúria, os quais excretavam grandes quantidades de ácido homogentísico na urina. Garrod observou que a alcaptonúria e outros EIM representavam exemplos de variáveis metabólicas determinadas pela hereditariedade, uma vez que verificou uma maior frequência dessas doenças em indivíduos pertencentes à mesma família e maior incidência de consanguinidade entre os pais dos pacientes. Garrod propôs um modelo de herança autossômica recessiva para a alcaptonúria baseando-se nas leis de Mendel e no fato dessa doença não ser encontrada nos pais dos indivíduos afetados (Scriver et al., 2001).

Até o presente momento mais de 500 diferentes EIM já foram identificados, sendo estes classificados em quatro grupos distintos: desordens de transporte, de armazenamento, de síntese e do metabolismo intermediário. Esse número vem crescendo constantemente à medida que novos conceitos e novas técnicas se tornam disponíveis para a identificação de fenótipos

bioquímicos (Scriver et al., 2001; Alfadhel et al., 2013). Embora individualmente raras, essas doenças alcançam em conjunto uma incidência de aproximadamente 1 a cada 500-1.000 recém nascidos vivos (Barić et al., 2001; Mak et al., 2013).

I.1.2. Sulfito oxidase (SO)

A enzima SO (EC 1.8.3.1) está localizada no espaço intermembranas da mitocôndria e catalisa a oxidação do sulfito a sulfato, sendo essa a reação final da rota de degradação dos aminoácidos sulfurados cisteína e metionina (Figura 1) (Johnson, 2003; Hobson et al., 2005). Além disso, essa enzima possui um importante papel na detoxificação do sulfito proveniente de fontes exógenas, uma vez que esse composto e derivados são amplamente utilizados em conservantes de alimentos e na indústria farmacêutica a fim de manter a estabilidade e a potência de alguns medicamentos (Gunnison e Palmes, 1973; Taylor et al., 1986; Chapman, 1993; Derin et al., 2006). A SO é um homodímero com massa molecular de aproximadamente 110 kDa, e cada monômero é dividido em três domínios, um heme citocromo b_5, um domínio de ligação do cofator molibdênio e um domínio C-terminal responsável pela dimerização (Kisker et al., 1997). O cofator molibdênio contém uma molécula de molibdopterina, à qual está ligado o molibdênio, sendo este responsável pela oxidação do sulfito com a redução do molibdênio (VI) para o molibdênio (IV). Os elétrons provenientes do sulfito são então transferidos um de cada vez através do heme b_5 da enzima para o citocromo *c* (Johnson e Duran, 2001). A

ação da SO mantém os níveis plasmáticos de sulfito em até 10 µM (Ji et al., 1995).

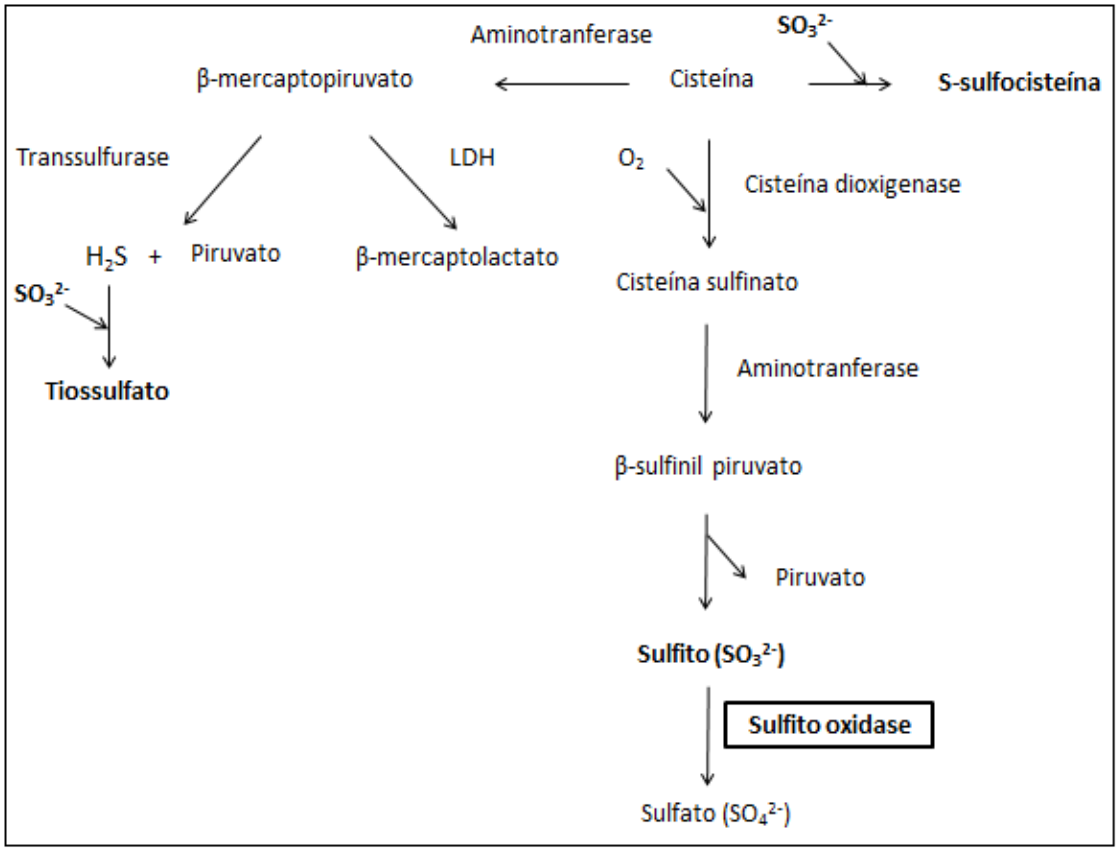

Figura 1. Via do catabolismo da cisteína (Adaptado de Johnson e Duran, 2001).

I.1.3. Deficiência da sulfito oxidase (SO)

A deficiência da SO é uma doença neurometabólica de caráter autossômico recessivo que ocorre devido a uma deficiência isolada da SO ou devido a uma deficiência na rota de biossíntese de seu cofator molibdênio. Além de ser necessário para a reação da SO, esse cofator também é essencial para a atividade das enzimas xantina oxidase e aldeído oxidase (Edwards et al., 1999; Tan et al., 2005). Portanto, os portadores da deficiência do cofator molibdênio são deficientes nas atividades dessas três enzimas.

I.1.3.1. Sintomatologia e Achados Bioquímicos

O quadro clínico da deficiência isolada da SO é similar ao da deficiência do cofator molibdênio (Chan et al., 2002). Entretanto, os sintomas na deficiência isolada da SO têm início mais tardio e são mais heterogêneos, enquanto que a deficiência do cofator molibdênio é uma doença geralmente fatal. Os sintomas incluem disfunção neurológica grave e progressiva, convulsões neonatais severas, hipotonia axial, hipertonicidade periférica, além de subluxação do cristalino e atraso no desenvolvimento, resultando geralmente em morte prematura (Edwards et al., 1999; Johnson e Duran, 2001).

As alterações bioquímicas tipicamente encontradas na deficiência da SO (deficiência isolada da SO e deficiência do cofator molibdênio) incluem excreção urinária excessiva de sulfito, tiossulfato e S-sulfocisteína (Basheer et al., 2007; Herken et al., 2009). Na deficiência do cofator molibdênio ainda são observados baixos níveis plasmáticos de ácido úrico e altos níveis de xantina e hipoxantina na urina, enquanto que na deficiência isolada da SO os níveis plasmáticos desses compostos são normais (Chan et al., 2002).

I.1.3.2. Achados Neuropatológicos

Os achados neuropatológicos característicos da deficiência isolada da SO incluem perda massiva de neurônios, desmielinização e proliferação glial com anormalidades na substância branca. Também já foi evidenciado atrofia cortical, leucoencefalopatia e anormalidades nos gânglios basais, tálamo e cerebelo (Rosenblum, 1968; Basheer et al., 2007; Bindu et al., 2011).

Já as investigações patológicas e neurorradiológicas para a deficiência do cofator molibdênio são mais numerosas. Estudos já demonstraram atrofia cortical grave, dilatação dos ventrículos e anormalidades nos gânglios basais e tálamo (Slot et al., 1993; Schuierer et al., 1995; Appignani et al., 1996; Vijayakumar et al., 2011). Com relação aos achados patológicos, foi verificada também perda massiva de neurônios do córtex cerebral, tálamo e gânglios basais, além de gliose, espongiose e desmielinização (Barth et al., 1985; Brown et al., 1989; Vijayakumar et al., 2011).

I.1.3.3. Diagnóstico

Todos os pacientes com a deficiência da SO possuem um perfil metabólico característico, embora os níveis excretados dos metabólitos acumulados variem devido a diferenças no consumo de proteína, catabolismo e produção de creatinina. Para o diagnóstico das duas formas de deficiência da SO, o sulfito é facilmente detectado na urina fresca através de uma fita indicadora de sulfito (Johnson e Duran, 2001). Entretanto, vários estudos indicam que o teste da fita de sulfito não é confiável devido à rápida oxidação do sulfito a sulfato em temperatura ambiente (Hobson et al., 2005; Ngu et al., 2009; Sass et al., 2010). Um teste mais preciso é a identificação de S-sulfocisteína na urina através da espectrometria de massas (Johnson e Rajagopalan, 1995; Hobson et al., 2005). O diagnóstico específico da deficiência do cofator molibdênio é realizado a partir da medida dos níveis plasmáticos e urinários de ácido úrico, xantina e hipoxantina, visto que as enzimas xantina oxidase e aldeído oxidase também estão deficientes neste

distúrbio. Caso sejam encontrados baixos níveis de ácido úrico e altas concentrações de xantina e hipoxantina, o diagnóstico é a deficiência do cofator molibdênio (Sass et al., 2010).

Embora a deficiência do cofator molibdênio possa ser geralmente diagnosticada através da detecção de hipouricemia, a deficiência isolada da SO geralmente não é detectada porque metabólitos relevantes como a S-sulfocisteína e o sulfito não são rotineiramente testados ou são instáveis (Sass et al., 2004). Dessa forma, a determinação da homocisteína total no plasma tem sido recomendada como um teste barato e de primeira linha (Sass et al., 2010). Os portadores da deficiência da SO ou deficiência do cofator molibdênio apresentam níveis plasmáticos indetectáveis de homocisteína, já que o acúmulo de sulfito nessas doenças pode resultar da degradação de compostos tiólicos tais como homocisteína (Sass et al., 2004).

A confirmação do diagnóstico pode ser feita através da medida da atividade da SO em fibroblastos cultivados e análise mutacional (Sass et al., 2010). O diagnóstico pré-natal também é possível através do monitoramento da atividade da SO em biópsia das vilosidades coriônicas (Johnson, 2003).

I.1.3.4. Tratamento

Atualmente, o tratamento para a deficiência da SO é bastante limitado. Na maioria dos casos, o tratamento sintomático é realizado com o objetivo de controlar as convulsões, apresentando geralmente pouco sucesso (Sass et al., 2010). Contudo, com a utilização de novas drogas antiepilépticas, tais como a vigabatrina, os pacientes têm apresentado melhora nas convulsões (Johnson e

Duran, 2001). Em alguns casos, uma dieta com baixa quantidade de proteína e teor reduzido de aminoácidos sulfurados tem sido utilizada. Em pacientes com uma deficiência moderada da SO, este tratamento diminuiu os níveis de tiossulfato e S-sulfocisteína na urina. Além disso, foi observado que estes pacientes apresentaram crescimento normal sem sinais de deterioração neurológica e progresso no desenvolvimento psicomotor (Touati et al., 2000).

I.1.3.5. Fisiopatologia

Apesar de os mecanismos responsáveis pelo aparecimento de sintomas neurológicos encontrados nos pacientes afetados pela deficiência da SO ainda não estarem totalmente estabelecidos, evidências apontam para uma ação tóxica do sulfito. Estudos mostraram que o sulfito pode sofrer auto-oxidação gerando radicais livres (Mottley e Mason, 1988; Abedinzadeh, 2001; Baker et al., 2002) e induzir estresse oxidativo em cérebro e fígado de ratos *in vitro* (Chiarani et al., 2008; Derin et al., 2009). Já em estudos com ratos deficientes para a enzima SO foi verificado que concentrações aumentadas de sulfito induzem lipoperoxidação no hipocampo e diminuem a capacidade antioxidante do plasma, além de causarem déficit cognitivo (Küçükatay et al., 2005; Herken et al., 2009). Zhang e colaboradores (2004) observaram que o sulfito diminui a biossíntese de ATP e o potencial de membrana mitocondrial quando o glutamato é utilizado como substrato e inibe a atividade da glutamato desidrogenase em mitocôndrias cerebrais de ratos. Já estudos em células Neuro-2a e PC12 evidenciaram que o sulfito aumenta a geração de espécies reativas de oxigênio (ERO) e causa diminuição dos níveis de ATP (Zhang et al.,

2004). Além disso, foi demonstrado que o sulfito causa morte neuronal no hipocampo e córtex somatossensorial de ratos (Kocamaz et al., 2012; Kencebay et al., 2013).

I.1.4 Metabolismo energético cerebral

O cérebro é um dos órgãos mais ativos metabolicamente, entretanto possui reservas energéticas extremamente reduzidas em relação à sua alta taxa metabólica (Dickinson, 1996). Em condições normais, o metabolismo energético no cérebro é mantido principalmente pelo metabolismo oxidativo da glicose, o qual ocorre mais rapidamente do que em outros órgãos como fígado, coração ou rins (Sokoloff, 1993; Dickinson, 1996). Os neurônios são altamente dependentes de lactato como substrato energético para o seu metabolismo oxidativo, o qual é fornecido principalmente por astrócitos que utilizam a maior parte da glicose (Pellerin, 2005).

Em contraste com outros tecidos como o músculo esquelético e o tecido adiposo, o cérebro não necessita de insulina para captar e oxidar a glicose. (Dickinson, 1996). A oxidação da glicose através da via glicolítica forma lactato nos astrócitos, o qual é direcionado para os neurônios, onde é oxidado no ciclo de Krebs e na cadeia transportadora de elétrons (CTE) (Pellerin, 2005). O acoplamento entre a CTE e a fosforilação oxidativa gera grande parte do ATP necessário ao cérebro (Erecińska e Silver, 1994).

I.1.4.1. Creatina quinase (CK)

Apesar de no cérebro a fosforilação oxidativa fornecer em torno de 95% de todo o ATP sintetizado na célula, outros mecanismos auxiliam na

manutenção dos níveis deste composto. Um destes importantes mecanismos é o sistema da enzima creatina quinase (CK), o qual está presente em tecidos com alta demanda energética, como o cérebro. A CK ocorre tanto no citosol quanto no espaço intermembranas mitocondrial, onde interage com a membrana mitocondrial interna e externa, e catalisa a reação reversível de transferência do grupamento fosfato do ATP para o grupamento guanidino da creatina, formando fosfocreatina e ADP. O sistema creatina/fosfocreatina/CK desempenha um papel fundamental na bioenergética e na função cerebral uma vez que é importante para o tamponamento energético, regenerando ATP e mantendo baixos níveis de ADP, e para a transferência de ATP de sítios de produção na mitocôndria para outros de consumo no citosol (Erecińska e Silver, 1994; Wendt et al., 2003).

I.1.4.2. Na^+,K^+-ATPase

A Na^+, K^+ -ATPase é uma enzima transmembrana que também desempenha um papel fundamental para o funcionamento do sistema nervoso. Essa enzima catalisa o transporte simultâneo de 3 íons Na^+ para fora e 2 íons K^+ para dentro da célula, garantindo a manutenção do gradiente de Na^+ e K^+ através da membrana plasmática, o qual é essencial para a manutenção do potencial de repouso da membrana e para a propagação do impulso nervoso (Kaplan, 2002; Hamada et al., 2003; Takeuchi et al., 2008). O transporte dos cátions Na^+ e K^+ através da membrana plasmática contra seus gradientes de concentração é dependente de ATP, sendo que cerca de 40 a 50 % do ATP

produzido no cérebro é consumido pela Na^+,K^+-ATPase para realizar este transporte (Erecińska e Silver, 1994).

I.1.5. Fosforilação Oxidativa e Homeostase Mitocondrial

A fosforilação oxidativa é o processo final do metabolismo produtor de energia nos organismos aeróbicos. Todas as vias de degradação oxidativa de aminoácidos, carboidratos e lipídios convergem para essa etapa, na qual a energia proveniente da oxidação e conservada em equivalentes reduzidos (NADH e o $FADH_2$) é utilizada para a síntese de ATP. Neste processo o NADH e $FADH_2$ doam elétrons para a CTE, os quais fluem sequencialmente através de uma série de complexos enzimáticos, que em eucariotos estão ancorados na membrana mitocondrial interna. Cada componente da CTE transfere elétrons para um aceptor com maior potencial de redução, sendo o oxigênio o aceptor final de elétrons, o qual é reduzido a H_2O. Sendo assim, a transferência de elétrons é impulsionada por um crescente potencial redox existente entre os equivalentes reduzidos, os complexos enzimáticos da CTE e o O_2. A energia liberada pela transferência de elétrons entre os complexos da CTE é utilizada para bombear prótons da matriz mitocondrial para o espaço intermembranas, possibilitando a produção de ATP a partir de ADP e fosfato inorgânico (Pi) em uma reação catalisada pela ATP sintase (Nelson e Cox, 2012).

A CTE é composta por uma série de complexos enzimáticos e uma coenzima lipossolúvel, os quais apresentam grupos prostéticos como flavina mononucleotídeo, centros ferro-enxofre e citocromos contendo ferro ou cobre, que permitem aos complexos o desempenho de sua função como aceptores e

doadores de elétrons (Di Donato, 2000; Lieberman e Marks, 2009). O complexo I, também chamado de NADH:ubiquinona oxidorredutase ou NADH desidrogenase recebe elétrons do NADH e os transfere para a coenzima Q (CoQ; ubiquinona), a qual também recebe elétrons do complexo II (succinato desidrogenase), provenientes do $FADH_2$ resultante da oxidação do succinato a fumarato no ciclo de Krebs. Os elétrons da ubiquinona reduzida são doados ao complexo III, também conhecido como complexo dos citocromos bc_1 ou ubiquinona:citocromo *c* oxidorredutase, que por sua vez reduz o citocromo *c*. Por fim, o complexo IV (citocromo *c* oxidase) catalisa a redução do O_2 a H_2O, utilizando os elétrons provenientes do citocromo *c*, sendo necessárias quatro moléculas de citocromo *c* para reduzir completamente uma molécula de O_2 (Abeles et al., 1992; Nelson e Cox, 2012).

É importante ressaltar que o NADH e o $FADH_2$ produzidos na matriz mitocondrial podem doar seus elétrons diretamente para a CTE. Por outro lado, o NADH produzido no citosol necessita de sistemas chamados de lançadeiras de elétrons para transferir elétrons do NADH do citosol para a CTE, uma vez que a membrana mitocondrial interna é impermeável às moléculas de NADH e $FADH_2$. Existem duas lançadeiras de elétrons para este propósito, designadas de lançadeira malato/aspartato e lançadeira do glicerol-3-fosfato (utilizada pelo cérebro). Uma vez formadas na mitocôndria, as moléculas de NADH e $FADH_2$ podem doar elétrons para o complexo I ou para a CoQ, respectivamente, suprindo a CTE (Lieberman e Marks, 2009; Nelson e Cox, 2012).

A transferência de elétrons através dos complexos da CTE até o O_2 está acoplada à síntese de ATP pela ATP sintase, de forma que a energia liberada no transporte de elétrons é utilizada para a formação de uma ligação de fosfato

de alta energia, dando origem a uma molécula de ATP, o que é explicado pelo modelo quimiosmótico. À medida que os elétrons são transferidos de um complexo da CTE para o outro há liberação de energia, uma vez que o potencial de redução de cada complexo está em um nível energético menor que o complexo anterior. Esta energia é utilizada pelos complexos I, III e IV para bombear prótons da matriz mitocondrial para o espaço intermembranas contra seu gradiente de concentração, gerando um gradiente eletroquímico através da membrana mitocondrial interna (MMI). Este gradiente possui dois componentes: o potencial de membrana (gradiente elétrico) e o gradiente de prótons (gradiente químico), e é também chamado de força próton-motora, uma vez que é a energia que impulsiona os prótons de volta para a matriz a fim de atingir o equilíbrio eletroquímico. A força próton-motora dirige a fosforilação do ADP à medida que os prótons retornam passivamente para a matriz a favor do gradiente eletroquímico através de um poro para prótons associado à ATP sintase (Lieberman e Marks, 2009; Nelson e Cox, 2012)

Considerando que o O_2 é oxidado a H_2O pelo complexo IV da CTE, pode-se estimar a respiração mitocondrial através da medida do consumo de O_2. Apesar de essa medida determinar diretamente apenas a velocidade da transferência de elétrons para o seu aceptor final, a molécula de O_2, muitas informações sobre diversos outros processos relacionados à mitocôndria podem ser obtidos simplesmente pela adaptação das condições de incubação. Através dessa medida pode-se investigar o transporte de substratos através da membrana mitocondrial, a atividade das desidrogenases, a atividade dos complexos da CTE, o transporte de nucleotídeos de adenina pela membrana

mitocondrial, a atividade da ATP sintase e a permeabilidade da membrana mitocondrial a H^+ (Nicholls e Ferguson, 2002).

A respiração mitocondrial pode ser dividida experimentalmente em 5 estágios, conforme ilustrado na figura 2. No estado 1 há apenas mitocôndrias na presença de Pi. Após a adição de substrato oxidável ocorre o estado 2, onde a respiração é lenta devido à falta de ADP. O estado 3 ou estado fosforilante representa o consumo de O_2 após a adição de ADP, o qual estimula o consumo de O_2 e a produção de ATP. Já o estado 4 ou estado não-fosforilante reflete o consumo de O_2 após as mitocôndrias já terem depletado o ADP disponível, resultando em uma diminuição da taxa de respiração e em uma ausência da produção de ATP. Por fim, o estado 5 representa a anóxia. É importante salientar que apenas os termos estado 3 e estado 4 são comumente utilizados (Nicholls e Ferguson, 2002). A transdução de energia entre a CTE e o gradiente eletroquímico de prótons é bem regulada, sendo que um pequeno desequilíbrio termodinâmico entre ambos pode resultar em uma alteração importante no transporte de elétrons pela cadeia respiratória. Dessa forma, quando há a dissipação do gradiente de prótons pela ação da ATP sintase devido à adição de ADP, há um desequilíbrio que estimula a transferência de elétrons pela cadeia respiratória e, consequentemente, o consumo de O_2. Portanto, é necessário que haja disponibilidade de ADP e um potencial de membrana suficientemente alto para que a ATP sintase esteja ativa (Nicholls e Ferguson, 2002; Nelson e Cox, 2012). Neste contexto, o acoplamento da respiração mitocondrial é definido como a capacidade da mitocôndria de gerar ATP quando exposta ao ADP, ou seja, acoplar os processos de oxidação e de fosforilação. No caso de ocorrer a dissipação do gradiente eletroquímico de

prótons devido a um dano ou aumento da permeabilidade da MMI ocorre o desacoplamento do transporte de elétrons e da síntese de ATP, resultando em um aumento do consumo de oxigênio (atividade respiratória aumentada) com reduzida produção de ATP (Nicholls e Ferguson, 2002).

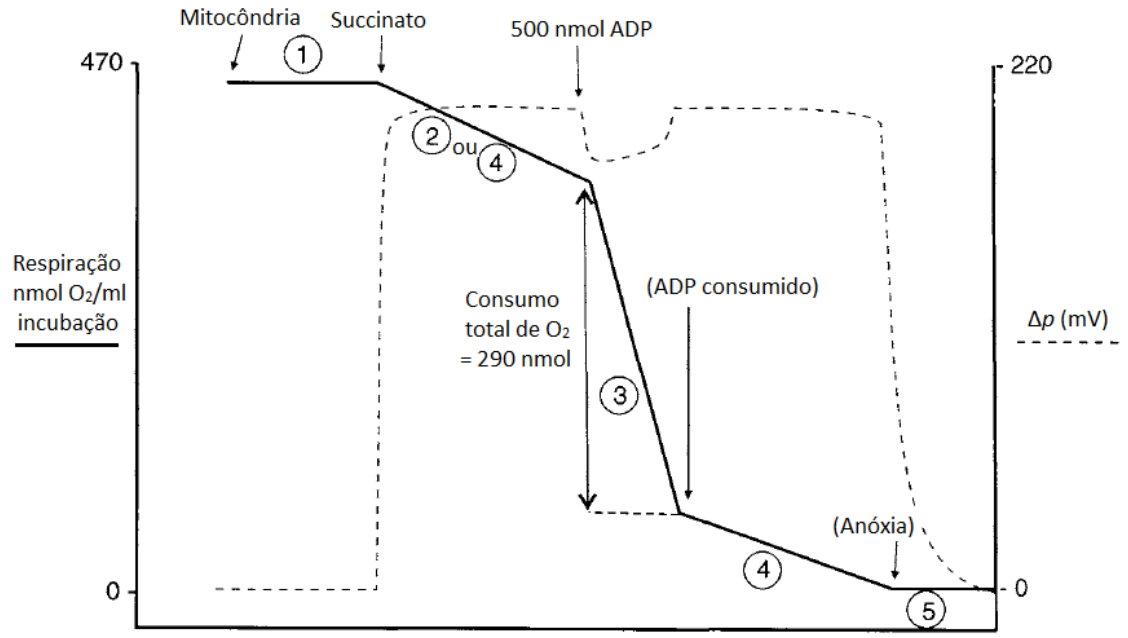

Figura 2. Estados da respiração mitocondrial (Adaptado de Nicholls e Ferguson, 2002).

Além de sua principal função de produção de energia na forma de ATP a mitocôndria desempenha outros importantes papeis. Essa organela é a principal fonte de ERO, uma vez que alguns centros redox dos complexos da CTE, como os presentes nos complexos I e III, podem ser diretamente oxidados pelo O_2, com a transferência de um único elétron para essa molécula, gerando ânions superóxido ($O_2^{\bullet -}$) na matriz mitocondrial e no espaço intermembranas. Por outro lado a mitocôndria também é a principal fonte de

defesas antioxidantes nas células (Cadenas e Davies, 2000; Han et al., 2001). Além disso, a mitocôndria é fundamental para a manutenção da homeostase do cálcio (Nicholls e Akerman, 1982) e está envolvida em diversos processos que levam à morte celular, incluindo a liberação de citocromo *c* (Liu et al., 1996).

I.1.6. Papel da Mitocôndria na Homeostase Celular do Ca^{2+}

A habilidade de acumular, reter e liberar Ca^{2+} é uma função ubíqua e fundamental das mitocôndrias de animais, sendo seu papel fundamental para o tamponamento do Ca^{2+} citosólico (Starkov, 2010) Em situações nas quais há um aumento nos níveis intracelulares de Ca^{2+}, devido a um prejuízo dos sistemas de remoção de Ca^{2+} ou devido à ativação de receptores N-metil-D-aspartato (NMDA) nos neurônios, essa sobrecarga citosólica de Ca^{2+} pode levar a uma ativação de enzimas líticas como a fosfolipase A2, proteases e endonucleases, resultando em degradação celular (Fiskum et al., 1999; Nicholls e Budd, 2000; Orrenius et al., 2003). Sob essas condições, a mitocôndria se torna essencial no sequestro de Ca^{2+} (Budd e Nicholls, 1996; Castilho et al., 1998; Stout et al., 1998).

O Ca^{2+} é captado e liberado pela mitocôndria através de transportadores em um processo que consome energia. Para acessar o espaço intermembranas ou ser liberado de volta no citosol, o Ca^{2+} utiliza os canais de ânions dependentes de voltagem (VDAC), localizados na membrana mitocondrial externa. Uma vez no espaço intermembranas, o Ca^{2+} é captado pela mitocôndria por um sistema uniporte de Ca^{2+} (MCU), sendo sua entrada

impulsionada pelo potencial de membrana negativo gerado pela atividade dos complexos da CTE. Já a liberação de Ca^{2+} da matriz é realizada pelos trocadores Na^{+}/Ca^{2+} (mNCX) e H^{+}/Ca^{2+} (mHCX) (Bernardi e von Stockum, 2012; Rizzuto et al., 2012; Brini et al., 2014) (Figura 3).

Na matriz mitocondrial, o Ca^{2+} acumulado forma precipitados osmoticamente inativos com o fosfato, permitindo que a mitocôndria seja capaz de acumular grandes concentrações de Ca^{2+}, podendo chegar a 300 μM dependendo do tecido (Starkov, 2010). Contudo, uma excessiva captação mitocondrial de Ca^{2+} pode ter como consequência a permeabilização mitocondrial não seletiva, conhecida como transição da permeabilidade mitocondrial (MPT), a qual resulta da abertura de um poro na MMI (Zoratti e Szabo, 1995; Adam-Vizi e Starkov, 2010; Starkov, 2010). O poro de transição da permeabilidade mitocondrial (MPTP) é formado por proteínas presentes na matriz mitocondrial ou na MMI e possui 2-3 nm de diâmetro. A composição proteica do MPTP ainda não é conhecida, porém alguns requerimentos mínimos são esperados para que uma proteína possa ser um candidato a componente do MPTP, tais como a capacidade de se ligar a membrana mitocondrial interna e abrir um canal de 2-3 nm de diâmetro que seja completamente reversível (transitório) (Starkov, 2010). O translocador de nucleotídeos adenina (ANT) tem sido sugerido como uma das mais prováveis proteínas formadoras do MPTP (Halestrap e Brenner, 2003; Leung e Halestrap, 2008). Evidências também apontam a ciclofilina D, uma proteína da matriz mitocondrial que interage com o ANT, como um componente chave para a abertura do poro (Figura 3). A ciclofilina D é um alvo da ciclosporina A (CsA),

um peptídeo inibidor do MPTP (Woodfield et al., 1998; Bernardi e von Stockum, 2012; Rizzuto et al., 2012; Brini et al., 2014).

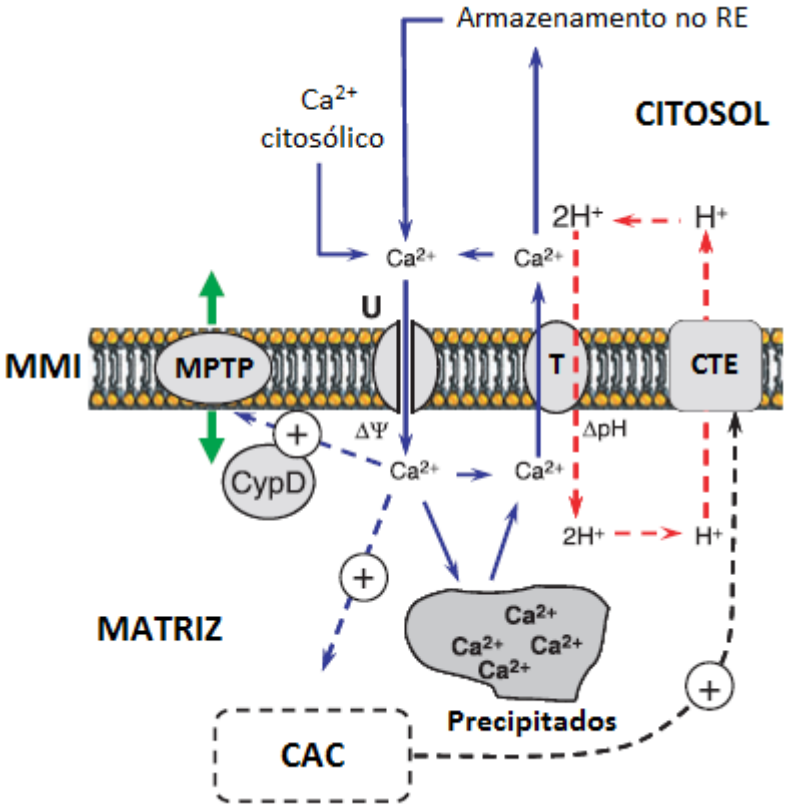

Figura 3. Modelo padrão da homeostase mitocondrial de Ca^{2+}. T: trocador; CTE: cadeia transportadora de elétrons; MMI: Membrana Mitocondrial Interna; CAC: ciclo do ácido cítrico (Adaptado de Starkov, 2010).

A abertura do MPTP leva à permeabilidade de solutos com uma massa molecular de até 1,5 kDa, resultando em liberação de Ca^{2+} para o citosol, inchamento mitocondrial devido à captação osmótica de água, dissipação do potencial de membrana ($\Delta\Psi m$), perda de nucleotídeos piridina e glutationa reduzida (GSH), comprometimento da síntese de ATP e liberação de fatores apoptogênicos intramitocondriais, como o citocromo *c* (Kim et al., 2003; Soane

et al., 2007; Starkov, 2010; Rasola e Bernardi, 2011; Bernardi e von Stockum, 2012; Rizzuto et al., 2012).

I.1.7. Radicais Livres e Defesas Antioxidantes

Radicais livres são espécies químicas que contêm um ou mais elétrons desemparelhados e que são capazes de existir de forma independente. Essas espécies encontram-se em uma situação energeticamente instável devido ao desemparelhamento de elétrons, o que as torna altamente reativas (Southorn e Powis, 1988; Halliwell e Gutteridge, 2007d).

Em condições fisiológicas do metabolismo celular aeróbico, o O_2 sofre redução tetravalente no complexo IV da CTE resultando na formação de H_2O. No entanto, cerca de 3 a 5% do O_2 não é completamente reduzido à H_2O, podendo ser convertido a ERO, tais como o radical superóxido ($O_2^{\bullet -}$) e hidroxila ($OH^{\bullet}$), além do não-radical peróxido de hidrogênio (H_2O_2), em um processo que pode ser exacerbado em condições patológicas (Boveris e Chance, 1973; Roede e Jones, 2010). Além das ERO, existem ainda as espécies reativas de nitrogênio (ERN), sendo as principais representantes o óxido nítrico ($NO^{\bullet}$) e o peroxinitrito ($ONOO^-$) (Halliwell e Gutteridge, 2007d).

As ERO e ERN estão presentes em processos fisiológicos e apresentam uma série de funções, tais como sinalização celular e síntese e regulação de proteínas (Ward e Peters, 1995; Irani, 2000; Wall et al., 2012), bem como a defesa do organismo contra infecções, realizada através da liberação de espécies reativas tóxicas pelos neutrófilos (Delanty e Dichter, 1998; Aratani et al., 2012). Entretanto, quando formadas excessivamente, essas espécies são

capazes de causar danos celulares através da oxidação de diversas biomoléculas, tais como, lipídios, proteínas, carboidratos e DNA (Halliwell e Gutteridge, 2007d).

A fim de evitar os efeitos danosos causados pelas espécies reativas, existem mecanismos eficientes para a detoxificação das mesmas: as defesas antioxidantes enzimáticas e antioxidantes não-enzimáticos endógenos ou derivados da dieta. Essas defesas estão amplamente distribuídas no organismo e compreendem agentes que removem cataliticamente os radicais livres, como as enzimas superóxido dismutase, catalase (CAT), glutationa peroxidase; proteínas que minimizam a disponibilidade de pró-oxidantes (íons de ferro e cobre, por exemplo), ao se ligarem aos mesmos como as transferrinas; agentes que aprisionam ERO e ERN, como GSH, α-tocoferol e ácido ascórbico; além de proteínas que protegem biomoléculas de danos por outros mecanismos (Halliwell e Gutteridge, 2007a).

I.1.8. Estresse Oxidativo

As espécies reativas produzidas fisiologicamente são na sua maioria neutralizadas pelos sistemas de defesa antioxidante presentes no organismo. Entretanto, em determinadas condições patológicas há um desequilíbrio entre a produção de espécies reativas e a sua remoção pelos mecanismos de defesa antioxidante, o que dá origem a uma situação denominada estresse oxidativo (Halliwell e Gutteridge, 2007b).

O estresse oxidativo pode resultar tanto de uma produção aumentada de espécies reativas quanto de uma diminuição das defesas antioxidantes,

podendo levar a lipoperoxidação, cujos produtos são altamente neurotóxicos, ao dano oxidativo a proteínas, levando à sua inativação e alterando a função celular, e ao dano oxidativo ao DNA e RNA, causando mutações somáticas e distúrbios de transcrição (Delanty e Dichter, 1998; Halliwell, 2001; Halliwell e Gutteridge, 2007b). Um nível moderado de estresse oxidativo pode ser tolerado pelas células, resultando geralmente em um aumento na síntese de enzimas antioxidantes com a finalidade de neutralizar as espécies reativas em excesso. Por outro lado, o estresse oxidativo exacerbado pode levar à morte celular por necrose ou apoptose, devido aos grandes danos celulares causados pelas espécies reativas (Halliwell e Gutteridge, 2007b).

I.2. JUSTIFICATIVA

Pacientes acometidos pela deficiência da SO apresentam principalmente encefalopatia, retardo psicomotor e convulsões neonatais severas (Edwards et al., 1999; Johnson e Duran, 2001), cuja fisiopatologia não está totalmente esclarecida. Uma vez que esta doença é bioquimicamente caracterizada pelo acúmulo de sulfito e tiossulfato nos tecidos e líquidos biológicos dos pacientes (Johnson e Duran, 2001), sugere-se que esses compostos sejam potencialmente neurotóxicos. Nesse contexto, nada se sabe sobre possíveis efeitos neurotóxicos do tiossulfato, ao passo que trabalhos anteriores demonstraram que o sulfito causa prejuízos à função mitocondrial (Vincent et al., 2004; Zhang et al., 2004). Também deve ser salientado aqui que alguns pacientes apresentam acidemia lática (Eichler et al., 2006; Basheer et al., 2007). Dessa forma, torna-se importante avaliar os efeitos desses compostos sobre parâmetros de bioenergética, homeostase redox e função mitocondrial, e investigar possíveis mecanismos envolvidos nesses efeitos em tecidos cerebrais a fim de melhor esclarecer os mecanismos responsáveis pelo dano neurológico apresentado pelos portadores da deficiência da SO.

I.3. OBJETIVOS

I.3.1. Objetivo Geral

O objetivo do presente trabalho foi investigar os efeitos *in vitro* do sulfito e do tiossulfato sobre parâmetros de metabolismo energético e homeostase redox e mitocondrial em cérebro de ratos jovens.

I.3.2 Objetivo Específicos

a) Avaliar os efeitos do sulfito e do tiossulfato sobre a atividade dos complexos I-III, II, II-III e IV da CTE em homogeneizado de córtex cerebral de ratos.

b) Avaliar os efeitos do sulfito e do tiossulfato sobre a atividade da CK total em homogeneizado de córtex cerebral de ratos.

c) Avaliar os efeitos do sulfito e do tiossulfato sobre a oxidação da diclorofluorescina (DCFH) em fatias de córtex cerebral de ratos e sobre a produção de peróxido de hidrogênio e a atividade da enzima aconitase em preparações mitocondriais de cérebro de ratos.

e) Avaliar os efeitos do sulfito e do tiossulfato sobre a atividade da Na^+,K^+-ATPase em membranas sinápticas isoladas de córtex cerebral de ratos.

f) Avaliar os efeitos do sulfito e do tiossulfato sobre os parâmetros respiratórios estado 3, estado 4, razão de controle respiratório (RCR) e estado induzido por carbonilcianeto m-clorofenil-hidrazona (CCCP) (estado desacoplado), medidos através do consumo de oxigênio em preparações mitocondriais de cérebro de ratos.

g) Avaliar os efeitos do sulfito sobre o ΔΨm, inchamento mitocondrial, retenção de Ca^{2+} pela mitocôndria, conteúdo de NAD(P)H na matriz mitocondrial, conteúdo de grupamentos tióis de proteínas de membrana da mitocôndria e liberação de citocromo *c* na presença ou ausência de Ca^{2+} em preparações mitocondriais de cérebro de ratos.

PARTE II

Artigos Científicos

Capítulo I

Disturbance of brain energy and redox homeostasis provoked by sulfite and thiosulfate: potential pathomechanisms involved in the neuropathology of sulfite oxidase deficiency

Mateus Grings, Alana Pimentel Moura, Belisa Parmeggiani, Gustavo Flora Marcowich, Alexandre Umpierrez Amaral, Angela Terezinha de Souza Wyse, Moacir Wajner, Guilhian Leipnitz

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Disturbance of brain energy and redox homeostasis provoked by sulfite and thiosulfate: Potential pathomechanisms involved in the neuropathology of sulfite oxidase deficiency

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ABSTRACT

Sulfite oxidase (SO) deficiency is biochemically characterized by tissue accumulation and high urinary excretion of sulfite, thiosulfate and S-sulfocysteine. Affected patients present severe neurological symptoms and cortical atrophy, whose pathophysiology is still poorly established. Therefore, in the present work we investigated the in vitro effects of sulfite and thiosulfate on important parameters of energy metabolism in the brain of young rats. We verified that sulfite moderately inhibited the activity of complex IV, whereas thiosulfate did not alter any of the activities of the respiratory chain complexes. It was also found that sulfite and thiosulfate markedly reduced the activity of total creatine kinase (CK) and its mitochondrial and cytosolic isoforms, suggesting that these metabolites impair brain cellular energy buffering and transfer. In contrast, the activity of synaptic Na^+,K^+-ATPase was not altered by sulfite or thiosulfate. We also observed that the inhibitory effect of sulfite and thiosulfate on CK activity was prevented by melatonin, reduced glutathione and the combination of both antioxidants, as well as by the nitric oxide synthase N^{ω}-nitro-L-arginine methyl ester, indicating the involvement of reactive oxygen and nitrogen species in these effects. Sulfite and thiosulfate also increased 2′,7′-dichlorofluorescin oxidation and hydrogen peroxide production and decreased the activity of the redox sensor aconitase enzyme, reinforcing a role for oxidative damage in the effects elicited by these metabolites. It may be presumed that the disturbance of cellular energy and redox homeostasis provoked by sulfite and thiosulfate contributes to the neurological symptoms and abnormalities found in patients affected by SO deficiency.

1. Introduction

Sulfite oxidase (SO; EC 1.8.3.1), encoded by *SUOX* gene, is an enzyme localized in the mitochondrial intermembrane space that catalyzes the oxidation of sulfite to sulfate, which is the terminal reaction of the metabolism of sulfur amino acids (Johnson, 2003). SO also detoxifies the sulfite derived from exogenous sources, such as sulfiting agents used in pharmaceuticals and as preservatives in food (Basheer et al., 2007; Chapman, 1993; Derin et al., 2006; Taylor et al., 1986). The importance of SO becomes obvious in cases of SO deficiency. The deficiency of this enzyme can be caused by mutations in the *SUOX* gene (isolated SO deficiency) or in any gene that affects the synthetic pathway of its molybdenum cofactor (molybdenum cofactor deficiency) (Sass et al., 2010).

Patients affected by SO deficiency (isolated SO deficiency and molybdenum cofactor deficiency) present severe neurological dysfunction characterized by encephalopathy, psychomotor retardation and generalized seizures and may have a fatal outcome at an early age. Dislocated lens and feeding difficulties are also commonly observed (Basheer et al., 2007; Chan et al., 2002; Edwards et al., 1999; Ngu et al., 2009; Tan et al., 2005). Neuropathological findings include massive neuronal loss and gliosis in cerebral cortex, atrophy in the cerebral white matter and abnormalities in basal ganglia and cerebellum (Appignani et al., 1996; Basheer et al., 2007; Bindu et al., 2011; Johnson and Duran, 2001; Schuierer et al., 1995; Slot et al., 1993; Vijayakumar et al., 2011). Biochemically, the disorder is characterized by tissue accumulation and high urinary excretion of sulfite and its related metabolites thiosulfate and S-sulfocysteine (Johnson and Duran, 2001).

The mechanisms underlying the pathogenesis of the severe brain damage in SO deficiency are still poorly elucidated. However, previous

Abbreviations: cCK, cytosolic creatine kinase; CK, creatine kinase; DCF, dichlorofluorescein; DCF-DA, 2′,7′-dichlorofluorescein diacetate; DCFH, 2′,7′-dichlorofluorescin; DCIP, 2,6-dichloroindophenol; GSH, reduced glutathione; L-NAME, N^{ω}-nitro-L-arginine methyl ester; mCK, mitochondrial creatine kinase; MEL, melatonin; Pi, inorganic phosphate; ROS, reactive oxygen species; SO, sulfite oxidase; tCK, total creatine kinase; TRO, trolox.

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studies demonstrated that sulfite induces lipid peroxidation and decreases antioxidant defenses in brain and erythrocytes of normal and SO-deficient rats (Chiarani et al., 2008; Derin et al., 2009; Herken et al., 2009; Küçükatay et al., 2005; Ozturk et al., 2010). Moreover, chemical studies demonstrated that sulfite generates a sulfite radical that could mediate lipid peroxidation, and leads to the formation of superoxide anion in the presence of oxygen (Baker et al., 2002; Mottley and Mason, 1988). It has been shown also that sulfite decreases mitochondrial membrane potential and ATP synthesis in mitochondria prepared from rat brain and kidney, as well as in Neuro-2a and PC12 cells (Vincent et al., 2004; Zhang et al., 2004). Finally, a recent study demonstrated that the administration of sulfite causes death of pyramidal neurons in the hippocampus of normal and SO-deficient rats (Kocamaz et al., 2012).

Considering that the potential neurotoxic effects of thiosulfate have not yet been investigated and that the exact neurotoxic mechanisms exerted by sulfite are not fully established, in the present study we evaluated the in vitro effects of these metabolites on bioenergetics, by determining the activities of the respiratory chain complexes I to IV (oxidative phosphorylation), creatine kinase (CK) (intracellular energy transfer) and Na^+,K^+-ATPase (neurotransmission) activities, as well as on redox homeostasis, by measuring 2′,7′-dichlorofluorescin (DCFH) oxidation, hydrogen peroxide production and the redox sensor aconitase activity (production of reactive species) in the brain of young rats.

2. Material and methods

2.1. Animals

Thirty-day-old male Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil, were used. The animals had free access to water and 20% (w/w) protein commercial chow (SUPRA, Porto Alegre, RS, Brazil) and were maintained on a 12:12 h light/dark cycle in an air conditioned constant temperature (22 °C ± 1 °C) colony room. The experimental protocol was approved by the Ethics Committee for Animal Research of the UFRGS, Porto Alegre, Brazil, and followed the "National Institutes of Health Guide for the Care and Use of Laboratory Animals" (NIH Publications No. 80-23, revised 1978). All efforts were made to minimize the number of animals used and their suffering.

2.2. Reagents

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA. Sulfite and thiosulfate were dissolved on the day of the experiments in the buffer used for each assay and the pH was adjusted to 7.4. The biochemical parameters were determined in the presence of various concentrations of sulfite and thiosulfate (1–500 μM), whereas control groups did not contain these metabolites in the incubation medium. Parallel experiments were carried out to detect any interference of sulfite and thiosulfate on the techniques utilized to measure the biochemical parameters.

2.3. Sample preparation

Animals were killed by decapitation and the brain was immediately removed, and kept on an ice plate. The olfactory bulb, pons and medulla were discarded and the cerebral cortex was dissected, weighed and kept chilled until homogenization. For the determination of the activities of the respiratory chain complexes I–III, II, II–III and IV, cerebral cortex was homogenized (1:20, w/v) in SETH buffer (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 IU·mL^{-1} heparin), pH 7.4. For total creatine kinase (tCK) activity determination, the cerebral cortex was homogenized (1:10 w/v) in an isosmotic saline solution. The homogenate was centrifuged at 800 *g* for 10 min and the supernatant was kept at −70 °C until being used for enzymatic activity determination. For the preparation of mitochondrial and cytosolic fractions, the homogenates were centrifuged at 800 *g* for 10 min at 4 °C and the pellet discarded (Ramirez and Jimenez, 2000). The supernatant was then centrifuged at 27,000 *g* for 30 min at 4 °C. The pellet containing the mitochondria was washed three times with saline solution and used as the mitochondrial fraction for the mitochondrial creatine kinase (mCK) enzymatic assay. The supernatants were further centrifuged at 125,000 *g* for 60 min at 4 °C, the microsomal pellet discarded, and the cytosol (supernatant) was used for the determination of cytosolic creatine kinase (cCK) activity. The period between tissue preparation and measurement of the parameters was always less than 5 days, except for mCK and cCK assays, which were performed in the same day of the preparations. We used samples containing approximately 0.01–1 mg protein in the assays. Tissue slices (25 mg) were also prepared from the cerebral cortex for DCFH oxidation measurement.

2.4. Preparation of mitochondrial fractions

For the measurement of aconitase activity and hydrogen peroxide production forebrain mitochondria were isolated from the brain of 30-day-old rats as described by Rosenthal et al. (1987), with slight modifications. Animals were killed by decapitation, had their brains rapidly removed and put into ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1% bovine serum albumin (free of fatty acids) and 10 mM HEPES, pH 7.2. The cerebellum, pons, medulla and olfactory bulbs were removed and the remaining material was used as the forebrain. The forebrain was cut into small pieces using surgical scissors, extensively washed to remove blood and homogenized 1:10 in a Dounce homogenizer using both a loose-fitting and a tight-fitting pestle. The homogenate was centrifuged for 3 min at 2000 *g*. After centrifugation, the supernatant was again centrifuged for 8 min at 12,000 *g*. The pellet was suspended in isolation buffer containing 10 μL of 10% digitonin and centrifuged for 8 min at 12,000 *g*. The supernatant was discarded and the final pellet gently washed and suspended in isolation buffer devoid of EGTA, at an approximate protein concentration of 20 mg·mL^{-1}. This preparation results in a mixture of synaptosomal and non-synaptosomal mitochondria similar to the brain composition.

2.5. Preparation of synaptic plasma membrane

For the measurement of Na^+,K^+-ATPase activity, cerebral cortex was homogenized (1:10, w/v) in a 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA. Synaptical plasma membranes were then prepared according to the method of Jones and Matus (1974), modified by Wyse et al. (1998) using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at 69,000 *g* for 2 h, the fraction at the 0.8–1.0 mM sucrose interface was taken as the membrane enzyme preparation.

2.6. Respiratory chain complex I–IV activities

The activities of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate/cytochrome c oxidoreductase (complexes II–III) were determined according to Fischer et al. (1985). The activity of NADH/cytochrome c oxidoreductase (complexes I–III) was assayed according to the method described by Schapira et al. (1990) and that of cytochrome c oxidase (complex IV) according to Rustin et al. (1994). The methods used to measure these activities were slightly modified, as described in detail in a previous report (da Silva et al., 2002). The samples were pre-incubated with sulfite or thiosulfate at 30 °C for 30 min. The controls did not contain the metabolites in the incubation medium. The activities of the respiratory chain complexes were calculated as

nmol·min^{-1}·mg protein^{-1} and the results were expressed as a percentage of control.

2.7. Creatine kinase (CK) activity

The activities of tCK, mCK and cCK were measured according to Hughes (1962) with slight modifications (da Silva et al., 2004). In brief, the reaction mixture consisted of 50 mM Tris buffer, pH 7.5, containing 7.0 mM phosphocreatine, 7.5 mM $MgSO_4$, and cortical preparations in a final volume of 0.1 mL. Sulfite or thiosulfate was added to the medium and submitted to a pre-incubation at 37 °C for 30 min. The reaction was then started by the addition of 4.0 mM ADP and stopped after 10 min by the addition of 0.02 mL of 50 mM p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 mL of 20% α-naphthol and 0.1 mL of 20% diacetyl in a final volume of 1.0 mL and read after 20 min at λ = 540 nm. Results were calculated as μmol of creatine·min^{-1}·mg protein^{-1} and expressed as a percentage of control. In some experiments, the antioxidants trolox (soluble α-tocopherol, TRO, 5 μM), melatonin (MEL, 1000 μM), the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 500 μM) or reduced glutathione (GSH, 10–100 μM) was co-incubated with 500 μM sulfite or thiosulfate.

2.8. Determination of Na^+,K^+-ATPase activity

The reaction mixture for the Na^+,K^+-ATPase assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, and 40 mM Tris–HCl buffer, pH 7.4, and purified synaptic membranes (approximately 3 μg of protein) previously incubated with sulfite or thiosulfate at 37 °C for 30 min. The enzymatic assay occurred at 37 °C during 5 min and started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. The reaction was stopped by the addition of 200 μL of 10% trichloroacetic acid. Mg^{2+}-ATPase ouabain-insensitive was assayed under the same conditions with the addition of 1 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays (Wyse et al., 1998). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Enzyme-specific activities were calculated as nmol Pi released·min^{-1}·mg protein^{-1} and expressed as a percentage of control.

2.9. 2′,7′-Dichlorofluorescin (DCFH) oxidation

The production of reactive species was determined according to the method of LeBel et al. (1992) with slight modifications. Cerebral cortex slices were exposed to sulfite or thiosulfate for 1 h at 37 °C. Afterwards, the tissue slices were incubated with 5 μM 2′,7′-dichlorofluorescein diacetate (DCF-DA), prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl, during 30 min at 37 °C. DCF-DA is permeable to the cell membrane and is deacetylated by esterases to DCFH in the intracellular medium. This nonfluorescent product is converted by reactive species into the highly fluorescent product dichlorofluorescein (DCF). After incubation, fluorescence was measured using wavelengths of 480 nm (excitation) and 535 nm (emission). The calibration curve was performed with standard DCF (1–100 μM). The production of reactive species was calculated as pmol DCF·g of tissue^{-1} and expressed as a percentage of control.

2.10. Mitochondrial hydrogen peroxide (H_2O_2) release

Mitochondrial preparations (0.5 mg protein·mL^{-1}) supported by 2.5 mM glutamate plus 2.5 mM malate were incubated in standard reaction medium in the presence of 10 μM Amplex red and 1 U·mL^{-1} horseradish peroxidase. The fluorescence was monitored over time on a Hitachi F-4500 spectrofluorometer operated at excitation and emission wavelengths of 563 and 587 nm, respectively, and slit width of 5 nm. Sulfite or thiosulfate (500 μM) was added to the reaction medium at the beginning of the assay. Antimycin A (AA) (0.1 μg·mL^{-1}) was added at the end of the measurement. Data were expressed as FAU.

2.11. Aconitase activity

The activity of aconitase was measured according to Morrison (1954) in brain mitochondrial fractions. Sulfite or thiosulfate (1–500 μM) was added to the medium containing 36 mM Tris buffer, pH 7.4, 0.07 mM sodium citrate, 0.08 mM L-cysteine, 1.3 mM manganese chloride, 0.5 U·mL^{-1} isocitrate dehydrogenase, 0.18 mM $NADP^+$ and mitochondrial preparations (0.5 mg protein·mL^{-1}) and submitted to a pre-incubation for 30 min at 37 °C. The enzyme activity was monitored following the reduction of $NADP^+$ at wavelengths of excitation and emission of 340 and 466 nm, respectively. Controls did not contain the metabolites in the incubation medium. The activity of aconitase was calculated as μmol NADPH·min^{-1}·mg protein^{-1} and the results were expressed as a percentage of control.

2.12. Protein determination

Protein content was measured by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

2.13. Statistical analysis

Results are presented as mean ± standard deviation. Assays were performed in triplicate and the mean was used for statistical calculations. Data were analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when F was significant. Linear regression analysis was also used to test dose-dependent effects. Only significant F values are shown in the text. Differences between groups were considered significant at $P < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

3. Results

3.1. Sulfite inhibits respiratory chain complex IV activity in rat cerebral cortex

First, we evaluated the effect of sulfite and thiosulfate on the activities of the respiratory chain complexes I to IV in rat cerebral cortex in an attempt to elucidate whether these compounds could alter the electron flow through the electron transfer chain. Sulfite significantly inhibited complex IV activity [$F_{(4,15)} = 4.438$, $P < 0.05$] in a dose-dependent manner [$\beta = -0.650$, $P < 0.01$], whereas no significant alterations were induced by this metabolite on the activities of the respiratory chain complexes I–III and II–III (Fig. 1). It should be stressed here that complex II activity could not be measured in the presence of sulfite because this compound interfered with the assay. Moreover, thiosulfate did not affect any of the complex activities (Fig. 1).

3.2. Sulfite and thiosulfate inhibit creatine kinase activity in rat cerebral cortex

The effect of sulfite and thiosulfate on tCK activity in cortical supernatants from young rats was also examined. We found that tCK activity was significantly inhibited by sulfite [$F_{(4,15)} = 36.717$, $P < 0.001$] and thiosulfate [$F_{(4,20)} = 250.306$, $P < 0.001$] in a dose-dependent manner [sulfite: $\beta = -0.721$, $P < 0.001$; thiosulfate: $\beta = -0.824$, $P < 0.001$] (Fig. 2A). We then investigated the effect of these compounds on the activity of mCK and cCK isoforms. Figs. 2B and C show, respectively, that mCK and cCK activities were inhibited by sulfite [mCK: $F_{(4,15)} = 55.250$, $P < 0.001$; cCK: $F_{(4,18)} = 15.368$, $P < 0.001$] and thiosulfate [mCK: $F_{(4,20)} = 58.537$, $P < 0.001$; cCK: $F_{(4,15)} = 33.312$, $P < 0.001$]. The inhibition induced by these metabolites on mCK and

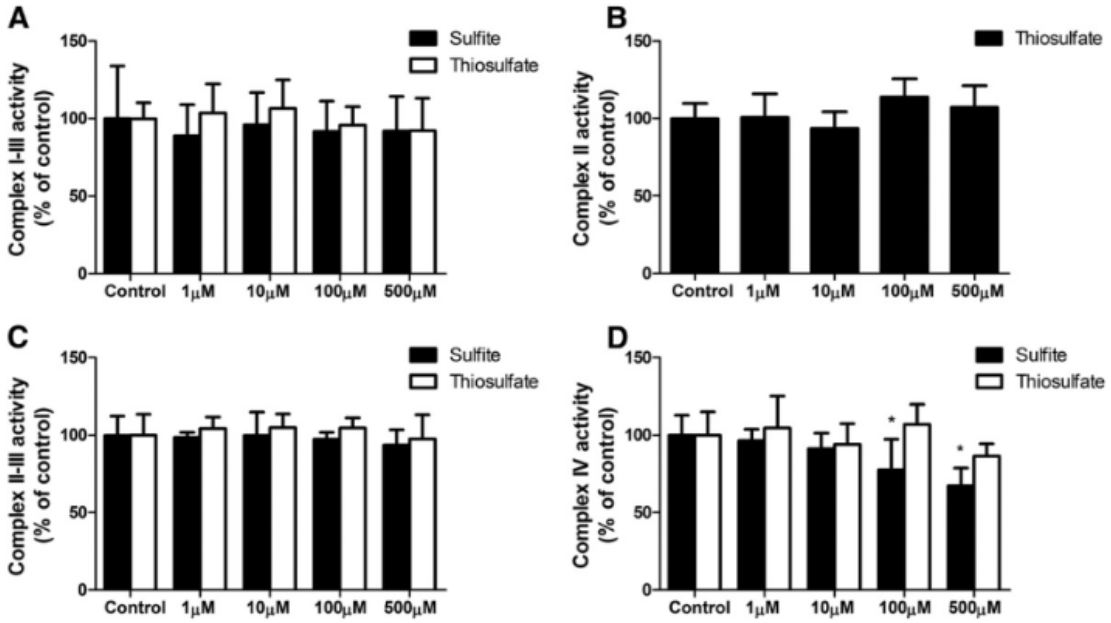

Fig. 1. Effect of sulfite and thiosulfate on the activities of the respiratory chain complexes I–IV in rat cerebral cortex. Cerebral cortex supernatants were incubated in the presence of sulfite or thiosulfate (1–500 μM). Values are means ± standard deviation for four to six independent experiments (animals) and are expressed as percentage of controls (controls: (A) Complex I–III activity [nmol cytochrome *c* reduced·min^{-1}·mg protein^{-1}]: sulfite: 4.14 ± 1.40, thiosulfate: 2.60 ± 0.27; (B) complex II activity [nmol DCIP reduced·min^{-1}·mg protein^{-1}]: thiosulfate: 9.97 ± 0.98; (C) complex II–III activity [nmol cytochrome *c* reduced·min^{-1}·mg protein^{-1}]: sulfite: 35.0 ± 4.32, thiosulfate: 27.4 ± 3.70; (D) complex IV activity [nmol cytochrome *c* oxidized·min^{-1}·mg protein^{-1}]: sulfite: 96.7 ± 12.3, thiosulfate: 67.1 ± 10.1). Controls did not contain the tested compounds in the incubation medium. *$P < 0.05$, compared to controls (Duncan multiple range test).

cCK activities presented a dose-dependent fashion [sulfite: mCK: $\beta = -0.687$, $P < 0.01$; cCK: $\beta = -0.653$, $P < 0.01$; thiosulfate: mCK: $\beta = -0.844$, $P < 0.001$; cCK: $\beta = -0.906$, $P < 0.001$].

3.3. Antioxidants prevent creatine kinase inhibition caused by sulfite and thiosulfate in rat cerebral cortex

In order to evaluate whether the significant reduction of tCK activity caused by sulfite and thiosulfate was mediated by the oxidation of critical groups of the enzyme that are susceptible to reactive species attack, cortical supernatants were pre-incubated with sulfite (500 μM) or thiosulfate (500 μM) in the presence of the antioxidants TRO (5 μM), MEL (1000 μM), L-NAME (500 μM) and GSH (10 μM). It was verified that MEL attenuated the inhibition of tCK activity caused by sulfite [$F_{(5,18)} = 34.794$, $P < 0.001$] (Fig. 3A). Furthermore, GSH fully prevented, whereas MEL and L-NAME attenuated the inhibitory effect induced by thiosulfate on tCK activity [$F_{(5,26)} = 50.402$, $P < 0.001$] (Fig. 3B). Thereafter, we tested whether higher doses of GSH (50 or 100 μM) or a combination of MEL (1000 μM) and GSH (10 or 50 μM) could prevent the sulfite-induced inhibition of tCK activity. We found that 50 μM GSH and a combination of 1000 μM MEL and 10 μM GSH attenuated tCK activity inhibition caused by sulfite, whereas 100 μM GSH and the combination of 1000 μM MEL and 50 μM GSH fully prevented this effect [GSH: $F_{(3,20)} = 52.358$, $P < 0.001$; MEL + GSH: $F_{(3,16)} = 56.345$, $P < 0.001$] (Figs. 4A and B).

3.4. Sulfite and thiosulfate do not change Na^+,K^+-ATPase activity in rat cerebral cortex

We also tested the effect of sulfite and thiosulfate on Na^+,K^+-ATPase activity from synaptic plasma membranes of cerebral cortex of rats. It can be observed in Table 1 that these metabolites did not change Na^+, K^+-ATPase activity.

3.5. Sulfite and thiosulfate increase reactive species production in rat brain

The next set of experiments was carried out to investigate the effect of sulfite and thiosulfate on reactive species production. Fig. 5 shows that both metabolites were able to increase DCFH oxidation in cortical slices [sulfite: $F_{(3,16)} = 5.862$, $P < 0.01$; thiosulfate: $F_{(3,16)} = 3.742$, $P < 0.05$] in a dose-dependent fashion [sulfite: $\beta = 0.524$, $P < 0.05$; thiosulfate: $\beta = 0.498$, $P < 0.05$]. We also found that, in the presence of glutamate and malate as substrates, H_2O_2 production was increased by sulfite in brain mitochondrial preparations, whereas thiosulfate did not modify this parameter (Fig. 6).

3.6. Sulfite and thiosulfate inhibit aconitase activity in rat brain

We then evaluated the effects of sulfite and thiosulfate on the activity of mitochondrial aconitase, which is considered a sensitive marker of oxidative stress. It can be observed in Fig. 7 that both compounds decreased aconitase activity [sulfite: $F_{(4,15)} = 36.717$, $P < 0.001$; thiosulfate: $F_{(4,15)} = 36.717$, $P < 0.001$] in a dose-dependent manner [sulfite: $\beta = -0.721$, $P < 0.001$; thiosulfate: $\beta = -0.824$, $P < 0.001$].

4. Discussion

Isolated SO deficiency and molybdenum cofactor deficiency are biochemically characterized by tissue accumulation and high urinary excretion of sulfite, thiosulfate and S-sulfocysteine (Johnson and Duran, 2001). Although severe neurological symptoms are observed in affected patients, the exact underlying mechanisms involved in the neuropathology of these disorders are poorly established. However, accumulation of high amounts of lactic acid in some affected patients indicates mitochondrial dysfunction (Basheer et al., 2007; Eichler et al., 2006). Therefore, in the present study we evaluated the in vitro effects of sulfite and thiosulfate on important parameters of energy metabolism, as well as on redox homeostasis in the brain of young rats.

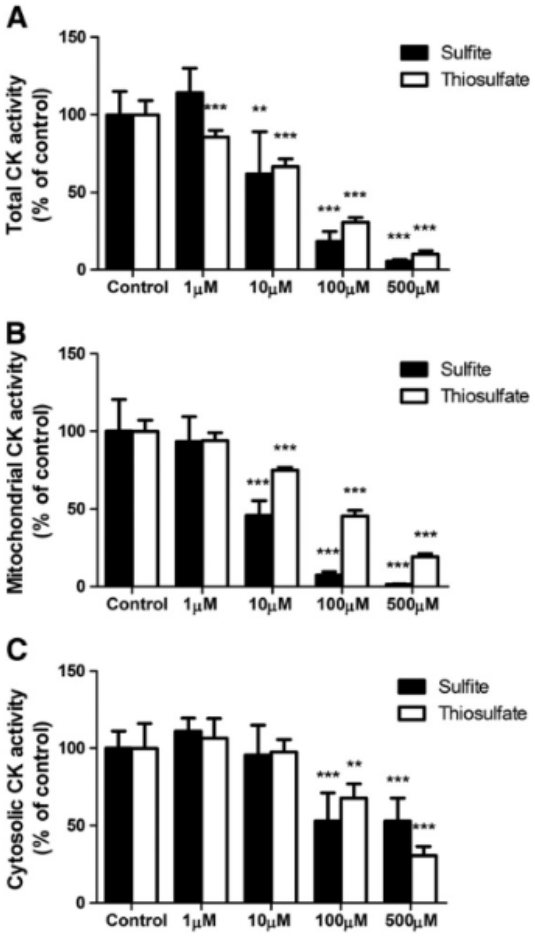

Fig. 2. Effect of sulfite and thiosulfate on total (A), mitochondrial (B) and cytosolic (C) creatine kinase (CK) activities in rat cerebral cortex. Cerebral cortex supernatants were incubated in the presence of sulfite or thiosulfate (1–500 μM). Values are means ± standard deviation for four to five independent experiments (animals) and are expressed as percentage of controls (controls: (A) total creatine kinase (tCK) activity [nmol creatine·min^{-1}·mg protein^{-1}]: sulfite: 5.20 ± 0.78, thiosulfate: 6.55 ± 0.59; (B) mitochondrial creatine kinase (mCK) activity [nmol creatine·min^{-1}·mg protein^{-1}]: sulfite: 1.73 ± 0.35, thiosulfate: 6.46 ± 1.02; (C) cytosolic creatine kinase (cCK) activity [nmol creatine·min^{-1}·mg protein^{-1}]: sulfite: 5.41 ± 0.60, thiosulfate: 6.52 ± 1.04). Controls did not contain the tested compounds in the incubation medium. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to controls (Duncan multiple range test).

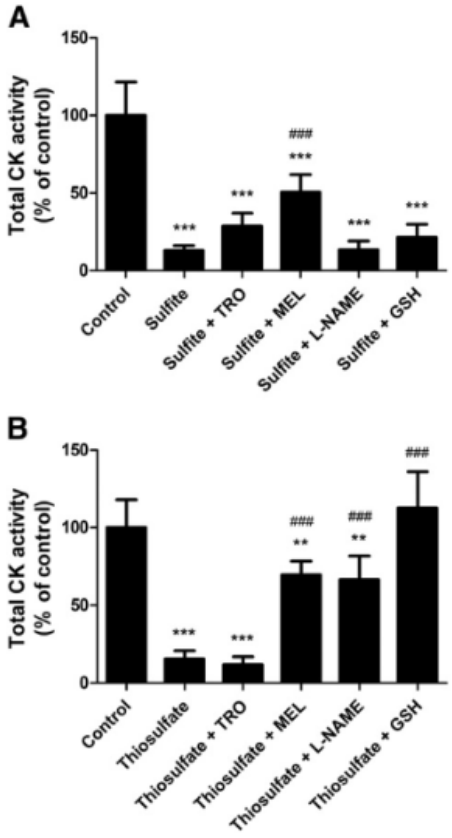

Fig. 3. Effect of antioxidants on the inhibition of total creatine kinase (tCK) activity induced by sulfite (A) and thiosulfate (B) in rat cerebral cortex. The antioxidants trolox (TRO, 5 μM), melatonin (MEL, 1000 μM), the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 500 μM) or reduced glutathione (GSH, 10 μM) was co-incubated with 500 μM sulfite or thiosulfate and the activity of the enzyme measured afterward. Values are means ± standard deviation for four to ten independent experiments (animals) and are expressed as percentage of controls (controls: tCK [nmol creatine·min^{-1}·mg protein^{-1}]: sulfite: 3.78 ± 0.81; thiosulfate: 6.72 ± 2.54). Controls did not contain the tested compounds in the incubation medium. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to controls; $^{###}P < 0.001$, compared to 500 μM sulfite or thiosulfate (Duncan multiple range test).

We initially verified that sulfite selectively inhibited complex IV activity of the respiratory chain. Since complex IV represents the rate-limiting enzyme of the mitochondrial respiratory chain, being a central site of regulation of oxidative phosphorylation, proton pumping efficiency and ATP production (Arnold, 2012), it may be suggested that this inhibition caused by sulfite could contribute, at least in part, to the decrease of ATP synthesis demonstrated in previous studies (Vincent et al., 2004; Zhang et al., 2004). Our present data indicate that oxidative phosphorylation is compromised by sulfite.

We also investigated the effect of sulfite and thiosulfate on the activities of CK and found that both compounds inhibited tCK, as well as the activities of the isoforms mCK and cCK in cerebral cortex. Considering that the CK system is essential for the transport of high-energy phosphate from the sites of energy production in the mitochondrial matrix to cytosolic sites of energy consumption, maintaining a stable ATP level in living cells (Du et al., 2013; Wendt et al., 2003), the present data indicate that sulfite and thiosulfate impair brain cellular energy buffering and transfer.

It should be emphasized here that CK has vulnerable amino acid residues, including cysteine thiol groups, which are very reactive to oxidants (Li et al., 2011; Wang et al., 2001; Wendt et al., 2003). Furthermore, it has been shown that brain CK activity decreases after exposure to agents that promote generation of free radicals, probably by oxidation of the cysteine residues of the enzyme that are critical for its functional activity (Arstall et al., 1998; Burmistrov et al., 1992; Konorev et al., 1998; Stachowiak et al., 1998; Wallimann et al., 1998; Wendt et al., 2003; Wolosker et al., 1996). Therefore, we evaluated the effect of antioxidants on the inhibitory effect caused by sulfite and thiosulfate on tCK activity and found that low concentrations of MEL attenuated the inhibitory effect of sulfite. We also observed that a high dose of GSH (100 μM) and the combination of relatively low doses of MEL (1000 μM) and GSH (50 μM) totally prevented sulfite-induced inhibition of tCK. These findings showing that GSH prevents the inhibitory effect caused by sulfite on CK activity suggest that sulfite probably mediates the oxidation of critical thiol groups of the enzyme that are protected by the antioxidant GSH, and that hydroxyl radical, which is mainly scavenged by MEL (Galano, 2011; Halliwell and Gutteridge, 2007; Reiter et al., 2001; Tamura et al., 2013), is probably involved in this effect. Regarding the thiosulfate-induced inhibition of tCK activity, we verified that, besides MEL and GSH, L-NAME also prevented this effect, implying that thiosulfate mediates the generation of reactive

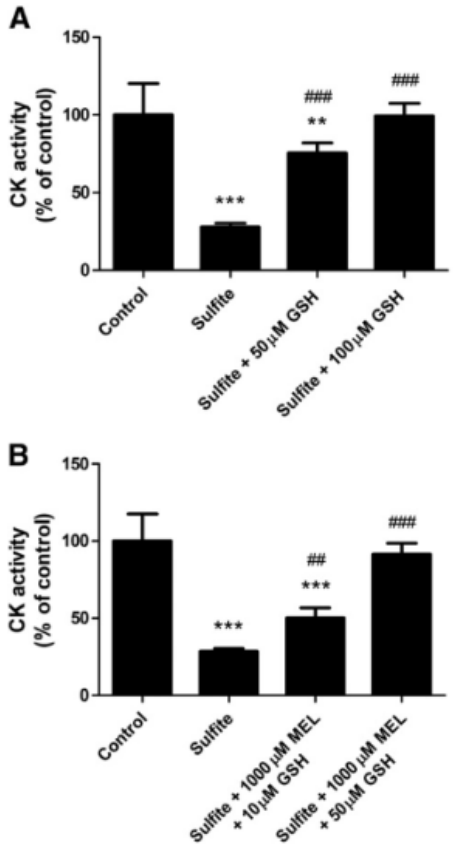

Fig. 4. Effect of increasing concentrations of glutathione (GSH) (A) and a combination of melatonin (MEL) and GSH (B) on sulfite-induced inhibition of total creatine kinase (tCK) in rat cerebral cortex. In these experiments, GSH (50 or 100 μM) (A) or a combination of MEL (1000 μM) and GSH (10 μM or 50 μM) (B) was co-incubated with 500 μM sulfite and the activity of the enzyme was measured afterward. Values are means ± standard deviation for five to six independent experiments (animals) and are expressed as percentage of controls (controls: tCK activity [nmol creatine·min^{-1}·mg protein^{-1}]: A: 7.04 ± 1.43; B: 7.38 ± 1.30). Controls did not contain the tested compounds in the incubation medium. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to controls; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared to 500 μM sulfite (Duncan multiple range test).

nitrogen species. This is in line with the previous studies showing that CK can be rapidly inactivated by peroxynitrite and nitric oxide-derived oxidants that mainly target the active cysteine sites of the enzyme (Konorev et al., 1998; Wendt et al., 2003).

Our results showing that sulfite and thiosulfate increased DCFH oxidation, which is a probe sensible to ROS oxidation, and only sulfite enhanced H_2O_2 production reinforce the role of reactive species in the inhibitions of tCK caused by sulfite and thiosulfate in rat brain. Although we cannot at present determine the exact mechanisms by which these reactive species are formed, it is well established that sulfite auto oxidation promotes the generation of sulfur trioxide anion, superoxide anion

Table 1
Effect of sulfite and thiosulfate on Na^+,K^+-ATPase activity in rat cerebral cortex.

Na^+,K^+-ATPase activity					
	Control	1 μM	10 μM	100 μM	500 μM
Sulfite	100 ± 27.6	116 ± 17.8	117 ± 16.0	108 ± 21.2	105 ± 13.7
Thiosulfate	100 ± 14.0	99.8 ± 10.8	98.0 ± 10.0	102 ± 16.4	101 ± 20.7

Values are means ± standard deviation for five to six independent experiments (animals) and are expressed as percentage of controls. (Controls: Na^+,K^+-ATPase activity [nmol Pi released·min^{-1}·mg of protein^{-1}]: sulfite: 715 ± 197; thiosulfate: 1250 ± 175). No significant differences between groups were detected (one-way ANOVA).

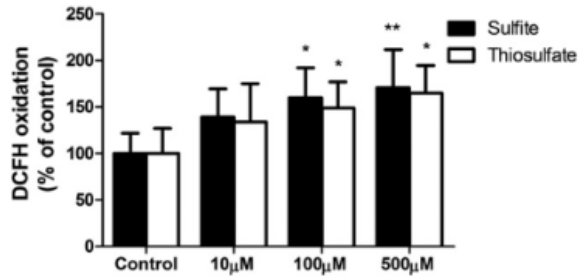

Fig. 5. Effect of sulfite and thiosulfate on 2′,7′-dichlorofluorescin (DCFH) oxidation in rat cerebral cortex. Cerebral cortex slices were incubated in the presence of sulfite or thiosulfate (1–500 μM). Values are means ± standard deviation for four to seven independent experiments (animals) and are expressed as percentage of controls (controls: DCFH oxidation [pmol/g of tissue]: sulfite: 0.47 ± 0.10; thiosulfate: 0.45 ± 0.12). Controls did not contain the tested compounds in the incubation medium $^{*}P < 0.05$, $^{**}P < 0.01$, compared to controls (Duncan multiple range test).

and hydrogen peroxide (Baker et al., 2002; Hayon et al., 1972; Hegg and Hobbs, 1978). In this particular, a large body of evidence also demonstrates that reactive species derived from sulfite auto oxidation induce lipid and protein oxidation and DNA damage (Baker et al., 2002; Hayatsu, 1976; Hayatsu and Miller, 1972; Peiser and Yang, 1979; Shi and Mao, 1994; Southerland et al., 1982; Yang, 1973).

Sulfite and thiosulfate also decreased the activity of mitochondrial aconitase, a citric acid cycle enzyme reported to be a redox sensor of reactive species (Gardner et al., 1994; Liang et al., 2012; Patel, 2004). Considering that aconitase is particularly susceptible to oxidative inactivation by superoxide anion, which oxidizes the 4Fe–4S cluster located at the active site of this enzyme releasing a labile iron with the concomitant generation of H_2O_2 (Bulteau et al., 2003; Cantu et al., 2011; Myers et al., 2010; Vasquez-Vivar et al., 2000) and that sulfite was shown to induce superoxide anion generation (Baker et al., 2002; Hayon et al., 1972; Mottley and Mason, 1988), it is conceivable that sulfite-induced aconitase inhibition was mediated by superoxide anion. It is also emphasized that the co-release of iron and hydrogen peroxide from aconitase during its inactivation facilitates the formation of hydroxyl radical via Fenton reaction (Cantu et al., 2011; Vasquez-Vivar et al., 2000), which is also in line with our results showing that MEL prevented the inhibitory effect of sulfite on CK activity. However, it should be noted that other reactive species are capable of inactivating aconitase (Gardner et al., 1997). Our findings demonstrating that thiosulfate inhibited aconitase activity, but did not alter hydrogen peroxide

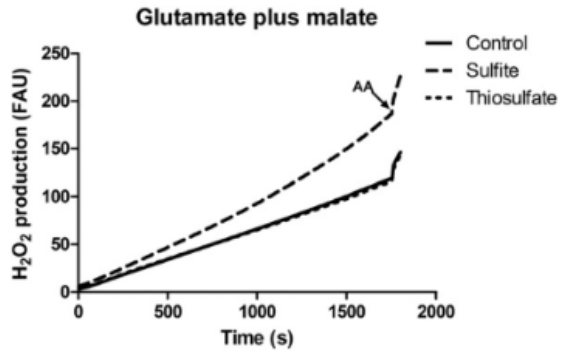

Fig. 6. Effect of sulfite and thiosulfate on hydrogen peroxide production in mitochondrial enriched fractions from rat brain. Brain mitochondrial preparations (0.5 mg protein·mL^{-1}), 2.5 mM glutamate plus 2.5 mM malate as substrates and sulfite or thiosulfate (500 μM) were added to the incubation medium in the beginning of the assay. The control group did not contain the tested compounds in the incubation medium. Antimycin A (AA) (0.1 μg·mL^{-1}) was added at the end of assays, as indicated in the figure. Traces are representative of four independent experiments and were expressed as fluorescence arbitrary units (FAU).

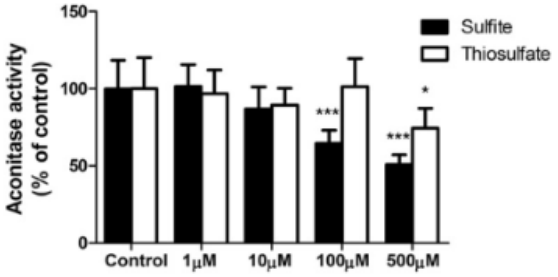

Fig. 7. Effect of sulfite and thiosulfate on aconitase activity in mitochondrial enriched fractions from rat brain. Mitochondrial enriched fractions (0.5 mg protein·mL^{-1}) were incubated in the presence of sulfite or thiosulfate (1–500 μM). Values are means ± standard deviation for six independent experiments (animals) and are expressed as percentage of controls (controls: aconitase activity [μmol NADPH·min^{-1}·mg protein^{-1}]: sulfite: 27.8 ± 5.16; thiosulfate: 18.1 ± 3.65). Controls did not contain the tested compounds in the incubation medium. *$P < 0.05$, ***$P < 0.001$, compared to controls (Duncan multiple range test).

generation, suggest that other reactive species, besides superoxide anion, are involved in this effect.

Finally, we observed that sulfite and thiosulfate did not affect Na^+, K^+-ATPase activity that corroborates with previous findings (Chiarani et al., 2008) and indicates that these compounds do not compromise the membrane potential generation and the cellular volume control which is highly dependent on Na^+,K^+-ATPase activity.

At the present, it is difficult to determine the pathophysiological relevance of our present data, since the concentrations of sulfite and thiosulfate in the brain of patients with SO deficiency are unknown. However, it should be stressed that the disturbance of energy metabolism and redox homeostasis verified in our study was achieved with micromolar concentrations of the evaluated metabolites. Although we did not determine whether sulfite or thiosulfate alters cell viability in the present work, this may be a possibility since it has been evidenced that sulfite-induced oxidative damage leads to neuron loss in rat hippocampus and somatosensory cortex (Kencebay et al., 2013; Kocamaz et al., 2012). It is also noteworthy that, among human brain structures, cerebral cortex presents the highest expression of SO, suggesting that this brain region needs higher enzyme levels, perhaps due to greater formation of sulfite during metabolism (Woo et al., 2003). Therefore, the deficiency of this enzyme may predispose the cerebral cortex to damage by excess of sulfite and thiosulfate, which is in accordance with the clinical evidence showing severe cortical damage in several patients (Woo et al., 2003). In this scenario, most of our results showing a disturbance of energy and redox homeostasis were carried out in cerebral cortex.

In conclusion, this is the first report showing that the metabolites accumulating in SO deficiency markedly inhibit CK activity and moderately impair the electron flow through the respiratory chain. The present study also evidences alterations of redox homeostasis caused by sulfite and thiosulfate in brain cortex. In case these findings are confirmed in vivo and in tissues from patients affected by isolated SO deficiency and molybdenum cofactor deficiency, it is tempting to speculate that bioenergetic and redox homeostasis dysfunction may contribute, at least in part, to the neurological damage found in these disorders.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

Appignani, B.A., Kaye, E.M., Wolpert, S.M., 1996. CT and MR appearance of the brain in two children with molybdenum cofactor deficiency. AJNR Am. J. Neuroradiol. 17, 317–320.
Arnold, S., 2012. The power of life—cytochrome c oxidase takes center stage in metabolic control, cell signalling and survival. Mitochondrion 12, 46–56.
Arstall, M.A., Bailey, C., Gross, W.L., Bak, M., Balligand, J.L., Kelly, R.A., 1998. Reversible S-nitrosation of creatine kinase by nitric oxide in adult rat ventricular myocytes. J. Mol. Cell. Cardiol. 30, 979–988.
Baker, M.T., Dehring, D.J., Gregerson, M.S., 2002. Sulfite supported lipid peroxidation in propofol emulsions. Anesthesiology 97, 1162–1167.
Basheer, S.N., et al., 2007. Isolated sulfite oxidase deficiency in the newborn: lactic acidaemia and leukoencephalopathy. Neuropediatrics 38, 38–41.
Bindu, P.S., Christopher, R., Mahadevan, A., Bharath, R.D., 2011. Clinical and imaging observations in isolated sulfite oxidase deficiency. J. Child. Neurol. 26, 1036–1040.
Bulteau, A.L., Ikeda-Saito, M., Szweda, L.I., 2003. Redox-dependent modulation of aconitase activity in intact mitochondria. Biochemistry 42, 14846–14855.
Burmistrov, S.O., Mashek, O.P., Kotin, A.M., 1992. The action of acute alcoholic intoxication on the antioxidant system and creatine kinase activity in the brain of rat embryos. Eksp. Klin. Farmakol. 55, 54–56.
Cantu, D., Fulton, R.E., Drechsel, D.A., Patel, M., 2011. Mitochondrial aconitase knockdown attenuates paraquat-induced dopaminergic cell death via decreased cellular metabolism and release of iron and H(2)O(2). J. Neurochem. 118, 79–92.
Chan, K.M., Delfert, D., Junger, K.D., 1986. A direct colorimetric assay for Ca2+-stimulated ATPase activity. Anal. Biochem. 157, 375–380.
Chan, K.Y., Li, C.K., Lai, C.K., Ng, S.F., Chan, A.Y., 2002. Infantile isolated sulphite oxidase deficiency in a Chinese family: a rare neurodegenerative disorder. Hong Kong Med. J. 8, 279–282.
Chapman, K., 1993. Sulfite-containing pharmaceuticals. Can. Med. Assoc. J. 148, 714.
Chiarani, F., Bavaresco, C.S., Dutra-Filho, C.S., Netto, C.A., Wyse, A.T., 2008. Sulfite increases lipoperoxidation and decreases the activity of catalase in brain of rats. Metab. Brain Dis. 23, 123–132.
da Silva, C.G., et al., 2002. Inhibition of cytochrome c oxidase activity in rat cerebral cortex and human skeletal muscle by D-2-hydroxyglutaric acid in vitro. Biochim. Biophys. Acta 1586, 81–91.
da Silva, C.G., et al., 2004. Inhibition of creatine kinase activity from rat cerebral cortex by D-2-hydroxyglutaric acid in vitro. Neurochem. Int. 44, 45–52.
Derin, N., Yargiçoğlu, P., Aslan, M., Elmas, O., Agar, A., Aicigüzel, Y., 2006. The effect of sulfite and chronic restraint stress on brain lipid peroxidation and anti-oxidant enzyme activities. Toxicol. Ind. Health 22, 233–240.
Derin, N., Akpinar, D., Yargicoglu, P., Agar, A., Aslan, M., 2009. Effect of alpha-lipoic acid on visual evoked potentials in rats exposed to sulfite. Neurotoxicol. Teratol. 31, 34–39.
Du, F., Cooper, A., Lukas, S.E., Cohen, B.M., Ongur, D., 2013. Creatine kinase and ATP synthase reaction rates in human frontal lobe measured by (31)P magnetization transfer spectroscopy at 4 T. Magn. Reson. Imaging 31, 102–108.
Edwards, M.C., et al., 1999. Isolated sulfite oxidase deficiency: review of two cases in one family. Ophthalmology 106, 1957–1961.
Eichler, F., Tan, W.H., Shih, V.E., Grant, P.E., Krishnamoorthy, K., 2006. Proton magnetic resonance spectroscopy and diffusion-weighted imaging in isolated sulfite oxidase deficiency. J. Child Neurol. 21, 801–805.
Fischer, J.C., et al., 1985. Differential investigation of the capacity of succinate oxidation in human skeletal muscle. Clin. Chim. Acta 153, 23–36.
Galano, A., 2011. On the direct scavenging activity of melatonin towards hydroxyl and a series of peroxyl radicals. Phys. Chem. Chem. Phys. 13, 7178–7188.
Gardner, P.R., Nguyen, D.D., White, C.W., 1994. Aconitase is a sensitive and critical target of oxygen poisoning in cultured mammalian cells and in rat lungs. Proc. Natl. Acad. Sci. U. S. A. 91, 12248–12252.
Gardner, P.R., Costantino, G., Szabo, C., Salzman, A.L., 1997. Nitric oxide sensitivity of the aconitases. J. Biol. Chem. 272, 25071–25076.
Halliwell, B., Gutteridge, J.M.C., 2007. Measurement of Reactive Species, 4th ed. Oxford University Press, Oxford.
Hayatsu, H., 1976. Bisulfite modification of nucleic acids and their constituents. Prog. Nucleic Acid Res. Mol. Biol. 16, 75–124.
Hayatsu, H., Miller Jr., R.C., 1972. The cleavage of DNA by the oxygen-dependent reaction of bisulfite. Biochem. Biophys. Res. Commun. 46, 120–124.
Hayon, E., Treinin, A., Wilf, J., 1972. Electronic spectra, photochemistry, and autoxidation mechanism of the sulfite–bisulfite–pyrosulfite systems. The SO_2^-, SO_3^-, SO_4^-, and SO_5^- radicals. J. Am. Chem. Soc. 94, 47–57.
Hegg, D.A., Hobbs, P.V., 1978. Oxidation of sulfur dioxide in aqueous systems with particular reference to the atmosphere. Atmos. Environ. 12, 241–253.
Herken, E.N., Kocamaz, E., Erel, O., Celik, H., Kucukatay, V., 2009. Effect of sulfite treatment on total antioxidant capacity, total oxidant status, lipid hydroperoxide, and total free sulfydryl groups contents in normal and sulfite oxidase-deficient rat plasma. Cell Biol. Toxicol. 25, 355–362.
Hughes, B.P., 1962. A method for estimation of serum creatine kinase and its use in comparing creatine kinase and aldolase activity in normal and pathological sera. Clin. Chim. Acta 7, 597–603.

Johnson, J.L., 2003. Prenatal diagnosis of molybdenum cofactor deficiency and isolated sulfite oxidase deficiency. Prenat. Diagn. 23, 6–8.
Johnson, J.L., Duran, M., 2001. Molybdenum Cofactor Deficiency and Isolated Sulfite Oxidase Deficiency. In: Scriver, C.R., Beaudet, A.L., Valle, D., Sly, W.S. (Eds.), The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York, pp. 3181–3217.
Jones, D.H., Matus, A.I., 1974. Isolation of synaptic plasma membrane from brain by combined flotation–sedimentation density gradient centrifugation. Biochim. Biophys. Acta 356, 276–287.
Kencebay, C., et al., 2013. Merit of quinacrine in the decrease of ingested sulfite-induced toxic action in rat brain. Food Chem. Toxicol. 52, 129–136.
Kocamaz, E., Adiguzel, E., Er, B., Gundogdu, G., Kucukatay, V., 2012. Sulfite leads to neuron loss in the hippocampus of both normal and SOX-deficient rats. Neurochem. Int. 61, 341–346.
Konorev, E.A., Hogg, N., Kalyanaraman, B., 1998. Rapid and irreversible inhibition of creatine kinase by peroxynitrite. FEBS Lett. 427, 171–174.
Küçükatay, V., Savcioğlu, F., Hacioğlu, G., Yargiçoğlu, P., Ağar, A., 2005. Effect of sulfite on cognitive function in normal and sulfite oxidase deficient rats. Neurotoxicol. Teratol. 27, 47–54.
LeBel, C.P., Ischiropoulos, H., Bondy, S.C., 1992. Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem. Res. Toxicol. 5, 227–231.
Li, C., et al., 2011. Hydrogen peroxide targets the cysteine at the active site and irreversibly inactivates creatine kinase. Int. J. Biol. Macromol. 49, 910–916.
Liang, L.P., Waldbaum, S., Rowley, S., Huang, T.T., Day, B.J., Patel, M., 2012. Mitochondrial oxidative stress and epilepsy in SOD2 deficient mice: attenuation by a lipophilic metalloporphyrin. Neurobiol. Dis. 45, 1068–1076.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Morrison, J.F., 1954. The activation of aconitase by ferrous ions and reducing agents. Biochem. J. 58, 685–692.
Mottley, C., Mason, R.P., 1988. Sulfate anion free radical formation by the peroxidation of (Bi)sulfite and its reaction with hydroxyl radical scavengers. Arch. Biochem. Biophys. 267, 681–689.
Myers, C.R., Antholine, W.E., Myers, J.M., 2010. The pro-oxidant chromium(VI) inhibits mitochondrial complex I, complex II, and aconitase in the bronchial epithelium: EPR markers for Fe–S proteins. Free Radic. Biol. Med. 49, 1903–1915.
Ngu, L.H., Afroze, B., Chen, B.C., Affandi, O., Zabedah, M.Y., 2009. Molybdenum cofactor deficiency in a Malaysian child. Singapore Med. J. 50, e365–e367.
Ozturk, O.H., Oktar, S., Aydin, M., Kucukatay, V., 2010. Effect of sulfite on antioxidant enzymes and lipid peroxidation in normal and sulfite oxidase-deficient rat erythrocytes. J. Physiol. Biochem. 66, 205–212.
Patel, M., 2004. Mitochondrial dysfunction and oxidative stress: cause and consequence of epileptic seizures. Free Radic. Biol. Med. 37, 1951–1962.
Peiser, G.D., Yang, S.F., 1979. Sulfite-mediated destruction of β-carotene. J. Agric. Food Chem. 27, 446–449.
Ramirez, O., Jimenez, E., 2000. Opposite transitions of chick brain catalytically active cytosolic creatine kinase isoenzymes during development. Int. J. Dev. Neurosci. 18, 815–823.
Reiter, R.J., Tan, D.X., Manchester, L.C., Qi, W., 2001. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species: a review of the evidence. Cell Biochem. Biophys. 34, 237–256.
Rosenthal, R.E., Hamud, F., Fiskum, G., Varghese, P.J., Sharpe, S., 1987. Cerebral ischemia and reperfusion — prevention of brain mitochondrial injury by lidoflazine. J. Cereb. Blood Flow Metab. 7, 752–758.
Rustin, P., et al., 1994. Biochemical and molecular investigations in respiratory chain deficiencies. Clin. Chim. Acta 228, 35–51.
Sass, J.O., et al., 2010. Functional deficiencies of sulfite oxidase: differential diagnoses in neonates presenting with intractable seizures and cystic encephalomalacia. Brain Dev. 32, 544–549.
Schapira, A.H.V., et al., 1990. Anatomic and disease specificity of NADH CoQ1 reductase (Complex I) deficiency in Parkinson's disease. J. Neurochem. 55, 2142–2145.
Schuierer, G., Kurlemann, G., Bick, U., Stephani, U., 1995. Molybdenum-cofactor deficiency: CT and MR findings. Neuropediatrics 26, 51–54.
Shi, X., Mao, Y., 1994. 8-Hydroxy-2′-deoxyguanosine formation and DNA damage induced by sulfur trioxide anion radicals. Biochem. Biophys. Res. Commun. 205, 141–147.
Slot, H.M., et al., 1993. Molybdenum-cofactor deficiency: an easily missed cause of neonatal convulsions. Neuropediatrics 24, 139–142.
Southerland, W.M., Akogyeram, C.O., Toghrol, F., Sloan, L., Scherrer, R., 1982. Interaction of bisulfite with unsaturated fatty acids. J. Toxicol. Environ. Health 10, 479–491.
Stachowiak, O., Dolder, M., Wallimann, T., Richter, C., 1998. Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. J. Biol. Chem. 273, 16694–16699.
Tamura, H., et al., 2013. Melatonin as a free radical scavenger in the ovarian follicle. Endocr. J. 60, 1–13.
Tan, W.H., et al., 2005. Isolated sulfite oxidase deficiency: a case report with a novel mutation and review of the literature. Pediatrics 116, 757–766.
Taylor, S.L., Higley, N.A., Bush, R.K., 1986. Sulfites in foods: uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity, and hypersensitivity. Adv. Food Res. 30, 1–76.
Vasquez-Vivar, J., Kalyanaraman, B., Kennedy, M.C., 2000. Mitochondrial aconitase is a source of hydroxyl radical. An electron spin resonance investigation. J. Biol. Chem. 275, 14064–14069.
Vijayakumar, K., et al., 2011. Clinical neuroimaging features and outcome in molybdenum cofactor deficiency. Pediatr. Neurol. 45, 246–252.
Vincent, A.S., et al., 2004. Sulfite-mediated oxidative stress in kidney cells. Kidney Int. 65, 393–402.
Wallimann, T., et al., 1998. Creatine kinase: an enzyme with a central role in cellular energy metabolism. MAGMA 6, 116–119.
Wang, P.F., McLeish, M.J., Kneen, M.M., Lee, G., Kenyon, G.L., 2001. An unusually low pK(a) for Cys282 in the active site of human muscle creatine kinase. Biochemistry 40, 11698–11705.
Wendt, S., Schlattner, U., Wallimann, T., 2003. Differential effects of peroxynitrite on human mitochondrial creatine kinase isoenzymes. Inactivation, octamer destabilization, and identification of involved residues. J. Biol. Chem. 278, 1125–1130.
Wolosker, H., Panizzutti, R., Engelender, S., 1996. Inhibition of creatine kinase by S-nitrosoglutathione. FEBS Lett. 392, 274–276.
Woo, W.H., Yang, H., Wong, K.P., Halliwell, B., 2003. Sulphite oxidase gene expression in human brain and in other human and rat tissues. Biochem. Biophys. Res. Commun. 305, 619–623.
Wyse, A.T., Brusque, A.M., Silva, C.G., Streck, E.L., Wajner, M., Wannmacher, C.M., 1998. Inhibition of Na+, K+-ATPase from rat brain cortex by propionic acid. Neuroreport 9, 1719–1721.
Yang, S.F., 1973. Destruction of tryptophan during the aerobic oxidation of sulfite ions. Environ. Res. 6, 395–402.
Zhang, X., Vincent, A.S., Halliwell, B., Wong, K.P., 2004. A mechanism of sulfite neurotoxicity: direct inhibition of glutamate dehydrogenase. J. Biol. Chem. 279, 43035–43045.

Capítulo II

Sulfite disrupts brain mitochondrial energy homeostasis and induces mitochondrial permeability transition pore opening via thiol group modification

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Sulfite disrupts brain mitochondrial energy homeostasis and induces mitochondrial permeability transition pore opening via thiol group modification

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Abstract

Sulfite oxidase (SO) deficiency is an inherited metabolic disease biochemically characterized by the accumulation of sulfite, thiosulfate and S-sulfocysteine in tissues and biological fluids of the affected patients. The main clinical symptoms include severe neurological dysfunction and brain abnormalities, whose pathophysiology is still unknown. The present study investigated the *in vitro* effects of sulfite and thiosulfate on mitochondrial homeostasis in isolated brain mitochondria from young rats in the presence or absence of Ca^{2+}. It was verified that sulfite *per se*, but not thiosulfate, decreased state 3, cyanide m-chlorophenyl hydrazine-stimulated state and respiratory control ratio in mitochondria respiring with glutamate plus malate, suggesting that the oxidation of these substrates could be impaired. In line with this, we found that sulfite inhibited the activities of glutamate and malate dehydrogenases. Sulfite also induced mitochondrial swelling and reduced mitochondrial membrane potential, Ca^{2+} retention capacity, matrix NAD(P)H pool and cytochrome *c* immunocontent when Ca^{2+} was present in the medium. These alterations were prevented by ruthenium red, cyclosporine A and ADP, supporting the involvement of mitochondrial permeability transition (MPT) in these effects. We further observed that, among various MPT inhibitors, only the thiol alkylating agent N-ethylmaleimide prevented the sulfite-elicited swelling and that sulfite decreased thiol group content in brain mitochondria. These findings indicate that sulfite acts directly on MPT pore containing thiol groups. Therefore, it may be presumed that disturbance of mitochondrial energy homeostasis and MPT induced by sulfite could be involved in the neuronal damage found in patients affected by SO deficiency.

Keywords: Sulfite oxidase deficiency; sulfite; bioenergetic dysfunction; mitochondrial permeability transition; calcium; brain mitochondria.

1. Introduction

Sulfite, thiosulfate and S-sulfocysteine accumulate in tissues and biological fluids of patients affected by sulfite oxidase (SO) deficiency, an autosomal recessive disorder which can arise either from the isolated deficiency of the enzyme SO or from defects in the biosynthetic pathway of its essential cofactor, a molybdenum containing pterin molecule [1, 2]. SO is a mitochondrial enzyme that catalyzes the final step in the oxidative degradation of the sulfur containing amino acids cysteine and methionine, playing also an important role in detoxifying exogenously supplied sulfite, since this metabolite may be generated from compounds that are used in food and pharmaceutical industries as preservatives and antimicrobial agents [3-5].

Both forms of SO deficiency are clinically characterized by progressive neurological dysfunction, severe neonatal seizures, lens subluxation, axial hypotonia, limb hypertonicity and failure to thrive, resulting often in early childhood death [1, 2, 6-9]. Neuropathological studies reveal severe encephalopathy with neuronal loss and demyelination in the cerebral white matter accompanied by gliosis and diffuse spongiosis. Marked atrophy of the cerebral cortex, basal ganglia, thalami, as well as myelin loss in cerebellum are also reported [1, 7, 10, 11]. Furthermore, MRI scans show hypoplasia of the corpus callosum, basal ganglia and brainstem, and cystic changes and calcifications in the basal ganglia [1, 8].

Although brain abnormalities are predominant features in patients affected by SO deficiency, the biochemical basis of the pathogenesis characteristic of this disorder is still unclear. Nevertheless, there is evidence that accumulation of sulfite and its derivatives may be responsible for the

clinical findings in affected patients. In this regard, it has been shown that sulfite can undergo oxidation generating free radicals [12-16], which induce oxidative stress in rat brain and erythrocytes [17-21]. Moreover, sulfite impairs the electron flow through the respiratory chain, whereas sulfite and thiosulfate inhibit the activity of creatine kinase in brain of rats [22]. Zhang and colleagues [23] also showed that sulfite causes a decrease in ATP biosynthesis and mitochondrial membrane potential, and inhibits the activity of glutamate dehydrogenase in rat brain, suggesting mitochondrial dysfunction. In this particular, it should be emphasized that lactic acidemia has been described in affected patients [2, 10, 24]. It was also shown that S-sulfocysteine, a sulfite derivative, triggers excessive Ca^{2+} influx through NMDA receptor overactivation [25]. Recent studies demonstrated that sulfite causes neuron loss in rat hippocampus and somatosensory cortex [26, 27].

Calcium homeostasis dysregulation has been suggested to play an important role in the pathophysiology of neurodegenerative disorders that are associated with excitotoxicity, bioenergetic dysfunction and oxidative stress [28-36]. Under such pathological conditions, excessive Ca^{2+} uptake by the mitochondrion directly result in organelle dysfunction characterized by exacerbated reactive oxygen species formation, dissipation of the membrane potential, altered redox potential and opening of the mitochondrial permeability transition (MPT) pore, which may lead to cell death [37, 38].

Since the effects of sulfite and thiosulfate on mitochondrial function are not totally elucidated, we examined the *in vitro* effects of these compounds on ADP-stimulated state (state 3), resting state (state 4) and carbonyl cyanide m-chlorophenyl hydrazine (CCCP)-stimulated state (uncoupled state) of

mitochondrial respiration, the respiratory control ratio (RCR), as well as the activities of glutamate, malate and α-ketoglutarate dehydrogenases in brain mitochondrial preparations from young rats. Considering that a mitochondrial dysfunction can compromise Ca^{2+} buffering system and that this may be involved in the pathogenesis of SO deficiency, we also investigated the influence of sulfite in the presence of micromolar concentrations of Ca^{2+} on mitochondrial membrane potential, swelling, Ca^{2+} retention capacity, matrix NAD(P)H content, membrane protein thiol group content and cytochrome *c* release.

2. Material and Methods

2.1 Reagents

All chemicals were purchased from Sigma (St. Louis, MO, USA), except for calcium green-5N that was obtained from Molecular Probes, Invitrogen (Carlsbad, CA), and mouse anti-cytochrome *c* monoclonal antibody and anti-mouse IgG peroxidase-linked antibody from Abcam (Cambridge, UK). Sulfite and thiosulfate were dissolved on the day of the experiments in the buffer used for each technique and the pH was adjusted to 7.4. The final concentrations of these metabolites in the incubation medium ranged from 1 to 500 μM.

2.2 Animals

Thirty-day-old male Wistar rats, obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, were used. The animals were maintained on a 12:12 h light/dark cycle (lights on 07:00-19:00 h) in air conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20 % (w/w) protein commercial chow (SUPRA, Porto Alegre, RS, Brazil). The experimental protocol was approved by the Ethics Committee for Animal Research of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, and followed the NIH Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

2.3 Preparation of mitochondrial fractions

Forebrain mitochondria were isolated from 30-day-old rats as previously described [39] with slight modifications [40]. Animals were killed by decapitation, had their brains rapidly removed and put into ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1 % bovine serum albumin (BSA; fatty acid free) and 10 mM HEPES, pH 7.2. The cerebellum, pons, medulla and olfactory bulbs were removed and the remaining material was used as the forebrain. The forebrain was cut into small pieces using surgical scissors, extensively washed to remove blood and homogenized 1:10 in a Dounce homogenizer using both a loose-fitting and a tight-fitting pestle. The homogenate was centrifuged for 3 min at 2000 *g*. After centrifugation, the supernatant was again centrifuged for 8 min at 12,000 *g*. The pellet was suspended in isolation buffer containing 10 µL of 10 % digitonin and centrifuged for 8 min at 12,000 *g*. The final pellet containing the mitochondria was gently washed and suspended in isolation buffer devoid of EGTA, at an approximate protein concentration of 20 $mg.mL^{-1}$. For the measurement of malate dehydrogenase (MDH), glutamate dehydrogenase (GDH) and α-ketoglutarate dehydrogenase (α-KGDH) activities, mitochondrial preparations were submitted to a pre-incubation at 37 ºC for 30 min in the absence or presence of sulfite.

We carried out parallel experiments with various blanks (controls) in the presence or absence of sulfite and thiosulfate, and also with or without mitochondrial preparations in the incubation medium in order to detect any interference (artifacts) in the techniques utilized to measure the mitochondrial parameters.

2.4 Determination of mitochondrial respiratory parameters by oxygen consumption

Oxygen consumption rate was measured according to Amaral et al. [41] using a Clark-type electrode in a thermostatically controlled (37 °C) and magnetically stirred incubation chamber using glutamate plus malate (2.5 mM each), succinate (5 mM) plus rotenone (2 μg.mL^{-1}), α-ketoglutarate (5 mM) or pyruvate plus malate (2.5 mM each) as substrates. Sulfite or thiosulfate was added to the reaction medium consisted of 0.3 M sucrose, 5 mM MOPS, 5 mM potassium phosphate, 1 mM EGTA and 0.1 % BSA, pH 7.4, and mitochondrial preparations (0.75 mg protein.mL^{-1} using glutamate plus malate, α-ketoglutarate or pyruvate plus malate and 0.5 mg protein.mL^{-1} using succinate). State 3 respiration was measured after the addition of 1 mM ADP to the incubation medium. In order to measure resting (state 4) respiration, 1 μg.mL^{-1} oligomycin A was added to the incubation medium. The respiratory control ratio (RCR: state 3/state 4) was then calculated. The uncoupled state was induced by the addition of the classical uncoupler CCCP (1 μM). States 3, 4 and CCCP-induced state were calculated as nmol O_2 consumed. min^{-1}.mg protein^{-1} and the results were expressed as percentage of control.

2.5 Determination of glutamate dehydrogenase (GDH) activity

GDH activity was assayed according to Colon et al. [42]. The reaction mixture contained mitochondrial preparations (60 μg protein.mL^{-1}), 50 mM triethanolamine buffer, pH 7.8, 2.6 mM EDTA, 105 mM ammonium acetate, 0.2 mM NADH, 10 mM α-ketoglutarate and 1.0 mM ADP. The reduction of NADH absorbance was monitored spectrophotometrically at 340 nm. GDH activity was

calculated as μmol NADH.min^{-1}.mg protein^{-1} and expressed as percentage of control.

2.6 Determination of malate dehydrogenase (MDH) activity

MDH activity was measured according to Kitto [43]. The incubation medium consisted of mitochondrial preparations (7 μg protein.mL^{-1}), 10 μM rotenone, 0.1 % Triton X-100, 0.14 mM NADH, 0.3 mM oxaloacetate and 50 mM potassium phosphate, pH 7.4. MDH activity was determined following the reduction of NADH fluorescence at wavelengths of excitation and emission of 366 and 450 nm, respectively. MDH activity was calculated as μmol NADH.min^{-1}.mg protein^{-1} and expressed as percentage of control.

2.7 Determination of α-ketoglutarate dehydrogenase (α-KGDH) complex activity

The activity of α-KGDH complex was evaluated according to Lai and Cooper [44] and Tretter and Adam-Vizi [45] with some modifications. The incubation medium contained mitochondrial preparations (0.25 mg protein.mL^{-1}), 50 mM K_2PO_4, 1 mM $MgCl_2$, 0.2 mM thiamine pyrophosphate, 0.4 mM ADP, 10 μM rotenone, 0.2 mM EGTA, 0.12 mM coenzyme A-SH, 1 mM α-ketoglutarate, 2 mM NAD^+ and 0.1 % Triton X-100 and the pH was adjusted to 7.35. The reduction of NAD^+ was recorded at wavelengths of excitation and emission of 366 and 450 nm, respectively. α-KGDH activity was calculated as nmol NADH.min^{-1}.mg protein^{-1} and expressed as percentage of control.

2.8 Determination of mitochondrial membrane potential (ΔΨm)

The ΔΨm was estimated according to Akerman and Wikstrom [46] and Figueira et al. [47], following the fluorescence of the cationic dye safranin O (5 µM) on a Hitachi F-4500 spectrofluorometer with magnetic stirring at an excitation and emission of 495 and 586 nm, respectively, using 2.5 mM glutamate plus 2.5 mM malate as substrates. Mitochondrial preparations (0.5 mg protein.mL^{-1}) were incubated at 37 °C with sulfite in 5 mM HEPES buffer, pH 7.2, containing 150 mM potassium chloride, 5 mM magnesium chloride, 0.015 mM EGTA, 5 mM potassium phosphate, 0.01 % BSA and 1 µg.mL^{-1} oligomycin A. $CaCl_2$ (10-30 µM) was added to the reaction medium 50 s after the beginning of the assay. In some experiments, the mitochondrial preparations were incubated with cyclosporin A (CsA; 1 µM), ADP (300 µM) or ruthenium red (RR; 1 µM). In the end of each measurement, maximal depolarization was induced by 1 µM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP). The data were expressed as fluorescence arbitrary units (FAU).

2.9 Determination of mitochondrial swelling

Mitochondrial swelling was assessed by measuring light scattering changes on a Hitachi F-4500 spectrofluorometer with magnetic stirring operating at excitation and emission of 540 nm, using 2.5 mM glutamate plus 2.5 mM malate as substrates. Mitochondrial preparations (0.5 mg protein.mL^{-1}) were incubated at 37 °C with 500 µM sulfite in 5 mM HEPES buffer, pH 7.2, containing 150 mM potassium chloride, 5 mM magnesium chloride, 0.015 mM EGTA, 5 mM potassium phosphate, 0.01 % BSA and 1 µg.mL^{-1} oligomycin A. $CaCl_2$ (40 µM) was added to the reaction medium 50 s after the beginning of the

assay. In some experiments the mitochondrial preparations were incubated with CsA (1 µM), ADP (300 µM), RR (1 µM), N-acetylcysteine (NAC; 1 mM), catalase (CAT, 500 $U.mL^{-1}$), melatonin (MEL, 1 mM), coenzyme Q_{10} (CoQ, 50 µM), trifluoperazine (TFZ; 20 µM), chlorpromazine (CP, 20 µM), quinacrine (QUIN; 200 µM), dithiothreitol (DTT, 3 mM) or N-ethylmaleimide (NEM, 20 µM). In the end of each measurement, maximal swelling was induced by the addition of alamethicin (40 $\mu g.mL^{-1}$), a pore-forming compound. The data were expressed as FAU.

2.10 Mitochondrial Ca^{2+} retention capacity

Ca^{2+} retention capacity was determined in mitochondria incubated at 37 ^{o}C with 500 µM sulfite in 5 mM HEPES buffer, pH 7.2, containing 150 mM potassium chloride, 5 mM magnesium chloride, 0.015 mM EGTA, 5 mM potassium phosphate, 0.01 % BSA, 30 µM ADP, 1 $\mu g.mL^{-1}$ oligomycin A, 2.5 mM glutamate and 2.5mM malate, and supplemented with 0.2 µM calcium green-5N. A low concentration of ADP (30 µM) was present in the incubation medium to achieve more consistent mitochondrial Ca^{2+} uptake responses [48]. Levels of external free Ca^{2+} were measured by recording the fluorescence of calcium green-5N on a temperature-controlled Hitachi F-4500 spectrofluorometer with magnetic stirring operating at excitation and emission wavelengths of 506 and 532 nm, respectively, and slit width of 5 nm. Two min after the addition of mitochondria (0.5 $mg.mL^{-1}$) to the cuvette, 20 µM $CaCl_2$ were added. In some experiments, the mitochondrial preparations were incubated with CsA (1 µM) and ADP (300 µM). The data were expressed as FAU.

2.11 Determination of NAD(P)H fluorescence

Mitochondrial matrix NAD(P)H autofluorescence was measured at 37 °C on a Hitachi F-4500 spectrofluorometer with magnetic stirring operating at an excitation wavelength of 366 nm and emission wavelength of 450 nm, using 2.5 mM glutamate plus 2.5 mM malate as substrates. Mitochondrial preparations (0.5 mg protein.mL^{-1}) were incubated at 37 °C with 500 µM sulfite in 5 mM HEPES buffer, pH 7.2, containing 150 mM potassium chloride, 5 mM magnesium chloride, 0.015 mM EGTA, 5 mM potassium phosphate, 0.01% BSA and 1 µg.mL^{-1} oligomycin A. $CaCl_2$ (20 µM) was added to the reaction medium 50 s after the beginning of the assay. In some experiments, the mitochondrial preparations were incubated with CsA (1 µM), ADP (300 µM) or RR (1 µM). In the end of the measurements, maximal NAD(P)H oxidation was induced by 1 µM FCCP. The data were expressed as FAU.

2.12 Determination of mitochondrial membrane protein thiol group content

The mitochondrial membrane protein thiol content in rat brain was measured according to Kowaltowski et al. [49] with slight modifications. After mitochondrial swelling experiments, the medium was centrifuged at 15,000 *g* for 2 min in order to sediment the mitochondria. The resultant pellet was resuspended in 5 mM HEPES buffer, pH 7.2, containing 150 mM potassium chloride, 5 mM magnesium chloride, 0.015 mM EGTA, 5 mM potassium phosphate and 0.01 % BSA and submitted to three subsequent freeze-thawing procedures to release matrix proteins. A centrifugation was then carried out during 2 min at 15,000 *g*. The pellet was treated with 200 µL of 6.5 % trichloroacetic acid and centrifuged at 15,000 *g* during 2 min in order to

precipitate the proteins. This procedure was repeated twice. The final pellet was suspended in 200 µL of a medium containing 0.5 mM EDTA and 0.5 M Tris, pH 8.3. Three hundred and forty micrograms of protein were added to 1 mL of a solution containing 100 µM 5,5'-dithio-bis(2-nitrobenzoic acid), 0.5 mM EDTA and 0.5 M Tris, pH 8.3. Absorption was measured at 412 nm. Thiol group content was calculated as nmol 5-thio-2-nitrobenzoic acid (TNB).mg protein^{-1} and expressed as percentage of control.

2.13 Cytochrome c immunocontent measurement

After mitochondrial swelling experiments, the medium was centrifuged at 12,000 *g* for 10 min in order to sediment the mitochondria. The resultant pellet was resuspended in 1 x RIPA buffer and centrifuged (10,000 *g* for 5 minutes at 4^{o}C). Equal amounts of protein (30 µg per well) for each sample prepared in Laemmli-sample buffer (62.5 mM Tris–HCl, pH 6.8, 1 % (w/v) SDS, 10% (v/v) glycerol) were fractionated by SDS-PAGE and electro-blotted onto nitrocellulose membranes with Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad (Hercules, CA, USA). Protein loading and electro-blotting efficiency were verified through Ponceau S staining, and the membrane was washed with Tween-Tris buffered saline (Tris 100 mM, pH 7.5, 0.9% NaCl and 0.1% Tween-20). Membranes were incubated for 20 min at room temperature in SNAP i.d.® 2.0 Protein Detection System Merck Millipore (Billerica, MA, USA) with cytochrome *c* primary antibody (1:500 dilution range) and washed with TTBS afterwards. Anti-mouse IgG peroxidase-linked secondary antibody was incubated with membrane for additional 20 min in SNAP (1:5,000 dilution range), washed again and the immunoreactivity was detected by enhanced

chemiluminescence using Supersignal West Pico Chemiluminescent kit from Thermo Scientific (Luminol/Enhancer and Stable Peroxide Buffer). Densitometric analysis of the films was performed with Image J software. Blots were developed to be linear in the range used for densitometry. The results were expressed as percentage of control.

2.14 Protein determination

Protein content was measured by the method of Bradford [50], using bovine serum albumin as a standard.

2.15 Statistical analysis

Results are presented as mean ± standard deviation. Assays were performed in duplicate or triplicate and the mean was used for statistical analysis. Data were analyzed by one-way ANOVA, followed by the post hoc Duncan multiple range test when F was significant. Only significant F values are shown in the text. Differences between groups were rated significant at $P<0.05$. All analyses were carried out using the Statistical Package for the Social Sciences (SPSS) software.

3. Results

3.1 Sulfite alters oxygen consumption in brain mitochondria supported by glutamate plus malate

First, we determined the effect of sulfite and thiosulfate on brain mitochondrial oxygen consumption using glutamate plus malate, succinate, α-ketoglutarate or pyruvate plus malate as substrates. Figure 1 shows that brain mitochondria incubated under our experimental conditions were well functioning, as indicated by the higher respiratory rates observed in the presence of ADP (state 3) relatively to those obtained after the addition of the ATP synthase inhibitor oligomycin A (state 4). It was verified that sulfite decreased state 3 respiration [$F_{(2,9)}$=15.546, P<0.01] (Figure 1A), CCCP-induced state respiration (uncoupled state) [$F_{(2,9)}$=30.413, P<0.001] (Figure 1C) and RCR [$F_{(2,9)}$=25.149, P<0.001] (Figure 1D), but not state 4 (Figure 1B), in mitochondria supported by glutamate plus malate. In contrast, sulfite did not alter mitochondrial respiration when succinate, α-ketoglutarate or pyruvate plus malate were used as substrates. Moreover, thiosulfate did not affect mitochondrial oxygen consumption regardless the substrate used (results not shown).

3.2 Sulfite inhibits GDH and MDH activities in brain mitochondria

We assessed the effect of sulfite on the activities of GDH, MDH and α-KGDH in mitochondrial preparations in order to evaluate whether the inhibition of brain mitochondrial respiration caused by this metabolite in the presence of glutamate plus malate as substrates could be explained by the impairment of glutamate and malate oxidation. Figures 2A and 2B show that sulfite inhibited

GDH [$F_{(4,15)}$=3.163, P<0.05] and MDH [$F_{(4,25)}$=17.192, P<0.001] activities. In contrast, α-KGDH activity was not altered by sulfite (Figure 2C).

3.3 Sulfite induces mitochondrial membrane potential (ΔΨm) dissipation and swelling in the presence of Ca^{2+} in brain mitochondria supported by glutamate plus malate

The next set of experiments was carried out to investigate the effect of sulfite on ΔΨm in state 4 respiring mitochondria in the absence or presence of Ca^{2+} (10-30 μM), using glutamate plus malate as substrates. It can be seen in Figure 3A that sulfite caused a progressive reduction of ΔΨm after the addition of increasing Ca^{2+} concentrations. In contrast, no effect could be observed in the absence of Ca^{2+} in the incubation medium. We then evaluated the effect of raising sulfite concentrations (10-500 μM) on ΔΨm in the presence of Ca^{2+} (20 μM) and observed that sulfite dissipates ΔΨm in a dose-dependent manner (Figure 3B). RR, a potent inhibitor of the mitochondrial Ca^{2+} uptake, was able to prevent sulfite-induced ΔΨm reduction, implying that sulfite effects are dependent on the entrance of Ca^{2+} into the mitochondria. Furthermore, the decrease of ΔΨm provoked by sulfite in the presence of Ca^{2+} was prevented by CsA and ADP, inhibitors of MPT (Figure 3C).

In order to further study a possible involvement of the non-selective inner membrane permeabilization due to MPT pore opening with sulfite-induced ΔΨm decrease, we measured mitochondrial swelling by following light scattering changes. It was verified that sulfite caused mitochondrial swelling in the presence of Ca^{2+}, being this effect fully prevented by RR, CsA and ADP, reinforcing therefore the induction of MPT by sulfite (Figure 4).

3.4 Sulfite reduces Ca^{2+} retention capacity in brain mitochondria supported by glutamate plus malate

We then investigated the influence of sulfite on mitochondrial Ca^{2+} retention, since MPT induction leads to a rapid release of Ca^{2+} from mitochondria. As shown in Figure 5, sulfite reduced the mitochondrial Ca^{2+} retention capacity compared with control mitochondria supported by glutamate plus malate. Furthermore, CsA and ADP prevented this effect, indicating the involvement of MPT induction in the mitochondrial Ca^{+2} handling disruption induced by sulfite.

3.5 Sulfite decreases mitochondrial matrix NAD(P)H pool in the presence of Ca^{2+} in brain mitochondria supported by glutamate plus malate

Sulfite reduced NAD(P)H fluorescence in the presence of Ca^{2+} in mitochondria supported by glutamate plus malate (Figure 6), suggesting that this metabolite decreased NAD(P)H pool due either to a oxidation of the reduced equivalents or their loss from the matrix. CsA, ADP and RR were able to prevent this effect, suggesting the involvement of MPT induction.

3.6 NEM prevents sulfite-induced swelling in the presence of Ca^{2+} in brain mitochondria supported by glutamate plus malate

In the next step, we evaluated whether compounds reported to inhibit the MPT, such as phospholipase A2 inhibitors (TFZ, CP and QUIN) [51, 52], antioxidants (NAC, CAT, MEL and CoQ) [53, 54], the reductant reagent DTT [49, 55, 56] and the hydrophobic thiol reagent NEM [49, 55-57], could prevent the mitochondrial swelling induced by sulfite. We found that the inhibitors of

phospholipase A2, antioxidants and DTT did not alter this effect (results not shown). In contrast, NEM prevented sulfite-induced swelling in the presence of Ca^{2+}, suggesting that sulfite exerts a direct effect on thiol groups of proteins involved in MPT pore formation (Figure 7).

3.7 Sulfite decreases the content of thiol groups in the presence of Ca^{2+} in brain mitochondria supported by glutamate plus malate

Our results also demonstrate that 500 µM sulfite significantly decreased the content of thiol groups in the presence of Ca^{2+} in brain mitochondria [$F_{(2,11)}$=34.591, P<0.001] (Figure 8), reinforcing the view that sulfite has a direct effect on these groups. We further observed that 200 µM NEM decreased this parameter in the same conditions (positive control) (Figure 8).

3.8 Sulfite induces cytochrome c release from brain mitochondria in the presence of Ca^{2+}

Finally, we measured the effect of sulfite on cytochrome *c* release from mitochondria. We found that sulfite markedly decreased mitochondrial cytochrome *c* immunocontent [$F_{(2,6)}$=119.171, P<0.001] (Figure 9), indicating an increase of cytochrome *c* release from the mitochondria. CsA attenuated this effect, implying that it occurred due to sulfite-induced MPT in the presence of Ca^{2+}.

4. Discussion

High tissue levels of sulfite and thiosulfate are characteristic of isolated SO deficiency and molybdenum cofactor deficiency. Affected patients predominantly present neonatal seizures, encephalopathy and psychomotor retardation, whose pathophysiology is uncertain. However, some evidences indicate that mitochondrial dysfunction caused by the accumulating metabolites may be involved in the neurological dysfunction of these diseases [2, 10, 22-24]. Therefore, in the present study we investigated the effect of sulfite and thiosulfate on brain mitochondrial homeostasis.

We first verified that sulfite, but not thiosulfate, decreased oxygen consumption in state 3 and uncoupled respiration in mitochondria supported by glutamate plus malate, that are electron donors of complex I, but not with succinate, which donates electrons to complex II, and other NADH-linked substrates (α-ketoglutarate or pyruvate plus malate). Our findings are in accordance with the results found by Zhang and collaborators [23], showing that sulfite causes ATP depletion in PC12 and Neuro-2a cells. Taken together, these data indicate that sulfite-induced decrease of mitochondrial respiration occurred due to a blockage of glutamate and/or malate oxidation. We further verified that sulfite inhibited GDH and MDH activities, whereas no alteration was observed in α-KGDH activity. In the brain, GDH reaction occurs mainly in the direction of oxidative deamination of glutamate forming NADH and α-ketoglutarate, which can be oxidized through the Krebs cycle [58]. MDH, in turn, is a Krebs cycle enzyme and also a component in the malate-aspartate shuttle system. Therefore, it is presumed that the mitochondrial respiration impairment caused by sulfite may be due to the inhibition of glutamate and malate oxidation through

GDH and MDH, which causes a decrease in the availability of intermediates of tricarboxylic acid cycle and a lack of reduced equivalents to supply the respiratory chain. These findings are in line with those observed by Zhang and colleagues [23], who showed that 100 μM sulfite, which was the highest dose tested, moderately inhibits MDH activity and markedly diminished GDH activity.

Next, we observed that sulfite decreased the ΔΨm and induced swelling in brain mitochondria in the presence of Ca^{2+}, but not in the absence of this cation. In addition, the prevention of these effects by RR, a potent inhibitor of the mitochondrial Ca^{2+} uptake [59], supports the importance of the involvement of Ca^{2+} in these effects. It is well known that mitochondria play a central role in cytosolic Ca^{2+} buffering, although an abnormal increase in mitochondrial Ca^{2+} uptake may induce MPT and lead to apoptosis in different cells [38, 60-62]. The MPT causes a non-selective traffic between the mitochondrial matrix and the cytosol, allowing the influx of water and substances up to 1.5 kDa into the matrix and leading to mitochondrial swelling, loss of metabolites (Ca^{2+}, glutathione, NADH and NADPH), ΔΨm collapse, impairment of oxidative phosphorylation and ATP synthesis, as well as release of proapoptotic factors, such as cytochrome *c* [63-68].

Our results also showed that the decrease of ΔΨm elicited by sulfite is due to a non-selective membrane permeabilization caused by MPT pore opening, since ΔΨm dissipation was accompanied by mitochondrial swelling and was inhibited by CsA and ADP. In this particular, CsA inhibits MPT by binding cyclophilin D, a mitochondrial matrix protein which has been reported to be a MPT pore modulator through its interaction with the adenine nucleotide translocator (ANT) [60, 69-71]. Furthermore, it is known that adenine

nucleotides inhibit MPT pore through their binding to ANT [38, 48]. Our data showing that sulfite reduced the mitochondrial Ca^{2+} retention capacity and that this effect was prevented by CsA plus ADP are also in line with the induction of MPT pore opening by synergistical effects of sulfite and Ca^{2+}.

We further verified that NEM prevented the mitochondrial swelling induced by sulfite in the presence of Ca^{2+}. NEM is an alkylating agent that reacts with sulfhydryl groups to form stable thioether bonds. Previous reports demonstrated that NEM has access to critical thiol groups on ANT that regulate the MPT pore opening [56, 72, 73]. Therefore, our data showing that NEM prevented the mitochondrial swelling caused by sulfite indicate that this compound modulates MPT by attacking thiol groups, which increases the probability of pore opening. This is reinforced by our findings showing that sulfite decreased the content of thiol groups in brain mitochondria and is in accordance with other evidences demonstrating that sulfite reacts directly with sulfhydryl groups [1]. On the other hand, since antioxidants did not prevent sulfite-induced swelling, it may be suggested that the reactive species generated from this metabolite do not alter critical groups involved in MPT pore opening. Indeed, it has been reported that other compounds, such as acetoacetate, phenylarsine oxide and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid, are able to induce MPT independently of reactive species [49, 56, 57, 72]. It is also of note that Costantini and collaborators [55] reported that at least two distinct sites containing thiol groups modulate MPT and that one site is insensitive to reductants like DTT.

Sulfite also diminished the mitochondrial matrix NAD(P)H levels in the presence of Ca^{2+}. The loss of the mitochondrial pool of pyridine nucleotides

could be explained by a release of NAD(P)H from the mitochondria through the MPT pore, which can lead to the impairment of mitochondrial redox homeostasis. In fact, the matrix NAD(P)H pool decrease elicited by sulfite was inhibited by CsA and ADP, implying that this effect was caused by MPT induction.

We also found that sulfite reduced mitochondrial cytochrome *c* immunocontent, which could be related to the MPT induced by this compound. In this regard, the release of cytochrome *c* from the intermembrane mitochondrial space into the cytosol typically accompanies the osmotic swelling and the physical rupture of the mitochondrial outer membrane provoked by MPT [74, 75]. The observation that the decrease of mitochondrial cytochrome *c* immunocontent was prevented by CsA supports this hypothesis. The release of cytochrome *c* from mitochondria into the cytosol plays a key role in the apoptosis induction by forming the apoptosome complex with Apaf-1 and procaspase-9 and initiating the caspase cascade [67, 76, 77]. Thus, our results suggest that sulfite may induce apoptosis by promoting MPT in the presence of Ca^{2+}.

It is noteworthy that neurons are more susceptible to the induction of MPT due to increases in intracellular Ca^{2+} levels after NMDA receptor overstimulation (excitotoxicity). In this context, it should be emphasized that S-sulfocysteine, a metabolite structurally similar to glutamate generated from sulfite and free cysteine, is able to activate NMDA receptors [78, 79], leading to high Ca^{2+} influx. Furthermore, the inhibition of GDH by sulfite could lead to high levels of glutamate in the synaptic cleft, contributing to the raise of intracellular Ca^{2+} concentrations. In addition, energy metabolism dysfunction induced by

sulfite could also play an important role in Ca^{2+} dyshomeostasis through the impairment of Ca^{2+} removal systems. Therefore, it is hypothesized that a failure in Ca^{2+} handling by the mitochondrion is potentially involved in the neuropathology of SO deficiency.

In conclusion, our study provides for the first time evidence that sulfite acts synergistically with Ca^{2+} inducing MPT in the brain mediated by a direct attack of sulfite on critical protein cysteinyl groups of the transition pore. Therefore, it is presumed that bioenergetic dysfunction and MPT induction may be important pathomechanisms underlying the pathogenesis of the neurological dysfunction observed in SO deficient patients. It is also conceivable that protective compounds targeting to mitochondrial thiol groups in MPT pore may be promising therapeutic strategies for SO deficiency.

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References

[1] J.L. Johnson, M. Duran, Molybdenum Cofactor Deficiency and Isolated Sulfite Oxidase Deficiency, in: B.A. Scriver CR, Valle D, Sly WS (Ed.) The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, New York, 2001, pp. 3181-3217.

[2] S.N. Basheer, P.J. Waters, C.W. Lam, C. Acquaviva-Bourdain, G. Hendson, K. Poskitt, J. Hukin, Isolated sulfite oxidase deficiency in the newborn: lactic acidaemia and leukoencephalopathy, Neuropediatrics 38 (2007) 38-41.

[3] C. Feng, G. Tollin, J.H. Enemark, Sulfite oxidizing enzymes, Biochim. Biophys. Acta 1774 (2007) 527-539.

[4] S.L. Taylor, N.A. Higley, R.K. Bush, Sulfites in foods: uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity, and hypersensitivity, Adv. Food Res. 30 (1986) 1-76.

[5] K. Chapman, Sulfite-containing pharmaceuticals, Can. Med. Assoc. J. 148 (1993) 714.

[6] K.Y. Chan, C.K. Li, C.K. Lai, S.F. Ng, A.Y. Chan, Infantile isolated sulphite oxidase deficiency in a Chinese family: a rare neurodegenerative disorder, Hong Kong Med. J. 8 (2002) 279-282.

[7] W.H. Tan, F.S. Eichler, S. Hoda, M.S. Lee, H. Baris, C.A. Hanley, P.E. Grant, K.S. Krishnamoorthy, V.E. Shih, Isolated sulfite oxidase deficiency: a case report with a novel mutation and review of the literature, Pediatrics 116 (2005) 757-766.

[8] M.C. Edwards, J.L. Johnson, B. Marriage, T.N. Graf, K.E. Coyne, K.V. Rajagopalan, I.M. MacDonald, Isolated sulfite oxidase deficiency: review of two cases in one family, Ophthalmology 106 (1999) 1957-1961.

[9] S. Rocha, A.C. Ferreira, A.I. Dias, J.P. Vieira, S. Sequeira, Sulfite oxidase deficiency - An unusual late and mild presentation, Brain Dev. (2013).

[10] C.A. Rupar, J. Gillett, B.A. Gordon, D.A. Ramsay, J.L. Johnson, R.M. Garrett, K.V. Rajagopalan, J.H. Jung, G.S. Bacheyie, A.R. Sellers, Isolated sulfite oxidase deficiency., Neuropediatrics 27 (1996) 299-304.

[11] K. Vijayakumar, R. Gunny, S. Grunewald, L. Carr, K.W. Chong, C. DeVile, R. Robinson, N. McSweeney, P. Prabhakar, Clinical neuroimaging features and outcome in molybdenum cofactor deficiency, Pediatr. Neurol. 45 (2011) 246-252.

[12] C. Mottley, T.B. Trice, R.P. Mason, Direct detection of the sulfur trioxide radical anion during the horseradish peroxidase-hydrogen peroxide oxidation of sulfite (aqueous sulfur dioxide), Mol. Pharmacol. 22 (1982) 732-737.

[13] Z. Abedinzadeh, Sulfur-centered reactive intermediates derived from the oxidation of sulfur compounds of biological interest., Can J Physiol Pharmacol 79 (2001) 166-170.

[14] X. Shi, Generation of SO3.- and OH radicals in SO3(2-) reactions with inorganic environmental pollutants and its implications to SO3(2-) toxicity, J. Inorg. Biochem. 56 (1994) 155-165.

[15] M.T. Baker, D.J. Dehring, M.S. Gregerson, Sulfite supported lipid peroxidation in propofol emulsions, Anesthesiology 97 (2002) 1162-1167.

[16] C. Mottley, R.P. Mason, Sulfate anion free radical formation by the peroxidation of (Bi)sulfite and its reaction with hydroxyl radical scavengers, Arch. Biochem. Biophys. 267 (1988) 681-689.

[17] F. Chiarani, C.S. Bavaresco, C.S. Dutra-Filho, C.A. Netto, A.T. Wyse, Sulfite increases lipoperoxidation and decreases the activity of catalase in brain of rats, Metab. Brain Dis. 23 (2008) 123-132.

[18] N. Derin, D. Akpinar, P. Yargicoglu, A. Agar, M. Aslan, Effect of alpha-lipoic acid on visual evoked potentials in rats exposed to sulfite, Neurotoxicol. Teratol. 31 (2009) 34-39.

[19] O.H. Ozturk, S. Oktar, M. Aydin, V. Kucukatay, Effect of sulfite on antioxidant enzymes and lipid peroxidation in normal and sulfite oxidase-deficient rat erythrocytes, J. Physiol. Biochem. 66 (2010) 205-212.

[20] V. Küçükatay, F. Savcioğlu, G. Hacioğlu, P. Yargiçoğlu, A. Ağar, Effect of sulfite on cognitive function in normal and sulfite oxidase deficient rats, Neurotoxicol. Teratol. 27 (2005) 47-54.

[21] E.N. Herken, E. Kocamaz, O. Erel, H. Celik, V. Kucukatay, Effect of sulfite treatment on total antioxidant capacity, total oxidant status, lipid hydroperoxide, and total free sulfydryl groups contents in normal and sulfite oxidase-deficient rat plasma, Cell. Biol. Toxicol. 25 (2009) 355-362.

[22] M. Grings, A.P. Moura, B. Parmeggiani, G.F. Marcowich, A.U. Amaral, A.T. de Souza Wyse, M. Wajner, G. Leipnitz, Disturbance of brain energy and redox homeostasis provoked by sulfite and thiosulfate: Potential pathomechanisms involved in the neuropathology of sulfite oxidase deficiency, Gene 531 (2013) 191-198.

[23] X. Zhang, A.S. Vincent, B. Halliwell, K.P. Wong, A mechanism of sulfite neurotoxicity: direct inhibition of glutamate dehydrogenase, J. Biol. Chem. 279 (2004) 43035-43045.

[24] F. Eichler, W.H. Tan, V.E. Shih, P.E. Grant, K. Krishnamoorthy, Proton magnetic resonance spectroscopy and diffusion-weighted imaging in isolated sulfite oxidase deficiency, J. Child Neurol. 21 (2006) 801-805.

[25] D.R. Lynch, R.P. Guttmann, Excitotoxicity: perspectives based on N-methyl-D-aspartate receptor subtypes, J. Pharmacol. Exp. Ther. 300 (2002) 717-723.

[26] E. Kocamaz, E. Adiguzel, B. Er, G. Gundogdu, V. Kucukatay, Sulfite leads to neuron loss in the hippocampus of both normal and SOX-deficient rats, Neurochem. Int. 61 (2012) 341-346.

[27] C. Kencebay, N. Derin, O. Ozsoy, D. Kipmen-Korgun, G. Tanriover, N. Ozturk, G. Basaranlar, P. Yargicoglu-Akkiraz, B. Sozen, A. Agar, Merit of quinacrine in the decrease of ingested sulfite-induced toxic action in rat brain, Food Chem. Toxicol. 52 (2013) 129-136.

[28] H. Du, L. Guo, F. Fang, D. Chen, A.A. Sosunov, G.M. McKhann, Y. Yan, C. Wang, H. Zhang, J.D. Molkentin, F.J. Gunn-Moore, J.P. Vonsattel, O. Arancio, J.X. Chen, S.D. Yan, Cyclophilin D deficiency attenuates mitochondrial and neuronal perturbation and ameliorates learning and memory in Alzheimer's disease, Nat. Med. 14 (2008) 1097-1105.

[29] L.J. Martin, B. Gertz, Y. Pan, A.C. Price, J.D. Molkentin, Q. Chang, The mitochondrial permeability transition pore in motor neurons: involvement in the pathobiology of ALS mice, Exp. Neurol. 218 (2009) 333-346.

[30] S. Gandhi, A. Wood-Kaczmar, Z. Yao, H. Plun-Favreau, E. Deas, K. Klupsch, J. Downward, D.S. Latchman, S.J. Tabrizi, N.W. Wood, M.R. Duchen, A.Y. Abramov, PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death, Mol. Cell 33 (2009) 627-638.

[31] J.P. Sheehan, R.H. Swerdlow, W.D. Parker, S.W. Miller, R.E. Davis, J.B. Tuttle, Altered calcium homeostasis in cells transformed by mitochondria from individuals with Parkinson's disease, J. Neurochem. 68 (1997) 1221-1233.

[32] J.P. Sheehan, R.H. Swerdlow, S.W. Miller, R.E. Davis, J.K. Parks, W.D. Parker, J.B. Tuttle, Calcium homeostasis and reactive oxygen species production in cells transformed by mitochondria from individuals with sporadic Alzheimer's disease, J. Neurosci. 17 (1997) 4612-4622.

[33] J.T. Yu, R.C. Chang, L. Tan, Calcium dysregulation in Alzheimer's disease: from mechanisms to therapeutic opportunities, Prog. Neurobiol. 89 (2009) 240-255.

[34] T.R. Rosenstock, A.C. Carvalho, A. Jurkiewicz, R. Frussa-Filho, S.S. Smaili, Mitochondrial calcium, oxidative stress and apoptosis in a neurodegenerative disease model induced by 3-nitropropionic acid, J. Neurochem. 88 (2004) 1220-1228.

[35] R.A. Quintanilla, G.V. Johnson, Role of mitochondrial dysfunction in the pathogenesis of Huntington's disease, Brain. Res. Bull. 80 (2009) 242-247.

[36] M.K. Jaiswal, W.D. Zech, M. Goos, C. Leutbecher, A. Ferri, A. Zippelius, M.T. Carri, R. Nau, B.U. Keller, Impairment of mitochondrial calcium handling in a mtSOD1 cell culture model of motoneuron disease, BMC Neurosci. 10 (2009) 64.

[37] I.B. Zavodnik, I.K. Dremza, V.T. Cheshchevik, E.A. Lapshina, M. Zamaraewa, Oxidative damage of rat liver mitochondria during exposure to t-butyl hydroperoxide. Role of Ca(2)(+) ions in oxidative processes, Life Sci. 92 (2013) 1110-1117.

[38] M. Crompton, The mitochondrial permeability transition pore and its role in cell death, Biochem. J. 341 (Pt 2) (1999) 233-249.

[39] R.E. Rosenthal, F. Hamud, G. Fiskum, P.J. Varghese, S. Sharpe, Cerebral ischemia and reperfusion - prevention of brain mitochondrial injury by lidoflazine, J. Cereb. Blood Flow Metab. 7 (1987) 752-758.

[40] S.R. Mirandola, D.R. Melo, P.F. Schuck, G.C. Ferreira, M. Wajner, R.F. Castilho, Methylmalonate inhibits succinate-supported oxygen consumption by interfering with mitochondrial succinate uptake, J. Inherit. Metab. Dis. 31 (2008) 44-54.

[41] A.U. Amaral, G. Leipnitz, C.G. Fernandes, B. Seminotti, P.F. Schuck, M. Wajner, Alpha-ketoisocaproic acid and leucine provoke mitochondrial bioenergetic dysfunction in rat brain, Brain Res. 1324 (2010) 75-84.

[42] A.D. Colon, A. Plaitakis, A. Perakis, S. Berl, D.D. Clarke, Purification and characterization of a soluble and a particulate glutamate dehydrogenase from rat brain, J. Neurochem. 46 (1986) 1811-1819.

[43] G.B. Kitto, Intra- and extramitochondrial malate dehydrogenase from chicken and tuna heart, Methods Enzymol. 13 (1969) 106-116.

[44] J.C. Lai, A.J. Cooper, Brain alpha-ketoglutarate dehydrogenase complex: kinetic properties, regional distribution, and effects of inhibitors, J. Neurochem. 47 (1986) 1376-1386.

[45] L. Tretter, V. Adam-Vizi, Generation of reactive oxygen species in the reaction catalyzed by alpha-ketoglutarate dehydrogenase, J. Neurosci. 24 (2004) 7771-7778.

[46] K.E. Akerman, M.K. Wikstrom, Safranine as a probe of the mitochondrial membrane potential, FEBS Lett. 68 (1976) 191-197.

[47] T.R. Figueira, D.R. Melo, A.E. Vercesi, R.F. Castilho, Safranine as a fluorescent probe for the evaluation of mitochondrial membrane potential in isolated organelles and permeabilized cells, Methods Mol. Biol. 810 (2012) 103-117.

[48] A. Saito, R.F. Castilho, Inhibitory effects of adenine nucleotides on brain mitochondrial permeability transition, Neurochem. Res. 35 (2010) 1667-1674.

[49] A.J. Kowaltowski, A.E. Vercesi, R.F. Castilho, Mitochondrial membrane protein thiol reactivity with N-ethylmaleimide or mersalyl is modified by Ca2+: correlation with mitochondrial permeability transition, Biochim. Biophys. Acta 1318 (1997) 395-402.

[50] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248-254.

[51] J.G. Pastorino, G. Simbula, K. Yamamoto, P.A. Glascott, Jr., R.J. Rothman, J.L. Farber, The cytotoxicity of tumor necrosis factor depends on induction of the mitochondrial permeability transition, J. Biol. Chem. 271 (1996) 29792-29798.

[52] K.M. Broekemeier, J.R. Iben, E.G. LeVan, E.D. Crouser, D.R. Pfeiffer, Pore formation and uncoupling initiate a Ca2+-independent degradation of mitochondrial phospholipids, Biochemistry 41 (2002) 7771-7780.

[53] M. Nishimura, Y. Okimura, H. Fujita, H. Yano, J. Lee, E. Suzaki, M. Inoue, K. Utsumi, J. Sasaki, Mechanism of 3-nitropropionic acid-induced membrane permeability transition of isolated mitochondria and its suppression by L-carnitine, Cell Biochem. Funct. 26 (2008) 881-891.

[54] C. Chinopoulos, A.A. Starkov, G. Fiskum, Cyclosporin A-insensitive permeability transition in brain mitochondria: inhibition by 2-aminoethoxydiphenyl borate, J. Biol. Chem. 278 (2003) 27382-27389.

[55] P. Costantini, B.V. Chernyak, V. Petronilli, P. Bernardi, Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites, J. Biol. Chem. 271 (1996) 6746-6751.

[56] V. Petronilli, P. Costantini, L. Scorrano, R. Colonna, S. Passamonti, P. Bernardi, The voltage sensor of the mitochondrial permeability transition pore is tuned by the oxidation-reduction state of vicinal thiols. Increase of the gating potential by oxidants and its reversal by reducing agents, J. Biol. Chem. 269 (1994) 16638-16642.

[57] T.S. Kim, D.W. Jeong, B.Y. Yun, I.Y. Kim, Dysfunction of rat liver mitochondria by selenite: induction of mitochondrial permeability transition through thiol-oxidation, Biochem. Biophys. Res. Commun. 294 (2002) 1130-1137.

[58] A. Kelly, C.A. Stanley, Disorders of glutamate metabolism, Ment. Retard. Dev. Disabil. Res. Rev. 7 (2001) 287-295.

[59] C.L. Moore, Specific inhibition of mitochondrial Ca++ transport by ruthenium red, Biochem. Biophys. Res. Commun. 42 (1971) 298-305.

[60] C. Yarana, J. Sripetchwandee, J. Sanit, S. Chattipakorn, N. Chattipakorn, Calcium-induced cardiac mitochondrial dysfunction is predominantly mediated by cyclosporine A-dependent mitochondrial permeability transition pore, Arch. Med. Res. 43 (2012) 333-338.

[61] S.S. Smaili, Y.T. Hsu, R.J. Youle, J.T. Russell, Mitochondria in Ca2+ signaling and apoptosis, J. Bioenerg. Biomembr. 32 (2000) 35-46.

[62] G. Hajnoczky, G. Csordas, S. Das, C. Garcia-Perez, M. Saotome, S. Sinha Roy, M. Yi, Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca2+ uptake in apoptosis, Cell calcium 40 (2006) 553-560.

[63] D.B. Zorov, M. Juhaszova, Y. Yaniv, H.B. Nuss, S. Wang, S.J. Sollott, Regulation and pharmacology of the mitochondrial permeability transition pore, Cardiovasc. Res. 83 (2009) 213-225.

[64] C. Martel, H. Huynh le, A. Garnier, R. Ventura-Clapier, C. Brenner, Inhibition of the Mitochondrial Permeability Transition for Cytoprotection: Direct versus Indirect Mechanisms, Biochem. Res. Int. 2012 (2012) 213403.

[65] W.G. Tatton, C.W. Olanow, Apoptosis in neurodegenerative diseases: the role of mitochondria, Biochim. Biophys. Acta 1410 (1999) 195-213.

[66] M. Zoratti, I. Szabo, The mitochondrial permeability transition, Biochim. Biophys. Acta 1241 (1995) 139-176.

[67] A. Rasola, P. Bernardi, Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis, Cell calcium 50 (2011) 222-233.

[68] A.A. Starkov, C. Chinopoulos, G. Fiskum, Mitochondrial calcium and oxidative stress as mediators of ischemic brain injury, Cell calcium 36 (2004) 257-264.

[69] J. Pottecher, M. Guillot, E. Belaidi, A.L. Charles, A. Lejay, A. Gharib, P. Diemunsch, B. Geny, Cyclosporine A normalizes mitochondrial coupling, reactive oxygen species production, and inflammation and partially restores skeletal muscle maximal oxidative capacity in experimental aortic cross-clamping, J. Vasc. Surg. 57 (2013) 1100-1108 e1102.

[70] E. Basso, L. Fante, J. Fowlkes, V. Petronilli, M.A. Forte, P. Bernardi, Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D, J. Biol. Chem. 280 (2005) 18558-18561.

[71] A. Tanveer, S. Virji, L. Andreeva, N.F. Totty, J.J. Hsuan, J.M. Ward, M. Crompton, Involvement of cyclophilin D in the activation of a mitochondrial pore by Ca2+ and oxidant stress, Eur. J. Biochem. 238 (1996) 166-172.

[72] G.P. McStay, S.J. Clarke, A.P. Halestrap, Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore, Biochem. J. 367 (2002) 541-548.

[73] A.P. Halestrap, K.Y. Woodfield, C.P. Connern, Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase, J. Biol. Chem. 272 (1997) 3346-3354.

[74] M. Crompton, Mitochondrial intermembrane junctional complexes and their role in cell death, J. Physiol. 529 Pt 1 (2000) 11-21.

[75] P.X. Petit, M. Goubern, P. Diolez, S.A. Susin, N. Zamzami, G. Kroemer, Disruption of the outer mitochondrial membrane as a result of large amplitude swelling: the impact of irreversible permeability transition, FEBS Lett. 426 (1998) 111-116.

[76] D.R. Green, J.C. Reed, Mitochondria and apoptosis, Science 281 (1998) 1309-1312.

[77] A. Andreyev, G. Fiskum, Calcium induced release of mitochondrial cytochrome c by different mechanisms selective for brain versus liver, Cell Death Differ. 6 (1999) 825-832.

[78] J.W. Olney, C.H. Misra, T. de Gubareff, Cysteine-S-sulfate: brain damaging metabolite in sulfite oxidase deficiency, J. Neuropathol. Exp. Neurol. 34 (1975) 167-177.

[79] B. Kagedal, M. Kallberg, B. Sorbo, A possible involvement of glutathione in the detoxication of sulfite, Biochem. Biophys. Res. Commun. 136 (1986) 1036-1041.

Legends to figures

Fig. 1. Effect of sulfite on ADP-stimulated respiration (state 3) (A), resting respiration (state 4) (B), CCCP-stimulated respiration (uncoupled state) (C) and on respiratory control ratio (RCR) (D) using brain mitochondria supported by glutamate plus malate (GM), succinate (SUC), α-ketoglutarate (α-KG) and pyruvate plus malate (PM). Sulfite (100 or 500 µM) was added at the beginning of incubation to the reaction medium containing the mitochondrial preparations (0.75 mg protein.mL^{-1} using glutamate plus malate, α-ketoglutarate or pyruvate plus malate and 0.5 mg protein.mL^{-1} using succinate). Values are means ± standard deviation for three to four independent experiments (animals) and are expressed as percentage of controls (Controls: (A) State 3 [nmol O_2.min^{-1}.mg of protein^{-1}]: GM: 80.7 ± 12.7; SUC: 72.2 ± 6.45; α-KG: 41.4 ± 6.54; PM: 124 ± 12.5; (B) State 4 [nmol O_2.min^{-1}.mg of protein^{-1}]: GM: 8.43 ± 1.54; SUC: 13.5 ± 0.87; α-KG: 11.6 ± 0.99; PM: 9.09 ± 0.50; (C) CCCP [nmol O_2.min^{-1}.mg of protein^{-1}]: GM: 85.9 ± 13.9; SUC: 67.0 ± 6.46; α-KG: 27.5 ± 2.59; PM: 124 ± 35.2; (D) RCR: GM: 9.67 ± 0.98; SUC: 5.32 ± 0.19; α-KG: 3.56 ± 0.31; PM: 13.7 ± 2.12. *P < 0.05, ** P < 0.01, *** P < 0.001, compared to controls (Duncan multiple range test).

Fig. 2. Effect of sulfite on glutamate dehydrogenase (GDH) (A), malate dehydrogenase (MDH) (B) and α-ketoglutarate dehydrogenase (α-KGDH) (C) activities in brain mitochondria. Sulfite (1-500 µM) was added to the reaction medium containing the mitochondrial preparations and incubated for 30 min. Values are means ± standard deviation for four to six independent experiments (animals) and are expressed as percentage of controls. (Controls: (A) GDH

[µmol NADH.min^{-1}.mg protein^{-1}]: 74.49 ± 4.470; (B) MDH [µmol NADH.min^{-1}.mg protein^{-1}]: 6377 ± 570.5; (C) α-KGDH [nmol NADH.min^{-1}.mg protein^{-1}]: 54.17 ± 11.04). *P < 0.05, *** P < 0.001, compared to controls (Duncan multiple range test).

Fig 3. Effect of sulfite on mitochondrial membrane potential (ΔΨm) using brain mitochondria supported by glutamate plus malate in the absence or presence of Ca^{2+}. Sulfite (500 µM) (trace b) was added at the beginning of incubation to the reaction medium containing the mitochondrial preparations (0.5 mg protein.mL^{-1}). Increasing concentrations of Ca^{2+} (0 µM, trace b; 10 µM, trace c; 20 µM, trace d; 30 µM, trace e) were added 50 s afterwards (A). Increasing concentrations of sulfite (10 µM, trace b; 100 µM, trace c; 250 µM, trace d; 500 µM, trace e) were added at the beginning of incubation to the reaction medium containing the mitochondrial preparations (0.5 mg protein. mL^{-1}). Ca^{2+} (20 µM) was added 50 s afterwards (B). Sulfite (500 µM) (trace b) was added at the beginning of incubation to the reaction medium containing the mitochondrial preparations (0.5 mg protein.mL^{-1}). Ca^{2+} (20 µM) was added 50 s afterwards. In some experiments, the mitochondrial preparations were incubated with sulfite plus ruthenium red (RR; 1 µM; trace c), cyclosporine A (CsA; 1 µM; trace d) or ADP (300 µM; trace e) (C). Control (trace a) in graphic A was performed in the absence of Ca^{2+} and sulfite, whereas controls (traces a) in graphics B and C were performed in the presence of Ca^{2+} (20 µM) and did not contain sulfite. FCCP (1 µM) was added at the end of the experiment, as indicated. Traces are representative of three independent experiments and were expressed as fluorescence arbitrary units (FAU).

Fig. 4. Effect of sulfite on mitochondrial swelling using brain mitochondria supported by glutamate plus malate in the presence of Ca^{2+}. Sulfite (500 µM) (trace b) was added at the beginning of incubation to the reaction medium containing the mitochondrial preparations (0.5 mg protein.mL^{-1}). Ca^{2+} (40 µM) was added 50 s afterwards. In some experiments, the mitochondrial preparations were incubated with sulfite plus ruthenium red (RR; 1 µM; trace c), cyclosporine A (CsA; 1 µM; trace d) or ADP (300 µM; trace e). Control (trace a) was performed in the presence of Ca^{2+} (40 µM) and did not contain sulfite. Alamethicin (Alam; 40 µg.mg of $protein^{-1}$) was added at the end of the experiment, as indicated. Traces are representative of three independent experiments and were expressed as fluorescence arbitrary units (FAU).

Fig. 5. Effect of sulfite on mitochondrial Ca^{2+} retention capacity using brain mitochondria supported by glutamate plus malate in the presence of Ca^{2+}. Sulfite (500 µM) (trace b) was added at the beginning of incubation to the reaction medium containing ADP (30 µM) and the mitochondrial preparations (0.5 mg protein.mL^{-1}). Ca^{2+} (20 µM) was added afterwards. In some experiments, the mitochondrial preparations were incubated with sulfite and cyclosporine A (CsA; 1 µM) plus ADP (300 µM; trace d). Controls (traces a and c) were performed in the presence of 20 µM Ca^{2+} and did not contain sulfite. Traces are representative of three independent experiments and were expressed as fluorescence arbitrary units (FAU).

Fig. 6. Effect of sulfite on mitochondrial NAD(P)H content using brain mitochondria supported by glutamate plus malate in the presence of Ca^{2+}.

Sulfite (500 µM) (trace b) was added at the beginning of incubation to the reaction medium containing the mitochondrial preparations (0.5 mg protein.mL^{-1}). Ca^{2+} (20 µM) was added 50 s afterwards. In some experiments, the mitochondrial preparations were incubated with sulfite plus ruthenium red (RR; 1 µM; trace c), cyclosporine A (CsA; 1 µM; trace d) or ADP (300 µM; trace e). Control (trace a) was performed in the presence of Ca^{2+} (20 µM) and did not contain sulfite. FCCP (1 µM) was added at the end of the experiment, as indicated. Traces are representative of three independent experiments and were expressed as fluorescence arbitrary units (FAU).

Fig. 7. Effect of N-ethylmaleimide (NEM; 20 µM) on sulfite-induced mitochondrial swelling using brain mitochondria supported by glutamate plus malate in the presence of Ca^{2+}. Sulfite (500 µM) (trace b) was added at the beginning of incubation to the reaction medium containing the mitochondrial preparations (0.5 mg protein.mL^{-1}). Ca^{2+} (40 µM) was added 50 s afterwards. In some experiments, the mitochondrial preparations were incubated with sulfite plus NEM (trace c). Control (trace a) was performed in the presence of Ca^{2+} (40 µM) and did not contain sulfite. Alamethicin (Alam; 40 µg.mg of protein^{-1}) was added at the end of the experiment, as indicated. Traces are representative of three independent experiments and were expressed as fluorescence arbitrary units (FAU).

Fig. 8. Effect of sulfite on thiol group content in brain mitochondria supported by glutamate plus malate in the presence of Ca^{2+}. Sulfite (500 µM) and Ca^{2+} (40 µM) were added to the incubation medium containing the mitochondrial

preparations (0.5 mg protein.mL^{-1}). In some experiments, the mitochondrial preparations were incubated with N-ethylmaleimide (NEM; 200 µM). Values are means ± standard deviation for four to five independent experiments (animals) and are expressed as percentage of control (Control: 540 ± 21.3 nmol TNB.mg protein^{-1}). ** P < 0.01, *** P < 0.001, compared to control (Duncan multiple range test).

Fig. 9. Effect of sulfite on cytochrome *c* immunocontent in brain mitochondria supported by glutamate plus malate in the presence of Ca^{2+}. Sulfite (500 µM) and Ca^{2+} (40 µM) were added to the incubation medium containing the mitochondrial preparations (0.5 mg protein.mL^{-1}). In some experiments, the mitochondrial preparations were incubated with sulfite plus cyclosporine A (CsA; 1 µM). A representative immunoblot of cytochrome *c* is also displayed. Values are means ± standard deviation for three to four independent experiments (animals) and are expressed as percentage of control (Control: 33091 ± 2660 arbitrary units). **P < 0.01, *** P < 0.001, compared to control; $^{###}$P<0.001, compared to sulfite (Duncan multiple range test).

Figure 1

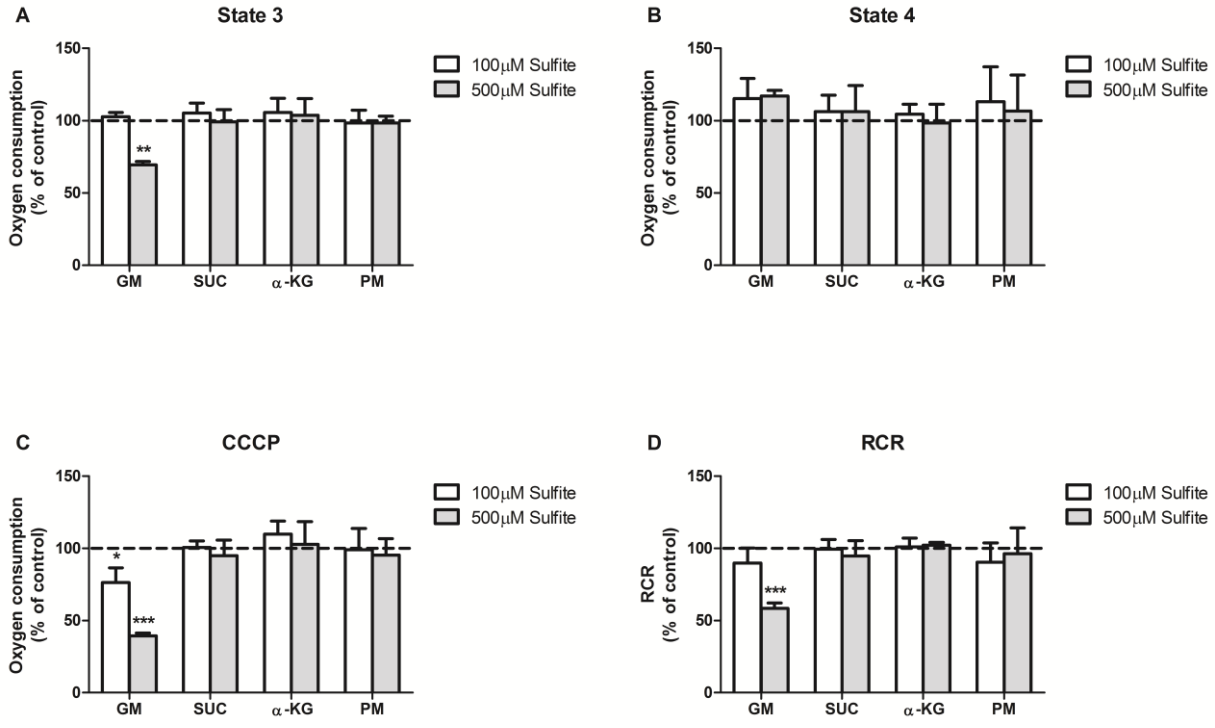


Figure 2

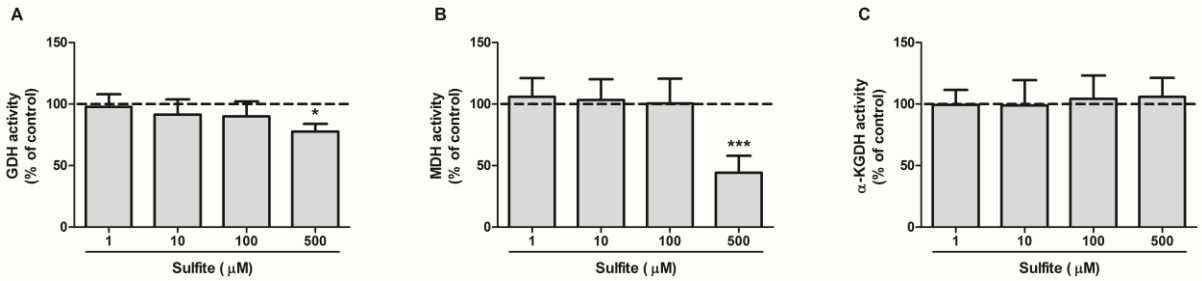


Figure 3

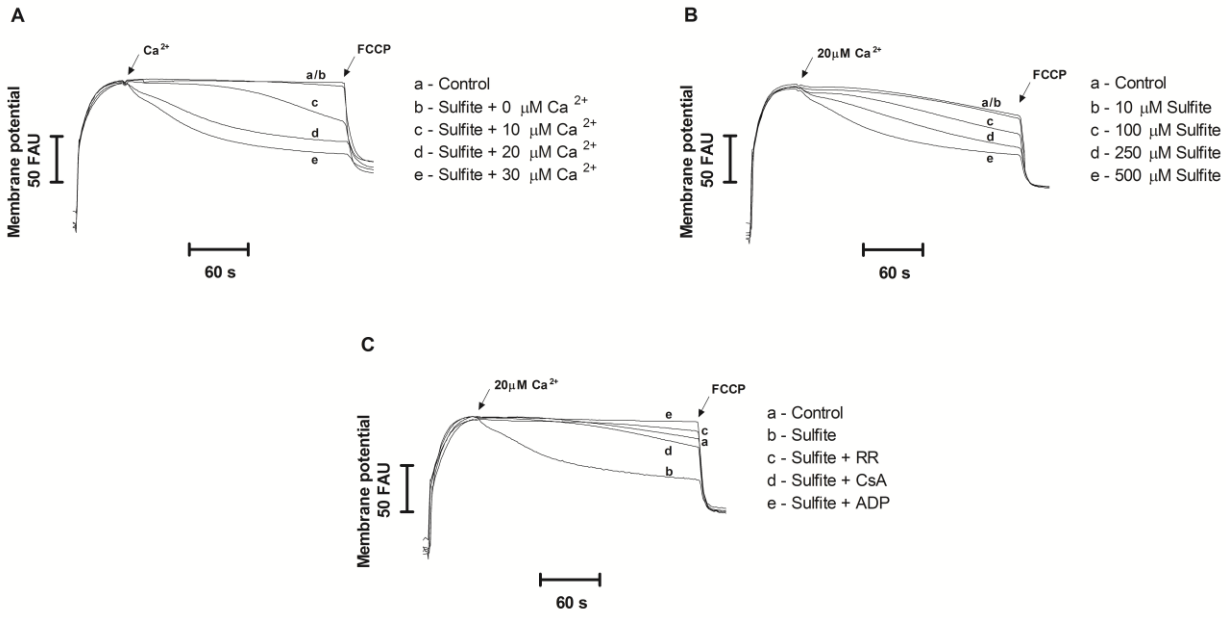


Figure 4

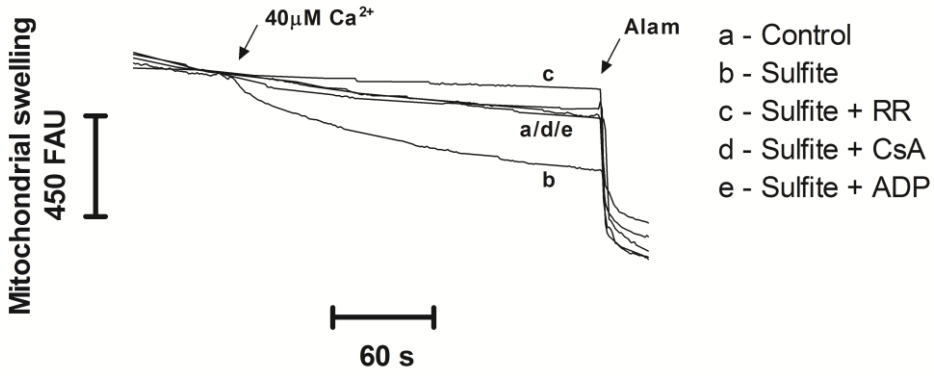


Figure 5

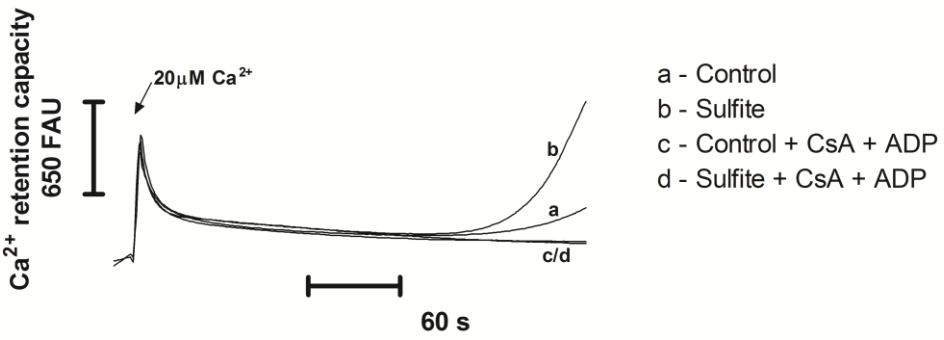


Figure 6

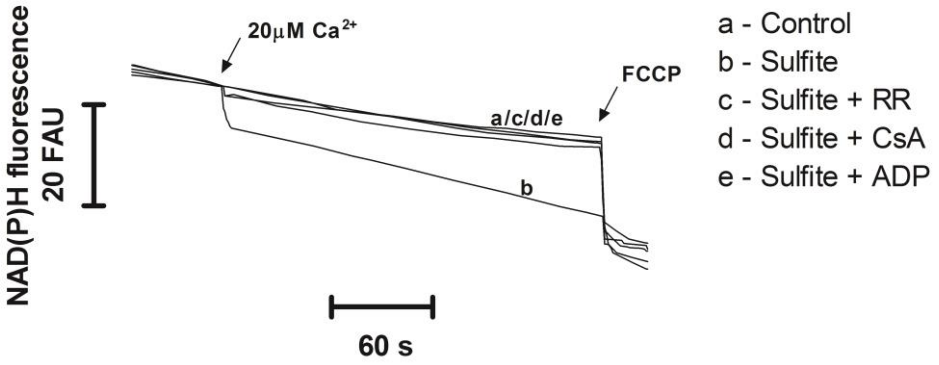


Figure 7

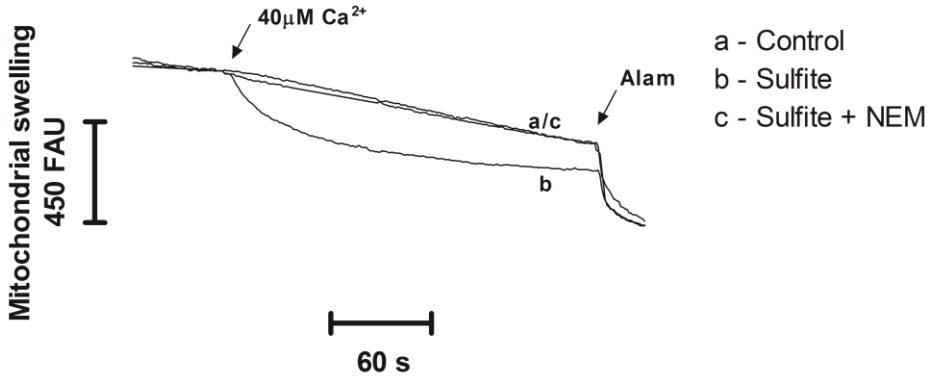


Figure 8

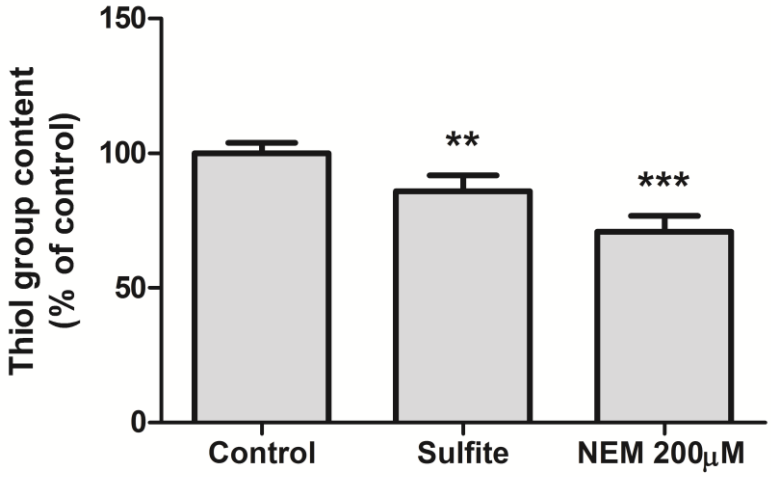
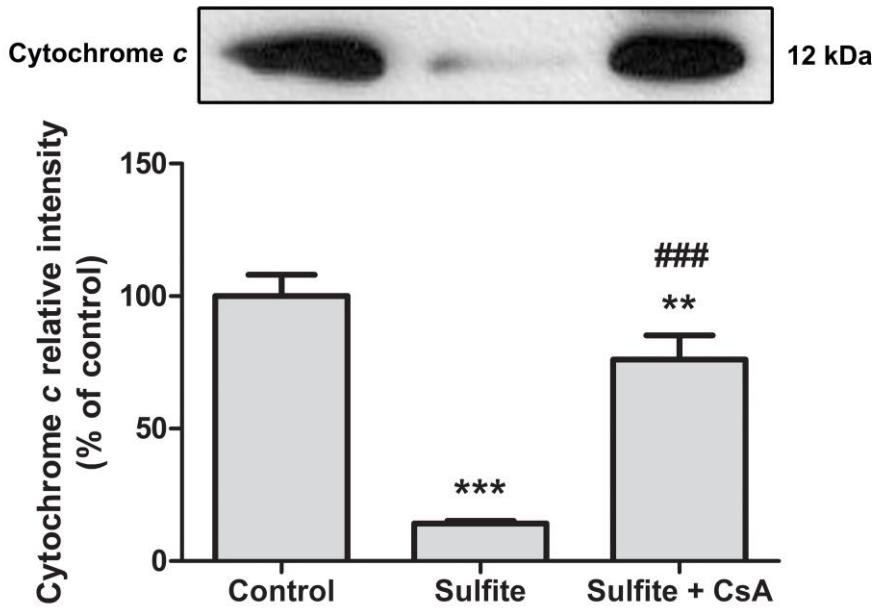


Figure 9


Cytochrome c
12 kDa
Cytochrome c relative intensity (% of control)
150
100
50
0
###
**
***
Control
Sulfite
Sulfite + CsA

PARTE III

Discussão e Conclusões

III.1 DISCUSSÃO

A deficiência da SO (tanto a deficiência isolada da enzima quanto a deficiência do cofator molibdênio) é caracterizada bioquimicamente por acúmulo tecidual e alta excreção urinária de sulfito, tiossulfato e S-sulfocisteína (Johnson e Duran, 2001). Os pacientes apresentam predominantemente encefalopatia, retardo psicomotor e convulsões neonatais severas (Tan et al., 2005; Basheer et al., 2007; Ngu et al., 2009), cuja patogênese tem sido atribuída à neurotoxicidade dos metabólitos acumulados (Küçükatay et al., 2005; Chiarani et al., 2008; Derin et al., 2009; Herken et al., 2009; Ozturk et al., 2010). Além disso, estudos anteriores sugerem o envolvimento de disfunção mitocondrial no aparecimento e progressão dos sintomas, o que é reforçado pela observação de acidemia lática em alguns pacientes (Vincent et al., 2004; Zhang et al., 2004; Eichler et al., 2006; Basheer et al., 2007). É fundamental ressaltar que a mitocôndria, além de desempenhar papel central na geração de energia para a homeostase dos processos celulares através da manutenção dos níveis de ATP, é importante fonte de espécies reativas, participa ativamente da manutenção da homeostase do Ca^{2+} (Nicholls e Akerman, 1982) e também pode estar envolvida na indução de morte celular em situações patológicas (Liu et al., 1996). Portanto, no presente estudo investigamos os efeitos do sulfito e do tiossulfato sobre importantes parâmetros bioquímicos de bioenergética e homeostase redox e mitocondrial com a finalidade de melhor esclarecer os mecanismos fisiopatogênicos do dano neurológico encontrado em portadores da deficiência da SO.

Incialmente, investigamos os efeitos do sulfito e do tiossulfato sobre a atividade dos complexos da CTE e observamos que o sulfito inibiu a atividade

do complexo IV, sem alterar a atividade dos demais complexos testados. O complexo IV é a enzima passo-limitante da CTE, desempenhando um papel central na regulação da fosforilação oxidativa, do bombeamento de prótons da matriz mitocondrial para o espaço intermembranas e da produção de ATP. Dessa forma, pode-se sugerir que a inibição do complexo IV causada pelo sulfito pode contribuir, ao menos em parte, para a diminuição dos níveis de ATP demonstrada em trabalhos anteriores (Vincent et al., 2004; Zhang et al., 2004). Por outro lado, verificamos que o tiossulfato não foi capaz de inibir a atividade dos complexos da cadeia respiratória.

Após, observamos que o sulfito e o tiossulfato inibiram fortemente a atividade da creatina quinase total (tCK), bem com de suas isoformas citosólica (cCK) e mitocondrial (mCK) isoladas. A CK possui um papel fundamental para a função cerebral através do transporte de ligações fosfato de alta energia dos seus sítios de produção, na matriz mitocondrial, para seus sítios de consumo, no citosol, mantendo os níveis de ATP estáveis na célula (Wendt et al., 2003; Du et al., 2013). A mCK utiliza ATP e creatina para produzir fosfocreatina, que atua como reserva e carreador de ligações fosfato de alta energia. A fosfocreatina é então transportada para o citosol, onde a cCK catalisa a reação reversa, reabastecendo o “pool” de ATP neste compartimento celular (Holtzman et al., 1997; Wendt et al., 2003). Sendo assim, o sulfito e o tiossulfato causam um grande prejuízo no transporte e tamponamento de energia intracelular inibindo tanto a produção de fosfocreatina na mitocôndria quanto o reestabelecimento do “pool” de ATP nos seus sítios de utilização no citosol.

É importante salientar que a CK possui resíduos de aminoácidos bastante vulneráveis, como a cisteína 282 do sítio ativo da enzima, que é muito reativa frente a oxidantes (Wang et al., 2001; Wendt et al., 2003; Li et al., 2011). Além disso, já foi demonstrado que a atividade da CK cerebral diminui após a exposição a agentes que induzem a produção de espécies reativas, provavelmente pela oxidação de resíduos de cisteína críticos para o funcionamento da enzima (Burmistrov et al., 1992; Wolosker et al., 1996; Arstall et al., 1998; Konorev et al., 1998; Stachowiak et al., 1998; Wallimann et al., 1998; Wendt et al., 2003). Tendo em vista essa suscetibilidade da CK frente a agentes oxidantes, foi investigado o efeito de antioxidantes sobre a inibição da enzima causada por sulfito e tiossulfato. Nestes experimentos, observamos que a melatonina (MEL; 1000 µM) atenuou a inibição induzida pelo sulfito, ao passo que 100 µM de GSH ou uma combinação de MEL (1000 µM) com uma menor concentração de GSH (50 µM) preveniram totalmente a diminuição da atividade da CK. Estes achados sugerem que a exposição ao sulfito leva à oxidação de grupamentos tióis fundamentais para a atividade da enzima, os quais podem ser protegidos pela GSH, um clássico protetor contra processos de oxidação de grupamentos sulfidrila (Halliwell e Gutteridge, 2007c). Através desses resultados também se pode inferir o envolvimento do radical hidroxila na inibição da atividade da CK pelo sulfito, uma vez que a MEL é um importante sequestrador deste radical (Halliwell e Gutteridge, 2007c; Galano, 2011; Tamura et al., 2013). Entretanto, não é possível descartar o envolvimento de outras espécies reativas nos efeitos do sulfito, uma vez que já foi demonstrado que a MEL também pode sequestrar outras espécies reativas, tais como peróxido de hidrogênio, oxigênio singlete e óxido nítrico (Reiter et al., 2001).

Quanto à inibição causada pelo tiossulfato sobre a atividade da CK, foi observado que, além da GSH e da MEL, o inibidor da óxido nítrico sintase N^{ω}-nitro-L-arginina metil éster (L-NAME) também atenuou este efeito, sugerindo o envolvimento de ERN. De fato, há evidências mostrando que a CK é rapidamente inativada por peroxinitrito e óxido nítrico, os quais têm como alvo principal resíduos de cisteína do sítio ativo (Konorev et al., 1998; Wendt et al., 2003).

Em seguida verificamos o efeito do sulfito e do tiossulfato sobre a oxidação do DCFH e observamos que ambos os compostos testados aumentaram este parâmetro em córtex cerebral. Este ensaio é uma medida inespecífica da produção de espécies reativas, uma vez que o DCFH pode ser oxidado por uma série delas, incluindo radicais peroxil, alcoxil, dióxido de nitrogênio, carbonato e hidroxila, bem como peroxinitrito (LeBel et al., 1992; Ischiropoulos et al., 1999; Bilski et al., 2002; Ohashi et al., 2002; Myhre et al., 2003). Dessa forma, nossos resultados indicam que o sulfito e o tiossulfato induzem a produção de espécies reativas. Além disso, verificamos que o sulfito, mas não o tiossulfato, aumentou a produção de peróxido de hidrogênio em mitocôndrias isoladas de cérebro. Tomando em conjunto, esses resultados reforçam o envolvimento de espécies reativas na inibição da CK pelo sulfito e pelo tiossulfato.

No presente momento, não podemos determinar os exatos mecanismos pelo quais ocorre a produção dessas espécies reativas, mas estudos anteriores mostram que o sulfito é capaz de sofrer auto-oxidação, levando à geração de espécies reativas, tais como o ânion trióxido de enxofre, ânion superóxido e peróxido de hidrogênio, através de reações de propagação (Hayon et al., 1972;

Hegg e Hobbs, 1978; Baker et al., 2002). Neste contexto, uma série de estudos também já demonstrou que espécies reativas derivadas da auto-oxidação do sulfito reagem com biomoléculas, causando a oxidação de nucleotídeos difosfopiridina e da metionina (Klebanoff, 1961; Yang, 1970), destruição do triptofano e do β-caroteno (Yang, 1973; Peiser e Yang, 1979), adição de ligação dupla em alquenos (Southerland et al., 1982), peroxidação de ácidos graxos (Lizada e Yang, 1981), modificação de ácidos nucleicos (Hayatsu, 1976) e dano ao DNA (Hayatsu e Miller, 1972).

Estudamos também o efeito do sulfito e do tiossulfato sobre a atividade da enzima aconitase, uma enzima que catalisa a estereoisomerização do citrato a isocitrato via *cis*-aconitato no ciclo de Krebs e que é considerada um sensor redox de espécies reativas (Gardner et al., 1994; Patel, 2004; Liang et al., 2012). Foi observado que ambos os metabólitos diminuíram a atividade da aconitase em preparações mitocondriais cerebrais. Levando em conta que a aconitase é suscetível à inativação por espécies reativas, principalmente pelo ânion superóxido, resultando na liberação de um Fe lábil do seu centro catalítico ferro-enxofre (4Fe-4S) com a concomitante formação de peróxido de hidrogênio (Vasquez-Vivar et al., 2000; Bulteau et al., 2003; Myers et al., 2010; Cantu et al., 2011), e que trabalhos anteriores demonstram que o sulfito induz a geração do ânion superóxido (Hayon et al., 1972; Mottley e Mason, 1988; Baker et al., 2002), pode-se sugerir que este radical livre esteja envolvido na inibição da aconitase induzida pelo sulfito. Ainda é importante ressaltar aqui que a liberação concomitante de peróxido de hidrogênio e Fe (II) do sítio ativo da aconitase pode levar ao aumento da formação do radical hidroxila através da reação de Fenton (Vasquez-Vivar et al., 2000; Cantu et al., 2011), o que

está de acordo com nossos resultados que demonstraram que MEL previne a inibição da CK induzida pelo sulfito. Quanto à inibição da aconitase pelo tiossulfato, pode-se inferir que há o envolvimento de outras espécies reativas, além do ânion superóxido, já que não foi observado um aumento na produção de peróxido de hidrogênio. De fato, já foi demonstrado que outras espécies reativas são capazes de inibir a atividade da aconitase, tais como óxido nítrico e peroxinitrito (Gardner et al., 1997; Tortora et al., 2007).

Por outro lado, observamos que o sulfito e o tiossulfato não alteraram a atividade da Na^+,K^+-ATPase, uma enzima importante para a geração do potencial de membrana e manutenção do volume celular, não sendo capazes, portanto, de comprometer o funcionamento destes processos celulares.

Deve ser enfatizado que dentre as estruturas cerebrais humanas, o córtex cerebral é aquela que apresenta a maior expressão de SO, o que sugere uma necessidade de níveis mais elevados dessa enzima especificamente nessa região, possivelmente devido a uma maior produção de sulfito (Woo et al., 2003). Sendo assim, a deficiência da SO pode predispor o córtex cerebral a um dano causado pelo excesso de sulfito e tiossulfato. De fato, evidências clínicas mostram dano cortical grave em diversos pacientes (Woo et al., 2003), estando de acordo com os nossos resultados que mostram distúrbios na homeostase energética e redox em córtex cerebral de ratos.

O próximo passo de nossa investigação teve como foco estudar o efeito do sulfito e do tiossulfato sobre parâmetros respiratórios e de homeostase mitocondrial em preparações mitocondriais obtidas de cérebro de ratos. Verificamos que o sulfito, mas não o tiossulfato, diminui o consumo de oxigênio no estado 3 e no estado induzido por CCCP (estado desacoplado), bem como

a RCR em mitocôndrias quando glutamato e malato (doadores de elétrons para o complexo I) foram utilizados como substrato. Por outro lado, quando succinato (doador de elétrons para o complexo II) ou α-cetoglutarato ou piruvato e malato (doadores de elétrons para o complexo I) foram ultilizados como substratos, não houve alteração no consumo de oxigênio. Nossos resultados ainda mostraram que o sulfito inibiu moderadamente a atividade da GDH e fortemente da MDH, não alterando a atividade da α-KGDH, o que está de acordo com os estudos de Zhang e colaboradores (2004). Sabe-se que o glutamato e a sua oxidação via GDH possuem um importante papel no sistema nervoso central. Além de ser o principal neurotransmissor excitatório do sistema nervoso central, o glutamato contribui significativamente como substrato para a respiração mitocondrial. No cérebro, a reação da GDH ocorre principalmente na direção da desaminação oxidativa do glutamato, formando NADH e α-cetoglutarato, o qual pode ser oxidado pelas reações subsequentes do ciclo de Krebs (Kelly e Stanley, 2001). Já a MDH, além de ser uma importante enzima do ciclo de Krebs, é de extrema importância na lançadeira de elétrons malato-aspartato. Assim, espera-se que a inibição da GDH e da MDH ocasione uma diminuição da disponibilidade de intermediários do ciclo de Krebs e de equivalentes reduzidos para fornecer elétrons à cadeira respiratória. Presume-se então que a diminuição do consumo de oxigênio causado pelo sulfito ocorra devido à inibição da GDH e MDH.

Em seguida, verificamos que o sulfito *per se* não alterou o ΔΨm utilizando glutamato e malato como substratos. Por outro lado, a adição de Ca^{2+} ao meio de incubação contendo sulfito ocasionou uma diminuição do ΔΨm de forma dose-dependente tanto de sulfito quanto de Ca^{2+}. Também

verificamos que a diminuição do ΔΨm causada pelo sulfito na presença de Ca^{2+} foi inibida pelo rutênio vermelho (RR), um potente inibidor da captação mitocondrial de Ca^{2+}, demonstrando que a diminuição do ΔΨm pelo sulfito é dependente da entrada de Ca^{2+} na mitocôndria. Além disso, os efeitos causados pelo sulfito sobre o ΔΨm foram totalmente prevenidos por CsA e ADP, dois clássicos inibidores do MPTP. A CsA inibe o MPTP através da sua ligação com a ciclofilina D, uma proteína da matriz mitocondrial que tem sido descrita como um modulador do MPTP através da sua interação com o ANT (Tanveer et al., 1996; Basso et al., 2005; Yarana et al., 2012; Pottecher et al., 2013). Já em relação ao ADP, estudos mostram que os nucleotídeos adenina são potentes inibidores do poro através da sua ligação ao ANT (Crompton, 1999; Saito e Castilho, 2010). Neste contexto, sabe-se que apesar de a mitocôndria possuir um papel central na homeostase do Ca^{2+}, sendo fundamental para o tamponamento do Ca^{2+} citosólico, um aumento excessivo da concentração deste cátion na matriz mitocondrial pode induzir a MPT e causar apoptose em diferentes tipos celulares (Crompton, 1999; Smaili et al., 2000; Hajnoczky et al., 2006; Yarana et al., 2012). A indução da MPT leva ao tráfego não seletivo entre a matriz mitocondrial e o citosol, o que permite o influxo de água e substâncias com até 1,5 kDa para a matriz, ocasionando uma série de consequências, tais como inchamento mitocondrial, perda de metabólitos (Ca^{2+}, Mg^{2+}, GSH, NADH e NADPH), dissipação do ΔΨm, prejuízo na fosforilação oxidativa e na síntese de ATP, bem como a liberação de fatores pró-apoptóticos, como o citocromo *c* (Zoratti e Szabo, 1995; Tatton e Olanow, 1999; Starkov et al., 2004; Zorov et al., 2009; Rasola e Bernardi, 2011; Martel et al., 2012).

No próximo passo da nossa investigação observamos que o sulfito induziu inchamento mitocondrial, o qual foi prevenido por RR, CsA e ADP. Estes achados sugerem que a dissipação do ΔΨm induzida por sulfito e Ca^{2+} está relacionada com a permeabilização não seletiva da membrana mitocondrial interna devido à abertura do MPTP.

No que diz respeito à capacidade de retenção de Ca^{2+} da mitocôndria, observamos que o sulfito diminuiu este parâmetro, sendo este efeito prevenido por CsA e ADP, o que está de acordo com a propriedade que o sulfito tem de promover a MPT na presença de Ca^{2+}. Neste contexto, é concebível que a abertura do MPTP permita a liberação de Ca^{2+} da matriz mitocondrial após este atingir um limiar de concentração que supera a capacidade de retenção da mitocôndria, resultando na permeabilização não seletiva da membrana mitocondrial interna (Zoratti e Szabo, 1995; Crompton et al., 1999; Kowaltowski et al., 2001; Bernardi e von Stockum, 2012).

Além disso, o sulfito causou a diminuição do conteúdo de NAD(P)H da matriz mitocondrial na presença de Ca^{2+}. Tendo em vista que a diminuição nos níveis de NAD(P)H induzida por sulfito foi inibida por CsA e ADP, presume-se que este efeito ocorreu devido à abertura do MPTP e consequente liberação de NAD(P)H da matriz mitocondrial, levando a um prejuízo na homeostase redox mitocondrial.

Avaliamos então se diversos compostos descritos como inibidores da MPT, como a trifluoperazina (TFZ), clorpromazina (CP), quinacrina (QUIN), N-acetilcisteína (NAC), CAT, melatonina (MEL), CoQ, ditiotreitol (DTT) e N-etilmaleimida (NEM), seriam capazes de prevenir o inchamento mitocondrial induzido por sulfito na presença de Ca^{2+}. Neste contexto, diversos trabalhos

utilizam TFZ, CP e QUIN como inibidores do poro devido à sua capacidade de inibir a fosfolipase A2 (Pastorino et al., 1996; Broekemeier et al., 2002). Estudos anteriores demonstraram que a ativação da fosfolipase A2 aumenta a geração de ácidos graxos insaturados, como o ácido araquidônico, o qual leva a produção de radicais livres, podendo induzir MPT (Nishimura et al., 2008). Já os antioxidantes NAC, CAT, MEL e CoQ são usados na prevenção de MPT, pois a geração de radicais livres pode ocasionar a oxidação de grupamentos tióis presentes em proteínas envolvidas no MPTP e induzir sua abertura (Kowaltowski et al., 2001; Chinopoulos et al., 2003; Nishimura et al., 2008; Adam-Vizi e Starkov, 2010). Em relação à utilização do DTT, este composto é um potente agente redutor, prevenindo a formação de pontes dissulfeto e tornando o MPTP menos suscetível à abertura (Petronilli et al., 1994; Costantini et al., 1996; Kowaltowski et al., 1997). Os nossos resultados demonstram que os inibidores da fosfolipase A2, antioxidantes e DTT não foram capazes de prevenir o inchamento mitocondrial induzido pelo sulfito na presença de Ca^{2+}. Por outro lado, a NEM preveniu totalmente este efeito. A NEM é um agente alquilante hidrofóbico que reage com grupamentos sulfidrila formando ligações tioéter estáveis. Estudos anteriores demonstraram que a NEM tem acesso a grupamentos tiol críticos de resíduos de aminoácidos do ANT, os quais regulam o MPTP deixando-o com maior ou menor suscetibilidade à abertura (Petronilli et al., 1994; Halestrap et al., 1997; McStay et al., 2002). Sendo assim, os nossos achados indicam que o sulfito age através da modulação de grupamentos tiol presentes em proteínas envolvidas na formação do MPTP. Verificamos também uma diminuição do conteúdo de grupamentos tiol de proteínas de membrana mitocondrial induzida pelo sulfito na presença de Ca^{2+},

reforçando que o sulfito induz MPT por modular tais grupamentos. Também se pode inferir que o sulfito possua um efeito direto sobre os grupamentos tiol, não sendo a diminuição do conteúdo destes uma consequência do ataque por espécies reativas produzidas pelo sulfito, uma vez que os antioxidantes testados não preveniram o inchamento mitocondrial induzido por este composto. De fato, já foi anteriormente demonstrado que o sulfito é capaz de reagir diretamente com grupamentos sulfidrila (Johnson e Duran, 2001). Além disso, já foi demonstrado que outros compostos, tais como acetoacetato, óxido de fenilarsina (OF) e ácido 4,4'-diisotiocianatostilbeno-2,2'-disulfônico (DIDS), induzem a abertura do poro de MPT através de mecanismos independentes de espécies reativas, como o "cross-linking" de grupamentos sulfidrila (Petronilli et al., 1994; Kowaltowski et al., 1997; Kim et al., 2002; McStay et al., 2002). Ainda, é importante salientar que Constatini e colaboradores (1996) sugeriram que pelo menos dois sítios distintos contento grupamentos tiol modulam o MPTP, sendo um destes sítios insensível a agentes redutores tais como o DTT. Da mesma forma, foi verificado que a diminuição no conteúdo de grupamentos tiol em proteínas de membrana mitocondrial causada pelo OF e pelo DIDS é parcialmente aditiva, indicando que moléculas indutoras de MPT podem reagir com diferentes grupamentos tiol da membrana mitocondrial (Kowaltowski et al., 1997).

Por fim, verificamos que o sulfito diminuiu o imunoconteúdo de citocromo *c* em preparações mitocondriais na presença de Ca^{2+}, podendo estar este resultado relacionado com a indução de MPT por este composto, uma vez que este efeito foi prevenido por CsA. A liberação do citocromo *c* pode ocorrer devido ao inchamento osmótico e consequente ruptura da membrana

mitocondrial externa provocada pela indução de MPT (Petit et al., 1998; Crompton, 2000). Quando liberado da matriz mitocondrial para o citosol, o citocromo *c* possui um papel fundamental na formação do apoptossomo e na ativação da apoptose, através da sua interação com o fator 1 de ativação de protease apoptótica (Apaf-1), recrutando e ativando a caspase-9, o que dá início à cascata de caspases (Green e Reed, 1998; Andreyev e Fiskum, 1999; Rasola e Bernardi, 2011). Portanto, os nossos resultados sugerem que o sulfito poderia induzir apoptose através da indução de MPT na presença de Ca^{2+}.

É difícil determinar a importância fisiopatológica dos nossos resultados, entretanto há uma série de evidências demonstrando que a abertura do MPTP e um prejuízo na homeostase do Ca^{2+} contribuem para a neurodegeneração observada em diversas doenças (Murphy et al., 1999; Friberg e Wieloch, 2002; Figueira et al., 2013). É importante enfatizar que os neurônios são particularmente vulneráveis à indução da MPT, pois em situações de privação energética severa, tais como isquemia/reperfusão e hipoglicemia, os níveis citosólicos de Ca^{2+} nos neurônios aumentam como uma consequência de um prejuízo nos sistemas de remoção de Ca^{2+} e da ativação de receptores de glutamato, principalmente os do tipo NMDA. Neste contexto, há evidências de que a S-sulfocisteína, um metabólito estruturalmente similar ao glutamato e derivado da reação de sulfito com cisteína livre, é capaz de ativar os receptores NMDA (Olney et al., 1975; Kagedal et al., 1986), levando a um aumento do influxo de Ca^{2+} na célula. Além disso, o sulfito poderia levar a um aumento dos níveis de glutamato na fenda sináptica devido à inibição da GDH, contribuindo ainda mais para o aumento dos níveis intracelulares de Ca^{2+}. Outro fator que pode contribuir para um desequilíbrio na homeostase do Ca^{2+} é um dano no

sistema de remoção deste cátion, ocasionado pela disfunção energética induzida pelo sulfito. Dessa forma, a indução de MPT pelo sulfito pode contribuir para a disfunção neurológica apresentada pelos pacientes com a deficiência da SO.

Concluindo, este estudo mostra que o sulfito e o tiossulfato causam dano na homeostase energética e redox do cérebro e que o sulfito age sinergicamente com o Ca^{2+} induzindo a MPT em mitocôndrias cerebrais através do ataque direto a grupamentos tiol de proteínas envolvidas na formação do MPTP. Caso estes resultados sejam observados *in vivo* e em tecidos de pacientes acometidos pela deficiência da SO, pode-se presumir que a disfunção bioenergética, o estresse oxidativo e a indução do MPT são importantes mecanismos fisiopatológicos envolvidos no dano cerebral apresentado pelos pacientes afetados por essa desordem. Além disso, é possível que inibidores da MPT possam representar potenciais estratégias terapêuticas para a prevenção do dano neurológico encontrado nestes pacientes.

III.2 CONCLUSÕES

- O sulfito inibe a atividade do complexo IV da CTE, indicando que este composto prejudica o fluxo de elétrons pela cadeia respiratória.
- O sulfito e o tiossulfato inibem a atividade da tCK, bem como de suas isoformas citosólica e mitocondrial isoladas, indicando que estes compostos comprometem a transferência e tamponamento energético celular.
- A inibição da tCK induzida por sulfito foi atenuada por MEL e totalmente prevenida por GSH e por uma combinação de MEL e doses mais baixas de GSH. Já a inibição da tCK induzida por tiossulfato foi totalmente prevenida por GSH e atenuada por MEL e L-NAME. Presume-se então que o sulfito e o tiossulfato causam a oxidação de grupamentos tiol críticos para a atividade da CK através da formação de espécies reativas.
- O sulfito e o tiossulfato induziram a produção de espécies reativas em córtex cerebral de ratos.
- O sulfito e o tiossulfato inibiram a atividade da aconitase em preparações mitocondriais de cérebro.
- O sulfito diminuiu o consumo de oxigênio no estado 3 e no estado induzido por CCCP (estado desacoplado) e o RCR em mitocôndrias cerebrais isoladas utilizando o glutamato e o malato com substratos.
- O sulfito inibiu a atividade da GDH e da MDH em mitocôndrias cerebrais isoladas, sugerindo que a diminuição do consumo de oxigênio causada

por este composto seja secundária à inibição da oxidação de glutamato e malato.

- O sulfito diminuiu o ΔΨm e induziu inchamento mitocondrial em mitocôndrias isoladas de cérebro utilizando glutamato e malato como substratos na presença de Ca^{2+}, porém não na ausência deste cátion. Estes efeitos foram prevenidos por RR, ADP e CsA, indicando que o sulfito induz MPT na presença de Ca^{2+}.
- O sulfito diminuiu a capacidade de retenção de Ca^{2+} em mitocôndrias isoladas de cérebro, sendo este efeito prevenido por CsA e ADP.
- O sulfito diminuiu os níveis de NAD(P)H em mitocôndrias isoladas de cérebro, sendo este efeito prevenido por RR, CsA e ADP.
- O inchamento mitocondrial induzido por sulfito foi totalmente prevenido por NEM. Além disso, o sulfito diminuiu o conteúdo de grupamentos tiol das proteínas de membrana mitocondrial na presença de Ca^{2+}, indicando que o sulfito induz a MPT através do ataque a grupamentos sulfidrila de proteínas envolvidas na formação do MPTP.
- O sulfito diminuiu o conteúdo de citocromo *c* em mitocôndrias isoladas de cérebro, sendo este efeito prevenido por CsA. Estes resultados indicam que o sulfito promove a liberação de citocromo *c* como consequência da indução de MPT.

III.3 PERSPECTIVAS

- Avaliar os efeitos *in vitro* do sulfito e do tiossulfato sobre parâmetros de metabolismo energético em diferentes estruturas, tais como estriado e cerebelo, uma vez que os pacientes afetados pela deficiência da SO apresentam, além de atrofia cortical, anormalidades nos gânglios da base e cerebelo.
- Avaliar os efeitos *ex vivo* do sulfito em córtex cerebral, estriado e cerebelo sobre parâmetros de metabolismo energético em ratos com deficiência para a enzima SO, induzida através de dieta pobre em molibdênio e adição de tungstênio na água de beber.
- Avaliar os efeitos *ex vivo* do sulfito em preparações mitocondriais de cérebro total sobre o consumo de oxigênio e a homeostase mitocondrial na presença e ausência de Ca^{2+} em ratos com deficiência para a enzima SO, induzida através de dieta pobre em molibdênio e adição de tungstênio na água de beber.
- Avaliar os efeitos *ex vivo* do sulfito sobre a morfologia, proliferação e morte de neurônios e astrócitos através de imunohistoquímica e da avaliação do imunoconteúdo de caspases em ratos com deficiência para a enzima SO, induzida através de dieta pobre em molibdênio e adição de tungstênio na água de beber.

III.4 REFERÊNCIAS

Abedinzadeh, Z., 2001. Sulfur-centered reactive intermediates derived from the oxidation of sulfur compounds of biological interest. Can J Physiol Pharmacol 79, 166-70.

Abeles, R., Frey, P. and Jencks, W., 1992. Biochemistry, Jones and Bartlett, London.

Adam-Vizi, V. and Starkov, A.A., 2010. Calcium and mitochondrial reactive oxygen species generation: how to read the facts. J Alzheimers Dis 20 Suppl 2, S413-26.

Alfadhel, M., Al-Thihli, K., Moubayed, H., Eyaid, W. and Al-Jeraisy, M., 2013. Drug treatment of inborn errors of metabolism: a systematic review. Arch Dis Child 98, 454-61.

Andreyev, A. and Fiskum, G., 1999. Calcium induced release of mitochondrial cytochrome c by different mechanisms selective for brain versus liver. Cell Death Differ 6, 825-32.

Appignani, B.A., Kaye, E.M. and Wolpert, S.M., 1996. CT and MR appearance of the brain in two children with molybdenum cofactor deficiency. AJNR Am J Neuroradiol 17, 317-20.

Aratani, Y., Miura, N., Ohno, N. and Suzuki, K., 2012. [Role of neutrophil-derived reactive oxygen species in host defense and inflammation]. Med Mycol J 53, 123-8.

Arstall, M.A., Bailey, C., Gross, W.L., Bak, M., Balligand, J.L. and Kelly, R.A., 1998. Reversible S-nitrosation of creatine kinase by nitric oxide in adult rat ventricular myocytes. J Mol Cell Cardiol 30, 979-88.

Baker, M.T., Dehring, D.J. and Gregerson, M.S., 2002. Sulfite supported lipid peroxidation in propofol emulsions. Anesthesiology 97, 1162-7.

Barić, I., Fumić, K. and Hoffmann, G., 2001. Inborn errors of metabolism at the turn of the millennium. Croat Med J 42, 379-83.

Barth, P.G., Beemer, F.A., Cats, B.P., Duran, M. and Wadman, S.K., 1985. Neuropathological findings in a case of combined deficiency of sulphite oxidase and xanthine dehydrogenase. Virchows Arch A Pathol Anat Histopathol 408, 105-6.

Basheer, S.N., Waters, P.J., Lam, C.W., Acquaviva-Bourdain, C., Hendson, G., Poskitt, K. and Hukin, J., 2007. Isolated sulfite oxidase deficiency in the newborn: lactic acidaemia and leukoencephalopathy. Neuropediatrics 38, 38-41.

Basso, E., Fante, L., Fowlkes, J., Petronilli, V., Forte, M.A. and Bernardi, P., 2005. Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. J Biol Chem 280, 18558-61.

Bernardi, P. and von Stockum, S., 2012. The permeability transition pore as a Ca(2+) release channel: new answers to an old question. Cell Calcium 52, 22-7.

Bickel, H., 1987. Early diagnosis and treatment of inborn errors of metabolism. Enzyme 38, 14-26.

Bilski, P., Belanger, A.G. and Chignell, C.F., 2002. Photosensitized oxidation of 2',7'-dichlorofluorescin: singlet oxygen does not contribute to the formation of fluorescent oxidation product 2',7'-dichlorofluorescein. Free Radic Biol Med 33, 938-46.

Bindu, P.S., Christopher, R., Mahadevan, A. and Bharath, R.D., 2011. Clinical and imaging observations in isolated sulfite oxidase deficiency. J Child Neurol 26, 1036-40.

Boveris, A. and Chance, B., 1973. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J 134, 707-16.

Brini, M., Cali, T., Ottolini, D. and Carafoli, E., 2014. Neuronal calcium signaling: function and dysfunction. Cell Mol Life Sci.

Broekemeier, K.M., Iben, J.R., LeVan, E.G., Crouser, E.D. and Pfeiffer, D.R., 2002. Pore formation and uncoupling initiate a Ca2+-independent degradation of mitochondrial phospholipids. Biochemistry 41, 7771-80.

Brown, G.K., Scholem, R.D., Croll, H.B., Wraith, J.E. and McGill, J.J., 1989. Sulfite oxidase deficiency: clinical, neuroradiologic, and biochemical features in two new patients. Neurology 39, 252-7.

Budd, S.L. and Nicholls, D.G., 1996. Mitochondria, calcium regulation, and acute glutamate excitotoxicity in cultured cerebellar granule cells. J Neurochem 67, 2282-91.

Bulteau, A.L., Ikeda-Saito, M. and Szweda, L.I., 2003. Redox-dependent modulation of aconitase activity in intact mitochondria. Biochemistry 42, 14846-55.

Burmistrov, S.O., Mashek, O.P. and Kotin, A.M., 1992. The action of acute alcoholic intoxication on the antioxidant system and creatine kinase activity in the brain of rat embryos. Eksp Klin Farmakol 55, 54-6.

Cadenas, E. and Davies, K.J., 2000. Mitochondrial free radical generation, oxidative stress, and aging. Free Radic Biol Med 29, 222-30.

Cantu, D., Fulton, R.E., Drechsel, D.A. and Patel, M., 2011. Mitochondrial aconitase knockdown attenuates paraquat-induced dopaminergic cell death via decreased cellular metabolism and release of iron and H(2)O(2). J Neurochem 118, 79-92.

Castilho, R.F., Hansson, O., Ward, M.W., Budd, S.L. and Nicholls, D.G., 1998. Mitochondrial control of acute glutamate excitotoxicity in cultured cerebellar granule cells. J Neurosci 18, 10277-86.

Chan, K.Y., Li, C.K., Lai, C.K., Ng, S.F. and Chan, A.Y., 2002. Infantile isolated sulphite oxidase deficiency in a Chinese family: a rare neurodegenerative disorder. Hong Kong Med J 8, 279-82.

Chapman, K., 1993. Sulfite-containing pharmaceuticals. CMAJ 148, 714.

Chiarani, F., Bavaresco, C.S., Dutra-Filho, C.S., Netto, C.A. and Wyse, A.T., 2008. Sulfite increases lipoperoxidation and decreases the activity of catalase in brain of rats. Metab Brain Dis 23, 123-32.

Chinopoulos, C., Starkov, A.A. and Fiskum, G., 2003. Cyclosporin A-insensitive permeability transition in brain mitochondria: inhibition by 2-aminoethoxydiphenyl borate. J Biol Chem 278, 27382-9.

Costantini, P., Chernyak, B.V., Petronilli, V. and Bernardi, P., 1996. Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. J Biol Chem 271, 6746-51.

Crompton, M., 1999. The mitochondrial permeability transition pore and its role in cell death. Biochem J 341 (Pt 2), 233-49.

Crompton, M., 2000. Mitochondrial intermembrane junctional complexes and their role in cell death. J Physiol 529 Pt 1, 11-21.

Crompton, M., Virji, S., Doyle, V., Johnson, N. and Ward, J.M., 1999. The mitochondrial permeability transition pore. Biochem Soc Symp 66, 167-79.

Delanty, N. and Dichter, M.A., 1998. Oxidative injury in the nervous system. Acta Neurol Scand 98, 145-53.

Derin, N., Akpinar, D., Yargicoglu, P., Agar, A. and Aslan, M., 2009. Effect of alpha-lipoic acid on visual evoked potentials in rats exposed to sulfite. Neurotoxicol Teratol 31, 34-9.

Derin, N., Yargiçoğlu, P., Aslan, M., Elmas, O., Agar, A. and Aicigüzel, Y., 2006. The effect of sulfite and chronic restraint stress on brain lipid peroxidation and anti-oxidant enzyme activities. Toxicol Ind Health 22, 233-40.

Di Donato, S., 2000. Disorders related to mitochondrial membranes: pathology of the respiratory chain and neurodegeneration. J Inherit Metab Dis 23, 247-63.

Dickinson, C., 1996. Cerebral oxidative metabolism in hypertension. Clin Sci (Lond) 91, 539-50.

Du, F., Cooper, A., Lukas, S.E., Cohen, B.M. and Ongur, D., 2013. Creatine kinase and ATP synthase reaction rates in human frontal lobe measured by (31)P magnetization transfer spectroscopy at 4T. Magn Reson Imaging 31, 102-8.

Edwards, M.C., Johnson, J.L., Marriage, B., Graf, T.N., Coyne, K.E., Rajagopalan, K.V. and MacDonald, I.M., 1999. Isolated sulfite oxidase deficiency: review of two cases in one family. Ophthalmology 106, 1957-61.

Eichler, F., Tan, W.H., Shih, V.E., Grant, P.E. and Krishnamoorthy, K., 2006. Proton magnetic resonance spectroscopy and diffusion-weighted imaging in isolated sulfite oxidase deficiency. J Child Neurol 21, 801-5.

Erecińska, M. and Silver, I., 1994. Ions and energy in mammalian brain. Prog Neurobiol 43, 37-71.

Figueira, T.R., Barros, M.H., Camargo, A.A., Castilho, R.F., Ferreira, J.C., Kowaltowski, A.J., Sluse, F.E., Souza-Pinto, N.C. and Vercesi, A.E., 2013. Mitochondria as a source of reactive oxygen and nitrogen species: from molecular mechanisms to human health. Antioxid Redox Signal 18, 2029-74.

Fiskum, G., Murphy, A.N. and Beal, M.F., 1999. Mitochondria in neurodegeneration: acute ischemia and chronic neurodegenerative diseases. J Cereb Blood Flow Metab 19, 351-69.

Friberg, H. and Wieloch, T., 2002. Mitochondrial permeability transition in acute neurodegeneration. Biochimie 84, 241-50.

Galano, A., 2011. On the direct scavenging activity of melatonin towards hydroxyl and a series of peroxyl radicals. Phys Chem Chem Phys 13, 7178-88.

Gardner, P.R., Costantino, G., Szabo, C. and Salzman, A.L., 1997. Nitric oxide sensitivity of the aconitases. J Biol Chem 272, 25071-6.

Gardner, P.R., Nguyen, D.D. and White, C.W., 1994. Aconitase is a sensitive and critical target of oxygen poisoning in cultured mammalian cells and in rat lungs. Proc Natl Acad Sci USA 91, 12248-52.

Green, D.R. and Reed, J.C., 1998. Mitochondria and apoptosis. Science 281, 1309-12.

Gunnison, A.F. and Palmes, E.D., 1973. Persistence of plasma S-sulfonates following exposure of rabbits to sulfite and sulfur dioxide. Toxicol Appl Pharmacol 24, 266-78.

Hajnoczky, G., Csordas, G., Das, S., Garcia-Perez, C., Saotome, M., Sinha Roy, S. and Yi, M., 2006. Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca2+ uptake in apoptosis. Cell Calcium 40, 553-60.

Halestrap, A.P. and Brenner, C., 2003. The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death. Curr Med Chem 10, 1507-25.

Halestrap, A.P., Woodfield, K.Y. and Connern, C.P., 1997. Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase. J Biol Chem 272, 3346-54.

Halliwell, B., 2001. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging 18, 685-716.

Halliwell, B. and Gutteridge, J., 2007a. Antioxidant defences: endogenous and diet derived, in: B, H. and JMC, G. (Eds.), Free Radicals in Biology and Medicine. Oxford University Press Inc., New York, pp. 79-185.

Halliwell, B. and Gutteridge, J., 2007b. Cellular responses to oxidative stress, in: B, H. and JMC, G. (Eds.), Free Radicals in Biology and Medicine. Oxford University Press Inc., New York, pp. 187-267.

Halliwell, B. and Gutteridge, J., 2007c. Measurement of reactive species, in: B, H. and JMC, G. (Eds.), Free Radicals in Biology and Medicine. Oxford University Press Inc., New York, pp. 268-340.

Halliwell, B. and Gutteridge, J., 2007d. Oxygen is a toxic gas-an introduction to oxygen toxicity and reactive species, in: B, H. and JMC, G. (Eds.), Free Radicals in Biology and Medicine. Oxford University Press Inc., New York, pp. 1-29.

Hamada, K., Matsuura, H., Sanada, M., Toyoda, F., Omatsu-Kanbe, M., Kashiwagi, A. and Yasuda, H., 2003. Properties of the Na+/K+ pump current in small neurons from adult rat dorsal root ganglia. Br J Pharmacol 138, 1517-27.

Han, D., Williams, E. and Cadenas, E., 2001. Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space. Biochem J 353, 411-6.

Hayatsu, H., 1976. Bisulfite modification of nucleic acids and their constituents. Prog Nucleic Acid Res Mol Biol 16, 75-124.

Hayatsu, H. and Miller, R.C., Jr., 1972. The cleavage of DNA by the oxygen-dependent reaction of bisulfite. Biochem Biophys Res Commun 46, 120-4.

Hayon, E., Treinin, A. and Wilf, J., 1972. Electronic spectra, photochemistry, and autoxidation mechanism of the sulfite-bisulfite-pyrosulfite systems. The SO_2^-, SO_3^-, SO_4^-, and SO_5^- radicals. J Am Chem Soc 94, 47-57.

Hegg, D.A. and Hobbs, P.V., 1978. Oxidation of sulfur dioxide in aqueous systems with particular reference to the atmosphere. Atmos Environ 12, 241-253.

Herken, E.N., Kocamaz, E., Erel, O., Celik, H. and Kucukatay, V., 2009. Effect of sulfite treatment on total antioxidant capacity, total oxidant status, lipid hydroperoxide, and total free sulfydryl groups contents in normal and sulfite oxidase-deficient rat plasma. Cell Biol Toxicol 25, 355-62.

Hobson, E.E., Thomas, S., Crofton, P.M., Murray, A.D., Dean, J.C. and Lloyd, D., 2005. Isolated sulphite oxidase deficiency mimics the features of hypoxic ischaemic encephalopathy. Eur J Pediatr 164, 655-9.

Holtzman, D., Meyers, R., Khait, I. and Jensen, F., 1997. Brain creatine kinase reaction rates and reactant concentrations during seizures in developing rats. Epilepsy Res 27, 7-11.

Irani, K., 2000. Oxidant signaling in vascular cell growth, death, and survival : a review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling. Circ Res 87, 179-83.

Ischiropoulos, H., Gow, A., Thom, S.R., Kooy, N.W., Royall, J.A. and Crow, J.P., 1999. Detection of reactive nitrogen species using 2,7-dichlorodihydrofluorescein and dihydrorhodamine 123. Methods Enzymol 301, 367-73.

Ji, A.J., Savon, S.R. and Jacobsen, D.W., 1995. Determination of total serum sulfite by HPLC with fluorescence detection. Clin Chem 41, 897-903.

Johnson, J.L., 2003. Prenatal diagnosis of molybdenum cofactor deficiency and isolated sulfite oxidase deficiency. Prenat Diagn 23, 6-8.

Johnson, J.L. and Duran, M., 2001. Molybdenum Cofactor Deficiency and Isolated Sulfite Oxidase Deficiency, in: Scriver CR, B.A., Valle D, Sly WS (Ed.), The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York, pp. 3181-3217.

Johnson, J.L. and Rajagopalan, K.V., 1995. An HPLC assay for detection of elevated urinary S-sulphocysteine, a metabolic marker of sulphite oxidase deficiency. J Inherit Metab Dis 18, 40-7.

Kagedal, B., Kallberg, M. and Sorbo, B., 1986. A possible involvement of glutathione in the detoxication of sulfite. Biochem Biophys Res Commun 136, 1036-41.

Kaplan, J.H., 2002. Biochemistry of Na,K-ATPase. Annu Rev Biochem 71, 511-35.

Kelly, A. and Stanley, C.A., 2001. Disorders of glutamate metabolism. Ment Retard Dev Disabil Res Rev 7, 287-95.

Kencebay, C., Derin, N., Ozsoy, O., Kipmen-Korgun, D., Tanriover, G., Ozturk, N., Basaranlar, G., Yargicoglu-Akkiraz, P., Sozen, B. and Agar, A., 2013. Merit of quinacrine in the decrease of ingested sulfite-induced toxic action in rat brain. Food Chem Toxicol 52, 129-36.

Kim, J.S., He, L. and Lemasters, J.J., 2003. Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. Biochem Biophys Res Commun 304, 463-70.

Kim, T.S., Jeong, D.W., Yun, B.Y. and Kim, I.Y., 2002. Dysfunction of rat liver mitochondria by selenite: induction of mitochondrial permeability transition through thiol-oxidation. Biochem Biophys Res Commun 294, 1130-7.

Kisker, C., Schindelin, H., Pacheco, A., Wehbi, W.A., Garrett, R.M., Rajagopalan, K.V., Enemark, J.H. and Rees, D.C., 1997. Molecular basis of sulfite oxidase deficiency from the structure of sulfite oxidase. Cell 91, 973-83.

Klebanoff, S.J., 1961. The sulfite-activated oxidation of reduced pyridine nucleotides by peroxidase. Biochim Biophys Acta 48, 93-103.

Kocamaz, E., Adiguzel, E., Er, B., Gundogdu, G. and Kucukatay, V., 2012. Sulfite leads to neuron loss in the hippocampus of both normal and SOX-deficient rats. Neurochem Int 61, 341-6.

Konorev, E.A., Hogg, N. and Kalyanaraman, B., 1998. Rapid and irreversible inhibition of creatine kinase by peroxynitrite. FEBS Lett 427, 171-4.

Kowaltowski, A.J., Castilho, R.F. and Vercesi, A.E., 2001. Mitochondrial permeability transition and oxidative stress. FEBS Lett 495, 12-5.

Kowaltowski, A.J., Vercesi, A.E. and Castilho, R.F., 1997. Mitochondrial membrane protein thiol reactivity with N-ethylmaleimide or mersalyl is modified by Ca2+: correlation with mitochondrial permeability transition. Biochim Biophys Acta 1318, 395-402.

Küçükatay, V., Savcioğlu, F., Hacioğlu, G., Yargiçoğlu, P. and Ağar, A., 2005. Effect of sulfite on cognitive function in normal and sulfite oxidase deficient rats. Neurotoxicol Teratol 27, 47-54.

LeBel, C.P., Ischiropoulos, H. and Bondy, S.C., 1992. Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 5, 227-31.

Leung, A.W. and Halestrap, A.P., 2008. Recent progress in elucidating the molecular mechanism of the mitochondrial permeability transition pore. Biochim Biophys Acta 1777, 946-52.

Li, C., Sun, S., Park, D., Jeong, H.O., Chung, H.Y., Liu, X.X. and Zhou, H.M., 2011. Hydrogen peroxide targets the cysteine at the active site and irreversibly inactivates creatine kinase. Int J Biol Macromol 49, 910-6.

Liang, L.P., Waldbaum, S., Rowley, S., Huang, T.T., Day, B.J. and Patel, M., 2012. Mitochondrial oxidative stress and epilepsy in SOD2 deficient mice: attenuation by a lipophilic metalloporphyrin. Neurobiol Dis 45, 1068-76.

Lieberman, M.A. and Marks, A.D., 2009. Marks' Basic Medical Biochemistry: A Clinical Approach, 3th ed. Lippincott Willians & Wilkins.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R. and Wang, X., 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86, 147-57.

Lizada, M.C.C. and Yang, S.F., 1981. Sulfite-induced lipid peroxidation. Lipids 16, 189-194.

Mak, C.M., Lee, H.C., Chan, A.Y. and Lam, C.W., 2013. Inborn errors of metabolism and expanded newborn screening: review and update. Crit Rev Clin Lab Sci 50, 142-62.

Martel, C., Huynh le, H., Garnier, A., Ventura-Clapier, R. and Brenner, C., 2012. Inhibition of the Mitochondrial Permeability Transition for Cytoprotection: Direct versus Indirect Mechanisms. Biochem Res Int 2012, 213403.

McStay, G.P., Clarke, S.J. and Halestrap, A.P., 2002. Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore. Biochem J 367, 541-8.

Mottley, C. and Mason, R.P., 1988. Sulfate anion free radical formation by the peroxidation of (Bi)sulfite and its reaction with hydroxyl radical scavengers. Arch Biochem Biophys 267, 681-9.

Murphy, A.N., Fiskum, G. and Beal, M.F., 1999. Mitochondria in neurodegeneration: bioenergetic function in cell life and death. J Cereb Blood Flow Metab 19, 231-45.

Myers, C.R., Antholine, W.E. and Myers, J.M., 2010. The pro-oxidant chromium(VI) inhibits mitochondrial complex I, complex II, and aconitase in the bronchial epithelium: EPR markers for Fe-S proteins. Free Radic Biol Med 49, 1903-15.

Myhre, O., Andersen, J.M., Aarnes, H. and Fonnum, F., 2003. Evaluation of the probes 2',7'-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation. Biochem Pharmacol 65, 1575-82.

Nelson, D.L. and Cox, M.M., 2012. Lehninger Principles of Biochemistry, 6th ed. Freeman, W. H. & Company.

Ngu, L.H., Afroze, B., Chen, B.C., Affandi, O. and Zabedah, M.Y., 2009. Molybdenum cofactor deficiency in a Malaysian child. Singapore Med J 50, e365-7.

Nicholls, D. and Akerman, K., 1982. Mitochondrial calcium transport. Biochimica et Biophysica Acta 683, 57-88.

Nicholls, D.G. and Budd, S.L., 2000. Mitochondria and neuronal survival. Physiol Rev 80, 315-60.

Nicholls, D.G. and Ferguson, S.J., 2002. The chemiosmotic proton circuit, in: Nicholls, D.G. and Ferguson, S.J. (Eds.), Bioenergetics 3. Academic Press, London, pp. 57-87.

Nishimura, M., Okimura, Y., Fujita, H., Yano, H., Lee, J., Suzaki, E., Inoue, M., Utsumi, K. and Sasaki, J., 2008. Mechanism of 3-nitropropionic acid-

induced membrane permeability transition of isolated mitochondria and its suppression by L-carnitine. Cell Biochem Funct 26, 881-91.

Ohashi, T., Mizutani, A., Murakami, A., Kojo, S., Ishii, T. and Taketani, S., 2002. Rapid oxidation of dichlorodihydrofluorescin with heme and hemoproteins: formation of the fluorescein is independent of the generation of reactive oxygen species. FEBS Lett 511, 21-7.

Olney, J.W., Misra, C.H. and de Gubareff, T., 1975. Cysteine-S-sulfate: brain damaging metabolite in sulfite oxidase deficiency. J Neuropathol Exp Neurol 34, 167-77.

Orrenius, S., Zhivotovsky, B. and Nicotera, P., 2003. Regulation of cell death: the calcium-apoptosis link. Nat Rev Mol Cell Biol 4, 552-65.

Ozturk, O.H., Oktar, S., Aydin, M. and Kucukatay, V., 2010. Effect of sulfite on antioxidant enzymes and lipid peroxidation in normal and sulfite oxidase-deficient rat erythrocytes. J Physiol Biochem 66, 205-12.

Pastorino, J.G., Simbula, G., Yamamoto, K., Glascott, P.A., Jr., Rothman, R.J. and Farber, J.L., 1996. The cytotoxicity of tumor necrosis factor depends on induction of the mitochondrial permeability transition. J Biol Chem 271, 29792-8.

Patel, M., 2004. Mitochondrial dysfunction and oxidative stress: cause and consequence of epileptic seizures. Free Radic Biol Med 37, 1951-62.

Peiser, G.D. and Yang, S.F., 1979. Sulfite-mediated destruction of β-carotene. J Agric Food Chem 27, 446-449.

Pellerin, L., 2005. How astrocytes feed hungry neurons. Mol Neurobiol 32, 59-72.

Petit, P.X., Goubern, M., Diolez, P., Susin, S.A., Zamzami, N. and Kroemer, G., 1998. Disruption of the outer mitochondrial membrane as a result of large amplitude swelling: the impact of irreversible permeability transition. FEBS Lett 426, 111-6.

Petronilli, V., Costantini, P., Scorrano, L., Colonna, R., Passamonti, S. and Bernardi, P., 1994. The voltage sensor of the mitochondrial permeability transition pore is tuned by the oxidation-reduction state of vicinal thiols. Increase of the gating potential by oxidants and its reversal by reducing agents. J Biol Chem 269, 16638-42.

Pottecher, J., Guillot, M., Belaidi, E., Charles, A.L., Lejay, A., Gharib, A., Diemunsch, P. and Geny, B., 2013. Cyclosporine A normalizes mitochondrial coupling, reactive oxygen species production, and inflammation and partially restores skeletal muscle maximal oxidative capacity in experimental aortic cross-clamping. J Vasc Surg 57, 1100-1108 e2.

Rasola, A. and Bernardi, P., 2011. Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis. Cell Calcium 50, 222-33.

Reiter, R.J., Tan, D.X., Manchester, L.C. and Qi, W., 2001. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species: a review of the evidence. Cell Biochem Biophys 34, 237-56.

Rizzuto, R., De Stefani, D., Raffaello, A. and Mammucari, C., 2012. Mitochondria as sensors and regulators of calcium signalling. Nat Rev Mol Cell Biol 13, 566-78.

Roede, J.R. and Jones, D.P., 2010. Reactive species and mitochondrial dysfunction: mechanistic significance of 4-hydroxynonenal. Environ Mol Mutagen 51, 380-90.

Rosenblum, W.I., 1968. Neuropathologic changes in a case of sulfite oxidase deficiency. Neurology 18, 1187-96.

Saito, A. and Castilho, R.F., 2010. Inhibitory effects of adenine nucleotides on brain mitochondrial permeability transition. Neurochem Res 35, 1667-74.

Sass, J.O., Gunduz, A., Araujo Rodrigues Funayama, C., Korkmaz, B., Dantas Pinto, K.G., Tuysuz, B., Yanasse Dos Santos, L., Taskiran, E., de Fátima Turcato, M., Lam, C.W., Reiss, J., Walter, M., Yalcinkaya, C. and Camelo Junior, J.S., 2010. Functional deficiencies of sulfite oxidase: Differential diagnoses in neonates presenting with intractable seizures and cystic encephalomalacia. Brain Dev 32, 544-9.

Sass, J.O., Nakanishi, T., Sato, T. and Shimizu, A., 2004. New approaches towards laboratory diagnosis of isolated sulphite oxidase deficiency. Ann Clin Biochem 41, 157-9.

Schuierer, G., Kurlemann, G., Bick, U. and Stephani, U., 1995. Molybdenum-cofactor deficiency: CT and MR findings. Neuropediatrics 26, 51-4.

Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., 2001. The Metabolic and Molecular Bases of Inherited Disease, 8 ed. McGraw-Hill, New York.

Slot, H.M., Overweg-Plandsoen, W.C., Bakker, H.D., Abeling, N.G., Tamminga, P., Barth, P.G. and Van Gennip, A.H., 1993. Molybdenum-cofactor deficiency: an easily missed cause of neonatal convulsions. Neuropediatrics 24, 139-42.

Smaili, S.S., Hsu, Y.T., Youle, R.J. and Russell, J.T., 2000. Mitochondria in Ca2+ signaling and apoptosis. J Bioenerg Biomembr 32, 35-46.

Soane, L., Kahraman, S., Kristian, T. and Fiskum, G., 2007. Mechanisms of impaired mitochondrial energy metabolism in acute and chronic neurodegenerative disorders. J Neurosci Res 85, 3407-15.

Sokoloff, L., 1993. Function-related changes in energy metabolism in the nervous system: localization and mechanisms. Keio J Med 42, 95-103.

Southerland, W.M., Akogyeram, C.O., Toghrol, F., Sloan, L. and Scherrer, R., 1982. Interaction of bisulfite with unsaturated fatty acids. J Toxicol Environ Health 10, 479-91.

Southorn, P.A. and Powis, G., 1988. Free radicals in medicine. I. Chemical nature and biologic reactions. Mayo Clin Proc 63, 381-9.

Stachowiak, O., Dolder, M., Wallimann, T. and Richter, C., 1998. Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. J Biol Chem 273, 16694-9.

Starkov, A.A., 2010. The molecular identity of the mitochondrial Ca2+ sequestration system. FEBS J 277, 3652-63.

Starkov, A.A., Chinopoulos, C. and Fiskum, G., 2004. Mitochondrial calcium and oxidative stress as mediators of ischemic brain injury. Cell Calcium 36, 257-64.

Stout, A.K., Raphael, H.M., Kanterewicz, B.I., Klann, E. and Reynolds, I.J., 1998. Glutamate-induced neuron death requires mitochondrial calcium uptake. Nat Neurosci 1, 366-73.

Takeuchi, A., Reyes, N., Artigas, P. and Gadsby, D.C., 2008. The ion pathway through the opened Na(+),K(+)-ATPase pump. Nature 456, 413-6.

Tamura, H., Takasaki, A., Taketani, T., Tanabe, M., Kizuka, F., Lee, L., Tamura, I., Maekawa, R., Asada, H., Yamagata, Y. and Sugino, N., 2013. Melatonin as a free radical scavenger in the ovarian follicle. Endocr J 60, 1-13.

Tan, W.H., Eichler, F.S., Hoda, S., Lee, M.S., Baris, H., Hanley, C.A., Grant, P.E., Krishnamoorthy, K.S. and Shih, V.E., 2005. Isolated sulfite oxidase deficiency: a case report with a novel mutation and review of the literature. Pediatrics 116, 757-66.

Tanveer, A., Virji, S., Andreeva, L., Totty, N.F., Hsuan, J.J., Ward, J.M. and Crompton, M., 1996. Involvement of cyclophilin D in the activation of a mitochondrial pore by Ca2+ and oxidant stress. Eur J Biochem 238, 166-72.

Tatton, W.G. and Olanow, C.W., 1999. Apoptosis in neurodegenerative diseases: the role of mitochondria. Biochim Biophys Acta 1410, 195-213.

Taylor, S.L., Higley, N.A. and Bush, R.K., 1986. Sulfites in foods: uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity, and hypersensitivity. Adv Food Res 30, 1-76.

Tortora, V., Quijano, C., Freeman, B., Radi, R. and Castro, L., 2007. Mitochondrial aconitase reaction with nitric oxide, S-nitrosoglutathione, and peroxynitrite: mechanisms and relative contributions to aconitase inactivation. Free Radic Biol Med 42, 1075-88.

Touati, G., Rusthoven, E., Depondt, E., Dorche, C., Duran, M., Heron, B., Rabier, D., Russo, M. and Saudubray, J.M., 2000. Dietary therapy in two patients with a mild form of sulphite oxidase deficiency. Evidence for clinical and biological improvement. J Inherit Metab Dis 23, 45-53.

Vasquez-Vivar, J., Kalyanaraman, B. and Kennedy, M.C., 2000. Mitochondrial aconitase is a source of hydroxyl radical. An electron spin resonance investigation. J Biol Chem 275, 14064-9.

Vijayakumar, K., Gunny, R., Grunewald, S., Carr, L., Chong, K.W., DeVile, C., Robinson, R., McSweeney, N. and Prabhakar, P., 2011. Clinical neuroimaging features and outcome in molybdenum cofactor deficiency. Pediatr Neurol 45, 246-52.

Vincent, A.S., Lim, B.G., Tan, J., Whiteman, M., Cheung, N.S., Halliwell, B. and Wong, K.P., 2004. Sulfite-mediated oxidative stress in kidney cells. Kidney Int 65, 393-402.

Wall, S.B., Oh, J.Y., Diers, A.R. and Landar, A., 2012. Oxidative modification of proteins: an emerging mechanism of cell signaling. Front Physiol 3, 369.

Wallimann, T., Dolder, M., Schlattner, U., Eder, M., Hornemann, T., Kraft, T. and Stolz, M., 1998. Creatine kinase: an enzyme with a central role in cellular energy metabolism. MAGMA 6, 116-9.

Wang, P.F., McLeish, M.J., Kneen, M.M., Lee, G. and Kenyon, G.L., 2001. An unusually low pK(a) for Cys282 in the active site of human muscle creatine kinase. Biochemistry 40, 11698-705.

Ward, R. and Peters, T., 1995. Free Radicals, in: Marshall, W. and Bangert, S. (Eds.), Clinical Biochemistry: Metabolic and Clinical Aspects. Churchill Livingstone, New York, pp. 765-777.

Wendt, S., Schlattner, U. and Wallimann, T., 2003. Differential effects of peroxynitrite on human mitochondrial creatine kinase isoenzymes. Inactivation, octamer destabilization, and identification of involved residues. J Biol Chem 278, 1125-30.

Wolosker, H., Panizzutti, R. and Engelender, S., 1996. Inhibition of creatine kinase by S-nitrosoglutathione. FEBS Lett 392, 274-6.

Woo, W.H., Yang, H., Wong, K.P. and Halliwell, B., 2003. Sulphite oxidase gene expression in human brain and in other human and rat tissues. Biochem Biophys Res Commun 305, 619-23.

Woodfield, K., Ruck, A., Brdiczka, D. and Halestrap, A.P., 1998. Direct demonstration of a specific interaction between cyclophilin-D and the adenine nucleotide translocase confirms their role in the mitochondrial permeability transition. Biochem J 336 (Pt 2), 287-90.

Yang, S.F., 1970. Sulfoxide formation from methionine or its sulfide analogs during aerobic oxidation of sulfite. Biochemistry 9, 5008-14.

Yang, S.F., 1973. Destruction of tryptophan during the aerobic oxidation of sulfite ions. Environ Res 6, 395-402.

Yarana, C., Sripetchwandee, J., Sanit, J., Chattipakorn, S. and Chattipakorn, N., 2012. Calcium-induced cardiac mitochondrial dysfunction is predominantly mediated by cyclosporine A-dependent mitochondrial permeability transition pore. Arch Med Res 43, 333-8.

Zhang, X., Vincent, A.S., Halliwell, B. and Wong, K.P., 2004. A mechanism of sulfite neurotoxicity: direct inhibition of glutamate dehydrogenase. J Biol Chem 279, 43035-45.

Zoratti, M. and Szabo, I., 1995. The mitochondrial permeability transition. Biochim Biophys Acta 1241, 139-76.

Zorov, D.B., Juhaszova, M., Yaniv, Y., Nuss, H.B., Wang, S. and Sollott, S.J., 2009. Regulation and pharmacology of the mitochondrial permeability transition pore. Cardiovasc Res 83, 213-25.