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Tese de Doutorado

CARACTERIZAÇÃO DA ATIVIDADE PRÓ-OXIDANTE DE DIFERENTES AGENTES E ESTUDO DO POTENCIAL ANTIOXIDANTE DE INTERMEDIÁRIOS DO CICLO DE KREBS SOBRE ALTERAÇÕES OXIDATIVAS INDUZIDAS *IN VITRO*

Robson Luiz Puntel

Santa Maria, RS, Brasil

2008

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por

Robson Luiz Puntel

Tese apresentada ao Programa de Pós-Graduação em Bioquímica Toxicológica, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
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Elaborada por **Robson Luiz Puntel** como requisito parcial para a obtenção do grau de **Doutor em Bioquímica Toxicológica**

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Santa Maria, Maio de 2008.

"A ciência se compõe de erros que,

por sua vez, são os passos até a verdade."

(Julio Verne)

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

CARACTERIZAÇÃO DA ATIVIDADE PRÓ-OXIDANTE DE DIFERENTES AGENTES E ESTUDO DO POTENCIAL ANTIOXIDANTE DE INTERMEDIÁRIOS DO CICLO DE KREBS SOBRE ALTERAÇÕES OXIDATIVAS INDUZIDAS *IN VITRO*

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CO-ORIENTADOR: João Batista Teixeira da Rocha
LOCAL E DATA DA DEFESA: Santa Maria, Maio de 2008.

Dados prévios da literatura têm mostrado que alguns intermediários do ciclo de Krebs podem agir como antioxidantes em diversos modelos, tanto *in vitro*, quanto *in vivo*. Porém, o(s) mecanismo(s) através dos qual(is) esses intermediários exercem suas atividades antioxidantes não são completamente entendidas. Considerando a escassez de dados na literatura a respeito do efeito dos intermediários do ciclo de Krebs durante situações de estresse oxidativo, o presente trabalho teve por objetivo determinar o efeito desses sob a peroxidação lipídica induzida por diferentes agentes pró-oxidantes *in vitro*, bem como investigar o(s) mecanismo(s) de ação dos mesmos. Além disso, faz-se necessário caracterizar o(s) mecanismos(s) pelo(s) qual(is) os diferentes pró-oxidantes agem nos sistemas *in vitro*. Os resultados dessa tese mostraram que a atividade pró-oxidante *in vitro* do malonato não foi modificada pela adição de cianeto de potássio, nem pelo MK-801. Por outro lado, o efeito pró-oxidante do ácido quinolínico foi significativamente prevenido pelo MK-801. Observamos ainda que o malonato, e também o oxalato foram capazes de formar complexos com íons ferrosos. Portanto, com base nos resultados encontrados, concluímos que o efeito pró-oxidante do malonato *in vitro* parece ser independente da excitotoxicidade secundária, conseqüência da ativação indireta dos receptores NMDA. Os resultados sugerem que o efeito do malonato e do oxalato nessas condições experimentais deve-se principalmente a sua capacidade de interagir com íons ferro, modulando uma razão Fe^{2+}/Fe^{3+} que favorece a geração de radicais livres. Por outro lado, o efeito do ácido quinolínico parece ser devido à ativação dos receptores NMDA. Porém, não podemos excluir a participação dos íons ferro para a toxicidade do mesmo nessas condições. Outro foco deste estudo foi investigar o efeito de alguns intermediários do ciclo de Krebs na produção de TBARS basal ou induzida por diferentes pró-oxidantes em S1 de cérebro de ratos *in vitro*, bem como investigar o(s) mecanismo(s) de ação dos mesmos. Os resultados mostraram que o oxaloacetato, o citrato, o sucinato e o malato foram capazes de reduzir significativamente a produção de TBARS basal, bem como a induzida por ácido quinolínico, ferro ou malonato. O fumarato, por sua vez, teve efeito antioxidante somente sobre a produção de TBARS induzida. Por outro lado, o α-cetoglutarato foi capaz de induzir *per se* um significativo aumento na produção de TBARS. O efeito antioxidante do fumarato e do sucinato foi completamente abolido quando o S1 foi submetido a um pré-tratamento por 10 min a 100ºC, enquanto que o efeito dos demais intermediários permaneceu inalterado. Da mesma forma, a adição de cianeto de potássio aboliu

completamente o efeito antioxidante do sucinato sem interferir significativamente no efeito dos demais intermediários estudados. Todos os intermediários estudados, exceto o sucinato e o fumarato, foram capazes de quelar íons ferro, porém somente o oxaloacetato e o α-cetoglutarato foram capazes de prevenir a degradação da desoxirribose induzida por peróxido de hidrogênio. Com base nos resultados obtidos, podemos concluir que o oxaloacetato, o malato, o sucinato, o fumarato e o citrato agem como antioxidantes sob determinadas condições, enquanto que o α-cetoglutarato age como um agente pró-oxidante *per se*. O mecanismo pelo qual o citrato, o malato e o oxaloacetato exercem seus efeitos antioxidantes parece ser devido à capacidade desses em interagir com íons ferro modulando o ciclo redox desse. Por outro lado, o efeito do sucinato e do fumarato parece ser devido a alguma atividade enzimática.

Palavras-chave: Intermediários do ciclo de Krebs, Malonato, Ácido Quinolínico, Ferro, Oxalato e Peroxidação Lipídica.

ABSTRACT

Thesis of Doctors's Degree
Post-Graduate Course in Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

EFFECT OF KREBS CYCLE INTERMEDIATES ON OXIDATIVE CHANGES INDUCED BY DIFFERENT OXIDANT AGENTS

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DATE AND PLACE OF THE DEFENSE: Santa Maria, May 2008

Previous data from the literature have shown that some Krebs cycle intermediates could act as antioxidant in several models, both *in vitro* and *in vivo*. However, the mechanism(s) involved in the antioxidant effect of Krebs cycle intermediates are not fully understood. Additionally, there are scarce data in the literature taking into account the *in vitro* effect of Krebs cycle intermediates during oxidative stress conditions. Thus, the aim of this study was to determine the effect of some Krebs cycle intermediates on lipid peroxidation induced *in vitro* by different pro-oxidant agents, and the mechanism(s) by which they act. Furthermore, it was necessary elucidate the mechanisms by which the different pro-oxidants acts under *in vitro* conditions. The present results showed that the malonate-induced TBARS production was not changed by potassium cyanide or MK-801. However, the pro-oxidant effect of quinolinic acid was significantly prevented by MK-801. In addition we found that both malonate and oxalate were able to form complexes with iron ions (Fe^{2+}). Based on the presented results, we conclude that malonate pro-oxidant activity *in vitro* seems to be independent of the secondary excitotoxicity via indirect NMDA receptors activation. Additionally, we suggest that both the malonate and oxalate effect, in these experimental conditions, is due to its ability to form complexes with iron ions, thus modulating an adequate ratio Fe^{2+}/Fe^{3+} that could cause an increase in free radicals generation. In contrast, the quinolinic acid effect seems to be dependent of the NMDA receptors activation. However, we can not rule out the involvement of iron ions in quinolinic acid toxicity under our assay conditions. Another objective of this study was to investigate the effect of some Krebs cycle intermediates against either basal or induced TBARS production, using rat brain S1 preparations and the mechanism(s) by which they act. The results showed that oxaloacetate, citrate, succinate, and malate were able to significantly prevent both basal and quinolinic acid-, iron- or malonate-induced TBARS production. On the other hand, fumarate prevented only malonate-induced TBARS production, without effect under basal conditions. However, α-ketoglutarate induced *per se* a significant increase in basal TBARS production. The antioxidant activity of fumarate and succinate were completely abolished when S1 was submitted to heat-treatment at 100ºC during 10 min. Likewise, potassium cyanide completely abolished the antioxidant effect of succinate. The effect of other Krebs cycle intermediates studied was unchanged with respect to heat-treatment, or cyanide. Except for succinate and fumarate, all intermediates used in this study were able to form complexes with iron (Fe^{2+}) ions, however only

oxaloacetate and α-ketoglutarate significantly prevented deoxyribose degradation induced by hydrogen peroxide. Based on the results presented, we concluded that oxaloacetate, malate, succinate, fumarate and citrate could act as antioxidants under such conditions, whereas α-ketoglutarate acts as a pro-oxidant agent *per se*. The mechanism(s) by which citrate, malate, and oxaloacetate acts seems to be related to their ability to form complexes with iron (Fe^{2+}) ions, thus modulating the iron redox cycle. In contrast, the succinate and fumarate antioxidant effect seems to be dependent of the some enzymatic system.

Keywords: Krebs cycle intermediates, Malonate, Quinolinic Acid, Iron, Oxalate and Lipid Peroxidation.

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LISTA DE ABREVIATURAS

ADP - Difosfato de Adenosina

AMP - Monofosfato de Adenosina

AQ – Ácido Quinolínico

ATP – Trifosfato de Adenosina

CAT – Catalase

DFO – Deferroxamina

DNA – Ácido Desoxirribonucleico

EROs – Espécies Reativas de Oxigênio

ERNs – Espécies Reativas de Nitrogênio

FAD – Dinucleotídeo de Flavina Adenina (forma oxidada)

GSH – Glutationa Reduzida

GSSG - Glutationa Oxidada

GTP – Trifosfato de Guanosina

H_2O_2 – Peróxido de Hidrogênio

HPLC – Cromatografia Liquida de Alto Desempenho

KCN – Cianeto de Potássio

MDA – Malondialdeído

NAD^+ – Dinucleotídeo de Nicotinamida Adenina (forma oxidada)

NADPH - Dinucleotídeo Fosfato de Nicotinamida Adenina (forma reduzida)

NMDA – N-metil-D-aspartato

NO – Óxido Nítrico

$O^{-}_{2}{}^{\bullet}$ – Radical Ânion Superóxido

$OH^{\bullet}$ – Radical Hidroxil

$ONOO^{-}$ – Peroxinitrito

PUFA – Ácidos Graxos Poliinsaturados

RL – Radical Livre

S1 – Sobrenadante após centrifugação a 4000 g por 10 min (4ºC)

SDH _ Sucinato Desidrogenase

SH – Grupos Tióis

SNC – Sistema Nervoso Central

SOD – Superóxido Dismutase

TBA – Ácido Tiobarbitúrico

TBARS – Espécies Reativas ao Ácido Tiobarbitúrico

TBHP – *tert*-butil Hidroperóxido

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APRESENTAÇÃO

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos, os quais encontram-se no item **ARTIGOS CIENTÍFICOS**. Esse item, por sua vez, está subdividido em Capitulo I e Capitulo II. Tendo em vista que migrei do Mestrado para o Doutorado na condição de continuar meu estudo, o Capitulo I está constituído pelos artigos **1** e **2** que fizeram parte de minha Dissertação de Mestrado, a qual foi apresentada ao Programa de Pós Graduação em Bioquímica Toxicológica da UFSM. No capitulo II estão apresentados os artigos **3** e **4** os quais representam a continuação de meu projeto inicial. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos e representam a íntegra deste estudo.

Os itens, **DISCUSSÃO E CONCLUSÕES**, encontrados no final desta tese, apresentam interpretações e comentários gerais sobre os artigos científicos contidos neste trabalho.

No item **PERSPECTIVAS** estão expostos os possíveis estudos para continuação do estudo do autor, referente a esse assunto.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO, REVISÃO BIBLIOGRÁFICA** e **DISCUSSÃO** desta tese.

1. INTRODUÇÃO

O estresse oxidativo é definido como sendo um desequilíbrio entre a produção de espécies reativas de oxigênio (EROs) ou nitrogênio (ERNs), e os mecanismos de defesa antioxidantes (Frei, 1994). As EROs são geradas durante o metabolismo aeróbico em todos os organismos vivos que utilizam oxigênio (Silva e cols., 2005). Contudo, acredita-se que a excessiva geração de EROs possa estar envolvida na patogênese de várias desordens via diferentes mecanismos, tais como mutação no DNA, oxidação de proteínas e também, peroxidação lipídica (Finkel e Holbrook, 2000; Valko e cols., 2004, 2006, 2007). Além disso, certas condições patológicas tais como episódios isquêmicos são acompanhados de um aumento excessivo na geração de EROs (Cao e cols., 1988; Sakamoto e cols., 1991; Porciúncula e cols., 2003).

Nesse contexto, vários estudos têm abordado o uso de compostos antioxidantes, os quais têm se mostrado eficazes contra uma variedade de modelos, tanto *in vitro* quanto *in vivo,* de patologias humanas, inclusive em modelos de neuro-toxicidade (Bastianetto e Quirion, 2002; Burget e cols., 2004; Wagner e cols., 2006; Williams e cols., 2004; Patel e cols., 2007).

Tendo em vista que os níveis dos intermediários metabólicos encontram-se alterados durante episódios isquêmicos e também em situações de disfunção metabólica (Hoyer and Krier, 1986; Folbergrová et al., 1974; Medvedeva et al., 2002), estudos são necessários para determinar o efeito desses intermediários nessas situações, uma vez que essas são acompanhadas tanto de um aumento acentuado na geração de EROs (Cao e cols., 1988; Sakamoto e cols., 1991; Porciúncula e cols., 2003), bem como na liberação intracelular dos íons ferro (Oubidar et al., 1994).

Dados recentes na literatura têm relatado que alguns intermediários do ciclo de Krebs podem agir como potentes antioxidantes, tanto *in vitro*, quanto *in vivo*, em diversos sistemas pró-oxidantes. Porém, o(s) mecanismo(s) através dos quais os intermediários do ciclo de Krebs exercem suas atividades antioxidantes não são completamente entendidas.

Baseado no exposto acima, estudar o efeito de diferentes intermediários do ciclo de Krebs sobre a peroxidação lipídica induzida por diferentes agentes pro-oxidantes, bem como o(s) mecanismo(s) pelo(s) qual(is) esses intermediários agem torna-se extremamente importante.

2. REVISÃO BIBLIOGRÁFICA

2.1. O Ciclo de Krebs

2.1.1. A descoberta do ciclo

O Ciclo dos Ácidos Tricarboxílicos foi postulado primeiramente por Hans Krebs, em 1937, sob o nome original de "Ciclo do Ácido Cítrico". Essa importante descoberta rendeu a Krebs o Prêmio Nobel, em 1953.

Já em 1936, Krebs, com base em estudos prévios, começou a estudar as inter-relações no metabolismo oxidativo dos vários ácidos di e tricarboxílicos em suspensões de músculos de vôo de pombos triturados. Em particular, ele procurou o significado biológico desses ácidos na oxidação da glicose (Lehninger, 1976).

A partir de uma série de experimentos simples, e argumentos inteligentes, Krebs postulou o Ciclo do Ácido Cítrico como a via principal para a oxidação dos carboidratos no músculo. Devido à incerteza, durante muitos anos, de ser ou não o ácido cítrico o primeiro ácido tricarboxílico formado na reação entre o piruvato e o oxaloacetato, o nome do ciclo foi mudado para ciclo do Ácido Tricarboxílico. Atualmente são usados como sinônimos os nomes "Ciclo dos Ácidos Tricarboxílicos", "Ciclo do Ácido Cítrico", ou "Ciclo de Krebs" (Lehninger, 1976).

2.1.2. Considerações Gerais sobre o Ciclo de Krebs

O Ciclo de Krebs é a mais importante via metabólica celular. O ciclo compreende uma série de reações químicas de importância central para todas as células que utilizam oxigênio durante o processo de respiração celular (organismos aeróbios). Nesses organismos, o Ciclo de Krebs é parte central das vias metabólicas envolvidas na conversão química de carboidratos, ácidos graxos e proteínas em dióxido de carbono, água e energia útil para a(s) célula(s) (Stryer, 1988; Campbell, 1999; Nelson e Cox, 2002).

O Ciclo de Krebs está associado à cadeia respiratória, ou seja, um complexo de compostos transportadores de prótons (H^+) e elétrons que consomem o oxigênio (O_2) absorvido por mecanismos respiratórios, sintetizando água e gerando ATPs através do processo de fosforilação oxidativa (Stryer, 1988; Campbell, 1999; Nelson e Cox, 2002).

Esses processos ocorrem dentro das mitocôndrias, com as enzimas do Ciclo de Krebs dispersas na matriz e os transportadores de elétrons fixos nas cristas mitocondriais.

O Ciclo de Krebs pode ser dividido em oito etapas consecutivas:

1. **INÍCIO:** ***condensação da aceti com o oxaloacetato, gerando citrato*** (catalisada pela *citrato-sintase*).
2. ***Isomerização do citrato em isocitrato*** (catalisada pela *aconitase*).
3. ***Oxidação do citrato a α-cetoglutarato*** (catalisada pela *isocitrato-desidrogenase*, utiliza o NAD^+ como transportador de dois elétrons liberados na reação, havendo o desprendimento de uma molécula de CO_2).
4. ***Descarboxilação oxidativa do α-cetoglutarato a sucinil-CoA*** (catalisada pelo complexo enzimático *α-cetoglutarato-desidrogenase* e utiliza o NAD^+ como transportador de dois elétrons liberados na reação, havendo o desprendimento de mais uma molécula de CO_2).
5. ***Desacilação do sucinil-CoA até sucinato*** (catalisada pela *sucinil-CoA sintase*, gera um GTP que é convertido, posteriormente, a ATP).
6. ***Oxidação do succinato a fumarato*** (catalisada pela *sucinato-desidrogenase* (SDH), utiliza o FAD como transportador de dois elétrons liberados na reação)
7. ***Hidratação do fumarato a malato*** (catalisada pela *fumarase*).
8. **TÉRMINO:** ***desidrogenação do malato com a regeneração do oxaloacetato*** (catalisada pela enzima *malato-desidrogenase,* utiliza o NAD^+ como transportador de dois elétrons liberados na reação).

O Ciclo de Krebs fornece ainda precursores para uma série de moléculas, como certos aminoácidos e glicose. Sendo assim, algumas das reações do Ciclo de Krebs também são importantes para organismos anaeróbios (por exemplo, aqueles que fazem fermentação) (Stryer, 1988; Campbell, 1999; Nelson e Cox, 2002).

A Figura 1 (a seguir) ilustra esquematicamente todas as reações de uma volta completa no Ciclo de Krebs, bem como as enzimas envolvidas e os produtos que são liberados em cada uma das reações.

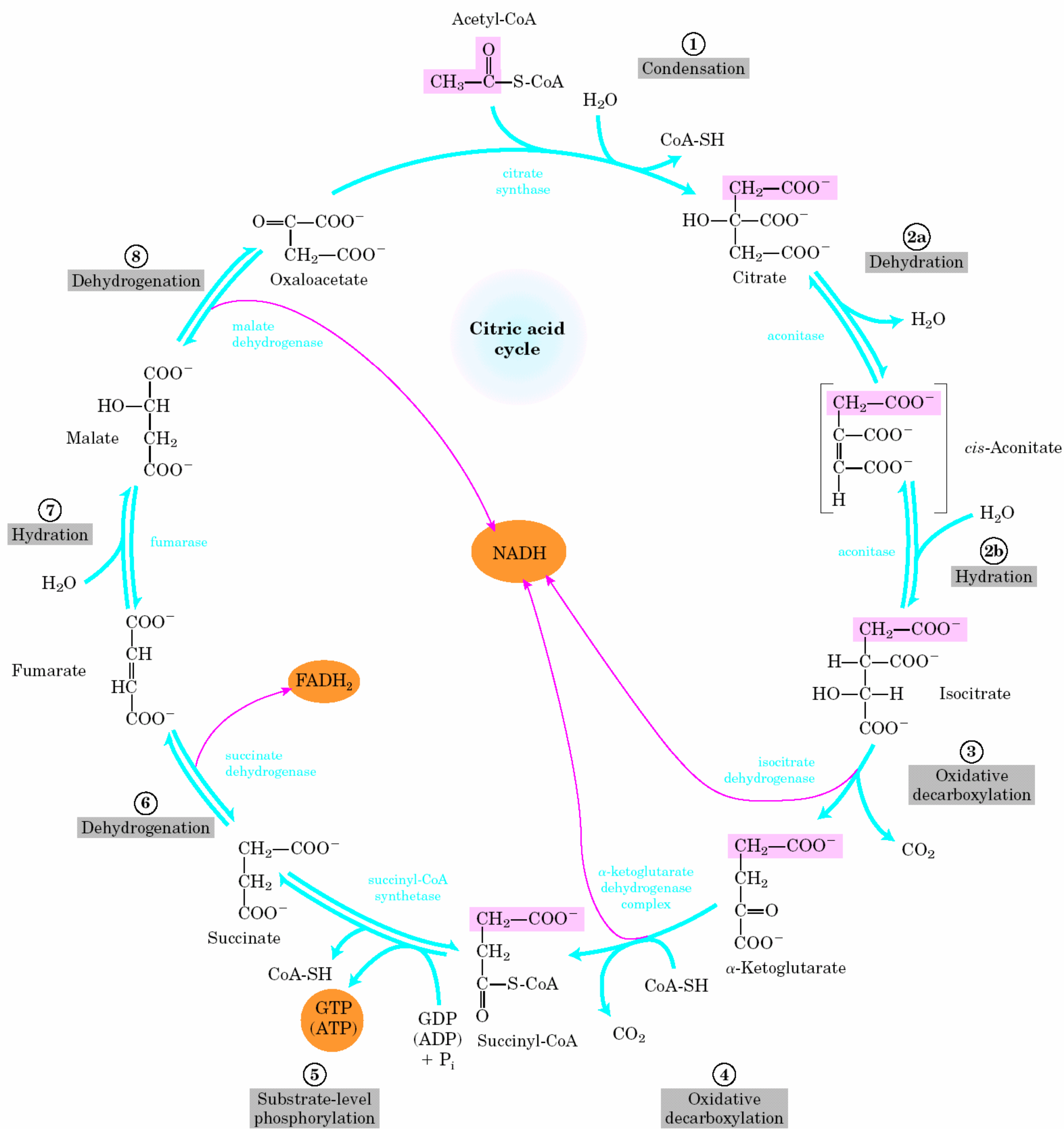

Figura 1. Representação esquemática das reações do Ciclo de Krebs (Nelson e Cox, 2002).

2.2. Os Intermediários do Ciclo de Krebs Estudados

2.2.1. α-Cetoácidos

O α-cetoglutarato é uma molécula composta por cinco átomos de carbono. O mesmo é formado a partir da descarboxilação oxidativa do isocitrato, numa reação catalisada pela *isocitrato-desidrogenase.* O α-cetoglutarato é um importante composto biológico, e um intermediário chave do Ciclo de Krebs, o qual é encontrado naturalmente dentro das células. Uma de suas funções é combinar-se com a amônia para formar glutamato e posteriormente glutamina. Outra função do mesmo é combinar-se com o nitrogênio liberado dentro das células. O α-cetoglutarato é um dos mais importantes transportadores de nitrogênio nas rotas metabólicas. Os grupos amino dos aminoácidos são transferidos para o α-cetoglutarato por transaminação, e por esse transportado até o fígado onde ocorre o ciclo da uréia (Stryer, 1988; Campbell, 1999; Nelson e Cox, 2002).

O oxaloacetato, assim como a maioria dos intermediários do Ciclo de Krebs, é constituído por quatro carbonos. O oxaloacetato é formado a partir do malato, numa reação catalisada pela *malato-desidrogenase.* Posteriormente, uma nova volta no ciclo pode ser iniciada com a condensação do oxaloacetato com o acetil, catalisada pela *citrato-sintase* (Stryer, 1988; Campbell, 1999; Nelson e Cox, 2002).

Nas plantas, o oxaloacetato pode, também, ser formado a partir da condensação do CO_2 com fosfoenol piruvato, catalisada pela *oxaloacetato descarboxilase*.

Dados recentes na literatura têm indicado que o α-cetoglutarato (intermediário do Ciclo de Krebs) inibe o estresse oxidativo induzido tanto *in vitro* (Desagher e cols., 1997; Sokolowska e cols., 1999) quanto *in vivo* (Velvizhi e cols., 2002 a; Velvizhi e cols., 2002 b). Da mesma forma, o α-cetoglutarato e o oxaloacetato (α-cetoácidos) podem prevenir danos ao DNA mitocondrial e convulsões induzidas por ácido caínico em camundongos (Yamamoto e Mohanan, 2003).

2.2.2. Citrato

O citrato (forma ionizada do ácido cítrico) é o primeiro intermediário do Ciclo de Krebs. Esse é formado a partir da condensação do oxaloacetato com o acetil, na reação catalisada pela *citrato-sintase* (primeira reação do Ciclo de Krebs). Como intermediário do Ciclo de Krebs, está envolvido na conversão metabólica de carboidratos, ácidos graxos e proteínas na maioria dos organismos vivos (Stryer, 1988; Campbell, 1999; Nelson e Cox, 2002).

O ácido cítrico (forma não-ionizada) é um ácido relativamente forte, e solúvel em água. O ácido cítrico bem como seus sais são amplamente usados, pois não são tóxicos e são facilmente biodegradáveis. Esse ácido é encontrado principalmente em frutas cítricas, mas também em outros tipos de frutas, em legumes e nos tecidos e fluídos animais, nos quais pode estar na forma ácida livre, ou complexado com íons ferro.

O ácido cítrico é amplamente usado nas indústrias farmacêuticas e alimentícias. Nessas, é usado para quelar metais essenciais, além de ajudar na redução do pH. Dessa forma, esse ácido contribui para retardar a atividade enzimática ajudando na conservação dos alimentos. Já na indústria farmacêutica, entre outros se destaca o uso do ácido cítrico como anticoagulante.

O citrato forma complexos com íons ferrosos (Fe^{2+}) ou férricos (Fe^{3+}), os quais podem ser ativos em sistemas biológicos (Baker e Gebicki, 1986). Esse ácido orgânico pode baixar os níveis de ferro através da quelação, ou aumentar sua disponibilidade através de reações redox (Abrahamson e cols., 1994). Têm-se relatos que o aumento no ciclo redox dos complexos de ferro pode levar à geração de radicais livres catalisada por ferro, à peroxidação lipídica, à distrofia axonal, à necrose e à morte celular apoptótica (Chiueh e cols., 1993; Gutteridge, 1994).

2.2.3. Sucinato e Malato

O sucinato é uma molécula com quatro carbonos. O sucinato é formado a partir do sucinil-CoA, na reação catalisada pela *sucinil-CoA sintase*. O sucinato é posteriormente oxidado a fumarato pela SDH.

Estudos prévios têm mostrado que o sucinato inibe a peroxidação lipídica induzida por uma variedade de agentes pró-oxidantes (Takayanagi e cols., 1980; Bindoli e cols.,

1982; Cavallini e cols., 1984). Em todos esses casos, a proteção oferecida por sucinato é atribuída à redução da coenzima Q (ubiquinol - coenzima Q no estado reduzido) através da atividade da SDH. O ubiquinol, por sua vez age como antioxidante em sistemas biológicos (Bindoli e cols., 1982; Cavallini e cols., 1984).

Além disso, recentemente Nowak e cols (2008) mostraram que o sucinato foi capaz de prevenir o déficit energético e a disfunção do complexo I mitocondrial em células do túbulo proximal renal expostas ao *tert*-butil hidroperóxido (TBHP) (Nowak e cols 2008).

O malato é constituído por quarto carbonos. O mesmo é originado a partir do fumarato pela ação da enzima *fumarase*. Posteriormente, o malato é oxidado a oxaloacetato por ação da *malato-desidrogenase* (Stryer, 1988; Campbell, 1999; Nelson e Cox, 2002).

O ácido málico é um ácido dicarboxílico de sabor azedo. Na sua forma ionizada, forma o malato, que juntamente com os demais intermediários do Ciclo de Krebs está envolvido na conversão metabólica de carboidratos, ácidos graxos e proteínas na maioria dos organismos vivos (Stryer, 1988; Campbell, 1999; Nelson e Cox, 2002).

Da mesma maneira que o sucinato, o malato age como antioxidante prevenindo a peroxidação lipídica. Seu efeito é também atribuído à redução da coenzima Q (Vianello e cols., 1986). Porém, nesse caso, a formação do ubiquinol é devido à atividade da enzima *malato-desidrogenase*.

2.2.4. Fumarato

O fumarato, por sua vez, é uma molécula composta por quatro carbonos. O fumarato é formado a partir do sucinato, na reação catalisada pela *sucinato desidrogenase (SDH),* o qual é posteriormente convertido em malato, na reação catalisada pela *fumarase* (Stryer, 1988; Campbell, 1999; Nelson e Cox, 2002).

Dados da literatura têm demonstrado que o fumarato é capaz de formar complexos bio ativos com íons ferro (Younes e cols., 1990; Lachili e cols., 2001), aumentando assim o ciclo redox do ferro, e conseqüentemente a geração de radicais livres. Nesse contexto, Lachili e cols., (2001) demonstraram que mulheres grávidas que receberam suplementação com complexos ferro/fumarato apresentaram um significativo aumento nos níveis de peróxidos lipídicos plasmático (Lachili e cols., 2001).

2.3. Agentes pró-oxidantes usados nesse estudo

2.3.1. Malonato

O cérebro contém concentrações substanciais de malonato livre (0,2 mM), mas pouco é conhecido a respeito da origem desse ácido dicarboxílico e sua importância biológica.

O malonato é um inibidor reversível da SDH (EC 1.3.99.1), uma enzima chave no metabolismo oxidativo, devido à sua semelhança estrutural com o substrato da enzima, o sucinato (Maragos e Silverstain, 1995; Shulz e cols., 1996). Sendo assim, seu acúmulo prejudica o metabolismo aeróbio.

O potencial de membrana é um importante modulador de excitotoxicidade, e sua manipulação tem sido proposta como um mecanismo pra explicar a ligação entre inibição metabólica e excitotoxicidade (Beal e cols., 1993; Greene e Greenamyre, 1996).

O malonato ao inibir a SDH, leva a uma interrupção da fosforilação oxidativa e, por conseqüência, a uma depleção nos níveis de adenosina trifosfato (ATP). Nessas condições, ocorre uma despolarização pela falência de várias ATPases, principalmente da Na^{+}/K^{+}/ATPase (Greene e cols., 1993), que mantêm a tensão e o gradiente de íons através da membrana plasmática. Ou seja, a inibição da SDH causada pelo malonato leva à disfunção mitocondrial, geração de radicais livres, excitotoxicidade secundária e apoptose (Dedeoglu e cols., 2002).

A neurotoxicidade associada com a administração intraestriatal de malonato é mediada quase que exclusivamente pela ativação indireta dos receptores N-metil-D-aspartato (NMDA) (Beal e cols., 1993; Greene e cols, 1993; Henshaw e cols., 1994; Greene e Greenamyre, 1995). Porém, o envolvimento dos receptores NMDA na toxicidade causada por malonato é controverso.

Estudos têm demonstrado que a administração do antagonista de receptores NMDA (MK-801) reduz o volume das lesões estriatais induzida por malonato. Porém, esse não é capaz de bloquear a geração de espécies reativas de oxigênio (EROs), o que sugere que a ativação de tais receptores não leva a um aumento na geração de EROs (Ferger e cols., 1999; Zeevalk e cols., 2000). Por outro lado, estudos demonstram claramente que a ativação dos receptores NMDA pode estar envolvida na geração de EROs, os quais

contribuiriam para o estresse oxidativo induzido por malonato (Santamaría e Rios, 1993; Rodríguez- Martinez e cols., 2000).

2.3.2. Ácido Quinolínico

A rota da quinurenina é a principal rota do metabolismo do triptofano em mamíferos e ocorre principalmente no fígado. Um dos metabólitos dessa rota é o ácido quinolínico (AQ) (Moroni, 1999).

O AQ é encontrado no cérebro de ratos e humanos e pode estar envolvido na etiologia de algumas doenças neurodegenerativas de humanos, como a doença de Huntington e a epilepsia (Moroni e cols., 1986; Schwarz e cols., 1988). De particular importância, a administração intracerebral de AQ causa neurodegeneração em mamíferos (Rodriguez-Martinez e cols., 2000). Sendo assim, o mecanismo através do qual o AQ induz neurotoxicidade parece envolver a superativação dos receptores NMDA, tanto por estimular pré-sinapticamente a liberação de aminoácidos neurotransmissores excitatórios, ou por ação pós-sináptica (Santamaría e Ríos, 1993). Essa ativação causaria uma entrada excessiva de cálcio nos neurônios (Rodriguez-Martinez e cols., 2000), promovendo a peroxidação lipídica e ativando uma série de processos intracelulares dependentes de cálcio (Rodriguez-Martinez e cols., 2000).

A participação dos receptors NMDA na toxicidade induzida pelo AQ é sustentada pelo fato do MK-801 (antagonista seletivo do receptor NMDA) bloquear a peroxidação lipídica induzida pela administração intraestriatal de AQ (Santamaría e Ríos, 1993; Rodriguez-Martinez e cols., 2000).

Contudo, a atividade pró-oxidante do AQ in vitro parece também ser dependente dos íons ferrosos (Stípek e cols., 1997).

2.3.3. Ferro

O ferro desempenha um papel importante nos processos metabólicos dos animais, sendo um constituinte vital nas células de todos os mamíferos. O ferro está também presente em algumas enzimas que catalisam mecanismos de oxidação celular. No homem, os órgãos mais ricos em ferro são o fígado e o baço. Embora em menor quantidade, é encontrado também nos ossos, na medula, nos rins e nos intestinos.

O ferro é de grande importância nos sistemas biológicos, onde participa de uma grande variedade de reações de transporte de elétrons, geralmente no estado de oxidação II e III. Porém, esses íons podem estimular a produção de radicais livres por diferentes mecanismos (Braughler e cols., 1986; Minotti e Aust, 1987; Minotti e Aust, 1992):

a) por degradar hidroperóxidos lipídicos pré-existentes (ROOH) nos tecidos, formando o radical lipídico alcoxil ($RO^{\cdot}$);

b) por participar nas reações do tipo Fenton produzindo radicais hidroxil ($OH^{\cdot -}$); ou

c) por formar complexos com oxigênio, tal como os complexos Fe^{2+}-O_2-Fe^{3+}, os quais são responsáveis por iniciar as reações de peroxidação lipídica (Oubidar e cols., 1996).

Além disso, dados na literatura sugerem que a razão Fe^{2+}/Fe^{3+} é um fator fundamental na iniciação e propagação das reações de peroxidação lipídica (Braughler e cols., 1986). Assim, compostos capazes de interagir com íons ferrosos (II) ou férricos (III) podem ajustar indiretamente a atividade desses íons por modular diferentes razões Fe(II)/ Fe(III).

2.3.4. Oxalato

O ácido oxálico (ácido dicarboxílico estruturalmente relacionado aos intermediários do ciclo de Krebs) é um constituinte natural das plantas e, várias espécies são capazes de acumular elevados níveis desse ácido dicarboxílico. A mais notável característica química desse ácido é sua capacidade de quelar cátions multivalentes. Nesse contexto, o ácido oxálico pode diminuir a bio disponibilidade mineral, sendo por isso considerado um anti-nutriente (Libert e Franceschi, 1987; Massey e cols., 2001).

A hiperoxaluria é um dos principais fatores de risco no desenvolvimento do cálculo renal em humanos. O oxalato, o principal constituinte formador do cálculo, é conhecido por induzir peroxidação lipídica (Selvan e Kurien, 1987). A exposição a elevadas concentrações de oxalato pode induzir o estresse oxidativo, demonstrado por: (i) peroxidação lipídica aumentada (TBARS) (Selvan e Kurien, 1987), (ii) diminuição na concentração de glutationa reduzida (Muthukumar e Selvan, 1998), (iii) aumento na geração de radicais livres (Scheid e cols., 1996), e (iv) aumento na liberação de ácido araquidônico via fosfolipase-A_2 (Kohjimoto e cols., 1999). Relativamente pouco é sabido sobre a origem das espécies reativas de oxigênio durante a exposição ao oxalato, porém

dados da literatura tem mostrado que a origem dessas espécies durante exposição ao oxalato é devida, principalmente, à produção mitocondrial (Khand e cols., 2002; Veena e cols., 2008). Além disso, Veena e cols. (2008) demonstraram recentemente uma relação entre hiperoxalúria e a disfunção mitocondrial (Veena e cols., 2008), sugerindo que as mitocôndrias podem estar envolvidas na etiologia do cálculo renal.

2.4. Espécies Reativas de Oxigênio e Peroxidação Lipídica (Estresse Oxidativo)

2.4.1.Espécies Reativas de Oxigênio

As células estão continuamente produzindo radicais livres e espécies reativas de oxigênio (EROs) como parte do processo metabólico. Tais espécies são capazes de gerar estresse oxidativo em conseqüência de suas propriedades oxidantes.

As principais EROs vinculadas ao estresse oxidativo são o radical ânion superóxido ($O^{-}_{2}{}^{\cdot}$), o radical hidroxil ($OH^{\cdot}$), o peróxido de hidrogênio (H_2O_2), o óxido nítrico (NO) e o peroxinitrito ($ONOO^{-}$). Estes são neutralizados por um elaborado sistema de defesa antioxidante que pode ser enzimático (a catalase, a superóxido dismutase, a glutationa peroxidase) ou não-enzimático (as vitaminas A, E, e C, flavonóides, ubiquinonas e a glutationa reduzida- GSH) (Alexi e cols., 1998; Gianni e cols., 2004). Neste contexto, o estado de estresse oxidativo pode resultar tanto de um aumento na produção de EROs quanto da redução da capacidade antioxidante celular total. Ou seja, a ocorrência de um dano oxidativo depende de um desequilíbrio entre a produção de EROs e a atividade das defesas antioxidantes (Halliwel, 1992; Dawson e Dawson, 1996).

2.4.2.Peroxidação Lipídica

As membranas biológicas apresentam uma estrutura geral comum (Figura 2). Essas são constituídas de uma bicamada lipídica as quais estão associadas a proteínas. As proteínas presentes na membrana celular são responsáveis pelo transporte de moléculas específicas através da bicamada lipídida. Além disso, essas proteínas podem agir como catalisadoras de reações associadas às membranas, como a síntese de ATP (Alberts e cols., 1994).

As membranas biológicas são constituídas principalmente por fosfolipídeos, os quais possuem uma cabeça polar e duas caudas hidrofóbicas. Geralmente, as caudas hidrofóbicas são compostas por ácidos graxos, que podem diferir no comprimento e na configuração em que se apresentam, podendo uma das caudas apresentar uma ou mais ligações duplas (insaturações) (Alberts e cols., 1994; Halliwell e Gutteridge, 1989).

Muitos compostos são metabolicamente ativados para intermediários reativos que são responsáveis por iniciar eventos tóxicos. Um tipo particular de intermediário reativo é o radical livre (RL).

Os RL são moléculas que tem um elétron ímpar (desemparelhado) na sua órbita externa (derivadas geralmente do oxigênio). Esta molécula se caracteriza por ser altamente instável, por ter uma vida média muito curta, medida em microssegundos, e por procurar sua estabilidade através do pareamento de seus elétrons. Os RL são espécies eletrofílicas extremamente reativas que podem reagir com os componentes celulares (Josephy, 1997; Timbrell, 2000). Os RL são gerados por uma variedade de processos, podendo atacar uma diversidade de biomoléculas alvo, tais como o DNA, os lipídeos e as proteínas.

Conforme mencionado anteriormente, uma fonte importante desses elétrons são os ácidos graxos insaturados encontrados na dupla camada de lipídeos das membranas celulares, que são vitais para o funcionamento da célula. Quando os RL reagem com esses ácidos graxos insaturados modificam os lipídeos (Figura 3, esquema das reações de peroxidação lipídica) e a membrana perde suas características arquitetônicas, tornando-se menos firme e menos flexível, criando-se verdadeiras fendas iônicas que alteram sua semipermeabilidade, o que favorece a entrada e saída indiscriminada de metabólitos e detritos da célula, provocando sua ruptura e lise com necrose (Josephy, 1997; Timbrell, 2000).

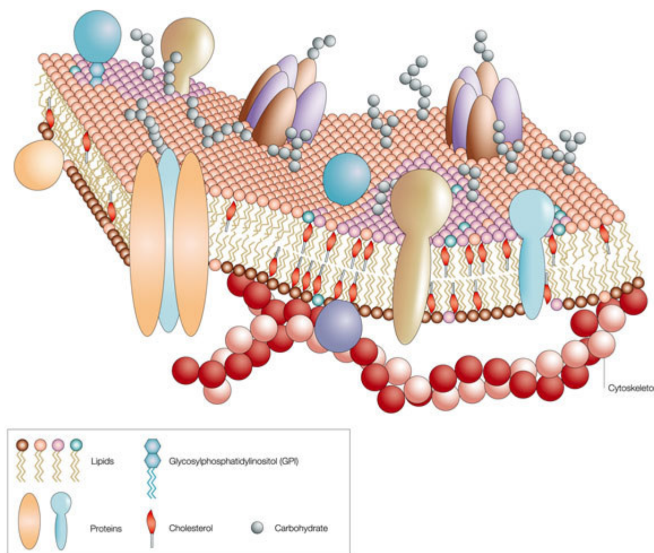

Figura 2. Estrutura geral das membranas biológicas (Fonte http://www.nature.com/horizon/livingfrontier/background/images/membrane_f2.jpg, em 02/04/2008).

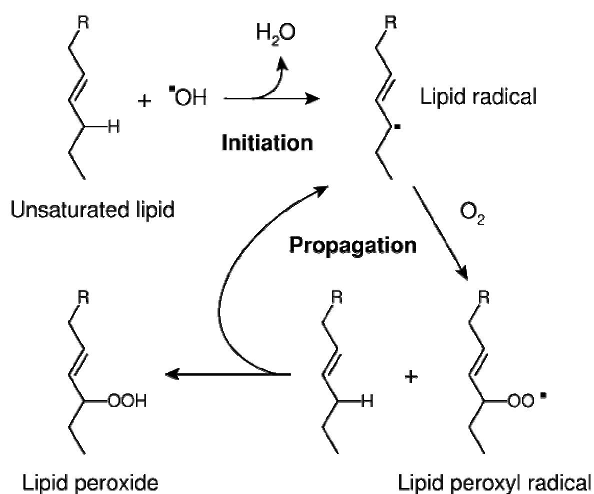

Figura 3. Esquema geral das reações de peroxidação lipídica (Fonte http://upload.wikimedia.org/wikipedia/commons/thumb/9/9e/Lipid_peroxidation.svg/711px-Lipid_peroxidation.svg.png, em 02/04/2008).

2.4.2.1. Quantificação da Peroxidação Lipídica: O Método do TBARS (Espécies Reativas ao Ácido Tiobarbitúrico)

A reação do malondialdeído (MDA) com o ácido tiobarbitúrico (TBA) para formar de um complexo colorido (TBA-MDA), o qual pode ser quantificado espectrofotometricamente em 532 nm (Figura 4) é a base do método mais comum para acessar peroxidação lipídica em materiais biológicos dos últimos 40-50 anos. Porém, esse método tem sido "alvo" de criticas generalizadas, por vários motivos. Outros aldeídos, que não o MDA, podem reagir com o TBA formando complexos coloridos, outros que não o TBA-MDA. Do mesmo modo, muitos tecidos de plantas e animais contêm pigmentos endógenos que absorvem na mesma região que o complexo TBA-MDA (532 nm) (Draper e cols., 1993).

Além disso, a reação entre o TBA e o MDA dá-se em condições acídicas em elevadas temperaturas. Nessas condições, o MDA também pode ser formado pela oxidação dos ácidos graxos poliinsaturados (PUFA; polyunsaturated fatty acids) e também pela decomposição dos lipóxidos pré-existentes nas amostras. Da mesma forma, alguns açucares, tais como a sacarose e 2-desoxirribose, formam MDA quando sujeitas à condições em que é gerado o radical hidroxil ($OH^{\bullet -}$) (Draper e cols., 1993).

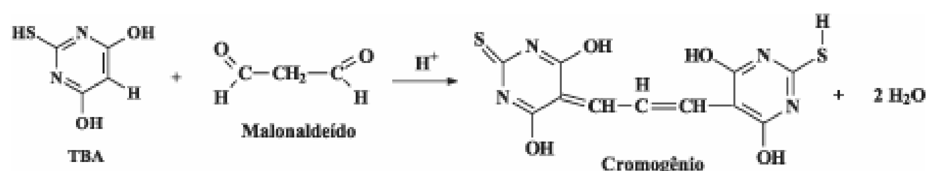

Figura 4. Reação de teste de TBARS entre o ácido 2-tiobarbitúrico e o malondialdeído, formando o complexo colorido TBA-MDA, medido espectrofotometricamente a 532 nm.

Com o objetivo de aperfeiçoar a especificidade do método do TBA para a determinação de MDA, Bird e cols, (1983) desenvolveram um procedimento para isolamento e quantificação dos complexos TBA-MDA por cromatografia liquida de alto desempenho (HPLC). Com a introdução do HPLC tanto o problema a especificidade do

método colorimétrico quanto a sensibilidade da medida de MDA foram resolvidos. Desde então, vários outros métodos de HPLC para isolar e quantificar complexos TBA e MDA foram desenvolvidos (Yu e cols., 1986; Esterbauer e Zollner, 1989; Kosugi e cols., 1989; Tatum e cols., 1990; Lepage e cols., 1991; Kikugawa e cols., 1992). Embora todos os métodos desenvolvidos sejam específicos para MDA, existem grandes diferenças entre os mesmos, principalmente no que diz respeito aos métodos de preparação das amostras, condições usadas para a reação com o TBA e técnicas utilizadas para isolamento e quantificação dos complexos TBA-MDA.

2.5. EROs e neurodegeneração

Trabalhos têm atribuído a perda celular associada às doenças neurodegenerativas à apoptose, um processo que está intimamente associado à sinalização celular envolvendo intermediários reativos de oxigênio (Beal, 1996; Mailly e cols., 1999). Estudos bioquímicos sugerem que as reações de oxidação podem ser importantes em patologias cerebrais (Ames e cols., 1993), e estão associadas com um desbalanço da regulação redox no sistema nervoso central (SNC) (Weber, 1999).

A neurodegeneração pode ocorrer como uma conseqüência da interação cooperativa de pelo menos três mecanismos neurotóxicos: o comprometimento metabólico, a excitotoxicidade e o estresse oxidativo (Alexi e cols., 1998; Kowaltowski e cols., 2001).

2.6. Isquemia e Intermediários metabólicos

2.6.1. Isquemia

Sabe-se que episódios isquêmicos são acompanhados de um aumento na geração de EROs (Cao e cols., 1988; Sakamoto e cols., 1991). Sob condições normais o sistema antioxidante endógeno (tanto enzimático quanto não-enzimático) é capaz de neutralizar as EROs produzidas durante o metabolismo basal. Porém, em situações patológicas, tais como durante os episódios isquêmicos esse sistema de defesa não é capaz de neutralizá-las completamente. Essa "ineficiência" do sistema antioxidante é devido tanto à superprodução

dessas EROs, quanto à inativação das enzimas antioxidantes (catalase - CAT e superóxido dismutase - SOD) e ao consumo dos antioxidantes não enzimáticos (vitaminas C e E) (Chan, 1996).

Demopoulos e colaboradores, em 1977, foram os primeiros a evidenciar o papel patogênico do processo de peroxidação lipídica induzido por EROs durante danos cerebrais isquêmicos (Demopoulos e cols., 1977). Desde então, muitos trabalhos (Haliwell, 1992; Blomgren e cols., 2003; Wang e Lo, 2003; Adibhatla e Hatcher, 2006; Gottlieb e cols., 2006; Gupta e Sharma, 2006) têm demonstrado o envolvimento dessas nos danos associados a essas condições.

Piantadosi e Zhang demonstraram que as EROs são geradas principalmente nas mitocôndrias dos tecidos injuriados (Piantadosi e Zhang, 1996). Desde então, tem-se demonstrado que existe uma correlação entre os danos isquêmicos e a disfunção mitocôndrial (Zoratti e Szabo, 1995). Além das mitocôndrias, acredita-se também que células inflamatórias, e também enzimas (i.e. xantina oxidase e ciclooxigenases) (Piantadosi e Zhang, 1996) possam estar envolvidas na geração de EROs durante episódios isquêmicos. Acredita-se, ainda, que o aumento na concentração extracelular de glutamato e aspartato possa contribuir para o aumento na geração de EROs durante os episódios isquêmicos (Yang e cols., 1996).

A acidose metabólica resultante desses períodos de isquemia também pode levar a um aumento na geração de EROs. Esse aumento na geração de EROs está relacionado principalmente a liberação do ferro de seus sítios de ligação (Siesjö e cols., 1985; Rehncrona e cols., 1989; Bralet e cols., 1992). Nesse contexto, Siesjö e colaboradores demonstraram que uma diminuição no pH do tecido cerebral (durante episódios de disfunção metabólica – tais como a isquemia) é acompanhado de um aumento na formação de espécies reativas ao ácido tiobarbitúrico (TBARS) e de dienos conjugados (Siesjö e cols., 1985).

Existem ainda evidências mostrando que a superatividade do sistema de transporte dos aminoácidos excitatórios e a estimulação excessiva dos receptores NMDA estão envolvidas na injuria neuronal isquêmica (Chiang e cols., 2006).

Os antioxidantes são capazes de amenizar os danos observados durante episódios isquêmicos, confirmando o envolvimento das EROs no processo degenerativo associado a

essas condições (Cao e cols., 1988; Liu e cols., 1989; Oliver e cols., 1990). Considerando o que foi exposto anteriormente acredita-se que intervenções bioquímicas e farmacológicas que tenham por objetivo diminuir ou mesmo prevenir a disfunção mitocondrial associada aos danos isquêmicos possa ser terapeuticamente viável para reduzir os danos associados a essas condições (Suleiman e cols., 2001). Nesse contexto, Sakamoto e colaboradores, demonstraram o papel protetor do sucinato em um modelo de isquemia/reperfusão cardíaca (Sakamoto e cols., 1998).

2.6.2. Nível dos Intermediários e Episódios Isquêmicos

Sabe-se que os episódios isquêmicos são acompanhados de uma diminuição no metabolismo energético (Gibson e cols., 1981). Goldberg e colaboradores demonstraram que os níveis dos intermediários do Ciclo de Krebs são alterados durante essas condições (Goldberg e cols., 1966). Estes autores também demonstraram que os níveis do citrato, α-cetoglutarato e oxaloacetato diminuíram de 15 a 60%, enquanto os níveis de sucinato e fumarato aumentaram 40%. Porém os dados encontrados na literarura são contraditórios. Alguns trabalhos mostram que os episódios isquêmicos são seguidos de uma aparente depleção nos níveis de glicose, oxaloacetato, ATP, fosfato de creatina, piruvato, citrato e α-cetoglutarato e de um acúmulo de frutose-1, 6-difosfato, lactato, sucinato, ADP e AMP (Folbergrová e cols., 1974; Hoyer e Krier, 1986). Contudo, Medvedeva e colaboradores mostraram que os níveis de citrato estão aumentados, enquanto o de sucinato diminuído após o período isquêmico (Medvedeva e cols., 2002).

Tendo em vista que os níveis dos intermediários metabólicos encontram-se alterados durante episódios os isquêmicos e também em situações de disfunção metabólica, estudos são necessários para determinar o efeito desses intermediários nessas situações, uma vez que essas são acompanhadas de um aumento acentuado na geração de EROs, bem como na liberação intracelular dos íons ferro.

3. OBJETIVOS

3.1. Objetivo Geral

Identificar os mecanismos envolvidos na atividade pró-oxidante *in vitro* do malonato, ácido quinolínico e oxalato. Além disso, investigar o efeito, bem como o(s) mecanismo(s) através do(s) qual(is) o citrato, sucinato, malato, oxaloacetato, fumarato e α-cetoglutarato (Intermediários do Ciclo de Krebs) exercem seus efeitos antioxidantes em diferentes sistemas pró- oxidantes *in vitro*.

3.2. Objetivos Específicos

- Identificar o(s) mecanismo(s) através do(s) qual(is) o malonato e o ácido quinolínico exercem sua atividade pró-oxidante *in vitro*;

- Averiguar o(s) mecanismo(s) envolvido na atividade pró-oxidante do oxalato em diferentes tecidos in vitro;

- Determinar o efeito do citrato, sucinato, malato, oxaloacetato, fumarato e α-cetoglutarato sobre a produção de TBARS basal, induzida por ferro(II), ácido quinolínico, ou malonato;

- Investigar o(s) mecanismo(s) através do(s) qual(is) esses intermediários exercem seus efeitos.

- Validar o método colorimétrico para a determinação de TBARS por meio de um estudo comparativo com análises de HPLC.

4. ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos, os quais encontram-se aqui organizados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos. Os **artigos 1, 2, 3** e **4** estão dispostos na forma que foram publicados na edição da revista científica.

O item 4 (Artigos Científicos) encontra-se sub-dividido em dois capítulos. No Capitulo I constam os artigos **1** e **2**, os quais já foram apresentados ao Programa de Pós Graduação em Bioquímica Toxicológica da Universidade Federal de Santa Maria, como minha dissertação de mestrado. Já no Capitulo II, encontram-se os artigos **3** e **4**, os quais representam a continuação de meu estudo, tendo em vista que migrei do mestrado para o doutorado.

4.1. ARTIGOS CIENTÍFICOS: CAPITULO I

CARACTERIZAÇÃO DA ATIVIDADE PRÓ-OXIDANTE *IN VITRO* DO MALONATO E DO ÁCIDO QUINOLÍNICO E ESTUDO DO POTENCIAL ANTIOXIDANTE DOS INTERMEDIÁRIOS DO CICLO DE KREBS SOBRE A GERAÇÃO DE TBARS INDUZIDAS POR Fe^{2+} OU ÁCIDO QUINOLÍNICO.

4.1.1. – Artigo 1 - N-METHYL-D-ASPARTATE RECEPTORS ARE INVOLVED IN THE QUINOLINIC ACID, BUT NOT IN THE MALONATE PRO-OXIDATIVE ACTIVITY IN VITRO

4.1.2. – Artigo 2 - KREBS CYCLE INTERMEDIATES MODULATE THIOBARBITURIC ACID REACTIVE SPECIES (TBARS) PRODUCTION IN RAT BRAIN IN VITRO

4.1.1. – CARACTERIZAÇÃO DA ATIVIDADE PRÓ-OXIDANTE *IN VITRO* DO MALONATO E DO ÁCIDO QUINOLÍNICO

Artigo 1

N-METHYL-D-ASPARTATE RECEPTORS ARE INVOLVED IN THE QUINOLINIC ACID, BUT NOT IN THE MALONATE PRO-OXIDATIVE ACTIVITY IN VITRO

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N-methyl-D-aspartate Receptors are Involved in the Quinolinic Acid, but not in the Malonate Pro-oxidative Activity *in vitro*

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Oxidative stress plays a significant role in the neurotoxicity of a variety of agents that interact with the *N*-methyl-D-aspartate (NMDA) receptors. Here we investigated in a comparative way the pro-oxidative effects of quinolinic acid (QA) and malonate, two neurotoxic substances that act through distinct primary molecular mechanisms on the production of thiobarbituric acid reactive species (TBARS) by brain homogenates. In fact, QA is thought to activate directly the NMDA receptor, whereas malonate seems to act primarily by inhibiting oxidative metabolism. The malonate-induced TBARS formation was not modified by cyanide (CN^-) or 2,4-dinitrophenol. MK-801 did not reduce basal or malonate induced-TBARS production in fresh tissues preparations. However, in heat-treated preparations a significant effect of MK-801 against basal TBARS production was observed, but not on the malonate induced-TBARS production. QA induced-TBARS production was significantly prevented by MK-801 either in fresh or heat-treated preparations. The antioxidant effect of MK-801 on basal and QA-induced TBARS production increased as the temperatures used to treat S1 were increased. Succinate dehydrogenase (SDH) was inhibited by malonate but not by QA. Malonate was able to chelate iron(II) and the malonate-iron complex(es) is(are) active as measured by its(their) activity on deoxyribose degradation assay. These findings indicate that direct interactions of malonate with NMDA receptors are not involved in malonate pro-oxidative activity *in vitro*. QA pro-oxidative activity *in vitro* was related, at least in part, to its capability in stimulate NMDA receptors. Taken together, these findings indicated that malonate pro-oxidative activity *in vitro* could be attributed to its capability of changing the ratio Fe^{2+}/Fe^{3+}, which is essential to TBARS production.

KEY WORDS: Iron; malonate; NMDA receptors; pro-oxidant; quinolinic acid.

INTRODUCTION

Malonate is a reversible inhibitor of mitochondrial succinate dehydrogenase (SDH) and, consequently can induce mitochondrial dysfunction. In fact, malonate can trigger the generation of superoxide radicals, secondary excitotoxicity mediated by Ca^{2+} influx, and apoptosis (1). Several earlier studies have demonstrated that the neurotoxicity associated with intrastriatal injection of malonate is mediated almost exclusively by the indirect activation of *N*-methyl-D-aspartate (NMDA) receptors (2–5). The mechanism underlying malonate induced-neurotoxicity seems to involve ATP exhaustion, which causes depolarization. However, although MK-801 reduced the malonate-induced lesion volume in the striatum,

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it did not block the generation of reactive oxygen species (ROS), indicating that NMDA receptor activation occurs but does not result in the generation of ROS in malonate toxicity (6, 7). In contrast with this, other studies have shown that NMDA receptors activation could be involved in the observed increased in ROS levels (8, 9), thus contributing to oxidative stress due to malonate.

In the rat, acute (2, 4, 5, 10) and chronic (11) intrastriatal administration of malonate produces dose-dependent lesions with features similar to those produces by quinolinic acid (QA), an agonist of NMDA receptor (12). In line with this, malonate exacerbates the neurotoxicity of glutamate and NMDA (10, 13). Thus, although some discrepancies still exist in literature about the involvement of ROS generation via NMDA activation on the neurotoxicity of malonate, a consensus has emerged in the literature connecting glutamate receptor activation with neuronal damage due to malonate (2, 13).

The mechanism underlying QA induced-neurotoxicity seems to involve direct activation of the NMDA receptor, either by pre-synaptically stimulating the release of the excitatory amino acid transmitter or by direct post-synaptic action, which in turn produces excitotoxicity (8, 14). In fact, it has been proposed that over stimulation of excitatory amino acid receptors is involved in the pathophysiology of acute brain injury and chronic neurodegenerative disease (15, 16) that can be associated, at least in part, with the over production of reactive oxygen and nitrogen species caused by glutamate, NMDA or QA (17–20).

Taking into account that *in vivo* and *in vitro* QA can exacerbate considerably the production of ROS and lipid peroxidation (17, 21, 22) and that lipid peroxidation is initiated by the attack of free radicals against unsaturated membrane lipids, it is reasonable assume that QA toxicity is mediated by ROS formed after NMDA receptors activation (8, 9). In addition, QA-induced lipid peroxidation is blocked by MK-801 and a variety of antioxidants (9, 22–28) giving further support for NMDA receptor activation and ROS overproduction on its toxicity. However, there are still controversies in the literature on whether NMDA receptors activation and/or ROS are involved in the neuronal cell death caused by malonate (6, 13).

Therefore, due to inconsistent data reported in the literature with respect to the involvement of NMDA receptors activation in the malonate neurotoxicity and ROS overproduction (6, 13, 29), the aim of this study was to investigate whether NMDA receptors are involved in the malonate and QA pro-oxidative activity measured *in vitro* by their ability in stimulating brain lipid peroxidation. In the present study, we used MK-801, to investigate the NMDA receptor involvement in the toxicity of these neurotoxic agents. Furthermore, the possible involvement of respiratory chain reactions on the pro-oxidative activity of these compounds was investigated by inactivating brain homogenates with heating.

MATERIAL AND METHODS

Tissue Preparation

Adult rats (inbreed Wistar strain) from our own breeding colony were maintained in an air-conditioned room (20–25°C) under natural lighting conditions (the luminosity of the colony room was determined by the environmental situation), with water and food (Guabi, Ribeirão Preto, SP, Brazil) *ad libitum*. Animals were anesthetized with ether and killed by decapitation. The brain was quickly removed, placed on ice, and homogenized within 10 min, in 10 volumes of cold 100 mmol/l NaCl. The homogenate was centrifuged at 4000 × *g* at 4°C for 10 min to yield a low speed supernatant fraction (S1) that was used immediately for thiobarbituric acid reactive species (TBARS) assay.

Lipid Peroxidation Assay

Lipid peroxidation was determined by measuring TBARS as described by Ohkawa et al. (30). Brain homogenates were prepared by homogenization as described above. Aliquots of the homogenate (100 µl) from brain were incubated at 37°C in a water bath for 60 min in a medium containing 10 mM Tris/HCl buffer, pH 7.4 in the presence of other reagents at concentrations indicated in each figure legend. The reaction was stopped by 0.5 ml of acetic acid buffer and lipid peroxidation products were measured by the addition of 0.5 ml of TBA 0.6% and 0.2 ml of SDS 8.1%. The color reaction was developed by incubating tubes in boiling water for 60 min. TBARS levels were measured at 532 nm using a standard curve of MDA. The values are expressed in (µmol MDA/g of tissue. Were indicated, the S1 fraction was pre-treated for 10 min at indicated temperature before the incubation at 37°C for 60 min.

SDH Activity Assay

SDH activity was estimated as described by (31). The reaction mixture containing 50 mmol/l potassium phosphate buffer (pH 7.4), 0.5 mmol/l succinate (pH 7.2), 10 mmol/l sodium azide, and 0.8 mmol/l INT was warmed for 1 min in a water bath at 37°C. Then 100 µl of the tissue (S1) was added to initiate the reaction. The total volume of the reaction mixture was 0.5 ml. After incubation for 10 min, 1.5 ml of 95% alcohol was added. After 15 min on ice, tubes were centrifuged for 10 min at 800 × *g* at room temperature. The absorbance of 1 ml of clear alcohol-H_2O extract was read at 458 nm (formazan formatted) in a spectrophotometer, the zero-reference cuvette contained H_2O. Basal reduction of INT was determined in control tubes in which

succinate was omitted from the reaction mixture, and this value was subtracted from the absorbance that was measured for the experimental tube. Where indicated, S1 was pre-treated at 100°C for 10 min and, after SDH activity was assayed.

Chelating Properties of the Malonate Assay

To examine iron chelating properties of malonate, we used the *o*-phenantroline method as previously described (32, 33). Iron (II) was added to the buffered medium containing 25 mmol/l NaCl and 10 mmol/l Tris/HCl buffer, the mixture containing Fe^{2+} 150 mol/l and malonate at indicated concentrations was allowed approximately by 5 min to complex(es) formation between iron(II) and malonate, than *o*-phenantroline solution was added to determine the presence of the colored complex(es) formed between *o*-phenantroline and free iron (II). A 0.25% *o*-phenantroline solution in ethanol was used in the analyses. Absorbance values were proportional to the amount of free Fe^{2+}; therefore a decrease in absorbance indicates that the malonate exhibited chelating properties.

The absorbance was recorded at 510 nm. The values are expressed in percentage of control determined in the absence of malonate. Solutions of $FeSO_4$ were made just before use in distilled water.

Degradation of Deoxyribose Assay

Reactions mixtures contained, in a final volume of 0.8 ml, the following reagents at the final concentrations stated: deoxyribose 3 mmol/l, KH_2PO_4 buffer 50 mmol/l, pH 7.4, $FeSO_4$ 50 µmol/l and H_2O_2 500 µmol/l. Solutions of $FeSO_4$ and H_2O_2 were made just before use in distilled water. Reaction mixtures were incubated at 37°C for 30 min and stopped by the addition of 0.8 ml of TCA 2.8%, followed by the addition of 0.4 ml of TBA 0.6% solution. Tubes were incubated in boiling water for 20 min and then the absorbance was recorded at 532 (34, 35). Standard curves of MDA were made in each experiment. The values are expressed in (µmol MDA/l.

Statistical Analysis

Data were analyzed by one-way ANOVA, followed by Tukey Multiple Range Test when appropriate. Differences between groups were considered to be significant when $P \leq 0.05$.

RESULTS

Effect of Malonate on Basal-TBARS Production

Malonate induced a concentration dependent increase in the TBARS production (Fig. 1), which was significant from 1 mmol/l onwards. The malonate effect was not modified by the addition of potassium cyanide (KCN) 1 mmol/l (Fig. 1). Comparisons between groups of the curve obtained in the absence of KCN with that of groups of the curve obtained in the presence of KCN were done by one-way ANOVA followed by Tukey multiple range test.

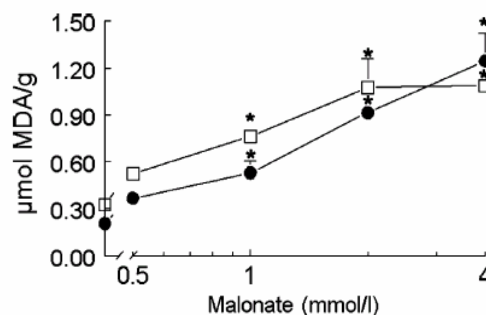

Fig. 1. Effect of malonate on basal-TBARS production. Low-speed supernatant (S1) from brain was incubated for 60 min in a medium containing 10 mmol/l Tris/HCl buffer, pH 7.4, in the presence of the indicated concentrations of malonate. TBARS are expressed as (µmol of MDA per g of tissue. —●— no addition, —□— KCN 1 mmol/l. The determinations were done in the absence of any other Krebs cycle substrate. Data are expressed as means ± SEM ($n = 3$). One-way ANOVA followed by Tukey multiple range test revealed no significant differences between the groups in the curve with cyanide, when compared to the groups in the curve without cyanide. *Indicate a $P < 0.05$ from respective control by Tukey multiple range test.

Statistical analysis revealed no significant difference between KCN groups and non-cyanide groups. 2,4 dinitrophenol (0.1–10 µmol/l) did not modify basal or malonate (4 mmol/l) induced-TBARS production (data not shown).

Effect of Succinate on Basal or Malonate–induced TBARS Production

Succinate (8 mmol/l), a substrate of SDH, attenuated the malonate (4 mmol/l) induced increase in TBARS production (Fig. 2). However, succinate did not revert malonate-induced TBARS to basal values and succinate reduced significantly the basal-TBARS production.

Effect of MK-801 on Basal, Malonate or QA-induced TBARS Production

In order to investigate the involvement of NMDA receptors in the malonate pro-oxidative activity, we measured malonate induced-TBARS production in the presence or absence of the MK-801 in fresh- and heat-treated brain S1 (at 100°C for 10 min). Malonate (4 mmol/l) caused a marked increase in TBARS production either in fresh (444.7% compared to fresh basal values) or heat-treated (197.5% compared to heat-treated basal values) preparations (Table I). These results support the conclusion that malonate effect was at least in part

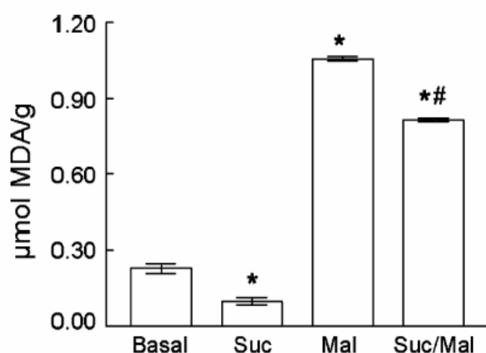

Fig. 2. Effect of succinate on basal or malonate–induced TBARS production**.** Antioxidant effect of succinate against basal or malonate induced TBARS production. The values are expressed as µmol of MDA per gram of tissue. Abbreviations: Suc: succinate (8 mmol/l); Mal: malonate (4 mmol/l). Data are expressed as means ± SEM ($n = 3$). $^{*}P < 0.05$ from basal and $^{\#}P < 0.05$ from malonate by Tukey multiple range test.

independent of respiratory chain reactions because this pre-treatment is expected to cause complete enzymatic inactivation. Accordingly, SDH an enzyme of Krebs cycle and component of respiratory chain was completely inactivated by this treatment (Fig. 3a). Furthermore, we also assayed lactate dehydrogenase of 100°C-treated S1 and its activity was completely inhibited by heat treatment (data not shown). The addition of MK-801 (1 mmol/l) was ineffective in prevent basal or malonate induced-TBARS production in fresh preparations (Table I). However, when we used heat-treated preparations, MK-801 significantly prevented basal TBARS production without abolishing malonate-induced TBARS production (Table I). These results suggested that NMDA receptors activation is not involved in the malonate induced TBARS production, and that oxidative stress due to malonate is not a downstream consequence of a secondary excitotoxicity in our *in vitro* conditions.

QA (1 mmol/l) caused a significantly increase in TBARS production either in fresh (62.5% compared to basal values) or heat-treated (35.4% compared to basal values) preparations (Table II). MK-801 (1 mmol/l) was ineffective against basal TBARS production in fresh preparations; however, exhibited a significant effect against basal-TBARS production in heat-treated preparations (Table II). MK-801 significantly reduced QA-induced TBARS production, either in fresh or heat-treated preparations (Table II). These results confirm the involvement of NMDA receptors activation in the QA pro-oxidative activity *in vitro*.

Effect of MK-801 on Basal or QA-induced TBARS Production in S1 Pre-treated at Different Temperatures

Exposure of S1 for 10 min to increasing temperatures caused an increase in basal-TBARS production (Table III) and a proportional decrease of QA-induced TBARS production. Note that at higher temperatures the QA-induced TBARS production was lower than that observed at lower temperatures (compare the 22.9% increase at 100°C with 41.3% at lower temperatures).

However, in the S1 treated at 100°C the reduction on basal TBARS caused by MK-801 was proportionally higher (56.1% of reduction) than that observed in S1 preparations pre-treated at lower temperatures (approximately 20%) (Table III). Similarly, MK-801 antagonizes more efficiently the QA-induced TBARS production in S1 pre-treated at 100°C than in those S1 preparations pre-treated at lower temperatures. After 10 min of pre-treatment of S1 at 100°C, the TBARS production in the presence of MK-801 plus QA was even lower than that of basal value (Table III).

Effect of Malonate and QA on SDH Activity

Malonate cause a significant inhibition of brain SDH (Fig. 3a) and the inhibition was significant from 0.1 mmol/l onwards.

QA has two carboxylic groups separated apart by about 0.2–0.4 nm, which is somewhat similar to

Table I. Effect of MK-801 on Basal or Malonate Induced-TBARS Production Either in Fresh or Brain S1 Preparations Treated at 100°C for 10 min

	Fresh	Heat-treated
Basal	212.33 ± 2.91 (100%)	499.47 ± 5.01[a](100%)
Malonate	1156.72 ± 196.44* (544%)	1486.05 ± 164.82* (297%)
MK-801	201.43 ± 2.15 (94%)	265.56 ± 52.32* (53%)
Malonate + MK-801	1117.81 ± 185.32* (526%)	1436.73 ± 213.89* (287%)

Data are expressed as mean ± S. E. ($n = 3$). Values are expressed as nmol MDA/g.
*From respective basal; [a]Different of respective control fresh by one way ANOVA following by Tukey test.

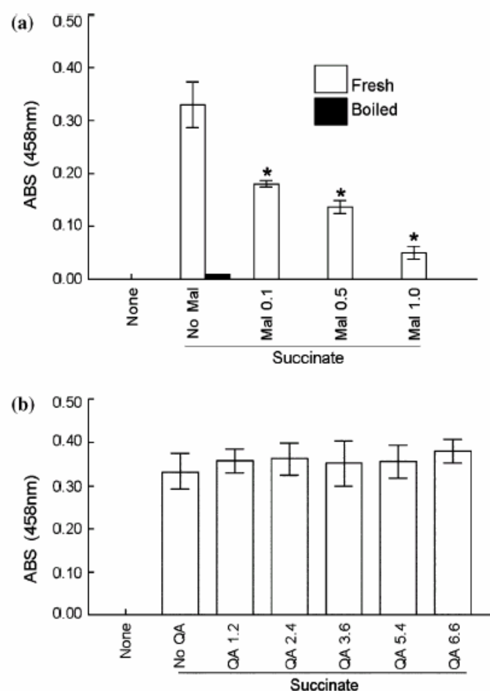

Fig. 3. Malonate and QA effect on the SDH activity. (a) Effect of malonate at indicated concentrations (mmol/l) on SDH activity. Values represent the difference between ABS (458 nm) without succinate and ABS with succinate (0.5 mmol/l). None: without succinate. Were indicated, S1 was pre-treated for 10 min at 100°C before SDH activity assay. Data are expressed as means ± SEM (n = 3). *P < 0.05 from control without malonate by Tukey multiple range test. (b) Effect of QA at indicated concentrations (mmol/l) on SDH activity. Values represent the difference between ABS (458 nm) without succinate and ABS with succinate (0.5 mmol/l). None: without succinate. Data are expressed as means ± SEM (n = 4).

that for other dicarboxylic acids, such as malonate, that are inhibitors of SDH. So, we tested whether QA could also inhibit SDH. However, QA (up to 6.6 mmol/l) did not inhibit SDH (Fig. 3b). Pretreatment of S1 by heating at 100°C for 10 min completely inhibited SDH activity (Fig. 3a).

Table II. Effect of MK-801 on Basal or QA Induced-TBARS Production either in Fresh or Brain S1 Preparations Treated at 100°C for 10 min

	Fresh	Heat-treated
Basal	215.63 ± 2.67 (100%)	425.37 ± 20.29[a] (100%)
QA	350.43 ± 4.94* (162%)	575.95 ± 23.76*[a] (135%)
MK-801	170.81 ± 4.94 (79%)	196.12 ± 19.65* (46%)
QA+MK-801	274.06 ± 7.86*[b] (127%)	418.01 ± 27.65 [ab] (98%)

Data are expressed as mean ± S. E. (n = 3). Values are expressed as nmol MDA/g.
*From respective basal; [a]Different of respective control fresh; [b]Different of respective QA by one way ANOVA following by Tukey test.

Chelating Properties of Malonate

Literature data indicates that iron ions (particularly the ratio Fe^{2+}/Fe^{3+}) are an important factor that regulates lipid peroxidation *in vitro* (36, 37). Furthermore, the formation of complexes of iron with negative charged compounds can enhance the ability of iron to produce oxidative stress, possibly by changing the ratio Fe^{2+}/Fe^{3+}. So, we investigate whether malonate could chelate iron (Fig. 4) and whether the complex(es) formed with Fe^{2+} could enhance its pro-oxidative activities by measuring deoxyribose degradation (Fig. 5), a well-established model for studying iron driven Fenton reaction (34, 35). Malonate chelated Fe(II) in a concentration-dependent manner and this was significant from 4 mmol/l onwards (Fig. 4). The maximum reduction in free Fe^{+2} in the medium reaction (65%) was obtained at 32 mmol/l of malonate.

Effect of Malonate on Deoxyribose Degradation

Fe(II) or H_2O_2 stimulated deoxyribose degradation. However, in the presence of iron and H_2O_2 deoxyribose degradation was higher than that determined in the presence of compounds separately. Malonate did not modify the stimulatory effect of iron on deoxyribose degradation.

DISCUSSION

The results presented in this paper suggest that the pro-oxidative activity of malonate *in vitro* was at least in part independent of mitochondrial respiratory chain reaction(s), because heat treatment (which inactivated completely SDH and lactate dehydrogenase activity) did not abolish malonate effect. Furthermore, KCN and 2,4-dinitrophenol did not change malonate-induced TBARS production (data not shown).

In contrast to the malonate effect, the protective effect of succinate (Fig. 2) against either basal or malonate-induced TBARS production supports a role for SDH activity on antioxidant properties of succinate. However, the succinate-induced decreases in the basal and malonate groups were of similar magnitude. These suggest that the entire decline was due to a decline in the basal TBARS production. We hypothesized that succinate antioxidant activity under our conditions could be attributed at least in part to ubiquinol (38). In line with this, ubiquinol has

Table III. Effect of MK-801 on Basal or QA-Induced TBARS Production in S1 Treated at different Temperatures for 10 min

	0°C	25°C	37°C	50°C	100°C
Basal	214.96 ± 3.61 (100%)	222.85 ± 6.83 (100%)	217.77 ± 7.19 (100%)	300.32 ± 10.39 (100%)	409.75 ± 14.95 (100%)
QA	303.63 ± 4.55* (141%)	323.47 ± 7.93* (145%)	329.25 ± 17.30* (151%)	387.96 ± 5.49* (129%)	503.62 ± 32.48* (122%)
MK-801	173.67 ± 2.80 (80%)	185.16 ± 8.57 (83%)	182.45 ± 7.83 (83%)	243.23 ± 10.15* (80%)	180.08 ± 16.83* (43%)
QA + MK-801	266.76 ± 10.21*[a] (124%)	271.17 ± 13.05*[a](121%)	275.26 ± 16.03 (126%)	318.42 ± 11.33[a] (106%)	330.99 ± 45.12[a] (80%)

Data are expressed as mean ± S. E. ($n = 4$). Values are expressed as nmol MDA/g.
* from respective basal; [a] different of respective QA by Tukey test.

potent antioxidant activity in biological systems (39, 40).

The absence of a protective effect of MK-801 against malonate pro-oxidative activity may indicate that secondary excitotoxicity is not involved in the effect of malonate in brain homogenates. These results are in accordance with previous study showing that NMDA receptor over-stimulation does not contribute significantly to the oxidative stress in cultured cells exposed to malonate (7). The malonate-induced TBARS production under conditions in which biological activity has been inactivated by heat treatment were unexpected. In fact, one could expect that heat treatment could cause non-specific increase in TBARS production by accelerating the oxidation of polyunsaturated fatty acids from brain lipids. However, the increase was specific for some treatments (for instance, malonate and QA) and reversed by MK-801. The effect of malonate even when all of the enzymes have been inactivated suggests that malonate pro-oxidative activity can not be attribute exclusively to inhibition of SDH and possibly is related to its iron-chelating properties. In fact, heat-treatment could increase the release of iron from storage proteins, accelerating the ROS generation, and consequently TBARS production.

Based on iron-chelating malonate properties, we hypothesized that malonate pro-oxidative activity *in vitro* can be attributed to its interaction with endogenous free iron present in the medium of preparation. These complexes, such as iron-citrate (41), are active in our system, thus increasing free radicals generations due to redox cycle of iron. However, malonate did not change deoxyribose degradation caused by iron and H_2O_2, indicating that the iron-malonate complexes are active when a pure chemical system was used.

Our results confirm the hypothesis that QA pro-oxidative activity is mediated by a direct activation of NMDA receptors (8, 9), because MK-801 significantly prevented QA-induced TBARS production. How QA can work in heat-treated samples is not clear. It would appear that the heat treatment did not inactivate the glutamate receptors. The data obtained with heat-treated preparations indicate that heat-treatment activate NMDA receptor. In line with this,

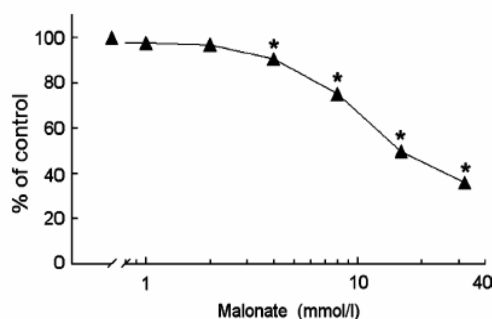

Fig. 4. Chelating property of malonate. Effect of malonate on colored iron-phenantroline complex formation. The values are expressed as percentage of control. Absorbance obtained by reaction between free Fe^{2+} with *o*-phenantroline in the absence of malonate is considered 100%. Data are expressed as means ± SEM ($n = 3$). *$P < 0.05$ from respective control by Tukey multiple range test.

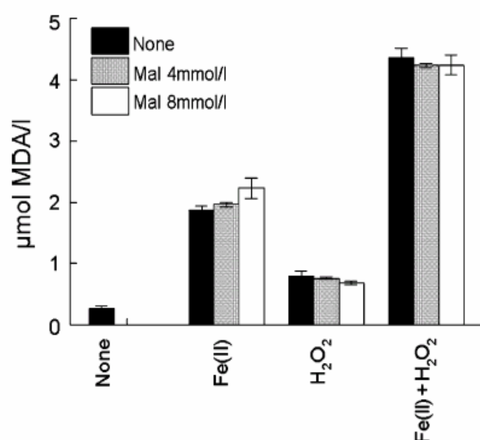

Fig. 5. Effect of malonate on deoxyribose degradation. Effect of malonate against deoxyribose degradation induced by Fe^{2+} 50 µmol/l and/or hydrogen peroxide 500 µmol/l. The values are expressed as (µmol MDA per liter. Data are expressed as means ± SEM ($n = 3$).

MK-801 was more efficient against either basal or QA-induced TBARS production when samples were exposed to increasing temperatures in comparison to fresh samples (see Table III). However, we did not exclude the potential intrinsic oxidative properties of QA (17), which can be related, at least in part with iron ions (42). These could partially explain how QA work in heat-treated samples. In spite of this, the protection afforded by MK-801 strongly suggests that part of its effect is mediated by NMDA receptor activation. These results are coincident with a previous study showing that the probability of the NMDA channel opening increases steeply with temperature up to near 50°C (43). To the best of our knowledge, there are no data on the literature about the effects of higher temperatures on NMDA activation. Furthermore, *in vivo* studies have demonstrated that hyperthermia aggravates seizures episodes, increasing brain damage mediated in part by NMDA receptors activation (44, 45). This result indicated that probably heat-treatment alters conformational structure of NMDA receptors leading to a permanent activation of NMDA receptor, which is prevented by MK-801. In line with these, the proportional decrease in the QA-induced TBARS production with temperature can be attributed to direct over activation of NMDA receptors by temperature.

In conclusion, our data suggest that malonate pro-oxidant activity *in vitro* is due to its ability to interact with endogenous iron, forming active complexes, thus contributing to maintenance of adequate ratio Fe^{2+}/Fe^{3+}. In addition, the results presented in this paper confirm the involvement NMDA receptors activation in the QA pro-oxidant activity *in vitro*. However, we cannot exclude that iron also modulates QA pro-oxidative activity *in vitro* by changing the ratio between Fe^{2+}/Fe^{3+} (42). In short, the effect of higher temperatures can involve an increase in the activation of NMDA receptor, inhibition of respiratory chain (ubiquinol formation), and release of iron from iron-binding proteins, whereas the pro-oxidative effect of QA involves NMDA receptor activation and the malonate pro-oxidative effect is mediated by a different mechanism that does not involves NMDA receptor.

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REFERENCES

1. Dedeoglu, A., Ferrante, R. J., Andreassen, O. A., Dillmann, W. H., and Beal, M. F. 2002. Mice overexpressing 70-kda heat shock protein show increased resistance to malonate and 3-nitropropionic acid. Exp. Neurol. 176:262–265.
2. Greene, J. G., Porter, R. H. P., Eller, R. V., and Greenamyre, J. T. 1993. Inhibition of succinate dehydrogenase by malonic acid produces an excitotoxic lesion in rat striatum. J. Neurochem. 61:1151–1154.
3. Greene, J. G., and Greenamyre, J. T. 1995. Characterization of the excitotoxic potential of the reversible succinate dehydrogenase inhibitor malonate. J. Neurochem. 64:430–436.
4. Beal, M. F., Brouillet, E., Jenkins, B., Henshaw, R., Rosen, B., and Hyman, B. T. 1993. Age-dependent striatal excitotoxic lesions produced by the endogenous mitochondrial inhibitor malonate. J. Neurochem. 61:1147–1150.
5. Henshaw, R., Jenkins, B. G., Schulz, J. B., Ferrante, R. J., Kowall, N. W., Rosen, B. R., and Beal, M. F. 1994. Malonate produces striatal lesions by indirect NMDA receptor activation. Brain Res. 647:161–166.
6. Ferger, B., Eberhardt, O., Teismann, P., Groote, C., and Schulz, J. B. 1999. Malonate-induced generation of reactive oxygen species in rat striatum depends on dopamine release but not on NMDA receptor activation. J. Neurochem. 73:1329–1332.
7. Zeevalk, G. D., Bernard, L. P., and Nicklas, W. J. 2000. Oxidative stress during energy impairment in mesencephalic cultures is not a downstream consequence of a secondary excitotoxicity. Neuroscience. 96:309–316.
8. Santamaría, A., and Ríos, C. 1993. MK-801, an *N*-methyl-D-aspartate receptor antagonist, blocks quinolinic acid-induced lipid peroxidation in rat corpus striatum. Neurosci. Lett. 159:51–54.
9. Rodríguez-Martínez, E., Camacho, A., Maldonado, P. D., Pedraza-Chaverrí, J., Santamaría, D., Galván-Arzate, S., and Santamaría, A. 2000. Effect of quinolinic acid on endogenous antioxidants in rat corpus striatum. Brain Res. 858:436–439.
10. Greene, J. G., and Greenamyre, J. T. 1995. Exacerbation of NMDA, AMPA and L-Glutamate excitotoxicity by the succinate dehydrogenase inhibitor malonate. J. Neurochem. 64:2332–2338.
11. Brazzett, T. J., Falik, R. C., Becker, J. B., and Albin, R. L. 1995. Chronic administration of malonic acid produces selective neural degeneration and transient changes in calbindin immunoreactivity in rat striatum. Exp. Neurol. 134:1–10.
12. Bazzett, J. T., Falik, R. C., Becker, J. B., and Albin, R. L. 1996. Synergistics effects of chronic exposure to subthreshold concentrations of quinolinic acid and malonate in the rat striatum. Brain Res. 718:228–232.
13. Kanki, R., Nakamizo, T., Yamashita, H., Kihara, T., Sawada, H., Uemura, K., Kawamata, J., Shibasaki, H., Akaike, A., and Shimohama, S. 2004. Effects of mitochondrial dysfunction on glutamate receptor-mediated neurotoxicity in cultured rat spinal motor neurons. Brain Res. 1015:73–81.
14. Tsuzuki, K., Lino, M., and Ozawa, S. 1989. Change in calcium permeability caused by quinolinic acid in cultured rat hippocampal neurons. Neurosci. Lett. 105:269–274.
15. Cabrera, J., Reiter, R. J., Tan, D., Qi, W., Sainz, R. M., Mayo, J. C., Garcia, J. J., Kim, S. J., and El-Sokkary, G. 2000. Mel-

atonin reduces oxidative neurotoxicity due to quinolinic acid:*In vitro* and *in vivo* findings. Neuropharmacology. 39:507–514.

16. Porciúncula, L. O., Rocha, J. B. T., Boeck, C. R., Vendite, D., and Souza, D. O. 2001. Ebselen prevents excitotoxicity provoked by glutamate in rat cerebellar granule neurons. Neurosci. Lett. 299:217–220.
17. Santamaría, A., Jimenez-Capdeville, M. E., Camacho, A., Rodriguez-Martinez, E., Flores, A., and Galvan-Arzate, S. 2001. *In vivo* hydroxyl radical formation after quinolinic acid infusion into rat corpus striatum. Neuroreport. 12:2693–2696.
18. Monje, M. L., Chatten-Brown, J., Hye, S. E., and Raley-Susman, K. M. 2000. Free radicals are involved in the damage to protein synthesis after anoxia/aglycemia and NMDA exposure. Brain Res. 857:172–182.
19. Gunasekar, P. G., Kanthasamy, A. G., Borowitz, J. L., and Isom, G. E. 1995. NMDA receptor activation produces concurrent generation of nitric oxide and reactive oxygen species: implication for cell death. J Neurochem. 65:2016–2021.
20. Hayashi, Y., Ueda, Y., Nakajima, A., Yokoyama, H., Mitsuyama, Y., Ohya-Nishiguchi, H., and Kamada, H. 2002. Nitric oxide and hydroxyl radicals initiate lipid peroxidation by NMDA receptor activation. Brain Res. 941:107–112.
21. Pérez-Severiano, F., Rodríguez-Pérez, M., Pedraza-Chaverrí, J., Maldonado, P. D., Medina-Campos, O. N., Ortíz-Plata, A., Sánchez-García, A., Villeda-Hernández, J., Galván-Arzate, S., Aguilera, P., and Santamaría, A. 2004. S-Allylcysteine, a garlic-derived antioxidant, ameliorates quinolinic acid-induced neurotoxicity and oxidative damage in rats. Neurochem. Int. 45:1175–1183.
22. Rossato, J. I., Ketzer, L. A., Centurião, F. B., Silva, S. J. N., Ludtke, D. S., Zeni, G., Braga, A. L., Rubin, M. A., and Rocha, J. B. T. 2002. Antioxidant properties of new chalcogenides against lipid peroxidation in rat brain. Neurochem Res. 27:297–303.
23. Rossato, J. I., Zeni, G., Mello, C. F., Rubin, M. A., and Rocha, J. B. T. 2002. Ebselen blocks the quinolinic acid-induced production of thiobarbituric acid reactive species but does not prevent the behavioral alterations produced by intra-striatal quinolinic acid administration in the rat. Neurosci. Lett. 318:137–140.
24. Santamaría, A., Santamaria, D., Diaz-Munoz, M., Espinoza-Gonzalez, V., and Ríos, C. 1997. Effects of *N* Omega-nitro-L-arginine and L-arginine on quinolinic acid- induced lipid peroxidation. Toxicol. Lett. 93:117–124.
25. Santamaría, A., Salvatierra-Sanchez, R., Vazquez-Roman, B., Santiago-Lopez, D., Villeda-Hernandez, J., Galvan-Arzates, S., Jiménez-Capdeville, M. E., and Ali, S. F. 2003. Protective effects of the antioxidant selenium on quinolinic acid-induced neurotoxicity in rats: *in vitro* and *in vivo* studies. J. Neurochem. 86:479–488.
26. Ríos, C., and Santamaría, A. 1991. Quinolinic acid is a potent lipid peroxidant in rat brain homogenates. Neurochem. Res. 16:1139–1141.
27. Medvedeva, L. V., Popova, T. N., Artyukhov, V. G., Matasova, L. V., and Akatova, R. V. 2002. Oxidative status and distribution of NADP-dependent isocitrate dehydrogenase and aconitate hydratase in rat cardiomyocytes under normal conditions and during ischemia. Bul. Exp. Biol. Med. 134:130–134.
28. Bellé, N. A. V., Dalmolin, G. D., Fonini, G., Rubim, M. A., and Rocha, J. B. T. 2004. Polyamines reduces lipid peroxidation induced by different pro-oxidant agents. Brain Res. 1008:245–251.
29. Connop, B. P., Boegman, R. J., Beninger, R. J., and Jhamandas, K. 1997. Malonate-induced degeneration of basal forebrain cholinergic neurons: Attenuation by lamotrigine, MK-801, and 7-nitroindazole. J. Neurochem. 68:1191–1199.
30. Ohkawa, H., Ohishi, N., and Yagi, K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95:351–358.
31. Green, J. D., and Narahara, H. T. 1980. Assay of succinate dehydrogenase activity by the tetrazolium method: evaluation of an improved technique in skeletal muscle fractions. J. Histochem. Cytochem. 28:408–412.
32. Minotti, G., and Aust, S. D. 1987. An investigation into the mechanism of citrate-Fe^{2+}-dependent lipid peroxidation. Free Rad. Biol. Med. 3:379–387.
33. Bucber, J. R., Tien, M., Morehouse, L. A., and Aust, S. D. 1983. Redox cycling and lipid peroxidation: The central role of iron chelates. Fundam. Appl. Toxicol. 3:222–226.
34. Halliwell, B., and Gutteridge, J. M. C. 1981. Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts: The role of superoxide and hydroxyl radicals. FEBS Lett. 128:347–352.
35. Gutteridge, J. M. C. 1981. Thiobarbituric acid-reactivity following iron-dependent free-radical damage to amino acids and carbohydrates . FEBS Lett. 128:343–346.
36. Caro, A. A., and Cederbaum, A. T. 2004. Antioxidant properties of *S*-adenosyl-L-methionine in Fe^{2+}- initiated oxidations. Free Radic. Biol. Med. 36:1303–1316.
37. Tang, L. X., Yang, J. L., and Shen, X. 1997. Effects of additional iron-chelators on Fe^{2+}- initiated lipid peroxidation: evidence to support the Fe^{2+}(Fe^{3+} complex as the initiator. J. Inorg. Biochem. 68:265–272.
38. Puntel, R. L., Nogueira, C. W., and Rocha, J. B. T. 2005. Krebs cycle intermediates modulate thiobarbituric acid reactive species (TBARS) production in rat brain *in vitro*. Neurochem. Res. 30:225–235.
39. Bindoli, A., Cavallini, L., and Jocelyn, P. 1982. Mitochondrial lipid peroxidation by cumene hydroperoxide and its prevention by succinate. Biochim. Biophys. Acta. 681:496–503.
40. Cavallini, L., Valente, M., and Bindoli, A. 1984. Comparison of cumene hydroperoxide – and NADPH/ Fe^{3+}/ADP-induced lipid peroxidation in heart and liver submitochondrial particles. Mechanisms of protection by succinate. Biochim. Biophys. Acta. 795:466–472.
41. Chiueh, C. C. 2001. Iron overload, oxidative stress, and axonal dystrophy in brain disorders. Pediatr. Neurol. 25:138–147.
42. Stípek, S., Stastný, F., Pláteník, J., Crkovská, J., and Zima, T. 1997. The effect of quinolinate on rat brain lipid peroxidation is dependent on iron. Neurochem. Int. 30:233–237.
43. Chung, S. H., and Kuyucak, S. 1995. Changes in the kinetics and conductance of *N*-methyl-D-aspartate (NMDA)-receptor activated single channels with temperature. Neurosci. Lett. 187:181–184.
44. Morimoto, T., Kida, K., Nagao, H., Yoshida, K., Fukuda, M., and Takashima, S. 1995. The pathogenic role of the NMDA receptor in hyperthermia-induced seizures in developing rats. Dev. Brain Res. 84:204–207.
45. Lundgren, J., Blennow, G., and Siesjo, B. K. 1994. Hyperthermia aggravates and hypothermia ameliorates epileptic brain-damage. Exp. Brain Res. 99:43–55.

4.1.2. – ESTUDO DO POTENCIAL ANTIOXIDANTE DOS INTERMEDIÁRIOS DO CICLO DE KREBS SOBRE A GERAÇÃO DE TBARS INDUZIDAS Fe^{2+} OU ÁCIDO QUINOLÍNICO

Artigo 2

KREBS CYCLE INTERMEDIATES MODULATE THIOBARBITURIC ACID REACTIVE SPECIES (TBARS) PRODUCTION IN RAT BRAIN IN VITRO

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Krebs Cycle Intermediates Modulate Thiobarbituric Acid Reactive Species (TBARS) Production in Rat Brain *In Vitro*

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(Accepted December 15, 2004)

The aim of this study was to investigate the effect of Krebs cycle intermediates on basal and quinolinic acid (QA)- or iron-induced TBARS production in brain membranes. Oxaloacetate, citrate, succinate and malate reduced significantly the basal and QA-induced TBARS production. The potency for basal TBARS inhibition was in the order (IC_{50} is given in parenthesis as mM) citrate (0.37) > oxaloacetate (1.33) = succinate (1.91) > > malate (12.74). α-Ketoglutarate caused an increase in TBARS production without modifying the QA-induced TBARS production. Cyanide (CN^-) did not modify the basal or QA-induced TBARS production; however, CN^- abolished the antioxidant effects of succinate. QA-induced TBARS production was enhanced by iron ions, and abolished by desferrioxamine (DFO). The intermediates used in this study, except for α-ketoglutarate, prevented iron-induced TBARS production. Oxaloacetate, citrate, α-ketoglutarate and malate, but no succinate and QA, exhibited significantly iron-chelating properties. Only α-ketoglutarate and oxaloacetate protected against hydrogen peroxide-induced deoxyribose degradation, while succinate and malate showed a modest effect against Fe^{2+}/H_2O_2-induced deoxyribose degradation. Using heat-treated preparations citrate, malate and oxaloacetate protected against basal or QA-induced TBARS production, whereas α-ketoglutarate induced TBARS production. Succinate did not offer protection against basal or QA-induced TBARS production. These results suggest that oxaloacetate, malate, succinate, and citrate are effective antioxidants against basal and iron or QA-induced TBARS production, while α-ketoglutarate stimulates TBARS production. The mechanism through which Krebs cycle intermediates offer protection against TBARS production is distinct depending on the intermediate used. Thus, under pathological conditions such as ischemia, where citrate concentrations vary it can assume an important role as a modulator of oxidative stress associated with such situations.

KEY WORDS: Krebs cycle intermediates; lipid peroxidation; quinolinic acid; iron; antioxidant.

INTRODUCTION

Increasing data from the literature have indicated that α-ketoglutarate, an intermediate of Krebs cycle, can inhibit oxidative stress *in vitro* when induced by hydrogen peroxide in human erythrocytes (1) and cultured striatal neurons (2), and after *in vivo* treatment with ammonium acetate (3) or chronic ethanol administration (4). In the same way, α-ketoglutarate and oxaloacetate can prevent damage to mitochondrial DNA and seizures induced by kainic acid in mice (5). The mechanism(s) underlying the antioxidant properties of Krebs cycle intermediates are not completely understood. However, recent studies have indicated that α-ketoacids non-enzymatically neutralize

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peroxides (1–6). Furthermore, Mallet and co-workers have shown that pyruvate (an α-ketoacid) protects post-ischemic myocardium by increasing the thiol status of the heart (6–8).

Citrate forms complexes with ferrous (Fe^{2+}) or ferric (Fe^{3+}) ions, which can be bioactive low molecular weight iron complexes (9). This organic acid can either lower iron levels through chelation, or increase its availability through redox reactions (10). Increased redox cycling of iron complexes may cause iron-catalyzed free radical generation, lipid peroxidation, axonal dystrophy, necrosis and apoptotic cell death (11,12).

Earlier studies indicated that succinate inhibits the NADH- and NADPH-dependent peroxidation (13) and offers protection against cumene hydroperoxide-induced lipid peroxidation in the mitochondria (14,15). Succinate can also prevent lipid peroxidation induced by chaotropic agents (16), such as Fe^{2+}/ADP (13,17) or NADPH/Fe^{3+}/ADP (15). In all cases, protection offered by succinate is attributed to ubiquinol formation, which behaves as a potent antioxidant in biological systems. In the same way, malate can act as a potent antioxidant against lipid peroxidation induced by Fe^{2+}/ascorbate in mitochondria and its effect is mainly ascribed to the reduction of the coenzyme *Q* (18).

In pathological conditions, such as ischemic episodes, there is an intensification of free radical production, which can lead to cell damage (19–21). Of particular importance, after a complete brain ischemia, an apparent depletion of glucose, oxaloacetate, ATP, creatine phosphate, pyruvate, citrate and α-ketoglutarate and a significant accumulation of fructose-1, 6-diphosphate, lactate, succinate, ADP and AMP occur (22,23). However, there are also indications that citrate levels can increase and succinate levels decrease after ischemia (24).

Quinolinic acid (QA) has been implicated in the pathogenesis of a broad spectrum of degenerative, infectious, inflammatory and non-inflammatory human neurological diseases (25–28). The mechanism underlying QA induced-neurotoxicity seems to involve over activation of the NMDA receptor, which in turn produces excitotoxicity (29). In fact, it has been proposed that over stimulation of excitatory amino acid receptors is involved in the pathophysiology of acute brain injury and chronic neurodegenerative disease (30,31). In addition, it has been reported that QA was able to stimulate lipid peroxidation in rat brain homogenates and its pro-oxidant effect is blocked by MK 801 and a variety of antioxidants (25,32–35). Of particular importance, the in vitro pro-oxidant activity of QA is dependent on iron ions [36, 37].

Iron is of great importance in biological systems and it participates in a wide variety of electron transport reactions. However, it can stimulate free radical production by different mechanisms (38,39): (a) via the breakdown of preexisting lipid hydroperoxides (ROOH) present in tissues forming lipid alkoxyl radical ($RO^{\bullet}$); (b) by entering a Fenton type reaction producing hydroxyl radical; or (c) via formation of iron oxygen complexes such as perferryl ions or ferrous–dioxygen–ferric complexes, which initiates lipid peroxidation (18,40).

The main objective of this study was to investigate the effect of citrate, succinate, malate, oxaloacetate, α-ketoglutarate (Krebs cycle intermediates) and oxalate (dicarboxilic acid) on basal, quinolinic acid-, and iron-induced TBARS production in order to know whether they can play a role as possible modulators of oxidative stress in brain tissues under different pro-oxidant situations. This investigation is of particular importance due to the fact that free radicals may be a primary cause of cerebral damage during ischemia and post-ischemic reperfusion (41,42) and because during these situations intracellular iron delocalization is thought to occur (20,43).

MATERIALS AND METHODS

Animals

Adult male Wistar rats from our own breeding colony (250–350 g) were maintained in an air-conditioned room (22–25°C) under natural lighting conditions, with water and food (Guabi, RS, Brazil) *ad libitum*. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil.

Tissue Preparation

Animals were anesthetized with ether and killed by decapitation. The whole brain was quickly removed, placed on ice, and homogenized within 10 min, in 10 volumes of 100 mM cold saline. The homogenate was centrifuged at $4000 \times g$ at 4°C for 10 min to yield a low speed supernatant fraction (S1) that was used immediately for TBARS assay.

Lipid Peroxidation Assay

Lipid peroxidation was determined by measuring thiobarbituric acid-reactive substances (TBARS) as described by Ohkawa

et al. (44). Brain homogenates were prepared by homogenization as described above. Aliquots of the homogenate (100–200 μl) from brain were incubated at 37°C in a water bath for 60 min in a medium containing 10 mM Tris/HCl buffer, pH 7.4, in the presence of indicated concentrations of Krebs cycle intermediates. The reaction was stopped by 0.5 ml of acetic acid buffer and lipid peroxidation products were measured by the addition of 0.5 ml of 0.6% TBA and 0.3 ml of 8.1% SDS. The color reaction was developed by incubating tubes in boiling water for 60 min. TBARS levels were measured at 532 nm using a standard curve of MDA. Solutions of $FeSO_4$ were made just before use in distilled water. The values are expressed in nmol MDA/g of tissue.

Degradation of Deoxyribose

Reaction mixtures contained, in a final volume of 0.8 ml, the following reagents at the final concentrations stated: 3 mM deoxyribose, 0.05 mM KH_2PO_4 buffer, pH 7.4, 50 μM $FeSO_4$ and 500 μM H_2O_2. Solutions of $FeSO_4$ and H_2O_2 were made just before use in distilled water. Reaction mixtures were incubated at 37°C for 30 min and terminated by the addition of 0.8 ml of 2.8% TCA, followed by the addition of 0.4 ml of 0.6% TBA solution. Tubes were incubated in boiling water for 20 min and then the absorbance was recorded at 532 nm (45,46). Standard curves of MDA were made in each experiment. The values are expressed in nmol MDA/l.

Chelating Properties of the Tricarboxilic Acid Intermediates Assay

To examine the iron chelating properties of Krebs cycle intermediates and quinolinic acid, we used the *o*-phenantroline method as previously described (47). The mixture containing Fe^{2+} (150 μM) and Krebs cycle intermediates or quinolinic acid at concentrations indicated in the figure, was allowed to react for 5 min and form complex(es) between Fe^{2+} and the related compounds. After that, *o*-phenantroline solution was added to determine the colored complex(es) formation between *o*-phenantroline and free Fe^{2+}.

The absorbance was recorded at 510 nm. The values are expressed in % of control determined in the absence of Krebs cycle intermediates or quinolinic acid. Solutions of $FeSO_4$ were made just before use in distilled water.

Thiol Assay

Total –SH content was assayed in brain S1 by the Ellman method as modified by Jacques-Silva et al. (48), which consisted of the reduction of 5,5″-dithio(bis-nithrobenzoic) acid (DTNB) in pH 7.4, measured at 412 nm. Brain S1 was incubated for 60 min in the presence of citrate or succinate, and then aliquots were sampled at 0, 30 and 60 min and added to two series of tubes, one containing Tris–HCl buffer and the other Tris–HCl buffer plus 1.2% SDS to analyze total –SH content. The absorbance recorded in the tube without DTNB was used to eliminate any interference.

Statistical Analysis

Data were analyzed by one-way ANOVA, followed by Duncan's Multiple Range Test when appropriate. Differences between groups were considered to be significant when $P \leq 0.05$.

RESULTS

Effect of Citrate, Succinate, Malate, Oxaloacetate, α-Ketoglutarate and Oxalate on Basal-TBARS Production in Brain S1

Oxaloacetate, citrate, succinate and malate (intermediates of Krebs cycle) reduced significantly the basal TBARS production in a concentration dependent manner. The antioxidant potency was in the order citrate > oxaloacetate ≥ succinate > malate. The effect of oxalate (a non-Krebs cycle intermediate dicarboxylic acid) was biphasic, causing a pro-oxidative effect at low concentrations (0.5– 4 mM) and an antioxidative effect at the highest concentration used (16 mM). α-Ketoglutarate caused a concentration dependent increase in TBARS production, which became apparent from 2 mM onwards (Fig. 1); however, only the highest concentration (16 mM) caused a significant increase in TBARS.

Antioxidant Effect of Citrate, Succinate, Malate and Oxaloacetate Against QA Induced Lipid Peroxidation

Citrate (0.5 mM) and succinate (4 mM) caused a significant reduction on basal and QA-induced TBARS production (Figs. 2a and b, respectively). Cyanide did not modify the basal TBARS production nor the effects of citrate or QA on TBARS produc-

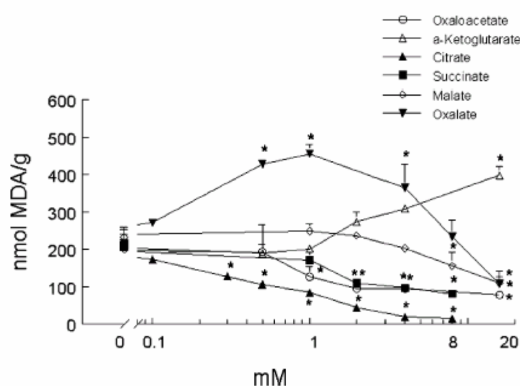

Fig. 1. Effect of citrate, succinate, malate, oxaloacetate, α-ketoglutarate and oxalate in basal- TBARS production on brain S1. Low-speed supernatant (S1) from brain was incubated for 60 min in a medium containing 10 mM Tris–HCl buffer, pH 7.4, in the presence of indicated concentrations of Krebs cycle intermediates. TBARS are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± SEM (n = 3–4). *P < 0.05 from respective control by Duncan's multiple range test.

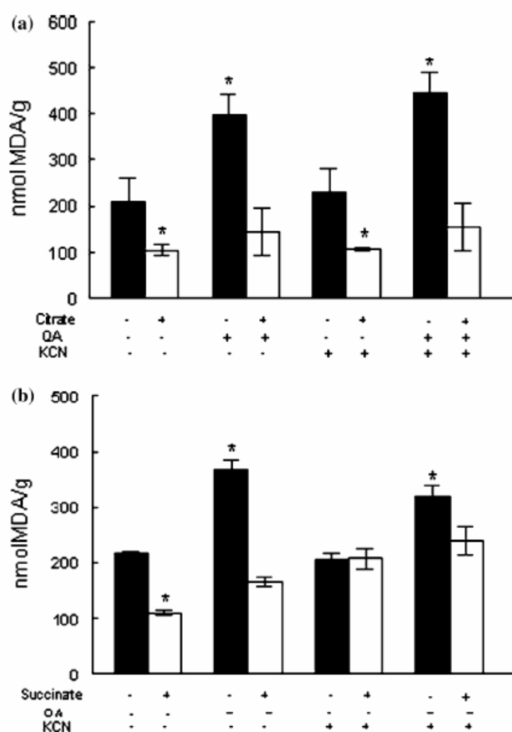

Fig. 2. Antioxidant effect of citrate and succinate against basal or QA induced lipid peroxidation. (a) Antioxidant effect of 0.5 mM citrate against basal TBARS production or 1 mM QA induced TBARS production, in the absence or presence of 1 mM KCN. (b) Antioxidant effect of 4 mM succinate against basal TBARS formation, or 1 mM QA induced TBARS production, in the presence or absence of 1 mM KCN. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± SEM (n = 3–4). *$P < 0.05$ from control by Duncan's multiple range test.

tion (Fig. 2a). Succinate reduced TBARS production induced by QA both in the presence and in the absence of cyanide. However, cyanide abolished the antioxidative effect of succinate measured under basal conditions (Fig. 2b).

Oxaloacetate caused a concentration-dependent reduction of the basal and QA-induced TBARS production (Fig. 3a). The effects of oxaloacetate measured in the absence or presence of QA was not significantly modified by cyanide (Fig. 3a). Malate at higher concentrations (8 and 16 mM) significantly reduced the basal TBARS production (Fig. 3b). Additions of NAD^+ (0.1 mM) did not modify the TBARS production and did not interfere on the malate effect. Cyanide did not modify the analyzed parameters.

Pro-oxidative Effect of α-Ketoglutarate and Oxalate on Rat Brain S1

α-Ketoglutarate did not modify the QA-induced TBARS production, but caused a significant increase of the basal TBARS production by brain S1. Cyanide did not modify the QA-induced or basal TBARS production (Fig. 3c). Oxalate caused an increase of the basal TBARS production by brain S1 at the intermediate concentrations tested (0.5–4 mM) (Fig. 3d). However, in the presence of 10 mM oxalate, the TBARS production returned to basal levels and a reduction of the QA-induced TBARS formation was observed.

Role of Iron on the Pro-oxidant Effect of QA in Rat Brain S1

Quinolinic acid (QA) 3 mM caused a significant increase in the TBARS production (Fig. 4a) when compared with basal values. The classical chelating iron agent, desferrioxamine (DFO) reduced the basal and QA-induced TBARS production at a concentration-dependent manner. DFO significantly decreased the basal TBARS production at 4 µM and its maximum effect was reached at 10 µM. In the QA-induced TBARS production assay, the maximum effect of DFO was reached with 10 µM and there was no significant effect at lower concentrations (Fig. 4a).

QA caused significant increase or decrease of the basal TBARS production, depending on its concentration. In fact, the increase of the basal TBARS production was significant at 1 and 2 mM, whereas it caused a significant reduction in the TBARS production when tested at 6 and 8 mM (Fig. 4b). Iron (0.3 µM) significantly increases the basal TBARS production and its enhancing oxidative effect was increased by QA. In the presence of iron, QA induced a significant increase on the TBARS production at 0.5 and 1 mM. However, at higher concentration (8 mM) QA significantly decreased the effect of iron.

Antioxidant Actions of Krebs Cycle Intermediates Against Iron-induced TBARS Production

Iron induced a concentration dependent increase in the TBARS production, which was significant from 0.5 µM onwards. The iron-induced TBARS production is significantly prevented in the presence of Krebs cycle intermediates used in this study. Citrate (0.5 mM; Fig. 5a), malate (16 mM, Fig. 5c), and oxaloacetate (4 mM, Fig. 5d) significantly

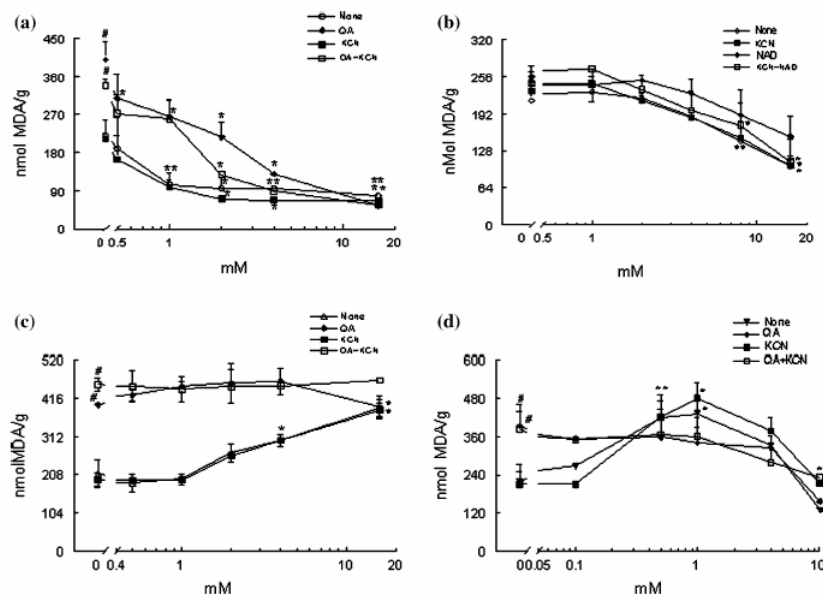

Fig. 3. Antioxidant effect of oxaloacetate and malate, and pro-oxidative effect of α-ketoglutarate and oxalate on rat brain S1. (a) Antioxidant effect of oxaloacetate (0.5 up to 16 mM) against basal or 1 mM QA- induced TBARS production, in the presence or absence of 1 mM KCN. (b) Antioxidant effect of malate (1 up to 16 mM) against basal TBARS production, in the presence or absence of 0.1 mM NAD^+ and/or 1 mM KCN. (c) Pro- oxidative effect of α-ketoglutarate (0.5 up to 16 mM) against basal and 1 mM QA-induced TBARS production in the presence or absence of 1 mM KCN. (d) Biphasic effect of oxalate (0.1 up to 10 mM) against basal or 1 mM QA-induced TBARS production, in the presence or absence of 1 mM KCN. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± SEM ($n = 3$). $^*P < 0.05$ from respective control (no intermediate) and $^\#P < 0.05$ of basal (no addition) by Duncan's multiple range test.

reduced the iron-induced TBARS production (Fig. 5a). Succinate (4 mM) reduced the iron induced TBARS production at all iron concentrations tested (Fig. 5b), while α-ketoglutarate (16 mM) did not modify the iron induced TBARS production (Fig. 5e).

Iron Chelating Properties of Krebs Cycle Intermediates

The iron chelating capacity of QA, succinate, malate, oxaloacetate, citrate and α-ketoglutarate, using *o*-phenantroline as a Fe^{2+} indicator is shown in Fig. 6. QA (up to 20 mM), and succinate (up to 20 mM) did not change the interaction of *o*-phenantroline with Fe^{2+}. α-Ketoglutarate (4 mM onwards) caused a modest reduction of about 30% of the formation of colored the Fe^{2+}–*o*-phenanthroline complex. Malate (2 mM onwards, Fig. 6a), oxaloacetate (0.3 mM onwards, Fig. 6b) and citrate (0.4 mM onwards, Fig. 6b) caused a concentration-dependent reduction of the colored iron–phenantroline complex. The maximum color reduction caused by these compounds was about 70%.

Protective Effect of Oxaloacetate and α-Ketoglutarate Against Deoxyribose Degradation Caused by Fe^{2+}, H_2O_2 and $H_2O_2 + Fe^{2+}$

Fe^{2+} (50 μM) caused a significant increase in deoxyribose degradation (Fig. 7a) and citatre (0.5 mM), succinate (4 mM), malate (4 mM), oxaloacetate (4 mM), and α-ketoglutarate (16 mM) did not reduce deoxyribose degradation stimulated by Fe^{2+} (Fig. 7a). Hydrogen peroxide (500 μM, Fig. 7b) stimulated deoxyribose degradation. Citrate, succinate, and malate did not modify the H_2O_2-induced deoxyribose degradation (Fig. 7b), whereas oxaloacetate and α-ketoglutarate produced a significant reduction of 33 and 68% in the deoxyribose degradation induced by H_2O_2, respectively. Association of Fe^{2+} and H_2O_2 caused an increase in deoxyribose degradation that was approximately equal to the sum of their isolated effect (Fig. 7c). Succinate and malate, which were devoid of effect against Fe^{2+} or H_2O_2, caused a modest statistically significant reduction of about 10% on deoxyribose degradation, when these two pro-oxidant agents were combined. The decrease in deoxyribose degradation

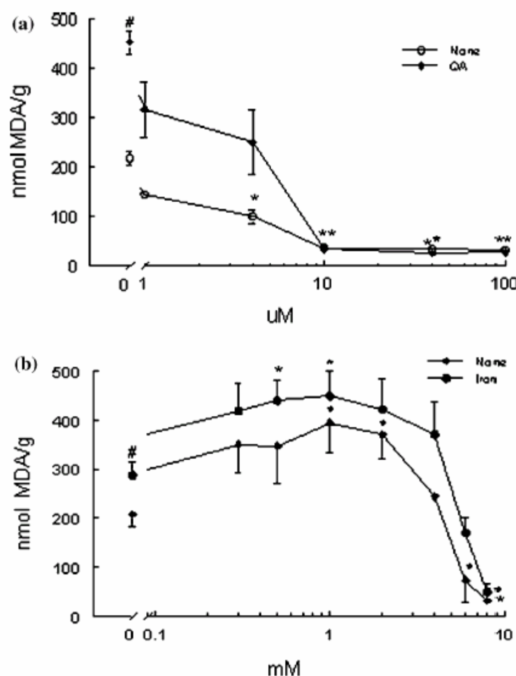

Fig. 4. Role of iron on the pro-oxidant effect QA in rat brain S1. (a) Effect of DFO (1 up to 100 μM) on basal or 3 mM QA-induced TBARS production. (b) Effect of QA (0.3 up to 10 mM) on basal and 0.3 μM iron- induced TBARS production. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± SEM ($n = 3$). $^{*}P < 0.05$ from respective control and $^{\#}P < 0.05$ of basal (no addition) by Duncan's multiple range test.

caused by oxaloacetate, and α-ketoglutarate in the presence of $Fe^{2+} + H_2O_2$ was similar to that observed when the pro-oxidant H_2O_2 was added separately to the assay medium (compare Fig. 7c with b).

The Antioxidant Properties of Citrate, Malate and Oxaloacetate are not Thermolabile, Whereas Those of Succinate Are

In order to investigate whether the mechanism involved in the protection against the basal and QA or Fe^{2+}-induced TBARS production caused by Krebs cycle intermediates depends on enzymatic components, we used heat-treated preparations (brain S1 was boiled for 10 min). In fresh tissue preparations (Fig. 8a), citrate (0.5 mM), succinate (4 mM), malate (16 mM) and oxaloacetate (4 mM) significantly prevented the basal and QA induced TBARS production. Similarly, in heat-treated preparations (Fig. 8b), citrate (0.5 mM), malate (16 mM) and oxaloacetate (4 mM) offered antioxidant protection against the basal or QA induced TBARS production, whereas α-ketoglutarate induced the TBARS production. However, in heat-treated preparations, succinate did not reduce the basal or QA-induced TBARS production.

Effect of Citrate and Succinate on Total–SH Content

In order to investigate whether citrate or succinate can alter total –SH content, we incubated S1 in the presence of these intermediates for 60 min. Total –SH content was not affected by citrate (1 mM) or succinate (4 mM- data not shown).

DISCUSSION

The antioxidant effect of Krebs cycle intermediates varies depending on the compound structure and on their concentration. In fact, oxaloacetate, malate, succinate, and citrate are effective antioxidants against the basal and iron (Fe^{2+}) or QA-induced TBARS production in rat brain S1 preparations. In contrast to other Krebs cycle intermediate, α-ketoglutarate has no antioxidant effect and, in fact, stimulates TBARS formation when tested at higher concentrations. Oxalate, which is not a Krebs cycle intermediate, has a dual effect: it stimulates TBARS production at lower (0.5 up to 4 mM) concentrations but protects against basal TBARS production at higher concentrations. The mechanism underlying the antioxidant effect of Krebs cycle intermediates against TBARS production is distinct depending on the intermediate used. In fact, succinate protection is thermolabile, being abolished when heat-treated preparations are used, whereas the protective effect of citrate, malate and oxaloacetate is not modified by heat treatment of brain tissue. Thus, taken together with the effect of cyanide, we can suppose that the effect of succinate depends on respiratory chain activity, whereas the other intermediates effects do not.

Iron chelating agents can reduce TBARS production and DFO, a classical iron chelator, reduced considerably TBARS production in brain S1. Krebs cycle intermediates, except for succinate, exhibited some iron chelating properties. However, only citrate and oxaloacetate exhibited iron-chelating activity at physiologically relevant concentrations. In fact, part of the antioxidant activity of citrate and oxaloacetate can be related to iron chelation. However, oxaloacetate was a better chelating agent than citrate, whereas citrate reduced TBARS production at concentrations

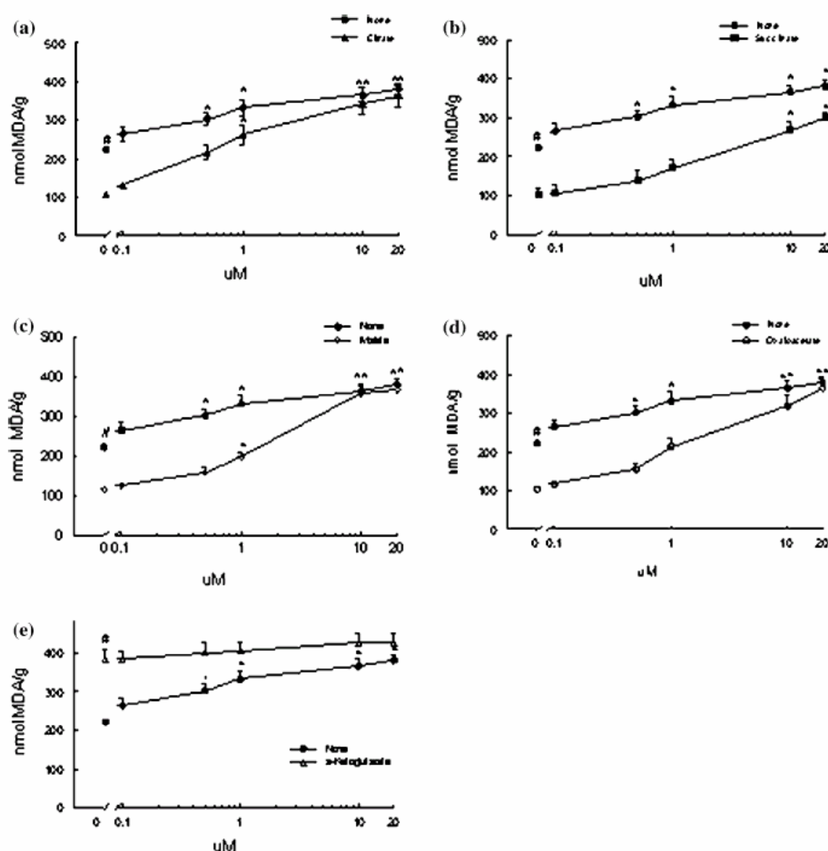

Fig. 5. Antioxidant actions of Krebs cycle intermediates against iron–induced TBARS production. (a) Effect of 0.5 mM citrate on basal or iron (0.1 up to 20 μM) induced TBARS formation. (b) Effect of 4 mM succinate on basal or iron (0.1 up to 20 μM) induced TBARS formation. (c) Effect of 16 mM malate on basal or iron (0.1 up to 20 μM) induced TBARS formation. (d) Effect of 4 mM oxaloacetate on basal or iron (0.1 up to 20 μM) induced TBARS formation. (e) Effect of 16 mM α-ketoglutarate on basal or iron (0.1 up to 20 μM) induced TBARS formation. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± SEM ($n = 4$). $^{*}P < 0.05$ from respective control (no intermediate) and $^{\#}P < 0.05$ of basal (no addition) by Duncan's multiple range test.

lower than oxaloacetate. Part of these discrepancies can be due to the fact that some iron-chelant complexes, such as citrate–Fe present redox activity (49).

In order to know whether Krebs cycle intermediates could form active complexes with iron, we examined their effect on deoxyribose degradation induced by iron (Fe^{2+}) and/or H_2O_2. Krebs cycle intermediates were ineffective against iron-induced deoxyribose degradation. Oxaloacetate and α-ketoglutarate significantly reduced the H_2O_2-induced deoxyribose degradation. In line with this, the α-ketoacids, oxaloacetate and α-ketoglutarate, are effective in detoxification of H_2O_2 (1–4,6). Consequently, inhibition of the Fenton reaction caused by oxaloacetate and α-ketoglutarate seems to be mediated by a direct reaction with H_2O_2. In fact, H_2O_2 can also accelerate the rate of deoxyribose degradation by an iron-independent pathway (50). Succinate and malate caused a modest reduction on deoxyribose degradation induced by Fe^{2+} plus H_2O_2. These results suggest that citrate did not interact with $OH^{\bullet}$ or with H_2O_2. Furthermore, the citrate–iron complex is effective in supporting deoxyribose degradation. Thus, citrate, which was effective against basal and QA- or iron-induced TBARS production, offered no protection against Fe^{2+} or Fe^{2+} plus H_2O_2-induced deoxyribose degradation. These findings suggest that iron–citrate complexes formed under our assay condition (10 times more citrate than iron) participate in the Fenton reaction though it does not enhance

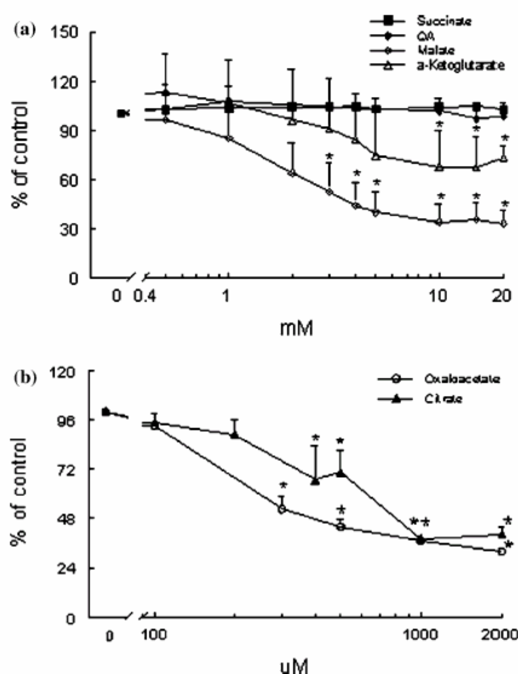

Fig. 6. Iron chelating properties of Krebs cycle intermediates. (a) Effect of QA, succinate, malate and α-ketoglutarate on colored iron–phenantroline complex formation. (b) Effect of oxaloacetate and citrate on colored iron–phenantroline complex formation. The values are expressed as % of control. Absorbance obtained by reaction between free Fe^{2+} with *o*-phenantroline in the absence of Krebs cycle intermediates or QA is considered 100%. Data are expressed as means ± SEM ($n = 4$). $*P < 0.05$ from respective control by Duncan's multiple range test.

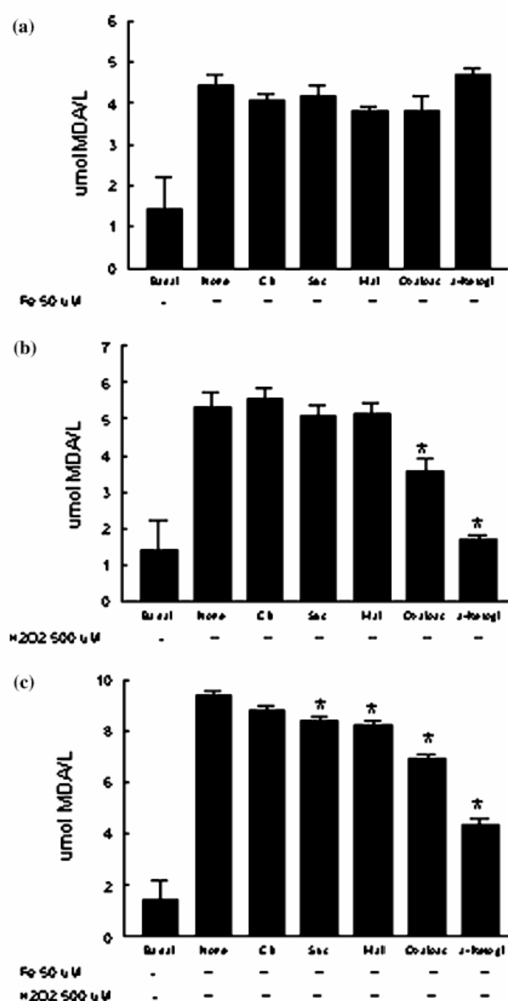

Fig. 7. Effect of Krebs cycle intermediates against deoxyribose degradation. (a) Effect of Krebs cycle intermediates against deoxyribose degradation induced by 50 μM Fe^{2+}. (b) Effect of Krebs cycle intermediates against deoxyribose degradation induced by 500 μM hydrogen peroxide. (c) Effect of Krebs cycle intermediates against deoxyribose degradation induced by 50 μM Fe^{2+} and 500 μM hydrogen peroxide. Final concentrations used: 0.5 mM citrate; 4 mM succinate; 4 mM malate; 4 mM oxaloacetate; and 16 mM α-ketoglutarate. The values are expressed as μM MDA per liter. Data are expressed as means ± SEM ($n = 6$). $*P < 0.05$ from induced by oxidant agents by Duncan's multiple range test.

deoxyribose degradation when compared to the system containing Fe^{2+} plus H_2O_2.These effects of citrate–iron complexes are in accordance with the oxidant effects of the Fe^{2+}–citrate complex frequently reported in the literature (47). In fact, the pro-oxidant or antioxidant effect of iron-chelanting agents depends on their ability to change the ratio of Fe^{2+}/Fe^{3+} present in the assay (20,47, 49, 51). The relative concentration of iron to chelating agents can change the ratio Fe^{2+}: Fe^{3+}, which is believed to have optimum activity at Fe^{2+}: Fe^{3+} of 1:1 (51,52). In addition, in biological systems the rate of oxidation of oxidizable substrate by "$Fe^{2+} + O_2$" could be as much as 10^8 times faster than the rate of oxidation by the Fenton reaction. This suggests that "$Fe^{2+} + O_2$" chemistry is probably the most important route for the free radical biology of iron (51,53). In line with this, iron chelators such as 2,2′-dipyridyl (which binds tightly to Fe^{2+} and thus inhibiting redox activity of Fe^{2+}), and desferrioxamine (which binds tightly only to Fe^{3+}-inhibiting redox cycling of iron and blocking its catalytic activity) possess strong antioxidant activities (54). Thus, we can suppose that citrate is active as an antioxidant when S1 is used by interfering with

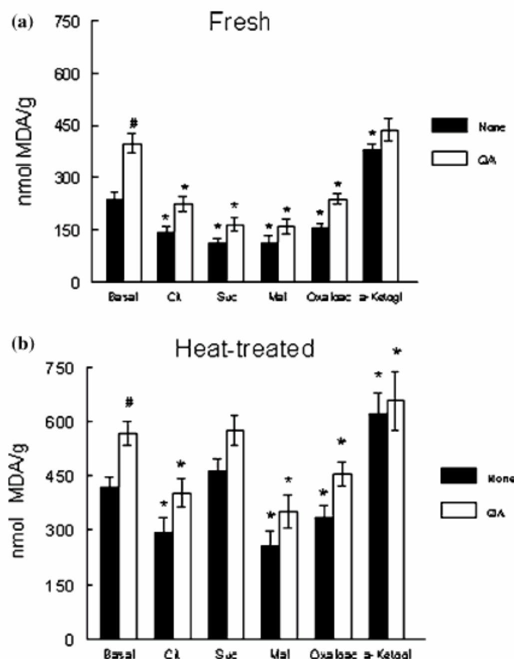

Fig. 8. Effect of Krebs cycle intermediates when heat-treated preparations are used. (a) Effect of Krebs cycle intermediates on basal and 1 mM QA induced TBARS production, using fresh tissue preparations. (b) Effect of Krebs cycle intermediates on basal and 1 mM QA induced TBARS production, using heat-treated preparations. 0.5 mM Citrate, 4 mM succinate, 16 mM malate, 4 mM oxaloacetate and 16 mM α-ketoglutarate. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± SEM (n = 10). *P < 0.05 from respective control (no intermediate) and $^{\#}P$ < 0.05 from basal without QA by Duncan's multiple range test.

"Fe^{2+} + O_2" chemistry, whereas it has no effect when the Fe^{2+} and/or H_2O_2 are used as a free radical generating system. These results are in accordance with previous studies in which iron–citrate complexes support deoxyribose degradation (55). However, Gutteridge showed that an increase in iron-citrate ratio could partially prevent deoxyribose degradation (55), thus, deoxyribose degradation induced by the Fe^{3+}–citrate complexes reaches a maximum effect when their ratio is close to 1 (55). In addition to the possibility of iron-chelation, citrate might contribute to change oxidative stress in neurons by modulating the NMDA receptor activation by interacting with Zn^{2+} or Mg^{2+} (56).

The results presented in this paper are in accordance with a previous study (36) where QA at high concentrations was able to abolish iron-induced TBARS production. In support, our data showed that DFO, a classical chelating of Fe^{3+}, completely abolished QA pro-oxidant activity, demonstrating iron ions involvement on QA-induced TBARS production. However, we did not find any chelating activity of QA up to 20 mM in our assay model. Thus, we can assume that the protective effect of Krebs cycle intermediates against QA-induced TBARS formation is due to its ability to modify the optimum ratio Fe^{2+}:Fe^{3+} necessary to induce TBARS production.

During and after pathological conditions such as cerebral ischemia a significant increase of ROS formation occurs which is thought to be associated with an increase in intracellular iron delocalization (20,43). Concomitantly, the concentrations of Krebs cycle intermediates vary depending on the intermediate considered. In fact, concentrations of citrate, malate and oxaloacetate apparently tend to decrease, while the succinate concentration tends to increase (22,23). However, there are only limited data on this subject and contradictory data can be found in the literature with respect to citrate concentration (22–24). The effective antioxidant concentrations of Krebs cycle intermediates oxaloacetate, malate, and succinate are relatively high and possibly without physiological significance. However, the effective concentrations of citrate are within the physiological range (56) and indicate that citrate may be an endogenous antioxidant. In line with this, Mallet and co-workers have recently demonstrated that citrate is an antioxidant for infracted myocardium. They showed that the protective effect of citrate is related to an increased in the thiol status caused by an inhibition of glycolysis at the level of phosphofructokinase. This causes the upstream intermediate glucose 6-phosphate accumulation, supplying substrate for the hexose monophosphate shunt, the major generator of NADPH, the reducing power that maintains GSH (6–8). In the present investigation, we observed no increase in total –SH content in the presence of citrate or succinate, thus under our assay condition an increase in thiol status can not explain the antioxidant effect of citrate. In the present study its antioxidant activity is related, at least in part, to iron chelating properties and is not linked to energetic metabolism. In fact, under our assay conditions of lack of oxygen and CO_2 (HCO_3^-), the Krebs cycle activity presented in the S1 homogenate should be fully impaired. Thus, it is extremely hard to see any effect that would be related to Krebs cycle activity. All possible effects would be due to direct reactions of the intermediates with free radicals or free radical formation-catalyzed reactions.

In conclusion, the results of the present investigation and those of Mallet and co-workers provide points of evidence in favor of a physiological antioxidant activity for citrate that is not related to its classical role as a Krebs cycle intermediate. Furthermore, under pathological conditions such as ischemia, where citrate concentrations vary (22–24) it can assume an important role as a modulator of oxidative stress associated with such situations.

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REFERENCES

1. Sokolowska, M., Oleszek, A., and Wodek, L. 1999. Protective effect of alpha-ketoacids on the oxidative hemolysis. Pol. J. Pharmacol. 51:429–434.
2. Desagher, S., Glowinski, J., and Premont, J. 1997. Pyruvate protects neurons against hydrogen peroxide-induced toxicity. J. Neurosci. 17:9060–9067.
3. Velvizhi, S., Dakshayani, K. B., and Subramanian, P. 2002. Effects of α-ketoglutarate on antioxidants and lipid peroxidation products in rats treated with ammonium acetate. Nutrition. 18:747–750.
4. Velvizhi, S., Nagalashmi, T., Mohamed Essa, M., Dakshayani, K. B., and Subramanian, P. 2002. Effects of α-ketoglutarate on lipid peroxidation and antioxidant status during chronic ethanol administration in Wistar rats. Pol. J. Pharmacol. 54:231–236.
5. Yamamoto, H., and Mohanan, P. V. 2003. Effect of α-ketoglutarate and oxaloacetate on brain mitochondrial DNA damage and seizures induced by kainic acid in mice. Toxicol. Lett. 143:115–122.
6. Mallet, R. T., and Sun, J. 2003. Antioxidant properties of myocardial fuels. Mol. Cell. Biochem. 253:103–111.
7. Mallet, R. T. 2000. Pyruvate: Metabolic protector of cardiac performance. Proc. Soc. Exp. Biol. Med. 223:136–148.
8. Mallet, R. T., and Sun, J. 1999. Mitochondrial metabolism of pyruvate is required for its enhancement of cardiac function and energetics. Cardiovasc. Res. 42:149–161.
9. Baker, M. S., and Gebicki, J. M. 1986. The effect of pH on yields of hydroxyl radicals produced from superoxide by potential biological iron chelators. Arch. Biochem. Biophys. 246:581–588.
10. Abrahamson, H. B., Rezvani, A. B., and Brushmiller, G. 1994. Photochemical and spectroscopic studies of complexes of iron (III) with citric acid and other carboxylic acids. Inorg. Chimica Acta. 226:117–127.
11. Chiueh, C. O., Miyake, H., and Peng, M. T. 1993. Role of dopamine autoxidation, hydroxyl radical generation, and calcium overload in underlying mechanisms involved in MPTP-induced parkinsonism. Adv. Neurol. 60:251–258.
12. Gutteridge, J. M. 1994. Hydroxyl radicals, iron, oxidative stress, and neurodegeneration. Ann. NY Acad. Sci. 738:201–213.
13. Takayanagi, R., Takeshige, K., and Minakami, S. 1980. NADH- and NADPH-dependent lipid peroxidation in bovine heart submitochondrial particles. Dependence on the rate of electron flow in the respiratory chain and an antioxidant role. Biochem. J. 192:853–860.
14. Bindoli, A., Cavallini, L., and Jocelyn, P. 1982. Mitochondrial lipid peroxidation by cumene hydroperoxide and its prevention by succinate. Biochim. Biophys. Acta. 681:496–503.
15. Cavallini, L., Valente, M., and Bindoli, A. 1984. Comparison of cumene hydroperoxide – and NADPH/ Fe^{3+} /ADP-induced lipid peroxidation in heart and liver submitochondrial particles. Mechanisms of protection by succinate. Biochim. Biophys. Acta. 795:466–472.
16. Hatefi, Y., and Hanstein, W. G. 1970. Lipid oxidation in biolo gical membranes .1. Lipid oxidation in submitochondrial particles and microsomes induced by chaotropic agents. Arch. Biochem. Biophys. 138:73–86.
17. Takeshige, K., and Minakami, S. 1975. Reduced nicotinamide adenine-dinucleotide phosphate-dependent lipid peroxidation by beef heart submitochondrial particles. J. Biochem. 77:1067–1073.
18. Vianello, A., Macri, F., Cavallini, L., and Bindoli, A. 1986. Induction of lipid peroxidation in Soybean mitochondria and protection by respiratory substrates. J. Plant Physiol. 125:217–224.
19. Ghisleni, G., Porciúncula, L. O., Cimarostia, H., Rocha, J. B. T., Salbego, C. G., and Souza, D. O. 2003. Diphenyl diselenide protects rat hippocampal slices submitted to oxygen–glucose deprivation and diminishes inducible nitric oxide synthase immunocontent. Brain Res. 986:196–199.
20. Oubidar, M., Boquillon, M., Marie, C., Schreiber, L., and Bralet, J. 1994. Ischemia-induced brain iron delocalization: Effect of iron chelators. Free Radic. Biol. Med. 16:861–867.
21. Porciúncula, L. O., Rocha, J. B. T., Cimarosti, H., Vinade, L., Ghisleni, G., Salbego, C. G., and Souza, D. O. 2003. Neuroprotective effect of ebselen on rat hippocampal slices submitted to oxygen-glucose deprivation: correlation with immunocontent of inducible nitric oxide synthase. Neurosci. Lett. 346:101–104.
22. Hoyer, S., and Krier, C. 1986. Ischemia and the aging brain. Studies on glucose and energy metabolism in rat cerebral cortex. Neurobiol. Aging. 7:23–29.
23. Folbergrová, J., Ljunggren, B., Norberg, K., and Siesjö, B. K. 1974. Influence of complete ischemia on glycolytic metabolites, citric acid cycle intermediates, and associated amino acids in the rat cerebral cortex. Brain Res. 80:265–279.
24. Medvedeva, L. V., Popova, T. N., Artyukhov, V. G., Matasova, L. V., and Akatova, R. V. 2002. Oxidative status and distribution of NADP-dependent isocitrate dehydrogenase and aconitate hydratase in rat cardiomyocytes under normal conditions and during ischemia. Bull. Exp. Biol. Med. 134:130–134.
25. Rossato, J. I., Zeni, G., Mello, C. F., Rubin, M. A., and Rocha, J. B. T. 2002. Ebselen blocks the quinolinic acid-induced production of thiobarbituric acid reactive species but does not prevent the behavioral alterations produced by intra-striatal quinolinic acid administration in the rat. Neurosci. Lett. 318:137–140.

26. Schwarz, R., Okuno, E., White, R. J., Bird, E. D., and Whetsell, W. O. Jr. 1988. 3-Hydroxyantranilat oxygenase activity is increased in the brain of Huntington disease victims. Proc. Natl. Acad. Sci. USA. 85:4079–4081.
27. Heyes, M. P., Saito, K., Crowley, J. S., Davis, L. E., Demitrack, M. A., Der, M., Dilling, L. A., Elia, J., Kruesi, M. J. P., Lackner, A., Larsen, S. A., Lee, K., Leonard, H. L., Markey, S. P., Martin, A., Milstein, S., Mouradian, M. M., Pranzatell, M. R., Quearry, B. J., Salazar, A., Smith, M., Strauss, S. E., Sunderland, T., Swedo, S. W., and Tourtellotte, W. W. 1992. Quinolinic acid and kynurenine pathway metabolism in inflammatory and non-inflammatory neurological disease. Brain. 115:1249–1273.
28. Heyes, M. P. 1996. The kynurenine pathway and neurological disease. Therapeutic strategies. Adv. Exp. Med. Biol. 398:125–129.
29. Santamaría, A., and Ríos, C. 1993. MK-801, an *N*-methyl-D-aspartate receptor antagonist, blocks quinolinic acid-induced lipid peroxidation in rat corpus striatum. Neurosci. Lett. 159:51–54.
30. Cabrera, J., Reiter, R. J., Tan, D., Qi, W., Sainz, R. M., Mayo, J. C., Garcia, J. J., Kim, S. J., and El- Sokkary, G. 2000. Melatonin reduces oxidative neurotoxicity due to quinolinic acid: In vitro and in vivo findings. Neuropharmacology. 39:507–514.
31. Porciúncula, L. O., Rocha, J. B. T., Boeck, C. R., Vendite, D., and Souza, D. O. 2001. Ebselen prevents excitotoxicity provoked by glutamate in rat cerebellar granule neurons. Neurosci. Lett. 299:217–220.
32. Santamaria, A., Salvatierra-Sanchez, R., Vazquez-Roman, B., Santiago-Lopez, D., Villeda-Hernandez, J., Galvan-Arzates, S., Jiménez-Capdeville, M. E., and Ali, S. F. 2003. Protective effects of the antioxidant selenium on quinolinic acid-induced neurotoxicity in rats: in vitro and in vivo studies. J. Neurochem. 86:479–488.
33. Rios, C., and Santamaria, A. 1991. Quinolinic acid is a potent lipid peroxidant in rat brain homogenates. Neurochem. Res. 16:1139–1141.
34. Rossato, J. I., Ketzer, L. A., Centurião, F. B., Silva, S. J. N., Ludtke, D. S., Zeni, G., Braga, A. L., Rubin, M. A., and Rocha, J. B. T. 2002. Antioxidant properties of new chalcogenides against lipid peroxidation in rat brain. Neurochem. Res. 27:297–303.
35. Bellé, N. A. V., Dalmolin, G. D., Fonini, G., Rubim, M. A., and Rocha, J. B. T. 2004. Polyamines reduces lipid peroxidation induced by different pro-oxidant agents. Brain Res. 1008:245–251.
36. Stípek, S., Stastný, F., Pláteník, J., Crkovská, J., and Zima, T. 1997. The effect of quinolinate on rat brain lipid peroxidation is dependent on iron. Neurochemistry 30:233–237.
37. Platenik, J., Stopka, P., Vejrazka, M., and Stipek, S. 2001. Quinolinic acid–iron(II) complexes: Slow autoxidation, but enhanced hydroxyl radical production in the Fenton reaction. Free Radic. Res. 34:445–459.
38. Braughler, J. M., Duncan, L. A., and Chase, R. L. 1986. The involvement of iron in lipid peroxidation. Importance of ferric to ferrous ratios in initiation. J. Biol. Chem. 261:10282–10289.
39. Minotti, G., and Aust, S. D. 1992. Redox cycling of iron and lipid peroxidation. Lipids 27:219–226.
40. Oubidar, M., Boquillon, M., Christine, M., Christine, B., Beley, A., and Bralet, J. 1996. Effect of intracellular iron loading on lipid peroxidation of brain slices. Free Radic. Biol. Med. 21:763–769.
41. Braughler, J. M., and Hall, E. D. 1989. Central nervous system trauma and stroke. I. Biochemical considerations for oxygen radical formation and lipid peroxidation. Free Radic. Biol. Med. 6:289–301.
42. Bromont, C., Marie, C., and Bralet, J. 1989. Increased lipid peroxidation in vulnerable brain regions after transient forebrain ischemia in rats. Stroke 20:918–924.
43. Krause, G. S., Joyce, K. M., Nayini, N. R., Zonia, C. L., Garritano, A. M., Hoehner, T. J., Evans, A. T., Indreri, R. J., Huang, R. R., Aust, S. D., and White, B. C. 1985. Cardiac arrest and resuscitation: Brain iron delocalization during reperfusion. Ann. Emerg. Med. 14:1037–1043.
44. Ohkawa, H., Ohishi, N., and Yagi, K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95:351–358.
45. Gutteridge, J. M. C. 1981. Thiobarbituric acid-reactivity following iron-dependent free-radical damage to amino acids and carbohydrates. FEBS Lett. 128:343–346.
46. Halliwell, B., and Gutteridge, J. M. C. 1981. Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts: The role of superoxide and hydroxyl radicals. FEBS Lett. 128:347–352.
47. Minotti, G., and Aust, S. D. 1987. An investigation into the mechanism of citrate–Fe^{2+}-dependent lipid peroxidation. Free Radic. Biol. Med. 3:379–387.
48. Jacqes-Silva, M. C., Nogueira, C. W., Broch, L. C., Flores, E. M. M., and Rocha, J. B. T. 2001. Diphenyl disselenides and ascorbic acid changes deposition of selenium and ascorbic acid in brain of mice. Phamacol Toxicol. 44:119–125.
49. Chiueh, C. C. 2001. Iron overload, oxidative stress, and axonal dystrophy in brain disorders. Pediatr. Neurol. 25:138–147.
50. Aruoma, O. I., Grootveld, M., and Halliwell, B. 1987. The role of iron in ascorbate-dependent deoxyribose degradation. Evidence consistent with a site-specific hydroxyl radical generation caused by iron ions bound to the deoxyribose molecule. J. Inorg. Biochem. 29:289–299.
51. Caro, A. A., and Cederbaum, A. T. 2004. Antioxidant properties of *S*-adenosyl-L-methionine in Fe^{2+}-initiated oxidations. Free Radic. Biol. Med. 36:1303–1316.
52. Tang, L. X., Yang, J. L., and Shen, X. 1997. Effects of additional iron-chelators on Fe^{2+}-initiated lipid peroxidation: Evidence to support the Fe^{2+}...Fe^{3+} complex as the initiator. J. Inorg. Biochem. 68:265–272.
53. Quian, S. Y., and Buettner, G. R. 1999. Iron and dioxygen chemistry is an important route to initiation of biological free radical oxidations: An electron paramagnetic resonance spin trapping study. Free Radic. Biol. Med. 26:1447–1456.
54. Huang, X., Dai, J., Fournier, J., Ali, A. M., Zhang, Q., and Frenkel, K. 2002. Ferrous ion autoxidation and its chelation in iron-loaded human liver HepG2 cells. Free Radic. Biol. Med. 32:84–92.
55. Gutteridge, J. M. C. 1991. Hydroxyl radical formation from the auto-reduction of a ferric citrate complex. Free Radic. Biol. Med. 11:401–406.
56. Westergaard, N., Banke, T., Wahl, P., Sonnewald, U., and Schousboe, A. 1995. Citrate modulates the regulation by Zn^{2+} of *N*-methyl-D-aspartate receptor-mediated channel current and neurotransmiter release. Proc. Natl. Acad. Sci. USA. 92:3367–3370.

4.2. ARTIGOS CIENTÍFICOS: CAPITULO II

CARACTERIZAÇÃO DA ATIVIDADE PRÓ-OXIDANTE DO OXALATO EM DIFERENTES TECIDOS E ESTUDO DO POTENCIAL ANTIOXIDANTE DOS INTERMEDIÁRIOS DO CICLO DE KREBS SOBRE A GERAÇÃO DE TBARS INDUZIDA POR MALONATO: ESTUDOS *IN VITRO*

4.2.1. – Artigo 3 - OXALATE MODULATES THIOBARBITURIC ACID REACTIVE SPECIES (TBARS) PRODUCTION IN SUPERNATANTS OF HOMOGENATES FROM RAT BRAIN, LIVER AND KIDNEY: EFFECT OF DIPHENYL DISELENIDE AND DIPHENYL DITELLURIDE

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4.2.1. – CARACTERIZAÇÃO DA ATIVIDADE PRÓ-OXIDANTE DO OXALATO EM DIFERENTES TECIDOS

Artigo 3

OXALATE MODULATES THIOBARBITURIC ACID REACTIVE SPECIES (TBARS) PRODUCTION IN SUPERNATANTS OF HOMOGENATES FROM RAT BRAIN, LIVER AND KIDNEY: EFFECT OF DIPHENYL DISELENIDE AND DIPHENYL DITELLURIDE

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Oxalate modulates thiobarbituric acid reactive species (TBARS) production in supernatants of homogenates from rat brain, liver and kidney: Effect of diphenyl diselenide and diphenyl ditelluride

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Abstract

The aim of this paper was to investigate the mechanism(s) involved in the sodium oxalate pro-oxidative activity *in vitro* and the potential protection by diphenyl diselenide ($(PhSe)_2$) and diphenyl ditelluride ($(PhTe)_2$) using supernatants of homogenates from brain, liver and kidney. Oxalate causes a significant increase in the TBARS (thiobarbituric acid reactive species) production up to 4 mmol/l and it had antioxidant activity from 8 to 16 mmol/l in the brain and liver. Oxalate had no effect in kidney homogenates. The difference among tissues may be related to the formation of insoluble crystal of oxalate in kidney, but not in liver and brain homogenates. $(PhSe)_2$ and $(PhTe)_2$ reduced both basal and oxalate-induced TBARS in rat brain homogenates, whereas in liver homogenates they were antioxidant only on oxalate-induced TBARS production. $(PhSe)_2$ showed a modest effect on renal TBARS production, whereas $(PhTe)_2$ did not modulate TBARS in kidney preparations. Oxalate at 2 mmol/l did not change deoxyribose degradation induced by Fe^{2+} plus H_2O_2, whereas at 20 mmol/l it significantly prevents its degradation. Oxalate (up to 4 mmol/l) did not alter iron (10 μmol/l)-induced TBARS production in the brain preparations, whereas at 8 mmol/l onwards it prevents iron effect. In liver preparations, oxalate amplifies iron pro-oxidant activity up to 4 mmol/l, preventing iron-induced TBARS production at 16 mmol/l onwards. These results support the antioxidant effect of organochalcogens against oxalate-induced TBARS production. In addition, our results suggest that oxalate pro- and antioxidant activity *in vitro* could be related to its interactions with iron ions.
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Keywords: Oxalate; Diphenyl diselenide; Diphenyl ditelluride; Iron; TBARS

1. Introduction

Hyperoxaluria is one of the main risk factors of human idiopathic calcium oxalate disease. Oxalate, the major stone-forming constituent is known to induce lipid peroxidation, which causes disruption of the cellular membrane integrity [1,2]. Oxidative stress may be caused by increased concentrations of free oxalate ions or by insoluble calcium oxalate interacting directly with renal epithelial cells, or may originate from other inflammatory event [3]. Exposure to high concentrations of oxalate can induce oxidative stress, as shown by (i) increased lipid peroxidation (thiobarbituric acid reactive species-TBARS) [4,5], (ii) decreased reduced glutathione concentrations [6], (iii) increase in free

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radical generation [7], and (iv) increases in arachidonic acid release via phospholipase-A_2 [3].

Although there is evidence that free oxalate ions can mediate an oxidative insult [7], relatively little is known about the origin of the reactive oxygen species (ROS) during oxalate exposure. However, data from the literature have shown that ROS generation during oxalate exposure is mainly attributed to mitochondrial production [8].

Lipid peroxidation is a free radical-induced process leading to oxidative deterioration of polyunsaturated lipids. This alters the fluidity of the biological membranes leading to dysfunction either in cellular [7] as in sub-cellular levels [9,10]. Earlier investigations have demonstrated that the involvement of enhanced lipid peroxidation reactions in cellular and sub-cellular levels, as an important factor for the etiology of stone formation [11–13]. Decreased functional efficiency in the antioxidant defense system and increased lipid peroxidation has been suggested to be one of the primary factors that contribute to lithogenesis [14,15]. In line with these, free radicals reaction can injure renal tubular cells and promote calcium oxalate crystallization [4,16]. Thus, antioxidants are necessary for preventing the formation of free radicals and the deleterious actions of the ROS [17–19].

Organic forms of selenium and tellurium have been suggested as possible antioxidant agents because they exhibit glutathione peroxidase-like activity and oxidize thiols during the reduction of H_2O_2 [20–22]. These organochalcogens also have antioxidant activities against oxidative stress induced by a variety of oxidants [23–27]. The mechanism(s) underlying either the toxic or protective effect of organochalcogens are not completely understood but certainly involves the reaction of chalcogenides with endogenous thiols [28–33]. It has been suggested that utilization of the redox activity of the selenium and, in particular, the tellurium atoms of such substances could provide antioxidants of considerable potency, suitable as tools in free radical biology [23,34,35]. Organoselenium and organotellurium compounds are readily oxidized from the divalent to the tetravalent state. This property makes them attractive as scavengers of reactive oxidizing agents such as hydrogen peroxide, hypochlorite, and peroxyl radicals, and as inhibitors of lipid peroxidation in chemical and biological systems [34,35].

Recent study showed that systemic administration of diphenyl diselenide induced a facilitation of formation of long-term object recognition memory [36]. In addition, we have recently showed that diphenyl diselenide protected rat hippocampal slices submitted to ischemic conditions in which enhanced free radicals generation take place [37]. Furthermore, diphenyl diselenide is also an anti-inflammatory and antinociceptive agent [38]. However, despite of several papers showing its beneficial effects, a recent study reported that diphenyl diselenide was a mutagenic agent, inducing simple and/or double strand breaks in DNA [39].

Based on that exposed above, the aim of this paper was to investigate whether organochalcogens (diphenyl diselenide and diphenyl ditelluride) could protect supernatants of homogenates from brain, liver and kidney of rats exposed to sodium oxalate *in vitro*. In addition, we sought to investigate the mechanism(s) involved in the oxalate pro-oxidative activity under *in vitro* conditions. First, we determined the relative concentrations of oxalate needed to cause an increase in the TBARS production using supernatants of homogenates from brain, liver and kidney. After, we determined the effect of organochalcogens against either basal or oxalate-induced TBARS production *in vitro*. Finally, we investigated the mechanism(s) involved in the oxalate pro-oxidative activity.

2. Material and methods

2.1. Animals

Adult male Wistar rats from our own breeding colony (250–350 g) were maintained in an air-conditioned room (22–25 °C) under natural lighting conditions, with water and food (Guabi, RS, Brazil) ad libitum. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil.

2.2. Tissue preparation

Animals were anesthetized with ether and killed by decapitation. The brain, liver and kidney were quickly removed, placed on ice, and homogenized within 10 min, in cold saline 100 mmol/l (brain in 10 volume; liver in 10 volume; and kidney in 5 volume). The homogenates were centrifuged at 4000 × *g* at 4 °C for 10 min to yield a low speed supernatant fraction (S1) that was used immediately for TBARS assay.

2.3. Lipid peroxidation assay

Lipid peroxidation was determined by measuring thiobarbituric acid- reactive species (TBARS) as described by Ohkawa et al. [40]. Tissues homogenates were prepared by homogenization as described above.

Aliquots of the homogenate (100 μl) from tissues were incubated for 60 min in a medium containing 10 mmol/l Tris/HCl buffer, pH 7.4, in the presence of other reagents at concentrations indicated in the legends. The mixtures were incubated at 37 °C in a water bath for 60 min. The reaction was stopped by addition of 0.5 ml of acetic acid buffer and lipid peroxidation products were measured by the addition of 0.5 ml of TBA 0.6% and 0.2 ml of SDS 8.1%. The color reaction was developed by incubating tubes in boiling water for 60 min. TBARS levels were measured at 532 nm using a standard curve of MDA. The values are expressed in nmol MDA/g of tissue. Where indicated, solutions of $FeSO_4$ were made just before use in distilled water.

2.4. Chelating properties of the oxalate assay

To examine iron chelating properties of sodium oxalate ($(COONa)_2$), we used the *o*-phenantroline method as previously described [41,42]. Fe^{2+} 150 μmol/l was added to the buffered medium containing 25 mmol/l NaCl and 10 mmol/l Tris/HCl buffer, sodium oxalate as indicated in the figure legends. Iron and oxalate were allowed to react for 5 min and then *o*-phenantroline solution was added to determine the colored complex(es) formation between *o*-phenantroline and free Fe^{2+}.

The absorbance was recorded at 510 nm. The values are expressed in % of control determined in the absence of sodium oxalate. Solutions of $FeSO_4$ were made just before use in distilled water.

2.5. Degradation of deoxyribose assay

Reactions mixtures contained, in a final volume of 0.8 ml, the following reagents at the final concentrations stated: deoxyribose 3 mmol/l, KH_2PO_4 buffer 50 mmol/l, pH 7.4, $FeSO_4$ 50 μmol/l, H_2O_2 500 μmol/l and sodium oxalate at concentrations indicated in the figure legend. Solutions of $FeSO_4$ and H_2O_2 were made just before use in distilled water. Reaction mixtures were incubated at 37 °C for 30 min and stopped by the addition of 0.8 ml of TCA 2.8% followed by the addition of 0.4 ml of TBA 0.6% solution. Tubes were incubated in boiling water for 20 min and then the absorbance was recorded at 532 [43,44]. Standard curves of MDA were made in each experiment. The values are expressed in nmol MDA/l.

2.6. Synthesis of organochalcogens

Diphenyl ditelluride was synthesized using the method described by Petragnani [45] and diphenyl diselenide using the method described by Paulmier [46]. Solutions of organochalcogens were prepared in dimethylsulfoxide (DMSO) and the final concentration of DMSO in all tubes was 3%.

2.7. IC_{50} calculation

IC_{50} (concentration inhibiting 50% of lipid peroxidation) for lipid peroxidation was determined by the method of Dixon and Webb [47].

2.8. Statistical analysis

Data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range test when appropriate. Differences between groups were considered to be significant when $P < 0.05$.

3. Results

3.1. Effect of oxalate on TBARS production in supernatants of homogenates from brain, liver and kidney of rats

Oxalate caused a biphasic effect on TBARS production in rat brain preparation. Oxalate caused a significant increased in TBARS production at 0.3 mmol/l, which was maximum at 2 mmol/l. However, using a higher concentration (16 mmol/l), oxalate was able to reduced TBARS production, reaching a value that was significantly lower that observed in basal conditions (Fig. 1).

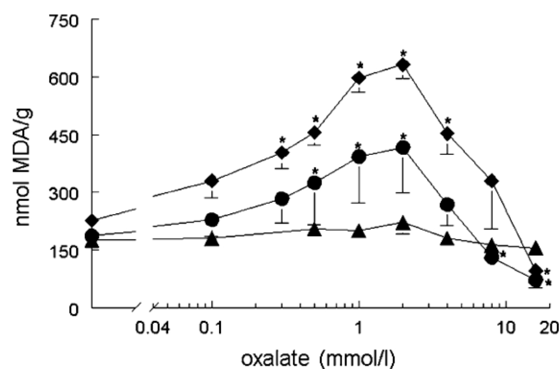


Fig. 1. Effect of oxalate on TBARS production in supernatants of homogenates from brain, liver and kidney of rats. Low-speed supernatant (S1) from tissues were incubated for 60 min in a medium containing 10 mmol/l Tris/HCl buffer, pH 7.4, in the presence of indicated concentrations of sodium oxalate (0.1–16 mmol/l). TBARS are expressed as nmol of MDA per gram of tissue. (▲) kidney, (♦) brain, and (●) liver. Data are expressed as means ± S.E.M. ($n = 3$). $^{*}p < 0.05$ from respective control by Duncan's multiple range test.

Likewise, oxalate caused a biphasic effect on TBARS production in rat liver preparation. Oxalate caused a significant increased in TBARS production at 0.5 up to 2 mmol/l. At higher concentrations (8 and 16 mmol/l) oxalate was able to reduce significantly basal-TBARS production in liver (Fig. 1).

Surprisingly, oxalate did not stimulated TBARS production in kidney supernatants (Fig. 1).

3.2. Incubation of sodium oxalate with supernatants of homogenates from kidney induces formation of insoluble crystals of oxalate

In an attempt to explain the absence of effect of sodium oxalate in kidney, we performed some experiments to verify the possible formation of insoluble crystals of oxalate in the presence of supernatants from brain, liver and kidney. Supernatants were incubated as described for TBARS assay and at the end of the incubation (1 h) an aliquot of 5 μl was placed in microscopy slide and observed in a Olympus BX-41 Microscope at a magnification of 400 times. Random fields (four per slide) were qualitatively analyzed by three experimenters who were blind to the treatment given to each slide. In the presence of brain and liver supernatants, small crystals of oxalate were not found at 2 mmol/l sodium oxalate (Fig. 2B and E, respectively) when compared to controls (Fig. 2A and D, respectively). In addition, they were rarely found at 16 mmol/l sodium oxalate (in fact, in Fig. 2C and F it is not possible to observe crystals because these fields were selected as a representative one by chance). In contrast, for the case of supernatants from kidney, a high incidence of oxalate crystals were found at 16 mmol/l (Fig. 2I) when compared to control (Fig. 2G). The presence of crystals were lower at 2 mmol/l (Fig. 2H), but the size and the form

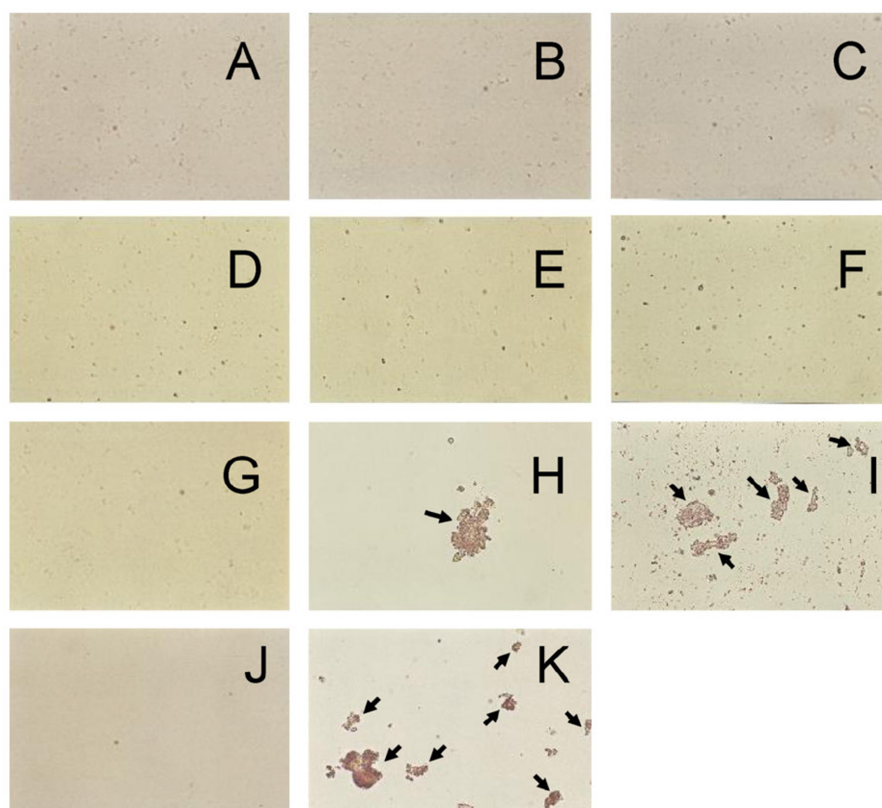


Fig. 2. Oxalate insoluble crystals formation in rat kidney supernatants. Low-speed supernatant (S1) from brain (A–C), liver (D–F) or kidney (G–I) were incubated during 60 min at 37 °C in a medium containing Tris/HCl 10 mmol/l, pH 7.4; without sodium oxalate (Ox) or with Ox at 2 or 16 mmol/l. After the incubation the photos were done in an optical microscope. (A) Brain supernatants without Ox; (B) brain supernatants with 2 mmol/l Ox; (C) brain supernatants with 16 mmol/l Ox; (D) liver supernatants without Ox; (E) liver supernatants with 2 mmol/l Ox; (F) liver supernatants with 16 mmol/l Ox; (G) kidney supernatants without Ox; (H) kidney supernatants with 2 mmol/l Ox; (I) kidney supernatants with 16 mmol/l Ox; (J) 16 mmol/l Ox; (K) 16 mmol/l calcium oxalate. The arrows indicate the oxalate insoluble crystals (magnitude 400×).

of crystals were similar to that observed at 16 mmol/l (compare Fig. 2H and I). In the absence of homogenates, sodium oxalate did not form crystals both at 2 mmol/l (data not shown) or 16 mmol/l (Fig. 2J). Fig. 2K shows the size and the form of calcium oxalate crystals (at 16 mmol/l).

3.3. Effect of diphenyl diselenide on basal or oxalate-induced TBARS production in supernatants of homogenates from brain, liver and kidney of rats

Diphenyl diselenide caused a significant reduction in basal and oxalate-induced TBARS production in

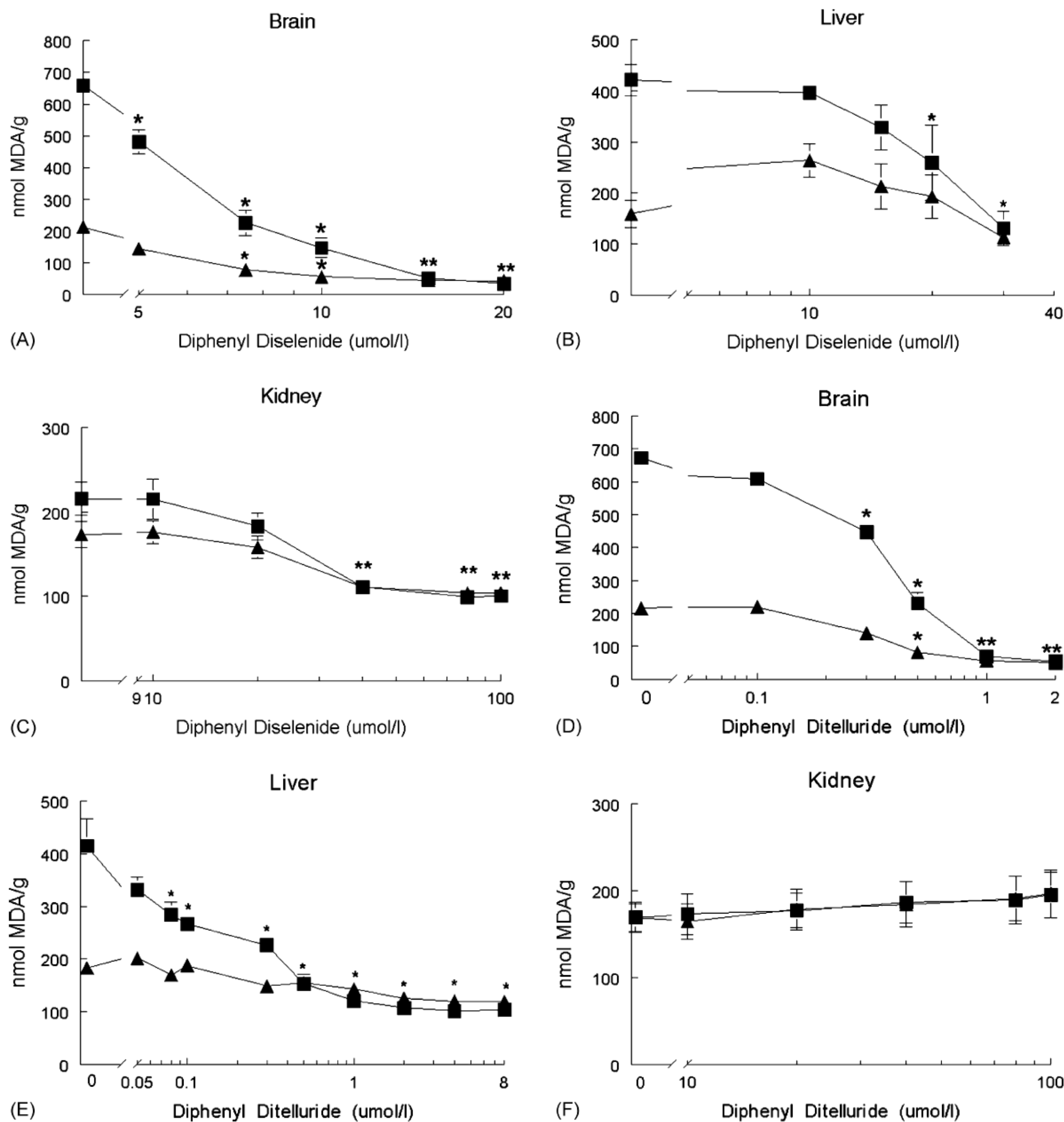


Fig. 3. Effect of diphenyl diselenide (A–C) and diphenyl ditelluride (D–F) on basal (▲) or oxalate-induced (■, 2 mmol/l) TBARS production in supernatants of homogenates from brain, liver and kidney of rats. Low-speed supernatant (S1) from tissues were incubated for 60 min in a medium containing 10 mmol/l Tris/HCl buffer, pH 7.4. (A and D) brain preparations; (B and E) liver; (C and F) kidney. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. ($n = 4$–5). $^{*}p < 0.05$ from respective control (no diphenyl diselenide or diphenyl ditelluride) by Duncan's multiple range test.

Table 1
IC_{50} (μmol/l) values for basal and oxalate-stimulated TBARS production for diphenyl diselenide and diphenyl ditelluride

	Diphenyl diselenide		Diphenyl ditelluride	
	Basal	Oxalate	Basal	Oxalate
Brain	5.36	6.09	0.27	0.36
Liver	>30	18.96	>8	0.14
Kidney	22.5	ND	>100	ND

ND: not determined.

rat brain preparations (Fig. 3A). Diphenyl diselenide effect against basal TBARS production was significant at 7.5 μmol/l onwards with maximum effect at 10 μmol/l. Similarly, diphenyl diselenide effect against oxalate-induced TBARS production was significantly at 5 μmol/l exhibiting a maximum effect at 15 μmol/l (Fig. 3A). The IC_{50} (concentration of diphenyl diselenide necessary to inhibit TBARS production by 50%) values for basal and oxalate-induced TBARS production were approximately 5.36 and 6.09 μmol/l respectively (Table 1).

In liver supernatants, diphenyl diselenide did not display anti-oxidant activity under non-stimulated assay conditions. However, diphenyl diselenide significantly reduced oxalate-induced TBARS production at higher concentrations (20 and 30 μmol/l) to basal values (Fig. 3B). The IC_{50} values of diphenyl diselenide for basal (>30 μmol/l) and oxalate (18.96 μmol/l)-induced TBARS production in the liver preparations are showed in Table 1.

In contrast to liver and brain preparations, oxalate did not induce TBARS production in supernatants of homogenates from kidney (Fig. 1). However, diphenyl diselenide significantly prevented TBARS production determined either in the presence or absence of oxalate. The maximum effect of diphenyl diselenide was obtained at 40 μmol/l (Fig. 3C). The IC_{50} value of diphenyl diselenide for basal TBARS production in the kidney preparations is showed in Table 1.

3.4. *Effect of diphenyl ditelluride on basal or oxalate-induced TBARS production in brain, liver and kidney preparations*

Diphenyl ditelluride significantly reduced either basal or oxalate-induced TBARS production in rat brain preparations (Fig. 3D). Diphenyl ditelluride effect against basal TBARS production was maximal at 0.5 μmol/l, whereas diphenyl ditelluride effect against oxalate-induced TBARS production was maximal at 1 μmol/l (Fig. 3D). The IC_{50} values of diphenyl ditelluride for basal (0.27 μmol/l) and oxalate (0.36 μmol/l)-induced TBARS production in the brain preparations are showed in Table 1.

Similarly to that observed with diphenyl diselenide, diphenyl ditelluride did not exert any effect against basal TBARS production when liver preparations were used (Fig. 3E). However, diphenyl ditelluride significantly reduced oxalate-induced TBARS production at 0.8 μmol/l with maximum effect at 1 μmol/l (Fig. 3E). The IC_{50} values of diphenyl ditelluride for basal (>8 μmol/l) and oxalate (0.14 μmol/l)-induced TBARS production in the liver preparations are showed in Table 1.

Diphenyl ditelluride did not exert any effect against TBARS production when kidney preparations were used either in the presence or in the absence of oxalate (Fig. 3F).

3.5. *Iron chelating properties of oxalate*

Since at higher concentrations oxalate reduced TBARS production in brain and in liver preparations, we examined whether this effect could be related to its iron chelating properties. Oxalate chelated Fe^{2+} in a concentration-dependent manner and this was significant from 0.5 mmol/l onwards. The maximum reduction in free Fe^{+2} in the medium reaction was obtained at the highest concentration of oxalate used (20 mmol/l) and was about 93% (data not shown). These results confirm the chelating properties of the oxalate, which can be responsible for the observed antioxidant activity of oxalate at elevated concentrations.

3.6. *Effect of oxalate on deoxyribose degradation*

In order to determine whether iron/oxalate complexes are active in biological system, we determine their effect on deoxyribose degradation assay (Fenton reaction). Fig. 4 shows that either Fe^{2+} or H_2O_2 are able to stimulate deoxyribose degradation. However, in the presence of Fe^{2+} and H_2O_2 deoxyribose degradation was higher than that determined in the presence of each compound alone (Fig. 4). Oxalate did not exert any effect on deoxyribose degradation when Fe^{2+} or H_2O_2 were used alone. However, oxalate at 20 mmol/l significantly prevented deoxyribose degradation induced by Fe^{2+} and H_2O_2 (Fig. 4). These results suggest that Fe^{2+}–oxalate complexes formed at lower oxalate concentrations are bioactive, since at 2 mmol/l oxalate was non-effective in preventing deoxyribose degradation. Alternatively, at low concentrations of oxalate, the ratio between free-to-complexed iron was sufficiently high to cause maximal deoxyribose degradation.

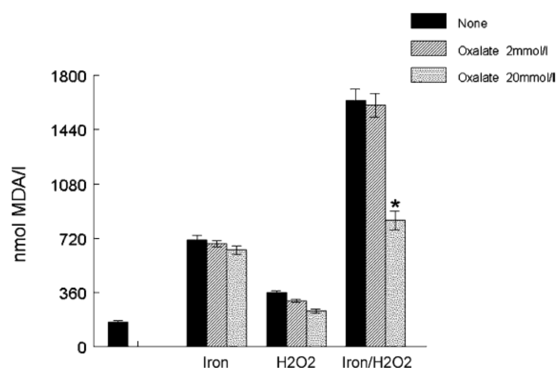


Fig. 4. Effect of oxalate on deoxyribose degradation. Effect of sodium oxalate against deoxyribose degradation induced by Fe^{2+} 50 μmol/l and/or hydrogen peroxide 500 μmol/l. The values are expressed as nmol MDA per liter. Data are expressed as means ± S.E.M. ($n = 6$). $^{*}p < 0.05$ from deoxyribose degradation induced by Fe^{2+} and hydrogen peroxide without oxalate by Duncan's multiple range test.

3.7. *Effect of oxalate on iron-induced TBARS production*

In order to clarify the role of oxalate chelating properties on modulation of TBARS production, we examined whether oxalate could modulate the effect of iron (10 μmol/l)-induced TBARS production in supernatants from rat brain (Fig. 5A) and liver (Fig. 5B) homogenates. Oxalate did not significantly modify iron-induced TBARS production (up to 4 mmol/l), whereas it caused significant reduction in Fe^{2+}-induced TBARS production at higher concentrations (8–32 mmol/l in rat brain preparations (Fig. 5A)). In liver homogenates, oxalate significantly enhanced iron-induced TBARS production at lower concentrations (1 up to 4 mmol/l) preventing iron-induced TBARS production at higher concentrations (16 and 32 mmol/l, Fig. 5B). These results confirm the involvement of oxalate chelating properties in modulating TBARS production.

3.8. *Effect of deferoxamine (DFO) on basal or oxalate-induced TBARS production*

To further substantiate that iron is involved in the pro-oxidative activity of oxalate, we also used a well-characterized iron chelator DFO as a positive control. DFO (10 μM) significantly prevented both basal and oxalate (2 mmol/l)-induced TBARS production in brain preparations (Fig. 6A). The same effect of DFO was observed in liver preparations (Fig. 6B). This results support the involvement of iron on oxalate pro-oxidative activity.

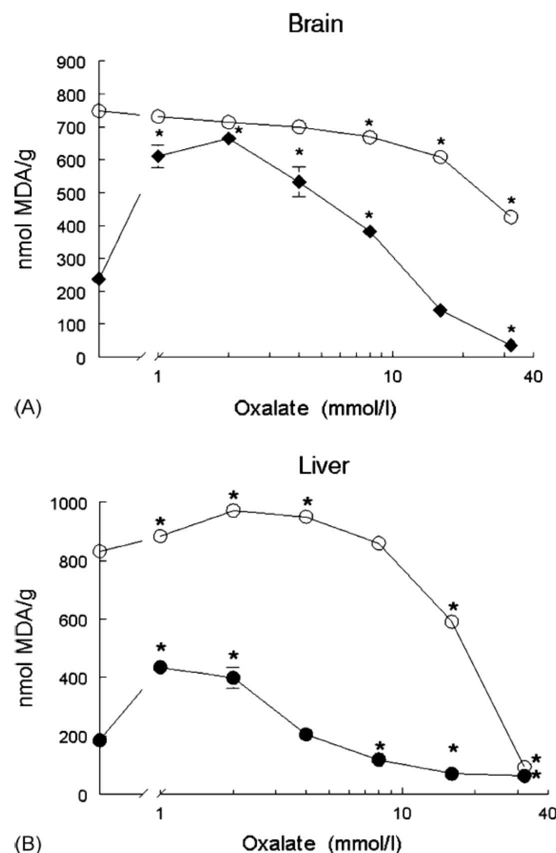


Fig. 5. Effect of oxalate on iron-induced TBARS production. Low-speed supernatant (S1) from tissues were incubated for 60 min in a medium containing 10 mmol/l Tris/HCl buffer, pH 7.4. (A) Effect of sodium oxalate (1 up to 32 mmol/l) on basal or iron-induced TBARS production in brain preparations. (♦) none, (○) iron 10 μmol/l; (B) effect of sodium oxalate (1 up to 32 mmol/l) on basal or iron-induced TBARS production in liver preparations. (●) none, (○) iron 10 μmol/l. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. ($n = 3$). $^{*}p < 0.05$ from respective control (no oxalate) by Duncan's multiple range test.

4. Discussion

The results presented in this paper showed that oxalate stimulated TBARS production in supernatants from homogenates of brain and in liver at lower concentrations used, whereas at higher studied concentrations it caused a significant reduction in the TBARS production. These antioxidant properties of oxalate against liver and brain preparations are in accordance with previous paper, showing that oxalate was able to prevent lipid peroxidation in rat liver microsomes and rat brain in a concentration dependent-fashion [48]. Furthermore, these authors showed that oxalate significantly prevent

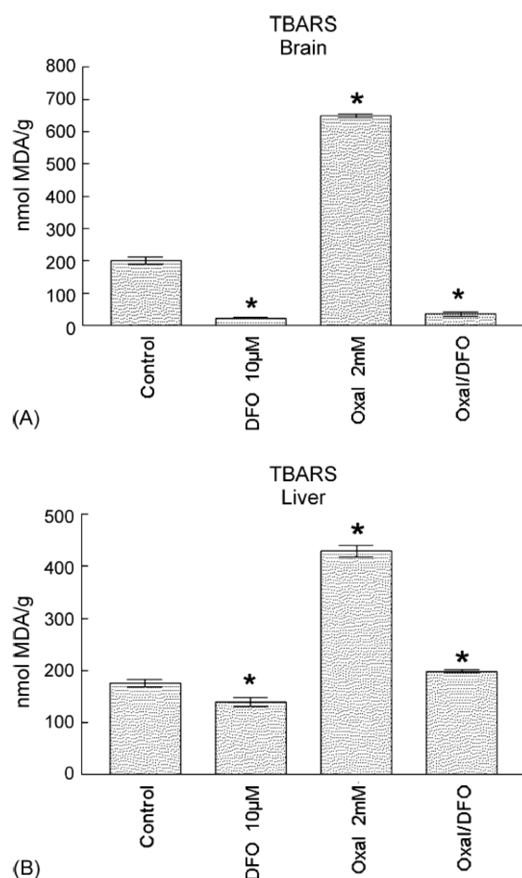


Fig. 6. Effect of DFO on basal or oxalate-induced TBARS production. Low-speed supernatant (S1) from brain (A) or liver (B) were incubated for 60 min in a medium containing 10 mmol/l Tris/HCl buffer, pH 7.4, in the presence or absence of oxalate (2 mmol/l) or DFO (10 μM). The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. ($n = 3$). $^{*}p < 0.05$ from respective control (no oxalate) by Duncan's multiple range test.

ascorbic acid oxidation induced by H_2O_2 and Cu^{2+} [48]. However, we demonstrated in this study, that oxalate did not induced TBARS production in the kidney preparations. The reasons for these unexpected results can be related to the fact that addition of sodium oxalate to kidney homogenates results in the formation of non-soluble oxalate crystals, a phenomenon not observed in brain or liver homogenates (see Fig. 2). Thus, the formation of crystals could reduce the effective free oxalate concentrations in kidney supernatants. From a theoretical point of view, the formation of a non-soluble crystal should reduce the area with contact with the surrounding medium and reduce its ability to induce specific chemical reaction (in the case of the present investigation TBARS formation). However, data from literature in this regard is somewhat controversial and it has been generally assumed that the crystals of oxalate are the forms that cause oxidative stress in intact systems [8,49]. In contrast, there are also data in literature supporting a role for soluble oxalate as a pro-oxidant [50–52].

On limitation of the present study was the use of homogenates and the relevance of the present results would dramatically increase if they could be reproduced the in cellular models (e.g., in primary cultures of the tissues). In spite of this, the results of the present investigation indicated a clear effect of renal supernatants as inductor of oxalate crystals (Fig. 2). Thus, taken together with some literature data showing that in intact cellular systems calcium oxalate crystals is more powerful than soluble oxalate as a renal toxic agent [8,53], we propose that *in vivo* oxalate is more toxic to kidney than to other organs such as brain and liver due to renal facility in promoting the formation of oxalate crystals. However, at the present moment its not possible to disregard a role for soluble oxalate on its renal toxicity.

The results presented in this paper confirm the antioxidant properties of diphenyl diselenide and diphenyl ditelluride against lipid peroxidation in the supernatants of homogenates from brain and liver under *in vitro* conditions. This antioxidant effect on TBARS production are in accordance with previous study of our group showing that diphenyl diselenide and diphenyl ditelluride are effective antioxidants against TBARS production induced by different pro-oxidant agents in the supernatants of homogenates from rat brain [25]. However, this is the first paper showing that diphenyl diselenide and diphenyl ditelluride can protect against TBARS production induced by oxalate ions in the liver and in the brain preparations. Furthermore, to the best of our knowledge, it is the first publication showing that the antioxidant activity of organochalcogenides varied considerably among supernatants of homogenates from different rat tissues. In fact, the antioxidant potency of chalcogenides was higher in brain than in liver preparations and nearly without antioxidant activity in kidney preparations.

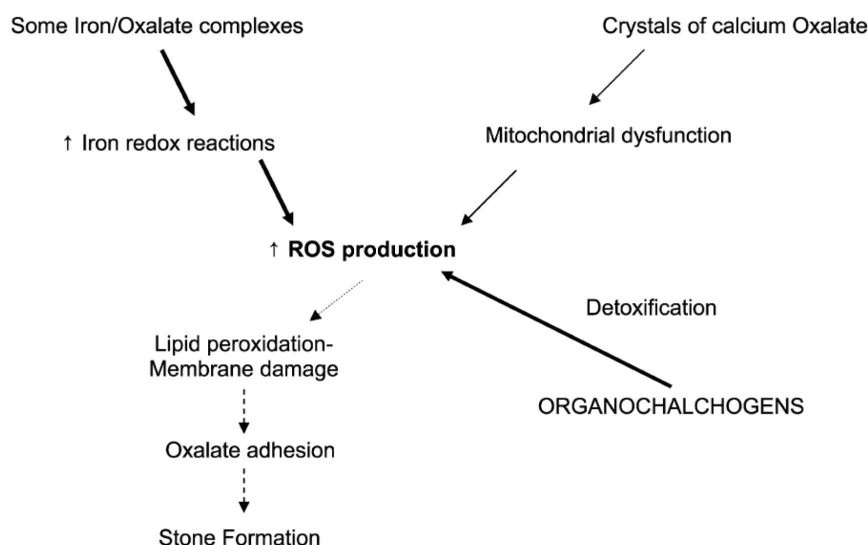
Deposition of calcium oxalate crystals in brain and meninges is exceptionally rare in primary oxalosis, however, a case of primary hyperoxaluria and oxalosis with chronic renal failure, crystalline myocarditis, and disseminated calcium oxalate crystal deposition in various tissues including the brain and meninges was described [54]. Here, we showed that oxalate could induce TBARS production in the brain preparations, in which accumulation of oxalate during severe episodes of hyperoxaluria could leads to brain lesion [54] will wait future studies.

In the same way, the liver can be recognized as a potential target to oxalate-induced damage during hyperoxaluria. In line with this, earlier studies showed that in the liver of hyperoxaluric rats a markedly increased in the activities of lactate dehydrogenase, glycolic acid oxidase and xanthine oxidase occur [55]. Most importantly, these authors showed that Vitamin E protected against the toxicity of oxalate [55] and decreased the level of malondialdehyde induced by oxalate [55,56]. Likewise, increase in lipid peroxidation and superoxide dismutase (SOD) activity, associated with a decrease in catalase activity and glutathione (GSH) level in liver of hyperoxaluric rats was related by Sumathi et al. [5]. Additionally, oxalate was also showed to inhibit the gluconeogenesis and glycolysis, and stimulate the glycogenolysis either in isolated hepatocytes or liver slices [57]. However, no data are reported in the literature considering the oxalate effect on the metabolic pathways in the brain or kidney.

The antioxidant activity of oxalate observed at higher used concentrations can be related to its iron chelating properties. In agreement with this, oxalate seems to be an effective gallium chelator (III) [58]. Here we found that oxalate was an effective Fe^{2+} chelator (not shown). We showed in this paper, that oxalate at lower studied concentrations was able to increase iron pro-oxidant activity in the liver (Fig. 5B) without change iron-induced TBARS production in the brain (Fig. 5A) preparations. Moreover, oxalate at higher studied concentrations was able to reduced iron-induced TBARS production both in brain and in liver (Fig. 5A and B) preparations. The involvement of iron on oxalate activity is strongly supported by the results showing that DFO suppress the oxalate-induced TBARS production, either in brain as in liver (Fig. 6) preparations. However, some ferric oxalate complexes are photocatalytic. Thus, it is possible that this played a role in the observed results.

The iron-oxalate complexes formed at lower used oxalate concentrations did not prevent deoxyribose degradation induced by Fe^{2+} and H_2O_2, indicating that at these concentrations they form bioactive complexes. In contrast, iron-oxalate complexes formed at higher used oxalate concentrations significantly prevent deoxyribose degradation induced by Fe^{2+} and H_2O_2 (Fig. 4). However, previous studies have demonstrated that oxalate can act as a hydroxyl radical scavenger yielding formate and superoxide [59]. Thus, the possibility of direct radical scavenging at high used oxalate concentrations should be considered as an alternative to iron complexation.

Based on the presented results, we could speculate that at higher studied concentrations, oxalate prevent TBARS production by modulate an inadequate ratio Fe^{2+}/Fe^{3+}, which is essential for free radicals generation, thus reducing free radicals generation due to iron redox cycle. However, at lower used concentrations, it is possible that an adequate ratio Fe^{2+}/Fe^{3+} is formed, that in turn increased iron redox cycle, free radicals levels and lipid peroxidation (TBARS). This conclusion is supported by earlier studies showing that adequate ratio Fe^{2+}/Fe^{3+} is essential for the TBARS production (free radicals generation due to iron redox cycle) [60,61]. In



Scheme 1. Scheme showing the possible site of action of organochalcogens.

addition, our conclusion is supported by previous studies showing that oxalate administered with Fe^{2+} cause an increase in the TBARS production [62,63].

In conclusion, our data support the protective role of diphenyl diselenide and diphenyl ditelluride against lipid peroxidation induced by oxalate. The presented results can be useful to further investigations *in vivo*, under hyperoxaluria conditions. We suppose that amelioration of lipid peroxidation is related to attenuation of calcium oxalate disease, once that the oxidative damage to biological membranes is essential to crystal formation (see Scheme 1 proposed). In addition, our results suggest that the anti- and pro-oxidant activity of oxalate *in vitro* is related to its ability on chelate Fe^{2+} ions. In short, we assume that at low used concentrations oxalate forms pro-oxidant complexes and at higher used oxalate concentrations form less-active complexes.

Acknowledgments

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References

[1] T. Bijikurien, R. Selvam, Induction of lipid peroxidation by oxalate in experimental rat urolithiasis, J. Biosci. 12 (1987) 367–373.

[2] N. Saravanan, D. Senthil, P. Varalakshmi, Effect of L-cysteine on lipid peroxidation in experimental urolithiatic rats, Pharmacol. Res. 32 (1995) 165–169.

[3] Y. Kohjimoto, L. Kennington, C.R. Scheid, T.W. Honeyman, Role of phospholipase A2 in the cytotoxic effects of oxalate in cultured renal epithelial cells, Kidney Int. 56 (1999) 1432–1441.

[4] S. Tamilselvan, K.J. Byer, R.L. Hackelt, S.R. Khan, Free radical scavengers, catalase and superoxide dismutase provide protection from oxalate associated injury to LLC-PK1 and MDCK cells, J. Urol. 164 (2000) 224–229.

[5] R. Sumathi, S. Jayanthi, V. Kalpanadevi, P. Varalakshmi, Effect of DL alpha-lipoic acid on tissue lipid peroxidation and antioxidant systems in normal and glycollate treated rats, Pharmacol. Res. 27 (1993) 309–318.

[6] A. Muthukumar, R. Selvam, Role of glutathione on renal mitochondrial status in hyperoxaluria, Mol. Cell Biochem. 185 (1998) 77–84.

[7] C. Scheid, H. Koul, A. Hill, J. Luber-Narod, L. Kennington, J. Jonassen, M. Menon, Oxalate toxicity in LLC-PK1[r] cells: role of free radicals, Kidney Int. 49 (1996) 413–419.

[8] F.D. Khand, M.P. Gordge, W.G. Robertson, A.A. Noronha-Dutra, J.S. Hothersall, Mitochondrial superoxide production during oxalate mediated oxidative stress in renal epithelial cells, Free Radic. Biol. Med. 32 (2002) 1339–1350.

[9] A.J. Kowaltowski, A.E. Vercesi, Mitochondrial damage induced by conditions of oxidative stress, Free Radic. Biol. Med. 26 (1999) 463–471.

[10] A.E. Vercesi, A.J. Kowaltowski, M.T. Grijalba, A.R. Meinicke, R.F. Castilho, The role of reactive oxygen species in mitochondrial permeability transition, Biosci. Rep. 17 (1997) 43–52.

[11] T. Bijikurien, R. Selvam, Induction of lipid peroxidation in calcium stone formation, Indian J. Exp. Biol. 27 (1989) 450–453.

[12] V. Ravichandran, R. Selvam, Increased lipid peroxidation in kidney of Vitamin B6 deficient rats, Biochem. Int. 21 (1999) 599–605.

[13] R. Selvam, D. Sridevi, Induction of oxalate binding by lipid peroxidation in rat kidney mitochondria, Biochem. Int. 23 (1991) 1007–1017.

[14] R. Selvam, T. Bijikurien, Methionine feeding prevents kidney stone deposition by restoration of free radical mediate changes in experimental rat urolithiasis, J. Nutr. Biochem. 2 (1991) 644–651.

[15] A. Muthukumar, R. Selvam, Effect of depletion of reduced glutathione and its supplementation by glutathione monoester on renal oxalate retention in hyperoxaluria, J. Nutr. Biochem. 8 (1997) 445–450.

[16] F. Grases, L. Garcia-Ferragut, A. Costa-Bauza, Development of calcium-oxalate crystals on urothelium: effect of free radicals, Nephron 78 (1998) 296–301.

[17] V. Perez-De La Cruz, C. Gonzalez-Cortes, J. Pedraza-Chaverri, P.D. Maldonado, L. Andres-Martinez, A. Santamaria, Protective effect of *S*-allylcysteine on 3-nitropropionic acid-induced lipid peroxidation and mitochondrial dysfunction in rat brain synaptosomes, Brain Res. Bull. 68 (2006) 379–383.

[18] F. Perez-Severiano, M. Rodriguez-Perez, J. Pedraza-Chaverri, P.D. Maldonado, O.N. Medina-Campos, A. Ortiz-Plata, A. Sanchez-Garcia, J. Villeda-Hernandez, S. Galvan-Arzate, P. Aguilera, A. Santamaria, *S*-Allylcysteine, a garlic-derived antioxidant, ameliorates quinolinic acid-induced neurotoxicity and oxidative damage in rats, Neurochem. Int. 45 (2004) 1175–1183.

[19] A. Santamaria, R. Salvatierra-Sanchez, B. Vazquez-Roman, D. Santiago-Lopez, J. Villeda-Hernandez, S. Galvan-Arzate, M.E. Jimenez-Capdeville, S.F. Ali, Protective effects of the antioxidant selenium on quinolinic acid-induced neurotoxicity in rats: in vitro and in vivo studies, J. Neurochem. 86 (2003) 479–488.

[20] A. Muller, H. Gabriel, H. Sies, A novel biologically active selenoorganic compound. IV. Protective glutathione-dependent effect of PZ51 (Ebselen) against ADP-Fe induced lipid peroxidation in isolated hepatocytes, Biochem. Pharmacol. 34 (1985) 1185–1189.

[21] A. Wendel, M. Fausel, H. Safayi, R. Otter, A novel biologically active seleno-organic compound. II. Activity of PZ 51 in relation to glutathione peroxidase, Biochem. Pharmacol. 33 (1984) 3241–3245.

[22] F.W. Santos, G. Zeni, J.B.T. Rocha, S.N. Weis, J.M. Fachinetto, A.M. Fávero, C.W. Nogueira, Diphenyl diselenide reverses cadmium-induced oxidative damage on mice tissues, Chem. Biol. Interact. 151 (2005) 159–165.

[23] L. Engman, J. Persson, K. Vessman, M. Ekstrom, M. Berglund, C. Andersson, Organotellurium compounds as efficient retards of lipid peroxidation in methanol, Free Radic. Biol. Med. 19 (1995) 441–452.

[24] T. Posser, M.B. Moretto, A.L. Dafre, M. Farina, J.B.T. Rocha, C.W. Nogueira, G. Zeni, J.S. Ferreira, R.B. Leal, J.L. Franco, Antioxidant effect of diphenyl diselenide against sodium nitruprusside (SNP) induced lipid peroxidation in human platelets and erythrocytes membranes: an in vitro evaluation, Chem. Biol. Interact. 164 (2006) 126–135.

[25] J.I. Rossato, L.A. Ketzer, F.B. Centurião, S.J.N. Silva, D.S. Ludtke, G. Zeni, A.L. Braga, M.A. Rubin, J.B.T. Rocha, Antiox-

idant properties of new chalcogenides against lipid peroxidation in rat brain, Neurochem. Res. 27 (2002) 297–303.

[26] L.P. Borges, C.W. Nogueira, R.B. Panatieri, J.B.T. Rocha, G. Zeni, Acute liver damage induced by 2-nitropropane in rats: effect of diphenyl diselenide on antioxidant defenses, Chem. Biol. Interact. 160 (2006) 99–107.

[27] F.W. Santos, T. Oro, G. Zeni, J.B.T. Rocha, P.C. Nascimento, C.W. Nogueira, Cadmium induced testicular damage and its response to administration of succimer and diphenyl diselenide in mice, Toxicol. Lett. 152 (2004) 255–263.

[28] F.C. Meotti, V.C. Borges, G. Zeni, J.B.T. Rocha, C.W. Nogueira, Potential renal and hepatic toxicity of diphenyl diselenide, diphenyl ditelluride and Ebselen for rats and mice, Toxicol. Lett. 143 (2003) 9–16.

[29] A.F. de Bem, R.D.L. Portella, J. Perottoni, E. Becker, D. Bohrer, M.W. Paixão, C.W. Nogueira, G. Zeni, J.B.T. Rocha, Changes in biochemical parameters in rabbits blood after oral exposure to diphenyl diselenide for long periods, Chem. Biol. Interact. 162 (2006) 1–10.

[30] C.W. Nogueira, L.N. Rotta, G. Zeni, D.O. Souza, J.B.T. Rocha, Exposure to Ebselen changes glutamate uptake and release by rat brain synaptosomes, Neurochem. Res. 3 (2002) 283–288.

[31] E.N. Maciel, R.C. Bolzan, A.L. Braga, J.B.T. Rocha, Diphenyl diselenide and diphenyl ditelluride differentially affect δ-aminolevulinate dehydratase from liver, kidney, and brain of mice, J. Biochem. Mol. Toxicol. 14 (2000) 310–319.

[32] N.B.V. Barbosa, J.B.T. Rocha, G. Zeni, T. Emanuelli, M.C. Beque, A.L. Braga, Effect of organic forms of selenium on δ-aminolevulinate dehydratase from liver, kidney and brain of adult rats, Toxicol. Appl. Pharmacol. 149 (1998) 243–253.

[33] C.W. Nogueira, G. Zeni, J.B.T. Rocha, Organoselenium and organotellurium compounds: toxicology and pharmacology, Chem. Rev. 104 (2004) 6255–6285.

[34] C.M. Anderson, A. Hallberg, R. Brattsand, I.A. Cotgreave, L. Engman, J. Person, Glutathione peroxidase-like activity of diaryl tellurides, Bioorg. Med. Chem. Lett. 3 (1993) 2553–2558.

[35] C.M. Anderson, R. Brattsand, A.R. Hallberg, L. Engman, J. Persson, P. Moldéus, I. Cotgreave, Diaryl tellurides as inhibitors of lipid peroxidation in biological and chemical systems, Free Radic. Res. 20 (1994) 401–410.

[36] R.M. Rosa, D.G. Flores, H.R. Appelt, A.L. Braga, J.A.P. Henriques, R. Roesler, Facilitation of long-term object recognition memory by pretraining administration of diphenyl diselenide in mice, Neurosci. Lett. 341 (2003) 217–220.

[37] G. Ghisleni, L.O. Porciúncula, H. Cimarostia, J.B.T. Rocha, C.G. Salbego, D.O. Souza, Diphenyl diselenide protects rat hippocampal slices submitted to oxygen-glucose deprivation and diminishes inducible nitric oxide synthase immunocontent, Brain Res. 986 (2003) 196–199.

[38] C.W. Nogueira, E.B. Quinhones, E.A.C. Jung, G. Zeni, J.B.T. Rocha, Anti-inflammatory and antinociceptive activity of diphenyl diselenide, Inflamm. Res. 52 (2003) 56–63.

[39] R.M. Rosa, K. Sulzbacher, J.N. Picada, R. Roesler, J. Saffi, M. Brendel, J.A.P. Henriques, Genotoxicity of diphenyl diselenide in bacteria and yeast, Mutat. Res. 563 (2004) 107–115.

[40] H. Ohkawa, N. Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95 (1979) 351–358.

[41] R.L. Puntel, C.W. Nogueira, J.B.T. Rocha, Krebs cycle intermediates modulate thiobarbituric acid reactive species (TBARS) production in rat brain *in vitro*, Neurochem. Res. 30 (2005) 225–235.

[42] R.L. Puntel, C.W. Nogueira, J.B.T. Rocha, *N*-Methyl-D-aspartate (NMDA) receptors are involved in the quinolinic acid (QA), but not in the malonate pro-oxidative activity *in vitro*, Neurochem. Res. 30 (2005) 417–424.

[43] B. Halliwell, J.M.C. Gutteridge, Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts: the role of superoxide and hydroxyl radicals, FEBS Lett. 128 (1981) 347–352.

[44] J.M.C. Gutteridge, Thiobarbituric acid-reactivity following iron-dependent free-radical damage to amino acids and carbohydrates, FEBS Lett. 128 (1981) 343–346.

[45] N. Petragnani, Tellurium in Organic Synthesis, Academic Press, San Diego, 1994, p. 248.

[46] C. Paulmier, Selenium Reagents and Intermediates in Organic Synthesis, Pergamon Press, New York, 1986, p. 463.

[47] M. Dixon, E.C. Webb, Enzymes, 2nd ed., Longmans, London and Colchester, 1964, p. 950.

[48] T. Kayashima, T. Katayama, Oxalic acid is available as a natural antioxidant in some systems, Biochim. Biophys. Acta 1573 (2002) 1–3.

[49] T. Umekawa, N. Chegini, S.R. Khan, Increased expression of monocyte chemoattractant protein-1 (MCP-1) by renal epithelial cells in culture on exposure to calcium oxalate, phosphate and uric acid crystals, Nephrol. Dial. Transplant. 18 (2003) 664–669.

[50] E. Meimaridou, J. Jacobson, A.M. Seddon, A.A. Noronha-Dutra, W.G. Robertson, J.S. Hothersall, Crystal and microparticle effects on MDCK cell superoxide production: oxalate-specific mitochondrial membrane potential changes, Free Radic. Biol. Med. 38 (2005) 1553–1564.

[51] T. Rashed, M. Menon, S. Thamilselvan, Molecular mechanism of oxalate-induced free radical production and glutathione redox imbalance in renal epithelial cells: effect of antioxidants, Am. J. Nephrol. 24 (2004) 557–568.

[52] S. Thamilselvan, K.R. Khan, M. Menon, Oxalate and calcium oxalate mediated free radical toxicity in renal epithelial cells: effect of antioxidants, Urol. Res. 31 (2003) 3–9.

[53] E. Meimaridou, J. Jacobson, A.M. Seddon, A.A. Noronha-Dutra, W.G. Robertson, J.S. Hothersall, Crystal and microparticle effects on MDCK cell superoxide production: oxalate-specific mitochondrial membrane potential changes, Free Radic. Biol. Med. 38 (2005) 1553–1564.

[54] M.T. Haqqani, Crystals in brain and meninges in primary hyperoxaluria and oxalosis, J. Clin. Pathol. 30 (1977) 16–18.

[55] M. Adhirai, R. Selvam, Effect of cyclosporin on liver antioxidants and the protective role of Vitamin E in hyperoxaluria in rats, J. Pharm. Pharmacol. 50 (1998) 501–505.

[56] M. Adhirai, R. Selvam, Effect of cyclosporin A on tissue lipid peroxidation and membrane bound phosphatases in hyperoxaluric rat and the protection by Vitamin E pretreatment, Jpn. J. Med. Sci. Biol. 1 (1997) 9–17.

[57] F.A. Tonon, F.S. Kemmelmeier, A. Bracht, E.L. Ishii-Iwamoto, E.A. Nascimento, Metabolic effects of oxalate in the perfused rat liver, Comparat. Biochem. Physiol. 121 (1998) 91–97.

[58] J.L. Domingo, J.M. Llobet, J. Corbella, Relative efficacy of chelating agents as antidotes for acute gallium nitrate intoxication, Arch. Toxicol. 59 (1987) 382–383.

[59] G.E. Adams, R.L. Willson, Pulse radiolysis studies on the oxidation of organic radicals in aqueous solution, Transact. Faraday Soc. 65 (1969) 2981–2987.

[60] A.A. Caro, A.T. Cederbaum, Antioxidant properties of *S*-adenosyl-L-methionine in Fe^{2+}-initiated oxidations, Free Radic. Biol. Med. 36 (2004) 1303–1316.
[61] L.X. Tang, J.L. Yang, X. Shen, Effects of additional iron-chelators on Fe^{2+}-initiated lipid peroxidation: evidence to support the $Fe^{2+}\ldots Fe^{3+}$ complex as the initiator, J. Inorg. Biochem. 68 (1997) 265–272.
[62] J.M.C. Gutteridge, G.J. Quindan, Malonaldehyde formation from lipid peroxides in TBA test: the role of lipid radicals, iron salts and metal chelators, J. Appl. Biochem. 5 (1983) 293–299.
[63] F. Ursini, M. Mariorino, P. Hochstein, L. Ernster, Microsomal lipid peroxidation: mechanisms of initiation. The role of iron and iron chelators, Free Radic. Biol. Med. 6 (1989) 31–36.

4.2.2. – ESTUDO DO POTENCIAL ANTIOXIDANTE DOS INTERMEDIÁRIOS DO CICLO DE KREBS SOBRE A GERAÇÃO DE TBARS INDUZIDA POR MALONATO: ESTUDOS *IN VITRO*

Artigo 4

ANTIOXIDANT PROPERTIES OF KREBS CYCLE INTERMEDIATES AGAINST MALONATE PRO-OXIDANT ACTIVITY IN VITRO: A COMPARATIVE STUDY USING THE COLORIMETRIC METHOD AND HPLC ANALYSIS TO DETERMINE MALONDIALDEHYDE IN RAT BRAIN HOMOGENATES

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Antioxidant properties of Krebs cycle intermediates against malonate pro-oxidant activity in vitro: A comparative study using the colorimetric method and HPLC analysis to determine malondialdehyde in rat brain homogenates

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Abstract

A variety of Krebs cycle intermediaries has been shown to possess antioxidant properties in different in vivo and in vitro systems. Here we examined whether citrate, succinate, malate, oxaloacetate, fumarate and α-ketoglutarate could modulate malonate-induced thiobarbituric acid-reactive species (TBARS) production in rat brain homogenate. The mechanisms involved in their antioxidant activity were also determined using two analytical methods: 1) a popular spectrophotometric method (Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry 95, 351–358.) and a high performance liquid chromatographic (HPLC) procedure (Grotto, D., Santa Maria, L. D., Boeira, S., Valentini, J., Charão, M. F., Moro, A. M., Nascimento, P. C., Pomblum, V. J., Garcia, S. C., 2006. Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography–visible detection. Journal of Pharmaceutical and Biomedical Analysis 43, 619–624.). Citrate, malate, and oxaloacetate reduced both basal and malonate-induced TBARS production. Their effects were not changed by pre-treatment of rat brain homogenates at 100 °C for 10 min. α-Ketoglutarate increased basal TBARS without changing malonate-induced TBARS production in fresh and heat-treated homogenates. Succinate reduced basal—without altering malonate-induced TBARS production. Its antioxidant activity was abolished by KCN or heat treatment. Fumarate reduced malonate-induced TBARS production in fresh homogenates; however, its effect was completely abolished by heat treatment. There were minimal differences among the studied methods. Citrate, oxaloacetate, malate, α-ketoglutarate and malonate showed iron-chelating activity. We suggest that antioxidant properties of citrate, malate and oxaloacetate were due to their ability to cancel iron redox activity by forming inactive complexes, whereas α-ketoglutarate and malonate pro-oxidant activity can be due to formation of active complexes with iron. In contrast, succinate and fumarate antioxidant activity was probably due to some enzymatic system.
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Keywords: Malonate; Iron; TBARS; HPLC; Krebs cycle intermediates; Free radicals

Introduction

Free radicals are now accepted as important mediators of tissue injury in several neurodegenerative states (Beal, 1996; Simonian and Coyle, 1996) and in some pathological conditions (Ji et al., 2003; Sun et al., 2005a,b). In fact, free radicals attack membrane lipids, proteins and nucleic acids, which can cause cell damage or death (Halliwell, 1992). In this scenario, antioxidants play a fundamental role as scavengers and antagonists of reactive oxygen species (ROS) (Puttfarcken et al., 1993; Farina et al., 2003; Jara-Prado et al., 2003; Santamaria et al., 2003; Perez-Severiano et al., 2004; Perez-De La Cruz et al., 2006; Sutherland et al., 2005, 2006; Posser et al., 2006).

Previous data from the literature have demonstrated that some Krebs cycle intermediates can act as antioxidants against a variety

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of in vitro and in vivo pro-oxidant situations (Hatefi and Hanstein, 1970; Takeshige and Minakami, 1975; Takayanagi et al., 1980; Bindoli et al., 1982; Cavallini et al., 1984; Baker and Gebicki, 1986; Vianello et al., 1986; Chiueh et al., 1993; Abrahamson et al., 1994; Gutteridge, 1994; Desagher et al., 1997; Mallet and Sun, 1999; Sokolowska et al., 1999; Mallet, 2000; Velvizhi et al., 2002a,b; Mallet and Sun, 2003; Yamamoto and Mohanan, 2003; Puntel et al., 2005a). In line with this, literature data have shown that α-ketoacids can non-enzymatically neutralize peroxides (Desagher et al., 1997; Sokolowska et al., 1999; Velvizhi et al., 2002a,b; Mallet and Sun, 2003; Yamamoto and Mohanan, 2003), protect post-ischemic myocardium (Mallet and Sun, 1999, 2003; Mallet, 2000), prevent damage to mitochondrial DNA (Yamamoto and Mohanan, 2003), and inhibit in vivo ammonium acetate- (Velvizhi et al., 2002a) and ethanol-induced oxidative stress (Velvizhi et al., 2002b). However the precise mechanism(s) by which they produce antioxidant activity remains unclear and more studies are necessary in order to clarify their effects.

Citrate, the first Krebs cycle intermediate, forms complexes with Fe (II) and (III) ions which are either more or less active than the free iron ions (Baker and Gebicki, 1986; Abrahamson et al., 1994). Consequently, iron chelation can change its availability to participate in reactions that initiate or propagate lipid peroxidation. In fact, in the presence of a chelator, the initiation of lipid peroxidation is strictly dependent on the ratio chelator to iron (Tang et al., 1997; Caro and Cederbaum, 2004). In this context, literature indicated that increased redox cycling of iron complexes can cause iron-catalyzed free radical generation, lipid peroxidation, axonal dystrophy, necrosis and apoptotic cell death (Chiueh et al., 1993; Gutteridge, 1994). Likewise, fumarate was previously shown to form complexes with iron ions that are bioactive (Younes et al., 1990; Lachili et al., 2001), thus enhancing the iron redox cycle, and consequently free radical generation. It has been reported that the supplementation with iron/fumarate to pregnant women is associated with a significant increase in lipid peroxide levels in the plasma of patients (Lachili et al., 2001).

Conversely, literature data have indicated that succinate inhibits the NADH- and NADPH-dependent lipid peroxidation (Takayanagi et al., 1980) and it offers protection against cumene hydroperoxide-induced mitochondrial lipid peroxidation (Bindoli et al., 1982; Cavallini et al., 1984). Succinate can also prevent lipid peroxidation induced by chaotropic agents (Hatefi and Hanstein, 1970), Fe(II)/ADP (Takeshige and Minakami, 1975; Takayanagi et al., 1980) or NADPH/Fe(III)/ADP (Cavallini et al., 1984). In all cases, the protective effect of succinate was attributed to ubiquinol formation, which can act as an antioxidant in biological systems (Vianello et al., 1986). Similarly, the antioxidant effect of malate against mitochondrial lipid peroxidation induced by Fe(II)/ascorbate was linked to coenzyme Q reduction (Vianello et al., 1986).

In line with these, we reported previously that citrate, succinate, malate and oxaloacetate exhibited antioxidant properties against either basal, quinolinic acid- or iron(II)-induced TBARS production, whereas α-ketoglutarate presented an effective pro-oxidant action. Furthermore, these intermediates, excepting succinate, exhibit iron-chelating properties. We suggest that these intermediates could modulate in vitro thiobarbituric acid-reactive species (TBARS) production by interacting with endogenous iron ions, thus adjusting the iron redox cycle and, consequently, free radicals generation (Puntel et al., 2005a). However, succinate effect can be related to succinate dehydrogenase (SDH) activity since its effect was abolished by high temperature pre-treatment (100 °C) or cyanide (Puntel et al., 2005b).

Malonate is a reversible inhibitor of SDH and can induce mitochondrial dysfunction, which in turn can trigger superoxide radicals generation, secondary excitotoxicity, and apoptosis (Dedeoglu et al., 2002). Thus, agents that restore mitochondrial function are thought to play an important role in preventing malonate pro-oxidant activity. Previous data from the literature have demonstrated that the neurotoxicity associated with intrastriatal injection of malonate is mediated almost exclusively by the indirect activation of *N*-methyl-D-aspartate (NMDA) receptor (Beal et al., 1993; Greene et al., 1993; Henshaw et al., 1994; Greene and Greenamyre, 1995). However, the literature data are contradictory with respect to the involvement of NMDA receptors in the malonate pro-oxidative activity (Ferger et al., 1999; Zeevalk et al., 2000).

The reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form colored complex TBA–MDA (a pink complex that has an absorption maximum at 532 nm) is the basis of the commonest method used to assess lipid peroxidation in biological materials for over last decades. However, in recent years this method has been subject to pervasive criticism on several grounds due to the existence of a large range of non-lipid oxidation products in this system that also react with TBA to form colored species that can interfere with this assay (Draper et al., 1993).

As a mean of improving the specificity of the TBA method for MDA determination, a high performance liquid chromatographic (HPLC) procedure for the isolation and measurement of the TBA–MDA complex was developed (Bird et al., 1983). The introduction of HPLC not only resolved the problem of specificity associated with the spectrophotometric method, but markedly increased the sensitivity of MDA measurement.

Based on the exposed, the aims of the present study were to investigate whether citrate, succinate, malate, oxaloacetate, fumarate and α-ketoglutarate (some Krebs cycle intermediates) could modulate malonate pro-oxidative activity under in vitro conditions and to investigate the possible mechanism(s) by which these Krebs cycle intermediates could act. Finally, based on the fact that spectrophotometric method is widely used, we did a comparative evaluation for the determination of MDA in rat brain homogenates using a popular spectrophotometric method (Ohkawa et al., 1979) and HPLC procedure (Grotto et al., 2006) in order to determine the effectiveness of the colorimetric method using brain homogenates.

Materials and Methods

Animals

Adult male Wistar rats (250–350 g) were used in this study. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil.

Tissue preparation

The low speed supernatant fraction (S1) from rat brain was prepared as previously described (Puntel et al., 2005a). Where indicated, the S1 fraction was pre-treated for 10 min at 100 °C (heat treatment) before the incubation at 37 °C for 60 min (see Table 2 and Fig. 3B).

In HPLC analysis, the experiment was done in quadruplicate (Figs. 2 and 6). After 1 h incubation at 37 °C, two tubes were used to colorimetric procedure, and other two tubes were analyzed by the HPLC method (see HPLC method details below).

Colorimetric lipid peroxidation assay

Lipid peroxidation was determined by measuring thiobarbituric acid-reactive species (TBARS) as described by Ohkawa et al. (1979), with minimal modifications (Puntel et al., 2005a). The color reaction was developed by incubating the tubes in boiling water for 60 min, both in the presence or the absence of 5 mmol/l BHT (to avoid further lipid oxidation during the heating step; Draper et al., 1993). BHT did not modify TBARS production during color development. The absence of BHT effect can be attributed to the presence of SDS. We observed that homogenization of brain tissue in SDS containing-medium reduces considerably the production of TBARS during color development. In fact, TBARS production was about 25–35% higher in tubes that were boiled in the absence of SDS (SDS added after color development—data not shown). TBARS levels were measured at 532 nm using a standard curve of MDA. The values are expressed in nmol MDA/g of tissue. Where indicated TBARS was extracted into a *n*-butanol–pyridine mixture (15:1 v:v) as previously described (Ohkawa et al., 1979).

Instrumentation and chromatographic conditions

The chromatographic equipment consisted of a gradient chromatography system Knauer® apparatus, WellChrom model, equipped with a quaternary pump, organizer of solvents, dynamic mixer, an online vacuum degassing solvents with four channels, manual injector with loop of 20 µl and UV–VIS detector. Chromatographic control, data collection and processing were carried out using EUROCHROM 2000 SOFTWARE® basic edition, 2.05 version to Windows.

The separation was achieved using a reverse-phase column: Eurospher-100 150 × 4 mm with 5 µm particle size and a guard column Eurospher-100 5 × 4 mm with 5 µm particle size.

The mobile phase was a mixture of KH_2PO_4–KOH (5 mM, pH 7.0) and methanol (50:50 v/v). The KH_2PO_4 solution was filtered through a 0.22 µm cellulose membrane filter before use.

The flow rate was maintained isocratically at 0.6 ml/min, the absorbance of the eluent was monitored at 532 nm and the total run time was 8 min. The column was thermostated at 40 °C in a thermostatization system to chromatographic column (Chromacon®).

Sample procedure

HPLC procedure was done as described by Grotto et al. (2006). Briefly a volume of 75 µl of homogenate was added to 25 µl of butylated hydroxytoluene (BHT) 10 mM, 25 µl of NaOH 3 N and 25 µl of water and incubated at 60 °C for 30 min in a shaking water bath. After that, it was added 125 µl of H_3PO_4 6% and 100 µl of TBA 0.8% and the mixture was heated at 90 °C for 45 min. Then, the mixture was cooled, 50 µl of 10% SDS was added and the extraction with 300 µl of *n*-butanol was carried out by vortex-mixing for 1 min and centrifuged at 3000 *g* for 10 min. Twenty microliters of the butanol layer was injected into HPLC with visible detector, using a reverse-phase column and eluted as described previously.

Iron-chelating property assay

To examine the iron-chelating property of Krebs cycle intermediates and malonate, we used the *o*-phenantroline method as previously described (Puntel et al., 2005a,b).

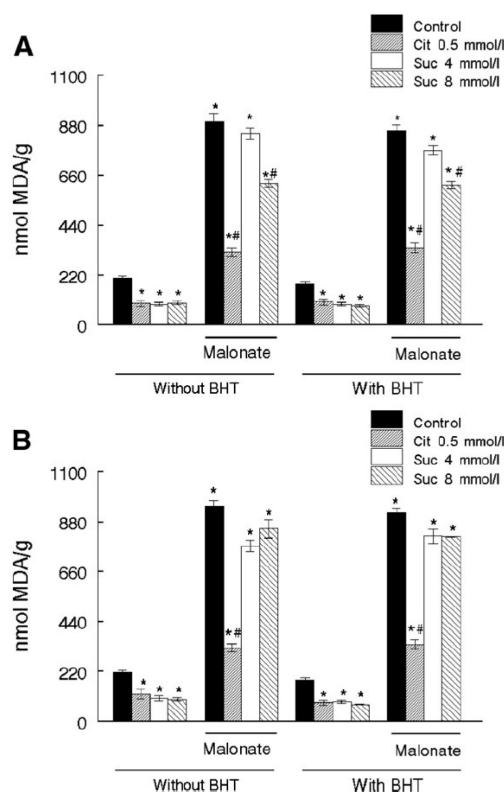


Fig. 1. Effect of citrate and succinate on basal or malonate-induced TBARS production. Low-speed supernatant (S1) from rat brain was incubated for 60 min in a medium containing 10 mM Tris/HCl buffer, pH 7.4. Effect of citrate (0.5 mmol/l) and succinate (4 or 8 mmol/l) against basal or malonate (4 mmol/l)-induced TBARS production by Ohkawa's method. (A) before and (B) after extraction into *n*-butanol–pyridine. Data are expressed as means ± SEM (n = 3–5). *p < 0.05 from basal (no addition); #p < 0.05 from malonate by Duncan's multiple range test.

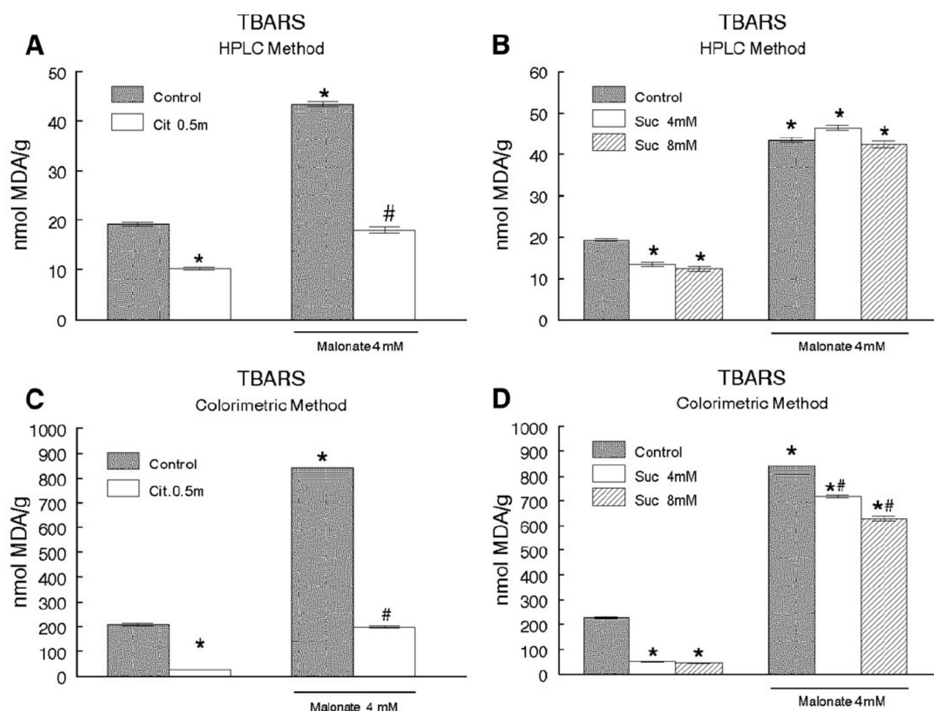


Fig. 2. Effect of citrate and succinate on basal or malonate-induced TBARS production. Effect of citrate (0.5 mmol/l) (A and C) and succinate (4 or 8 mmol/l) (B and D) against basal or malonate (4 mmol/l)-induced TBARS production. (A) Effect of citrate (0.5 mmol/l) against basal or malonate-induced TBARS production by HPLC analyzes. (B) Effect of succinate (4 or 8 mmol/l) against basal or malonate-induced TBARS production by HPLC analyzes. (C) Effect of citrate (0.5 mmol/l) against basal or malonate-induced TBARS production by Ohkawa's method. (D) Effect of succinate (4 or 8 mmol/l) against basal or malonate-induced TBARS production by Ohkawa's method. Data are expressed as means±SEM (n=3–5). *$p<0.05$ from basal (no addition); $^{\#}p<0.05$ from malonate by Duncan's multiple range test.

IC_{50} calculation

The IC_{50} (the concentration that inhibits 50% of lipid peroxidation) values for lipid peroxidation were determined by the method of Dixon (Dixon and Webb, 1964).

Statistical analysis

Data were analyzed statistically by one-way ANOVA, followed by Duncan's Multiple Range Test when appropriate. Differences between groups were considered to be significant when $p < 0.05$.

Results

Effect of citrate and succinate on basal or malonate-induced TBARS production

Succinate (4 and 8 mmol/l) and citrate (0.5 mmol/l) caused a significant reduction on basal-TBARS production that was not modified by addition of BHT during the color development (Fig. 1A). Citrate (0.5 mmol/l) prevented malonate-induced TBARS production, whereas succinate did not reduce malonate-induced TBARS production to the control level. These results were not modified by BHT addition during the color development (Fig. 1A).

The extraction of colored products into an organic phase (*n*-butanol–pyridine) did not significantly change the stimulatory effect of malonate (4 mmol/l) and the inhibitory effect of citrate (0.5 mmol/l) on TBARS production (Fig. 1B). However, the antioxidant effect of succinate at higher concentration (8 mmol/l) was completely abolished when the products were extracted into an organic solvent (Fig. 1B).

Results obtained with HPLC method are shown in Fig. 2A and B and similar experiments quantified colorimetrically are shown in Fig. 2C and D. It is possible to observe that citrate antioxidant effect against either basal or malonate-induced TBARS production was not changed (compare Fig. 2A and C), whereas the protective effect of succinate against malonate-induced TBARS production was completely abolished in the HLPC assay (compare Fig. 2B and D), similar to that observed after extraction in organic solvent (compare Figs. 1B and 2B).

Effect of citrate, succinate, malate, oxaloacetate and, α-ketoglutarate on basal or malonate-induced TBARS production

Malonate (4 mmol/l) caused a significant increase in the TBARS production in fresh tissue preparations (432% compared to basal, Table 1). The pro-oxidant activity of malonate was not significantly modified by the addition of potassium cyanide (KCN—1 mmol/l) or Triton X-100 (0.1%), either alone or simultaneously. In the same way, neither KCN nor Triton X-

Table 1
Effect of citrate, succinate, malate, oxaloacetate and, α-ketoglutarate on basal or malonate-induced TBARS production in fresh tissue preparations

	None	Citrate (0.5 mmol/l)	Malate (16 mmol/l)	Oxaloacetate (4 mmol/l)	Succinate (4 mmol/l)	α-ketoglutarate (16 mmol/l)
Basal	212.68±12.59 (100%)	86.90±10.57† (49%)	110.54±21.27† (62%)	68.02±16.76† (38%)	99.70±10.76† (56%)	461.05±32.88† (260%)
Malonate (4 mmol/l)	918.79±19.73* (432%)	276.02±17.11† (156%)	214.29±29.54† (121%)	153.64±19.22† (87%)	765.64±45.18* (432%)	688.71±61.57 (388%)
Malonate+KCN	862.46±24.59* (405%)	264.82±16.44† (149%)	195.87±16.41† (110%)	114.30±7.92† (64%)	704.02±49.15* (397%)	631.49±25.95 (356%)
Malonate+triton	957.80±20.93* (450%)	314.11±15.34*,† (177%)	241.90±18.41† (136%)	153.28±14.22† (86%)	798.97±41.14* (451%)	701.54±35.85* (396%)
Malonate+KCN+triton	901.44±24.94* (424%)	267.88±3.67† (151%)	207.13±18.93† (117%)	126.59±28.92† (71%)	713.50±41.54* (402%)	680.74±27.83 (384%)
KCN (1 mmol/l)	206.05±11.69 (97%)	68.28±10.74† (38%)	97.58±8.82† (55%)	74.87±21.77† (42%)	200.41±19.19* (113%)	457.67±45.76† (258%)
KCN+triton	234.73±12.47 (110%)	81.26±10.65† (46%)	104.54±17.99† (59%)	74.31±19.99† (42%)	254.26±12.75* (143%)	491.67±31.87† (277%)
Triton (0.1%)	208.05±13.12 (98%)	82.93±12.00† (47%)	134.48±22.13† (76%)	74.90±20.58† (42%)	127.95±14.98† (72%)	520.47±35.09† (294%)

The values are expressed in nmol MDA/g of tissue. Data are expressed as mean±S. E. (n=3–5); *from respective basal; †different of respective control (without intermediates) by one-way ANOVA following by Duncan's test.

Table 2
Effect of citrate, succinate, malate, oxaloacetate and, α-ketoglutarate on basal or malonate-induced TBARS production in heat-treated preparations

	None	Citrate (0.5 mmol/l)	Succinate (4 mmol/l)	Malate (16 mmol/l)	Oxaloacetate (4 mmol/l)	α-ketoglutarate (16 mmol/l)
Basal	454.89±5.69 (100%)	348.11±50.67† (76%)	486.95±49.48 (107%)	275.90±10.39† (61%)	354.55±43.59† (78%)	1038.13±82.87† (228%)
KCN (1 mmol/l)	552.17±22.99 (121%)	321.26±31.05† (71%)	560.19±20.77 (123%)	211.46±18.37† (46%)	329.95±33.29† (72%)	1028.18±80.98† (226%)
Malonate (4 mmol/l)	1165.74±50.84* (256%)	702.15±55.68*,† (154%)	1102.63±42.17* (242%)	415.04±41.27† (91%)	503.41±59.36† (111%)	1168.67±89.51 (257%)
KCN+malonate	1109.76±41.70* (244%)	647.31±64.03*,† (142%)	1012.18±57.58* (222%)	308.03±29.26† (68%)	425.49±35.10† (93%)	1184.12±90.23 (260%)

The values are expressed in nmol MDA/g of tissue. Data are expressed as mean±S. E. (n=4–6); *from respective basal; †different of respective control (without any intermediate) by one-way ANOVA following by Duncan's test.

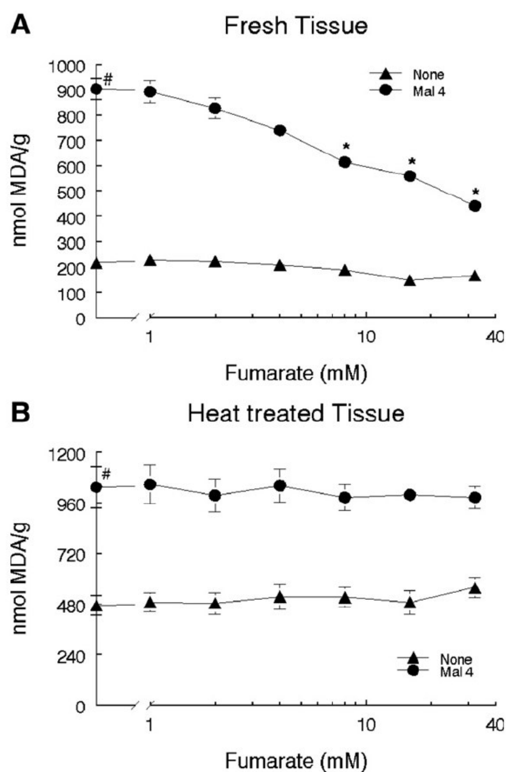


Fig. 3. Effect of fumarate on malonate-induced TBARS production. Low-speed supernatant (S1) from brain was incubated for 60 min in a medium containing 10 mM Tris/HCl buffer, pH 7.4. (A) Effect of fumarate against basal or malonate-induced TBARS production in fresh tissue preparations. –▲– None, –●– malonate 4 mmol/l. (B) Effect of fumarate against basal or malonate-induced TBARS production in heat-treated preparations.–▲– None, –●– malonate 4 mmol/l. Data are expressed as means±SEM (n=3–5). $^*p<0.05$ from malonate alone; $^{\#}p<0.05$ from basal by Duncan's multiple range test.

100 alone or concomitantly displayed any effect against basal-TBARS production (Table 1).

Citrate (0.5 mmol/l), malate (16 mmol/l), and oxaloacetate (4 mmol/l) significantly prevented both basal and malonate-induced TBARS production (Table 1). The antioxidant effect attributed to citrate, malate and oxaloacetate against basal and malonate-induced TBARS production was not modified either by KCN or Triton X-100 (Table 1).

Similar to the other Krebs cycle intermediates, succinate (4 mmol/l) significantly prevented basal-TBARS production. The antioxidant property against basal-TBARS production was not changed by the addition of Triton X-100. However, succinate antioxidant property against basal-TBARS production was completely abolished by the addition of KCN. In contrast to the basal condition, succinate did not exhibit antioxidant property against malonate-induced TBARS production (Table 1).

α-Ketoglutarate (16 mmol/l) significantly induced TBARS production, and KCN and/or Triton X-100 did not modify its pro-oxidant activity. Furthermore, α-ketoglutarate did not change significantly malonate pro-oxidant activity (Table 1).

Effect of citrate, succinate, malate, oxaloacetate and α-ketoglutarate on basal or malonate-induced TBARS production in heat-treated preparations

Malonate (4 mmol/l) caused a significant increase on TBARS production in heat-treated preparations (256% compared to the basal value, Table 2). KCN (1 mmol/l) did not change basal or malonate-induced TBARS production.

Citrate (0.5 mmol/l), malate (16 mmol/l), and oxaloacetate (4 mmol/l) significantly prevented both basal and malonate-induced TBARS production, regardless of KCN (1 mmol/l, Table 2). Conversely, citrate did not reduce malonate-induced TBARS production to basal levels, whereas malate (16 mmol/l) and oxaloacetate (4 mmol/l) reduced malonate-induced TBARS production to values that did not significantly differ from basal levels (Table 2).

Succinate (4 mmol/l), contrary to that observed using fresh preparations, did not reduce basal-TBARS production (Table 2). In line with this, succinate did not prevent

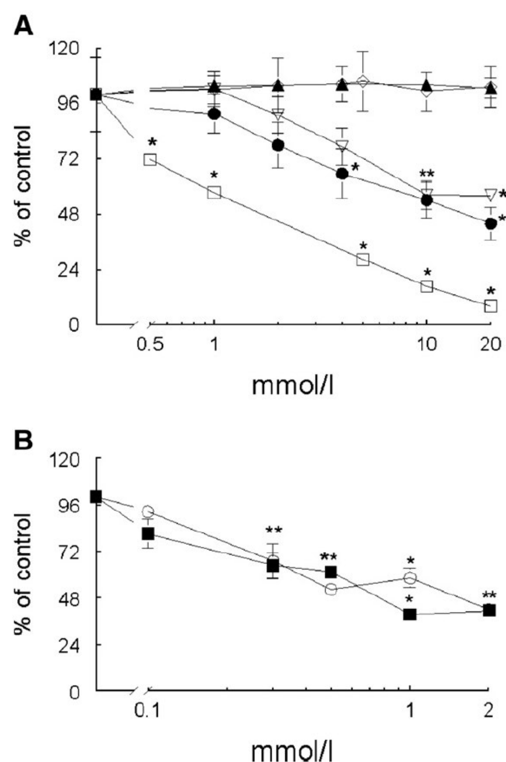


Fig. 4. Iron-chelating properties of Krebs cycle intermediates and malonate. (A) Effect of succinate, malate, α-ketoglutarate and malonate on colored iron–phenantroline complex formation.–▲– Succinate, –●– malate, –▽– α-ketoglutarate, –◇– fumarate and –□– malonate. (B) Effect of oxaloacetate and citrate on colored iron–phenantroline complex formation.–■– Citrate, –○– oxaloacetate. The values are expressed as % of control. Absorbance obtained by reaction between free Fe^{2+} with o-phenantroline in the absence of Krebs cycle intermediates or malonate is considered 100%. Data are expressed as means± SEM (n=4). $^*p<0.05$ from respective control by Duncan's multiple range test.

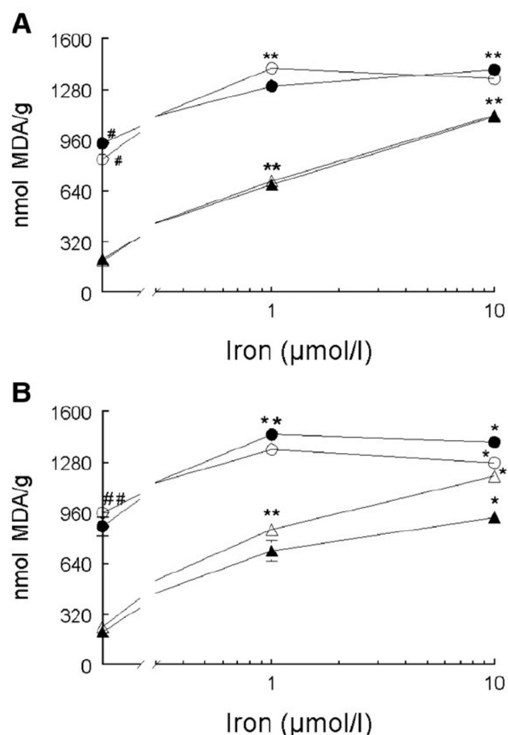


Fig. 5. Effect of iron at different concentrations on basal or malonate-induced TBARS production. Effect of iron (0–10 µmol/l) against basal or malonate (4 mmol/l)-induced TBARS production. (A) before extraction into *n*-butanol–pyridine and (B) after extraction into *n*-butanol–pyridine.–▲– iron; –△– iron and BHT (5 mmol/l) during heating step; –●– iron and malonate (4 mmol/l); –○– iron, malonate and BHT during heating step. Data are expressed as means ± SEM (n=3–5). *$p<0.05$ from respective control; $^{\#}p<0.05$ from respective basal by Duncan's multiple range test.

malonate-induced TBARS production. KCN did not alter per se the analyzed parameters (Table 2).

α-Ketoglutarate (16 mmol/l) induced basal-TBARS production, which was not changed by the addition of malonate and/or KCN (Table 2).

Effect of fumarate on basal or malonate-induced TBARS production

Fumarate significantly prevented the malonate-induced TBARS production at supra-physiological concentrations (at 8 mmol/l onwards) using fresh tissue preparations, without significant effect against the basal-TBARS production (Fig. 3A). Furthermore, the fumarate antioxidant effect was completely abolished when tissue preparations were boiled at 100 °C to inactivate the enzyme systems (Fig. 3B).

Iron-chelating property of Krebs cycle intermediates and malonate

Succinate did not display iron-chelating property up to 20 mmol/l (Fig. 4A). Malate exhibited a significant concentration-dependent iron-chelating property at 4 mmol/l onwards (Fig. 4A). α-Ketoglutarate exhibited a significantly iron-chelating property at 10 mmol/l, but this effect was not concentration-dependent (Fig. 4A). Malonate exhibited iron-chelating property, which was significantly from 0.5 mmol/l onwards. Malonate effect was concentration-dependent reaching maximal effect at 20 mmol/l (Fig. 4A). Similar to succinate, fumarate did not present iron-chelating activity up to 20 mmol/l (Fig. 4A).

Oxaloacetate and citrate chelated iron with a greater potency than did the other intermediates or malonate (compare Fig. 4A with B). Oxaloacetate and citrate showed a significant iron-chelating property from 0.3 mmol/l onwards (Fig. 4B).

Effects of iron on malonate-induced TBARS production

In order to investigate whether the malonate-iron complex (es) could be involved in malonate-induced TBARS production, we tested iron at different concentrations (0–10 µmol/l) on a system containing malonate (4 mmol/l). Fig. 5A shows that iron caused a significant increase in TBARS production from 1 µmol/l onwards, with maximum effect at 10 µmol/l. Malonate (4 mmol/l) caused a significant increase in TBARS production

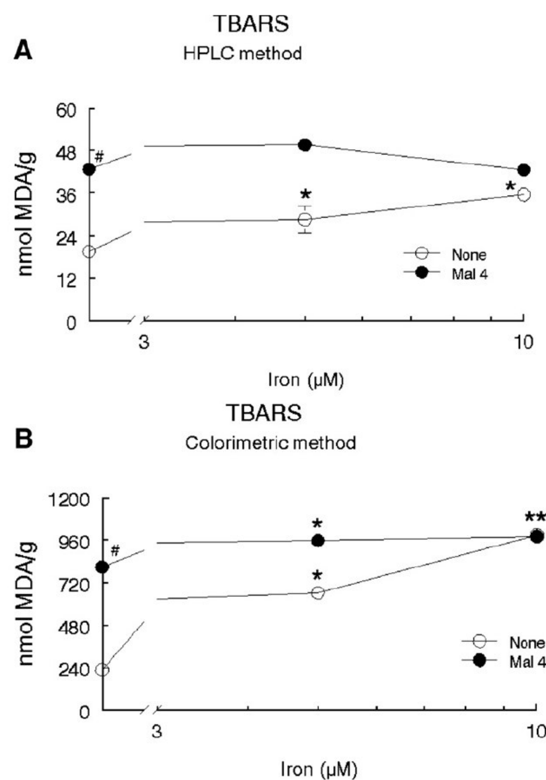


Fig. 6. Effect of iron at different concentrations on basal or malonate-induced TBARS production. Effect of iron (0–10 µmol/l) against basal or malonate (4 mmol/l)-induced TBARS production by HPLC analyzes (A), or by Ohkawa's method (B).–○– iron alone and –●– iron plus malonate (4 mmol/l). Data are expressed as means ± SEM (n=3–5). *$p<0.05$ from respective control; $^{\#}p<0.05$ from basal (no addition) by Duncan's multiple range test.

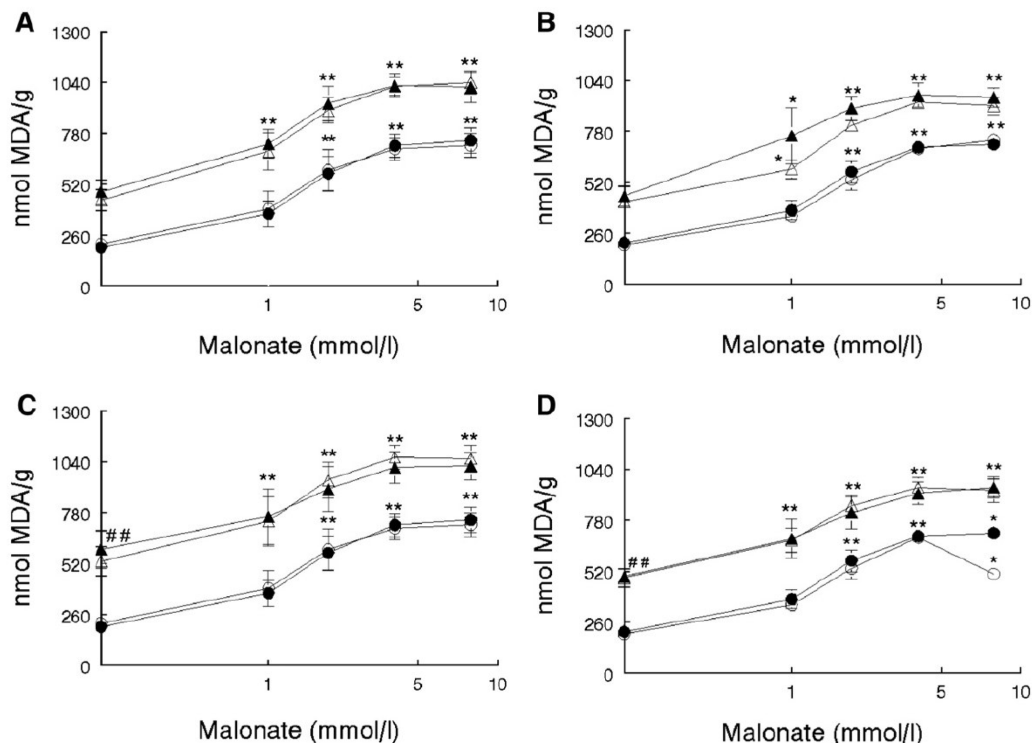


Fig. 7. Effects of malonate on basal or iron-induced TBARS production. (A and B) Effect of iron (0.1 μmol/l) against basal or malonate (0–8 mmol/l)-induced TBARS production. (A) before extraction into *n*-butanol–pyridine, and (B) after extraction into *n*-butanol–pyridine.–○– malonate; –●– malonate and BHT (5 mmol/l) during heating step; –△– malonate and iron 0.1 μmol/l;–▲– malonate, iron and BHT during heating step. (C and D) Effect of iron (0.5 μmol/l) against basal or malonate (0–8 mmol/l)-induced TBARS production. (C) before extraction into *n*-butanol–pyridine, and (D) after extraction into *n*-butanol–pyridine.–○– malonate; –●– malonate and BHT (5 mmol/l) during heating step; –△– malonate and iron 0.5 μmol/l; –▲– malonate, iron and BHT during heating step. Data are expressed as means±SEM (n=3–5). *$p<0.05$ from respective control; #$p<0.05$ from respective basal by Duncan's multiple range test.

without altering the iron (1 μmol/l) pro-oxidant activity (the result was approximately equal to the sum of their isolated effect). The addition of BHT during the color reaction development did not modify the effect of iron or malonate, indicating that lipid peroxidation was negligible during the heating step. Extraction of colored products with *n*-butanol–pyridine did not change the effects of iron or malonate (Fig. 5B). The HPLC analysis (Fig. 6A) confirms the results obtained by the colorimetric method (Fig. 6B).

Fig. 7A shows the effect of different malonate concentrations (0–8 mmol/l) on basal or iron (0.1 μmol/l)-induced TBARS production. Malonate caused a significantly increase in TBARS production from 2 mmol/l onwards with maximum effect at 4 mmol/l. Iron 0.1 μmol/l did not produce a significant increase in TBARS production. In the presence of iron (0.1 μmol/l) the effect of malonate (1 mmol/l) was higher (but not statistically significant) than that of malonate alone (approximately the sums of theirs effects alone). As indicated above, BHT and butanol–pyridine extraction did not modify the pro-oxidant effects of iron or malonate (Fig. 7A and B).

Fig. 7C demonstrates the effect of malonate (0–8 mmol/l) and/or iron (0.5 μmol/l) on brain TBARS production. As observed in Fig. 7A, malonate caused a significantly increase in TBARS production at 2 mmol/l onwards, with a maximum effect at 4 mmol/l. Iron (0.5 μmol/l) caused per se a significant increase in TBARS production. The simultaneous addition of malonate (0–8 mmol/l) and iron (0.5 μmol/l) confirm that there

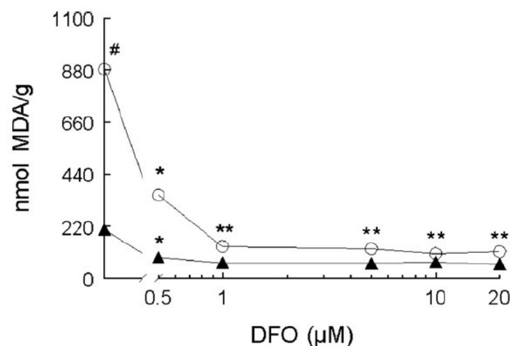


Fig. 8. Effect of deferoxamine on malonate-induced TBARS production. Effect of deferoxamine (DFO) against basal or malonate (4 mmol/l)-induced TBARS production in the brain tissue preparation. –▲– DFO alone, –○– malonate plus DFO. Data are expressed as means±SEM (n=3). *$p<0.05$ from respective control; #$p<0.05$ from basal (no addition) by Duncan's multiple range test.

was no additional effect of iron on malonate pro-oxidative activity. As observed in Figs. 5 (A and B) and 7 (A and B), the inclusion of BHT during color development or the extraction with *n*-butanol–pyridine did not modify the effects of malonate and iron (Fig. 7C and D).

Effect of deferoxamine on malonate-induced TBARS production

Deferoxamine (DFO) caused a significantly and maximal reduction in basal-TBARS production at 0.5 μmol/l. Malonate (4 mmol/l)-induced TBARS production was significantly reduced by DFO, with maximal effect at 1 μmol/l (Fig. 8).

Discussion

The results presented in this paper indicated that citrate, malate, fumarate and oxaloacetate can act as antioxidants against malonate-induced TBARS production. In general, antioxidants are usually effectives in the μmo/l range, for example ascorbate and urate (Chatterjee et al., 1975; Nyyssönen et al., 1997; Girard et al., 2005; Yanpallewara et al., 2005). However, ascorbate can reach higher concentrations (up to 0.9 mmol/l) in the plasma (Becker, 1993). One of the key features of antioxidants is that they are so reactive with oxidants that at low concentrations they will effectively out compete for the other biomolecules for the radical. Thus, for malate, oxaloacetate, fumarate and succinate we can not describe the effect as being as potent when compared to other well known antioxidants. In fact, we found significantly antioxidant activity for Krebs cycle intermediates only at supra-physiological (mmol/l) concentrations. Indeed, hepatic intracellular concentrations of Krebs cycle intermediate range from 100 to 700 μmol/l, depending on the intermediate (Mahler and Cordes, 1969). Thus, we assume that only citrate could have a significant effect at physiological range of concentrations. Similar to liver, plasma citrate concentration range from 70 to 700 μmol/l levels (Yonekawa et al., 2005). Oxaloacetate exhibits iron-chelating property at physiological concentration; however, it did not prevent basal, or malonate-induced TBARS production at physiological concentrations.

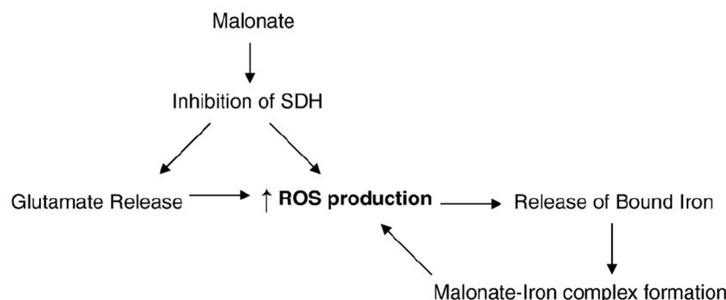
The citrate, malate, oxaloacetate and α-ketoglutarate effect against either basal or malonate-induced TBARS production was not changed by KCN or triton, or heat treatment (Tables 1 and 2), suggesting that the effect of these agents is not dependent on respiratory chain reactions. These results are similar to that obtained in our previous study, in which these intermediates were effective against either basal or quinolinic acid-induced TBARS production in heat-treated preparations (Puntel et al., 2005a), condition where a complete inhibition of respiratory chain reactions occurs (Puntel et al., 2005b). Thus, based on the presented results, we suggest that the antioxidant properties of these Krebs cycle intermediates, under our assay conditions, are related to their iron-chelating properties (Fig. 4A and B).

These conclusions are supported by results reported in this and in our previous paper, showing a significant iron-chelating property of citrate, malate and oxaloacetate. Furthermore, these interpretations are supported by earlier studies demonstrating that adequate ratio Fe(II)/Fe(III) is essential for the TBARS production (free radicals generation due to iron redox cycle) (Tang et al., 1997; Caro and Cederbaum, 2004). In fact, in the presence of a chelator the initiation of lipid peroxidation is strictly dependent on the chelator to iron ratio and, according to this ratio, the complex might act as stimulator or inhibitor of lipid peroxidation (Tang et al., 1997; Caro and Cederbaum, 2004). However, the significance of this metal binding, in comparison to other metal-binding agents in the homogenates (i.e. protein) must be considered. In a previous study, Fukuzawa et al. (2005) have shown that bovine serum albumin prevented membrane lipid peroxidation induced by iron and superoxide by decreasing the availability of iron responsible for the initiation of deleterious reactions. Based on previous results reported in the literature, we assume that the iron content present in rat brain S1 preparations was approximately 0.2 μg/g, corresponding to about 1% of the total iron content (21 μg/g) (Bralet et al., 1992; Oubidar et al., 1994). However, we can not rule out that the concentration of iron might be variable according to the preparations and treatments used.

The succinate antioxidant effect against basal-TBARS production was completely abolished by the addition of KCN or heat treatment. Based on these data, we suggest that the succinate antioxidant activity could be related to SDH activity. This conclusion is supported by some findings: (a) the succinate antioxidant activity is abolished by the addition of SDH activity inhibitor (malonate); (b) the succinate antioxidant activity is abolished by the heat treatment at 100 °C, which was previously shown to completely inhibit the SDH activity (Puntel et al., 2005b); and (c) the succinate antioxidant activity is abolished by the addition of KCN. In fact, in the presence of KCN, coenzyme Q is expected to be maximally reduced (Vianello et al., 1986) and, consequently, lipid peroxidation should be inhibited. However, one can speculate that in the reported experiments, KCN interacted with other sites in addition to cytochrome oxidase. In addition, we could speculate that the increase in ubiquinol pool, when KCN was present, can lead to an increase in the ubisemiquinone radical life span, and consequently an increase of ROS generation (Nohl et al., 1998), which could abolish the succinate effect.

In a similar way, the fumarate antioxidant effect against malonate-induced TBARS production was completely abolished by heat treatment, suggesting that its effect could be associated with some enzymatic systems. Based on the presented results (Fig. 4A) we rule out the possible interaction between iron (II) and fumarate as a responsible for modulating the free radical generation due to iron redox cycle.

The malonate pro-oxidant activity was not strictly related to inhibition of SDH, because malonate exhibited pro-oxidant properties even when heat-treated preparations were used. Thus, we assume that malonate pro-oxidant effect is related to its ability to form complex(es) with iron (Fukuzawa et al., 1995). Modulation of Fe(II)/Fe(III) ratio by malonate could increase the iron redox cycle, increasing free radicals generation. Accordingly, antioxidant properties of citrate, malate and oxaloacetate against malonate-induced TBARS production



Scheme 1. Proposed mechanism(s) involved in the malonate toxicity. In scheme 1, first malonate inhibits energetic metabolism by inhibit SDH, which causes excitotoxicity via glutamate release and also via an increase in ROS production due to mitochondrial dysfunction. ROS overproduction can release iron from proteins that complexes with malonate worsening ROS production and its neurotoxicity.

can be ascribed to their ability to form less active iron(II) ion complexes, which did not allow iron to form complex(es) with malonate. However, to activate an iron redox cycle is necessary, a source of reducing equivalents. In the reported experiments the endogenous reduced nucleotides, found in the preparations, could be considered as the promoters of iron redox cycle.

On the other hand, α-ketoglutarate pro-oxidative activity in basal-TBARS production can be related to its chelating properties, which could form active complexes analogous to that ascribed to malonate complexes. In fact, α-ketoglutarate was able to chelate iron (Fig. 4A).

Regarding the comparative study between common spectrophotometric method (Ohkawa et al., 1979) and the HPLC (Grotto et al., 2006) procedure for the determination of MDA in the rat brain S1, we found that there were minimal differences among the techniques. The major point to be discussed here is about the overestimation in the quantity of MDA found by the spectrophotometric assay as compared to values obtained with HPLC analysis. In fact, the values obtained by the spectrophotometric method were approximately 10–20 times higher than that obtained by HPLC. The identity of the non-MDA TBARS that account for the part of absorbance at 532 nm in the spectrophotometric method is unknown and undoubtedly varies among the biological materials (Draper et al., 1993). Except by the amount of TBARS determined, there were minimal differences among the results obtained by the HPLC and that from colorimetric method followed by extraction on organic phase. Thus, since the TBARS assay measures end-point oxidative damage, our TBARS system is useful to assess the overall impact of oxidative stress-inducing and neuroprotective agents, which is supported by recent data from literature (Dawn-Linsley et al., 2005).

In conclusion, these data suggest that citrate, malate and oxaloacetate were effective antioxidant agents against either basal, or malonate-induced TBARS production tentatively by chelating iron(II) ions and forming inactive complexes or by reducing iron redox cycle. The antioxidant effect of citrate, at physiological concentrations, can also indicate that this compound could be a modulator of oxidative stress under physiological and pathological conditions. The results presented here also indicate that in vitro the neurotoxicity induced by malonate could be attributed, at least in part, to formation of iron complex(es). Based on the presented results and on the literature data, we propose the following scheme (Scheme 1) for malonate toxicity.

Acknowledgments

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References

Abrahamson, H.B., Rezvani, A.B., Brushmiller, G., 1994. Photochemical and spectroscopic studies of complexes of iron (III) with citric acid and other carboxylic acids. Inorganica Chimica Acta 226, 117–127.

Baker, M.S., Gebicki, J.M., 1986. The effect of pH on yields of hydroxyl radicals produced from superoxide by potential biological iron chelators. Archives of Biochemistry and Biophysics 246, 581–588.

Beal, M.F., 1996. Mitochondria, free radicals, and neurodegeneration. Current Opinion in Neurobiology 6, 661–666.

Beal, M.F., Brouillet, E., Jenkins, B., Henshaw, R., Rosen, B., Hyman, B.T., 1993. Age-dependent striatal excitotoxic lesions produced by the endogenous mitochondrial inhibitor malonate. Journal of Neurochemistry 61, 1147–1150.

Becker, B.F., 1993. Toward the physiological function of uric acid. Free Radical Biology & Medicine 14, 615–631.

Bindoli, A., Cavallini, L., Jocelyn, P., 1982. Mitochondrial lipid peroxidation by cumene hydroperoxide and its prevention by succinate. Biochimica Et Biophysica Acta 681, 496–503.

Bird, R.P., Hung, S.O.S., Hadley, M., Draper, H.H., 1983. Determination of malondialdehyde in biological materials by high-pressure liquid chromatography. Analytical Biochemistry 128, 240–244.

Bralet, J., Schreiber, L., Bouvier, C., 1992. Effect of acidosis and anoxia on iron delocalization from brain homogenates. Biochemical Pharmacology 43, 979–983.

Caro, A.A., Cederbaum, A.T., 2004. Antioxidant properties of *S*-adenosyl-L-methionine in Fe^{2+}-initiated oxidations. Free Radical Biology & Medicine 36, 1303–1316.

Cavallini, L., Valente, M., Bindoli, A., 1984. Comparison of cumene hydroperoxide- and NADPH/Fe^{3+}/ADP-induced lipid peroxidation in heart and liver submitochondrial particles. Mechanisms of protection by succinate. Biochimica Et Biophysica Acta 795, 466–472.

Chatterjee, I.B., Majumder, A.K., Nandi, B.K., Subramanian, N., 1975. Synthesis and some major functions of vitamin C in animals. Annals of the New York Academy of Sciences 258, 24–47.

Chiueh, C.O., Miyake, H., Peng, M.T., 1993. Role of dopamine autoxidation, hydroxyl radical generation, and calcium overload in underlying mechanisms involved in MPTP-induced Parkinsonism. Advances in Neurology 60, 251–258.

Dawn-Linsley, M., Ekinci, F.J., Ortiz, D., Rogers, E., Shea, T.B., 2005. Monitoring thiobarbituric acid-reactive substances (TBARs) as an assay for oxidative damage in neuronal cultures and central nervous system. Journal of Neuroscience Methods 141, 219–222.

Dedeoglu, A., Ferrante, R.J., Andreassen, O.A., Dillmann, W.H., Beal, M.F., 2002. Mice overexpressing 70-kda heat shock protein show increased resistance to malonate and 3-nitropropionic acid. Experimental Neurology 176, 262–265.

Desagher, S., Glowinski, J., Premont, J., 1997. Pyruvate protects neurons against hydrogen peroxide-induced toxicity. Journal of Neuroscience 17, 9060–9067.

Dixon, M., Webb, E.C., 1964. Enzymes, 2nd ed. Longmans, London. 950p.

Draper, H.H., Squires, E.J., Mahmoodi, H., Wu, J., Agarwal, S., Hadley, M., 1993. A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. Free Radical Biology & Medicine 15, 353–363.

Farina, M., Brandao, R., de Lara, F.S., Pagliosa, L.B., Soares, F.A., Souza, D.O., Rocha, J.B.T., 2003. Profile of nonprotein thiols, lipid peroxidation and delta-aminolevulinate dehydratase activity in mouse kidney and liver in response to acute exposure to mercuric chloride and sodium selenite. Toxicology 184, 179–187.

Ferger, B., Eberhardt, O., Teismann, P., Groote, C., Schulz, J.B., 1999. Malonate-induced generation of reactive oxygen species in rat striatum depends on dopamine release but not on NMDA receptor activation. Journal of Neurochemistry 73, 1329–1332.

Fukuzawa, K., Suomi, K., Iemura, M., Goto, S., Tokumura, A., 1995. Dynamics of xanthine oxidase- and Fe^{3+}-ADP-dependent lipid peroxidation in negatively charged phospholipid vesicles. Archives of Biochemistry and Biophysics 10, 83–91.

Fukuzawa, K., Saitoh, Y., Akai, K., Kogure, K., Ueno, S., Tokumura, A., Otagiri, M., Shibata, A., 2005. Antioxidant effect of bovine serum albumin on membrane lipid peroxidation induced by iron chelate and superoxide. Biochimica Et Biophysica Acta 1668, 145–155.

Girard, A., Madani, S., El Boustani, E.S., Belleville, J., Prost, J., 2005. Changes in lipid metabolism and antioxidant defense status in spontaneously hypertensive rats and Wistar rats fed a diet enriched with fructose and saturated fatty acids. Nutrition 21, 240–248.

Greene, J.G., Greenamyre, J.T., 1995. Characterization of the excitotoxic potential of the reversible succinate dehydrogenase inhibitor malonate. Journal of Neurochemistry 64, 430–436.

Greene, J.G., Porter, R.H.P., Eller, R.V., Greenamyre, J.T., 1993. Inhibition of succinate dehydrogenase by malonic acid produces an excitotoxic lesion in rat striatum. Journal of Neurochemistry 61, 1151–1154.

Grotto, D., Santa Maria, L.D., Boeira, S., Valentini, J., Charão, M.F., Moro, A.M., Nascimento, P.C., Pomblum, V.J., Garcia, S.C., 2007. Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography–visible detection. Journal of Pharmaceutical and Biomedical Analysis 43, 619–624.

Gutteridge, J.M., 1994. Hydroxyl radicals, iron, oxidative stress, and neurodegeneration. Annals of the New York Academy of Sciences 738, 201–213.

Halliwell, B., 1992. Reactive oxygen species and the central nervous system. Journal of Neurochemistry 59, 1609–1623.

Hatefi, Y., Hanstein, W.G., 1970. Lipid oxidation in biological membranes. 1. Lipid oxidation in submitochondrial particles and microsomes induced by chaotropic agents. Archives of Biochemistry and Biophysics 138, 73–86.

Henshaw, R., Jenkins, B.G., Schulz, J.B., Ferrante, R.J., Kowall, N.W., Rosen, B.R., Beal, M.F., 1994. Malonate produces striatal lesions by indirect NMDA receptor activation. Brain Research 647, 161–166.

Jara-Prado, A., Ortega-Vazquez, A., Ruano, L.M., Rios, C., Santamaria, A., 2003. Homocysteine-induced brain lipid peroxidation: effects of NMDA receptor blockade, antioxidant treatment, and nitric oxide synthase inhibition. Neurotoxicity Research 5, 237–243.

Ji, X.Y., Tan, B.K.H., Zhu, Y.C., Linz, W., Zhu, Y.Z., 2003. Comparison of cardioprotective effects using ramipril and DanShen for the treatment of acute myocardial infarction in rats. Life Sciences 73, 1413–1426.

Lachili, B., Hininger, I., Faure, H., Arnaud, J., Richard, M.J., Favier, A., Roussel, A.M., 2001. Increased lipid peroxidation in pregnant women after iron and vitamin C supplementation. Biological Trace Element Research 83, 103–110.

Mahler, H.R., Cordes, E.H., 1969. Biological Chemistry. Harper and Row, New York. 872p.

Mallet, R.T., 2000. Pyruvate: metabolic protector of cardiac performance. Proceedings of the Society for Experimental Biology and Medicine 223, 136–148.

Mallet, R.T., Sun, J., 1999. Mitochondrial metabolism of pyruvate is required for its enhancement of cardiac function and energetics. Cardiovascular Research 42, 149–161.

Mallet, R.T., Sun, J., 2003. Antioxidant properties of myocardial fuels. Molecular and Cellular Biochemistry 253, 103–111.

Nohl, H., Gille, L., Kozlov, A.V., 1998. Antioxidant-derived prooxidant formation from ubiquinol. Free Radical Biology & Medicine 25, 666–675.

Nyyssönen, K., Porkkala-Sarataho, E., Kaikkonen, J., Salonen, J.T., 1997. Ascorbate and urate are the strongest determinants of plasma antioxidative capacity and serum lipid resistance to oxidation in Finnish men. Atherosclerosis 130, 223–233.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry 95, 351–358.

Oubidar, M., Boquillon, M., Marie, C., Schreiber, L., Bralet, J., 1994. Ischemia-induced brain iron delocalization: effect of iron chelators. Free Radical Biology & Medicine 16, 861–867.

Perez-De La Cruz, V., Gonzalez-Cortes, C., Pedraza-Chaverri, J., Maldonado, P.D., Andres-Martinez, L., Santamaria, A., 2006. Protective effect of *S*-allylcysteine on 3-nitropropionic acid-induced lipid peroxidation and mitochondrial dysfunction in rat brain synaptosomes. Brain Research Bulletin 68, 379–383.

Perez-Severiano, F., Rodriguez-Perez, M., Pedraza-Chaverri, J., Maldonado, P.D., Medina-Campos, O.N., Ortiz-Plata, A., Sanchez-Garcia, A., Villeda-Hernandez, J., Galvan-Arzate, S., Aguilera, P., Santamaria, A., 2004. *S*-allylcysteine, a garlic-derived antioxidant, ameliorates quinolinic acid-induced neurotoxicity and oxidative damage in rats. Neurochemistry International 45, 1175–1183.

Posser, T., Moretto, M.B., Dafre, A.L., Farina, M., da Rocha, J.B.T., Nogueira, C.W., Zeni, G., Ferreira, J.D.S., Leal, R.B., Franco, J.L., 2006. Antioxidant effect of diphenyl diselenide against sodium nitroprusside (SNP) induced lipid peroxidation in human platelets and erythrocyte membranes: an in vitro evaluation. Chemico-Biological Interactions 164, 126–135.

Puntel, R.L., Nogueira, C.W., Rocha, J.B.T., 2005a. Krebs cycle intermediates modulate thiobarbituric acid reactive species (TBARS) production in rat brain *in vitro*. Neurochemical Research 30, 225–235.

Puntel, R.L., Nogueira, C.W., Rocha, J.B.T., 2005b. *N*-methyl-D-aspartate (NMDA) receptors are involved in the quinolinic acid (QA), but not in the malonate pro-oxidative activity *in vitro*. Neurochemical Research 30, 417–424.

Puttfarcken, P.S., Getz, R.L., Coyle, J.T., 1993. Kainic acid-induced lipid-peroxidation-protection with butylated hydroxytoluene and U78517f in primary cultures of cerebellar granule cells. Brain Research 624, 223–232.

Santamaria, A., Salvatierra-Sanchez, R., Vazquez-Roman, B., Santiago-Lopez, D., Villeda-Hernandez, J., Galvan-Arzate, S., Jimenez-Capdeville, M.E., Ali, S.F., 2003. Protective effects of the antioxidant selenium on quinolinic acid-induced neurotoxicity in rats: in vitro and in vivo studies. Journal of Neurochemistry 86, 479–488.

Simonian, N.A., Coyle, J.T., 1996. Oxidative stress in neurodegenerative diseases. Annual Review of Pharmacology and Toxicology 36, 83–106.

Sokolowska, M., Oleszek, A., Wodek, L., 1999. Protective effect of alpha-ketoacids on the oxidative hemolysis. Polish Journal of Pharmacology 51, 429–434.

Sun, J., Huang, S.H., Tan, B.K.H., Whiteman, M., Zhu, Y.C., Wu, Y.J., Ng, Y., Duan, W., Zhu, Y.Z., 2005a. Effects of purified herbal extract of *Salvia miltiorrhiza* on ischemic rat myocardium after acute myocardial infarction. Life Sciences 76, 2849–2860.

Sun, J., Huang, S.H., Zhu, Y.C., Whiteman, M., Wang, M.J., Tan, B.K.H., Zhu, Y.Z., 2005b. Anti-oxidative stress effects of *Herba leonuri* on ischemic rat hearts. Life Sciences 76, 3043–3056.

Sutherland, B.A., Shaw, O.M., Clarkson, A.N., Jackson, D.M., Sammut, I.A., Appleton, I., 2005. Neuroprotective effects of (+)-epigallocatechin gallate after hypoxia–ischemia-induced brain damage: novel mechanisms of action. FASEB Journal 19, 258–260.

Sutherland, B.A., Rahman, R.M.A., Appleton, I., 2006. Mechanisms of action of green tea catechins, with a focus on ischemia-induced neurodegeneration. Journal of Nutritional Biochemistry 17, 291–306.

Takayanagi, R., Takeshige, K., Minakami, S., 1980. NADH- and NADPH-dependent lipid peroxidation in bovine heart submitochondrial particles. Dependence on the rate of electron flow in the respiratory chain and an antioxidant role. Biochemical Journal 192, 853–860.

Takeshige, K., Minakami, S., 1975. Reduced nicotinamide adenine-dinucleotide phosphate-dependent lipid peroxidation by beef heart submitochondrial particles. Journal of Biochemistry 77, 1067–1073.

Tang, L.X., Yang, J.L., Shen, X., 1997. Effects of additional iron-chelators on Fe^{2+}-initiated lipid peroxidation: evidence to support the Fe^{2+}–Fe^{3+}complex as the initiator. Journal of Inorganic Biochemistry 68, 265–272.

Vianello, A., Macri, F., Cavallini, L., Bindoli, A., 1986. Induction of lipid peroxidation in soybean mitochondria and protection by respiratory substrates. Journal of Plant Physiology 125, 217–224.

Velvizhi, S., Dakshayani, K.B., Subramanian, P., 2002a. Effects of α-ketoglutarate on antioxidants and lipid peroxidation products in rats treated with ammonium acetate. Nutrition 18, 747–750.

Velvizhi, S., Nagalashmi, T., Mohamed, E.M., Dakshayani, K.B., Subramanian, P., 2002b. Effects of α-ketoglutarate on lipid peroxidation and antioxidant status during chronic ethanol administration in Wistar rats. Polish Journal of Pharmacology 54, 231–236.

Yamamoto, H., Mohanan, P.V., 2003. Effect of α-ketoglutarate and oxaloacetate on brain mitochondrial DNA damage and seizures induced by kainic acid in mice. Toxicology Letters 143, 115–122.

Yanpallewara, S., Raib, S., Kumarb, M., Chauhanc, S., Acharya, S.B., 2005. Neuroprotective effect of *Azadirachta indica* on cerebral post-ischemic reperfusion and hypoperfusion in rats. Life Sciences 76, 1325–1338.

Yonekawa, C., Nakae, H., Tajimi, K., Asanuma, Y., 2005. Effectiveness of combining plasma exchange and continuous hemodiafiltration in patients with postoperative liver failure. Artificial Organs 29, 324–328.

Younes, M., Trepkau, H.D., Siegers, C.P., 1990. Enhancement by dietary iron of lipid-peroxidation in mouse colon. Research Communications in Chemical Pathology and Pharmacology 70, 349–354.

Zeevalk, G.D., Bernard, L.P., Nicklas, W.J., 2000. Oxidative stress during energy impairment in mesencephalic cultures is not a downstream consequence of a secondary excitotoxicity. Neuroscience 96, 309–316.

5. DISCUSSÃO

Um dos objetivos desse estudo foi a identificação do(s) mecanismo(s) envolvido(s) na atividade pró-oxidante *in vitro* do malonato, ácido quinolínico e oxalato. Os resultados apresentados no **Artigo 1** sugerem que a atividade pró-oxidante do malonato *in vitro* independe do efeito inibitório desse sobre a SDH. Essa conclusão é baseada nos resultados mostrados na Tabela I do referido Artigo. Os resultados indicam que o pré-tratamento do S1 de cérebro de ratos por 10 min à 100ºC leva a uma completa inibição dos sistemas enzimáticos (Figura 3, Artigo 1). Mesmo nessas condições (de inatividade enzimática) o malonato foi capaz de induzir um significativo aumento na geração de espécies reativas ao ácido tiobarbitúrico (TBARS), o que suporta nossa idéia inicial de que o efeito do malonato é independente de seu efeito direto sobre a enzima SDH. Além disso, nem a adição de cianeto de potássio (KCN - inibidor da cadeia respiratória; Figura 1, Artigo 1) nem do 2,4-dinitrofenol (desacoplador da cadeia respiratória) alteraram a produção de TBARS induzida por malonato (dados não mostrados). Esses resultados sugerem que a disfunção mitocondrial induzida por malonato não explica *per se* o aumento no TBARS *in vitro.*

Do mesmo modo, o antagonista de receptors NMDA (MK-801) não foi capaz de prevenir o aumento no TBARS induzido pelo malonato, indicando que a excitotoxicidade secundária não está envolvida na atividade pró-oxidante do malonato *in vitro* (Tabela I, Artigo 1). Esse resultado está de acordo com um estudo prévio o qual mostra que a estimulação dos receptores NMDA não contribui para o estresse oxidativo de células expostas ao malonato (Zeevalk e cols. 2000).

Baseado na propriedade do malonato de quelar ferro (Figura 4, Artigo 1) e nas observações feitas anteriormente, sugerimos que a atividade pró-oxidante do malonato *in vitro* pode ser devida à sua interação com os íons ferro (endógenos) presentes no S1. Esses complexos entre os íons ferro e malonato poderiam levar a um aumento na geração de EROs devido ao ciclo redox do ferro. Contudo, o malonato não teve efeito nos ensaios da degradação da desoxirribose induzida por ferro e peróxido de hidrogênio (Figura 5, Artigo 1), indicando que os complexos ferro/malonato são ativos quando um sistema puramente químico é usado. Sendo assim, assumimos que o malonato modula a produção de TBARS *in vitro* por manter uma adequada razão Fe^{2+}/ Fe^{3+}, a qual é um fator essencial para a geração de radicais livres. Essa idéia está de acordo com dados prévios que demonstraram

claramente que uma razão adequada Fe^{2+}/ Fe^{3+} é essencial para que o ferro exerça seus efeitos pró-oxidantes (Tang e cols., 1997; Caro e Cederbaum, 2004).

Por outro lado, nossos resultados confirmaram a hipótese de que a atividade pró-oxidante do ácido quinolínico (AQ) é mediada pela ativação direta dos receptores NMDA, uma vez que a adição do MK-801 reduziu a geração de TBARS induzida por esse (Tabela II, Artigo 1). Sendo assim, nossos resultados confirmam dados anteriores os quais demonstraram que a ativação dos receptores NMDA por AQ está associada a um aumento na geração de EROs e, conseqüentemente um aumento no TBARS (Santamaría e Rios, 1993; Rodríguez-Martínez e cols., 2000). Contudo, devemos considerar o envolvimento dos íons ferro na toxicidade *in vitro* do AQ, uma vez que a deferroxamina (DFO) aboliu completamente o efeito desse (Figura 4, Artigo 2).

O pré-tratamento do S1 de cérebro de ratos com diferentes temperaturas revelou um dado importante. Os resultados apresentados na Tabela III (Artigo 1) mostraram que o aumento na temperatura de pré-tratamento do S1 está relacionado com uma ativação dos receptores NMDA. Nesse contexto, nossos resultados mostraram que o efeito antioxidante do MK-801 é mais pronunciado em preparações pré-tratadas a 100ºC, quando comparado com o efeito desse em preparações mantidas a 37°C. Isso indica que a alta temperatura pode causar uma alteração conformacional na estrutura do receptor, levando a uma ativação permanente do mesmo, a qual pode ser prevenida pelo MK-801. Assim, a diminuição na eficiência do AQ em induzir TBARS (o AQ induz um aumento de aproximadamente 40 % em S1 pré-tratados até 37ºC, e apenas 22% em S1 pré-tratado a 100ºC; Tabela III- Artigo 1) pode ser atribuída à ativação direta dos receptores NMDA pela temperatura. Esse resultado está de acordo com o trabalho de Chung e Kuyucak, 1995, os quais demonstram que a atividade dos receptores NMDA está diretamente relacionada ao aumento da temperatura (Chung e Kuyucak, 1995). Além disso, estudos *in vivo* mostraram que o aumento na temperatura corpórea pode agravar os episódios convulsivos, levando a um aumento no dano cerebral o qual é, em parte, devido a um aumento na atividade dos receptores NMDA (Lundgren e cols., 1994; Morimoto e cols., 1995).

Da mesma forma, estudamos o(s) mecanismo(s) envolvido(s) na atividade pró-oxidante *in vitro* do oxalato, utilizando diferentes tecidos de ratos. Como mostrado no **Artigo 3,** o oxalato, em baixas concentrações (0.3 – 2 mmol/l), foi capaz de induzir a

produção de TBARS em homogeneizado de fígado e cérebro de ratos. Porém, em concentrações maiores (8 – 16 mmol/l) o oxalato reduziu significativamente a produção de TBARS. Essa propriedade antioxidante do oxalato no fígado e em cérebro está de acordo com dados da literatura, onde o oxalato foi capaz de prevenir a peroxidação lipídica em microssomas de fígado e cérebro de ratos (Kayashima e Katayama, 2002). Surpreendentemente, o oxalato não induziu a produção de TBARS em preparações de rim de ratos. A razão para esse resultado inesperado pode estar relacionada ao fato que em presença de homogeneizado de rim, o oxalato de sódio precipita na forma de cristais insolúveis, o que não é observado quando homogeneizado de fígado ou cérebro foram usadas (Figura 2, Artigo 3). Assim, a formação desses cristais insolúveis (em preparações de rim de ratos) pode estar associada a uma diminuição na concentração de oxalato livre e, consequentemente disponível para participar de reações químicas específicas.

A atividade antioxidante do oxalato nas maiores concentrações usadas pode estar relacionada à sua habilidade em quelar íons ferro (resultados não mostrados). Porém, como mostrado no **Artigo 3** o oxalato nas menores concentrações estudadas (1 – 3 mmol/l) foi capaz de aumentar a atividade pró-oxidante dos íons ferro em homogeneizado do fígado de ratos (Figura 5B, Artigo 3), sem contudo alterar a produção de TBARS induzida por ferro em homogeneizado de cérebro de ratos (Figura 5A, Artigo 3). Por outro lado, o oxalato nas maiores concentrações estudadas (8 – 32 mmol/l) foi capaz de reduzir a produção de TBARS induzida por ferro, tanto em preparações de fígado, quanto de cérebro de ratos (Figuras 5A e 5B, Artigo 3). O envolvimento dos íons ferro na atividade pró-oxidante *in vitro* do oxalato é também embasado quando consideramos o efeito inibitório da deferroxamina (DFO) sobre a produção de TBARS induzida por oxalato (Figura 6, Artigo 3).

Além do mais, os complexos ferro/oxalato formados nas situações em que usamos as menores concentrações de oxalato (2 mmol/l) não preveniram a degradação da desoxirribose induzida por Fe^{2+} e H_2O_2, sugerindo que nessa concentração de oxalato formam-se complexos bio ativos entre esse e os íons ferro. Por outro lado, os complexos formados quando usamos oxalato 20 mmol/l previnem a degradação da desoxirribose induzida pelo Fe^{2+} e H_2O_2 (Figura 4, Artigo 3). Contudo, um estudo prévio demonstrou que o oxalato pode agir como um “scavenger” de radicais hidroxil, produzindo formato e

superóxido (Adams e Willson, 1969). Assim, a possibilidade de neutralizar radicais hidroxil diretamente (nas maiores concentrações de oxalato usadas) pode ser considerada como uma alternativa, além da complexação de íons ferro.

Baseado nos resultados apresentados no **Artigo 3**, nós especulamos (da mesma forma que sugerido para o malonato) que o oxalato nas maiores concentrações estudadas previne a produção de TBARS por modular uma inadequada razão Fe^{2+}/Fe^{3+}, a qual é essencial para a geração de radicais livres. Porém, quando usamos concentrações menores de oxalato, é possível especular que uma razão Fe^{2+}/Fe^{3+} adequada seja formada, o que ocasionaria um aumento no ciclo redox do ferro e consequentemente na geração de radicais livres e peroxidação lipídica (TBARS).

Outro objetivo do nosso estudo foi investigar o efeito de alguns intermediários do ciclo de Krebs sobre a produção de TBARS basal, ou induzida por diferentes pró-oxidantes, bem como o(s) mecanismo(s) através do qual(is) esses intermediários exercem seus efeitos. Os resultados apresentados nos **Artigos 2** e **4** indicam que o efeito antioxidante dos intermediários do Ciclo e Krebs varia, dependendo da sua estrutura e da sua concentração. O citrato, o sucinato, o oxaloacetato, e o malato, preveniram significativamente a produção de TBARS basal, ou induzida por diferentes pró-oxidantes (Artigos 2 e 4) em S1 de cérebro de ratos, enquanto que o fumarato foi capaz de prevenir somente a produção de TBARS induzida, sem efeito significativo sobre a geração de TBARS basal (Artigo 4). O α-cetoglutarato, por sua vez, foi capaz de induzir *per se* um aumento na produção de TBARS (Artigos 2 e 4). Entretanto, o(s) mecanismo(s) pelo(s) qual(is) os intermediários do Ciclo de Krebs exercem seus efeitos parece ser distinto e depende do intermediário usado.

O efeito do sucinato e fumarato mostrou-se sensível à temperatura, sendo abolido quando o S1 de cérebro de ratos foi pré-tratado por 100ºC por 10 min. Além disso, o efeito do sucinato foi completamente abolido quando cianeto de potássio foi adicionado ao meio. Por outro lado, o efeito do citrato, malato, oxaloacetato e α-cetoglutarato não foram alterados pelo pré-tratamento a elevadas temperaturas (Artigos 2 e 4). Considerando o efeito do KCN (somente para o sucinato), bem como o efeito de pré-tratamento a 100ºC, supomos que o efeito tanto do sucinato quanto do fumarato é dependente de alguma atividade enzimática, enquanto que o efeito dos demais intermediários não.

Compostos capazes de quelar ferro podem modular a produção de TBARS. Assim a

DFO, um quelante clássico de íons férricos (Fe^{3+}), reduziu consideravelmente a produção de TBARS em S1 de cérebro de ratos (Artigos 2 e 4). Da mesma maneira que a DFO, os intermediários do Ciclo de Krebs, exceto o sucinato e fumarato, exibiram atividade quelante de íons ferrosos (Fe^{2+}) (Artigos 2 e 4). Porém, somente o citrato e o oxaloacetato exibiram atividade quelante em concentrações fisiológicas. Sendo assim, parte da atividade antioxidante desses intermediários pode ser atribuída a suas atividades quelantes de íons ferrosos. Contudo, o oxaloacetato foi um agente quelante mais eficiente que o citrato, enquanto que esse reduziu a produção de TBARS mais eficientemente que o oxaloacetato. Parte dessas discrepâncias pode ser atribuída ao fato que alguns complexos de ferro, tais como os complexos ferro/citrato, apresentam atividade redox (Chiueh, 2001).

Os resultados dos ensaios da degradação da desoxirribose induzida por ferro e peróxido de hidrogênio demonstram que os intermediários do Ciclo de Krebs não são capazes de prevenir a degradação induzida por ferro. Porém, o oxaloacetato e o α-cetoglutarato foram capazes de prevenir a degradação da desoxirribose induzida por peróxido de hidrogênio (Figura 7, Artigo 2). Esse resultado está de acordo com estudos prévios que mostram claramente que os α-cetoácidos são efetivos na detoxificação não enzimática do peróxido de hidrogênio (Desagher e cols., 1997; Sokolowska e cols., 1999; Oh e cols., 2002; Velvizhi e cols., 2002 a; Velvizhi, e cols., 2002 b; Mallet e Sun, 2003). Conseqüentemente, a inibição da reação de Fenton causada pelo oxaloacetato e pelo α-cetoglutarato parece ser devido à reação direta desses com o peróxido de hidrogênio. O sucinato e o malato preveniram a degradação da desoxirribose induzida por ferro e peróxido de hidrogênio, porém seus efeitos foram moderados, enquanto que o citrato não protegeu contra a degradação da desoxirribose induzida por ferro/peróxido de hidrogênio (Figura 7, Artigo 2). A partir desses resultados, sugerimos que os complexos ferro/citrato formados em nossas condições experimentais (razão ferro/citrato, 1/10) participam da reação de Fenton, embora não sejam capazes de aumentar a degradação da desoxirribose quando comparados ao sistema contendo apenas o ferro e o peróxido de hidrogênio. Esse efeito dos complexos ferro/citrato está de acordo com o efeito oxidante desses relatado na literatura (Minotti e Aust, 1987).

Compostos capazes de interagir com íons ferro (agentes quelantes) podem tanto inibir quanto induzir a produção de TBARS. O efeito pró ou antioxidante dos mesmos depende da

habilidade desses em modular uma adequada razão Fe^{2+}/Fe^{3+} no meio (Minotti e Aust, 1987; Oubidar e cols., 1994; Chiueh, 2001; Caro e Cederbaum, 2004). Sendo assim, a adição de um quelante pode alterar a razão Fe^{2+}/Fe^{3+}, a qual tem atividade máxima quando a proporção for 1:1 (Fe^{2+}: Fe^{3+}) (Tang e cols., 1997; Caro e Cederbaum, 2004), fazendo com que a geração de radicais livres aumente (se for razão 1:1) ou diminua. Por exemplo, o quelante 2,2' dipiridil (o qual liga-se firmemente com Fe^{2+} inibindo seu ciclo redox), e a DFO (a qual liga-se fortemente somente a Fe^{3+} inibindo o ciclo redox do ferro e bloqueando sua atividade catalítica) possuem atividade antioxidante por impedir a formação de uma adequada razão Fe^{2+}/ Fe^{3+} (Huang e cols., 2002), impedindo dessa forma a iniciação, bem como a propagação das reações de peroxidação lipídica dependentes de íons ferro.

Considerando o que foi exposto, sugerimos que o efeito pró-oxidante do α-cetoglutarato deve-se à capacidade desse composto em ajustar uma razão Fe^{2+}/Fe^{3+}, que favorece o ciclo redox do ferro, levando a um aumento na geração de EROs, e conseqüentemente na produção de TBARS. Por outro lado, o efeito antioxidante do citrato, do malato, e do oxaloacetato sobre a produção de TBARS basal ou induzida pode estar relacionado à capacidade de tais compostos interagirem com íons ferro modulando uma razão Fe^{2+}/Fe^{3+} inadequada para a iniciação e propagação das reações de peroxidação lipídica. Além disso, observamos que o citrato age como um potente antioxidante quando o S1 de cérebro de ratos é usado, porém não tem efeito quando o Fe^{2+} e/ou peróxido de hidrogênio são usados em um sistema para gerar radicais livres. Essas observações estão de acordo com os resultados de Gutteridge, nos quais os complexos ferro/citrato foram ativos frente à degradação da desoxirribose induzida por ferro e peróxido de hidrogênio. Gutteridge mostrou ainda que aumentando a razão ferro/citrato ocorre uma redução na degradação da desoxirribose, obtendo degradação máxima quando a razão ferro/citrato aproxima-se de 1:1 (Gutteridge, 1991).

As concentrações nas quais o oxaloacetato, o malato e o sucinato apresentaram o efeito antioxidante são relativamente elevadas e sem significância fisiológica, enquanto que as concentrações nas quais o citrato exerceu esse efeito são de relevância fisiológica. Sendo assim, podemos sugerir que o citrato pode agir como um antioxidante endógeno em situações isquêmicas, as quais estão relacionadas a um aumento na geração de EROs bem como na liberação intracelular dos íons ferro.

Nesse contexto, Mallet relatou o efeito antioxidante do citrato durante a reperfusão de tecidos cardíacos sujeitos a isquemia. Em seu trabalho, Mallet demonstrou que o efeito antioxidante do citrato está relacionado a um aumento no conteúdo de GSH (Mallet e Sun, 2003), uma vez que elevados níveis de citrato são capazes de inibir a glicólise ao nível da *fosfofrutoquinase*. Essa inibição ao nível de *fosfofrutoquinase* levaria a um acúmulo no intermediário glicose-6-fosfato, o qual é substrato da Via das Pentoses Fosfato. Com o aumento na velocidade dessa via, haveria um aumento na geração de NADPH (Mallet e Sun, 2003), o qual é usado pela *glutationa redutase* na regeneração da GSH a partir de GSSG. Porém nós não encontramos um aumento no conteúdo de –SH total, em presença de citrato ou sucinato, em nossas condições experimentais. Dessa forma, o efeito do citrato não pode ser atribuído a um aumento no conteúdo de –SH. Em nossas condições experimentais, a atividade antioxidante do citrato está relacionada principalmente à propriedade quelante e não ao seu papel no metabolismo energético.

Finalmente, tivemos por objetivo validar o método colorimétrico de TBARS (Ohkawa e cols., 1979) por meio de um estudo comparativo com analises em HLPC (Grotto e cols., 2006) (Artigo 4). Quando comparados os resultados obtidos com o método colorimétrico comumente usado e analises em HPLC, praticamente não encontramos diferenças entre as técnicas empregadas, principalmente quando no método colorimétrico o complexo TBA-MDA é extraído em solvente orgânico (butanol/piridina) (Artigo 4). O principal ponto a ser discutido, é com relação à super estimação na quantidade de MDA encontrada por meio do método colorimétrico (10 a 20 vezes maior), quando comparados aos valores obtidos por meio de analises com HPLC. As espécies reativas ao TBA que não são MDA, e que contribuem para a super estimação não são completamente conhecidos, além do que variam entre os diferentes materiais biológicos (Draper e cols., 1993). Entretanto, uma vez que o ensaio do TBARS é usado como um indicador final do dano oxidativo (Dawn-Linsley e cols., 2005), nosso sistema torna-se útil para acessar o impacto final de agentes indutores de estresse oxidativo, bem como de agentes protetores.

6. CONCLUSÕES

De acordo com os resultados apresentados nesta tese podemos inferir o que segue:

- O efeito pró-oxidante do malonato *in vitro* é independente de seu papel no metabolismo energético, e é devido principalmente à capacidade desse composto de interagir com íons ferro.

- O efeito do AQ *in vitro* parece estar diretamente relacionado à ativação dos receptores NMDA, bem como parece ser dependente dos íons ferro.

- O efeito pró- ou antioxidante do oxalato *in vitro* parece estar relacionado à habilidade do mesmo em formar complexos com íons ferro.

- O oxalato teve efeito pró-oxidante somente em preparações de cérebro e fígado de ratos, enquanto que em preparações de rim o oxalato precipitou na forma de cristais.

- O efeito antioxidante do citrato, do malato, e do oxaloacetato sobre a produção de TBARS basal ou induzida por AQ, Fe^{2+}, ou malonato é devido à capacidade de tais compostos interagirem com íons ferro modulando uma razão Fe^{2+}/Fe^{3+}, inadequada para a iniciação e propagação das reações de peroxidação lipídica.

- O efeito pró-oxidante do α-cetoglutarato deve-se à capacidade desse composto em ajustar uma razão Fe^{2+}/Fe^{3+}, que favorece o ciclo redox do ferro, levando a um aumento na geração de EROs, e conseqüentemente na produção de TBARS.

- O efeito antioxidante do sucinato, bem como do fumarato parece estar relacionado a alguma atividade enzimática, uma vez que o pré-tratamento com KCN (somente para o sucinato) ou em elevada temperatura (para o sucinato e fumarato) aboliu o efeito antioxidante de ambos os intermediários.

- Somente o citrato teve efeito antioxidante em concentrações fisiológicas, o que sugere que esse intermediário pode agir como um modulador do estresse oxidativo em situações fisiológicas e também durante certas situações patológicas, tais como durante episódios isquêmicos.

- O método colorimétrico comumente usado para a determinação de TBARS mostrou-se útil no nosso sistema, uma vez que esse é utilizado como um indicador final de dano oxidativo. Portanto, esse método se mostra adequado em pesquisas envolvendo tanto agentes pró quanto antioxidantes.

7. PERSPECTIVAS

Baseado nos resultados apresentados nessa tese faz-se necessário:

- Avaliar o efeito desses intermediários frente a outras medidas de estresse oxidativo, além do TBARS.

- Investigar o efeito desses intermediários sobre a produção de TBARS induzida por oxalato em preparações de diferentes tecidos de ratos.

- Caracterizar utilizando técnicas específicas a formação, bem como a reatividade dos complexos formados entre os íons ferro e os intermediários do Ciclo de Krebs.

- Avaliar a contribuição dos diferentes complexos, ferro-intermediários do Ciclo de Krebs, para a produção de TBARS, em ensaios *in vitro*.

8. REFERÊNCIAS BIBLIOGRÁFICAS

ABRAHAMSON, H. B., REZVANI, A. B., BRUSHMILLER, G. Photochemical and spectroscopic studies of complexes of iron (III) with citric acid and other carboxylic acids. **Inorg. Chimica Acta**, 226, 117-127, 1994.

ADAMS, G. E., WILLSON, R. L. Pulse radiolysis studies on the oxidation of organic radicals in aqueous solution. **Trans. Faraday Soc.**, 65, 2981-2987, 1969.

ADIBHATLA, R. M., HATCHER, J. F. Phospholipase A_2, reactive oxygen species, and lipid peroxidation in cerebral ischemia. **Free Radic. Biol. Med.**, 40, 376 – 387, 2006.

ALBERTS, B., BRAY, D., LEWIS, J., RAFF, M., ROBERTS, K., WATSON, J. D. **Molecular Biology of the cell**. 3ª ed, New York & London: Garland Publishing, 1994.

ALEXI, T., HUGHES, P. E., FAULL, R. L. M., WILLIAMS, C. E. 3-Nitropropionic acid's lethal triplet: cooperative pathway of neurodegeneration. **Neuroreport**, 9, R57-R64, 1998.

AMES, B. N., SHIGENAGA, M. K., HAGEN, T. M. Oxidants, antioxidants, and the neurodegenerative diseases of aging. **Proc. Natl. Acad. Sci. USA**, 90, 7915-7922, 1993.

BAKER, M. S., GEBICKI, J. M. The effect of pH on yields of hydroxyl radicals produced from superoxide by potential biological iron chelators. **Arch. Biochem. Biophys.**, 246, 581-588, 1986.

BASTIANETTO, S., QUIRION, R. Natural extracts as possible protective agents of brain aging. **Neurobiol. Aging**, 23, 891–897, 2002.

BEAL , M. F. Mitochondria, free radicals, and neurodegeneration. **Current Opinion in Neurobiology**, 6, 661-666, 1996.

BEAL, M. F., BROUILLET, E., JENKINS, B., HENSHAW. R., ROSEN, B., HYMAN, B. T. Age-dependent striatal excitotoxic lesions produced by the endogenous mitochondrial inhibitor malonate. **J. Neurochem.**, 61, 1147- 1150, 1993.

BINDOLI, A., CAVALLINI, L., JOCELYN, P. Mitochondrial lipid peroxidation by cumene hydroperoxide and its prevention by succinate. **Biochim. Biophys. Acta**, 681, 496-503, 1982.

BIRD, R. P., HUNG, S. O. S., HADLEY, M., DRAPER, H. H. Determination of malonaldehyde in biological materials by high-pressure liquid chromatography. **Anal. Biochem.**, 128, 240-244, 1983.

BLOMGREN, K., ZHU, C., HALLIN, U., HAGBERG, H. Mitrochondria and ischemic reperfusion damage in the adult and in the developing brain. **Biochem. Biophys. Res. Comm.**, 304, 551–559, 2003.

BRALET, J., SCHREIBER, L., BOUVIER, C. Effect of acidosis and anoxia on iron delocalization from brain homogenates. **Biochem. Pharmacol.**, 43, 979–983, 1992.

BRAUGHLER, J. M., DUNCAN, L. A., CHASE, R.L. The involvement of iron in lipid peroxidation. Importance of ferric to ferrous ratios in initiation. **J. Biol. Chem.**, 261, 10282-10289, 1986.

BURGER, M. E., FACHINETTO, R., CALEGARI, L., PAIXÃO, M. W., BRAGA, A. L., ROCHA, J. B. T., Effects of age on reserpine-induced orofacial dyskinesia and possible protection of diphenyl diselenide. **Brain Res. Bull.,** 64, 339–345, 2004.

CAMPBELL, M. K. **Bioquimica**. 3ªed., Porto Alegre, Artmed Editora, 1999.

CAO, W., CARNEY, J., DUCHON, A., FLOYD, R., CHEVION, M. Oxygen free radical involvement in ischemia and reperfusion injury to brain. **Neurosci. Lett.**, 88, 233–238, 1988.

CARO, A. A., CEDERBAUM, A. T. Antioxidant Properties of S-Adenosyl-L-Methionine in Fe^{2+}- Initiated oxidations. **Free Radic. Biol. Med.**, 36, 1303-1316, 2004.

CAVALLINI, L., VALENTE, M., BINDOLI, A. Comparison of cumene hydroperoxide – and NADPH/ Fe^{3+}/ ADP-induced lipid peroxidation in heart and liver submitochondrial particles. Mechanisms of protection by succinate. **Biochim. Biophys. Acta**, 795, 466-472, 1984.

CHAN, P. H. Role of oxidants in ischemic brain damage. **Stroke**, 27, 1124–1129, 1996.

CHIANG, Y. H., CHEN, G. J., SHEN, H., CHOU, J., WANG, Y. Histogranin reduced brain injury after transient focal ischemia in rats. **Neurosci. Lett.**, 406, 211-215, 2006.

CHIUEH, C. C. Iron overload, oxidative stress, and axonal dystrophy in brain disorders. **Pediatr. Neurol.**, 25, 138- 147, 2001.

CHIUEH, C. O., MIYAKE H., PENG, M. T. Role of dopamine autoxidation, hydroxyl radical generation, and calcium overload in underlying mechanisms involved in MPTP-induced parkinsonism. **Adv. Neurol.**, 60, 251-258, 1993.

CHUNG, S. H., KUYUCAK, S. Changes in the kinetics and conductance of N-methyl-D-aspartate (NMDA)-receptor activated single channels with temperature. **Neurosci. Lett.**, 187, 181-184, 1995.

DAWN-LINSLEY, M., EKINCI, F. J., ORTIZ, D., ROGERS, E., SHEA, T. B. Monitoring thiobarbituric acid-reactive substances (TBARs) as an assay for oxidative damage in neuronal cultures and central nervous system. **J. Neurosci. Meth.,** 141, 219-222, 2005.

DAWSON, V., L., DAWSON, T. M. Free radicals and neuronal cell death. **Cell Death and Differentiation**, 3, 71-78, 1996.

DEDEOGLU, A., FERRANTE, R. J., ANDREASSEN, O. A., DILLMANN, W. H., BEAL, M. F. Mice overexpressing 70-kda heat shock protein show increased resistance to malonate and 3-nitropropionic acid. **Exp. Neurol.**, 176, 262– 265, 2002.

DEMOPOULOS, H., FLAMM, E., SELIGMAN, M., POWER, R., PIETRONIGRO, D., RANSOHOFF, J. **Molecular pathology of lipids in CNS membranes**. In: Jöbsis, F., ed. *Oxygen and physiological function*. Dallas: Professional Information Library, 491–508, 1977.

DESAGHER, S., GLOWINSKI, J., PREMONT, J. Pyruvate protects neurons against hydrogen peroxide-induced toxicity. **J. Neurosci.**, 17, 9060-9067, 1997.

DRAPER, H. H., SQUIRES, E. J., MAHMOODI, H., WU, J., AGARWAL, S., HADLEY, M. A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. **Free Radic. Biol. Med.**, 15, 353-363, 1993.

ESTERBAUER, H., ZOLLNER, H. Methods for determination of aldehydic lipid peroxidation products. **Free Radic. Biol. Med.**, 7, 197-203, 1989.

FERGER, B., EBERHARDT, O., TEISMANN, P., GROOTE, C., SCHULZ, J. B. Malonate-induced generation of reactive oxygen species in rat striatum depends on dopamine release but not on NMDA receptor activation. **J. Neurochem.**, 73, 1329-1332, 1999.

FINKEL, T., HOLBROOK, N. J. Oxidants, oxidative stress and the biology of ageing. **Nature**, 408, 239-247, 2000.

FOLBERGROVÁ, J., LJUNGGREN, B., NORBERG, K. SIESJÖ, B. K. Influence of complete ischemia on glycolytic metabolites, citric acid cycle intermediates, and associated amino acids in the rat cerebral cortex. **Brain Res.**, 80, 265-279, 1974.

FREI, B. Reactive oxygen species and antioxidant vitamins: mechanisms of action. **Am. J. Med.**, 97, 5S-13S, 1994.

GIANNI, P., JAN, K. J., DOUGLAS, M. J., STUART, P. M., TARNOPOLSKY, M. A. Oxidative stress and the mitochondrial theory of aging in human skeletal muscle. **Exp. Gerontol.**, 39, 1391-1400, 2004.

GIBSON, G. E., PULSINELLI, W., BLASS, J. P., DUFFY, T. E. Brain dysfunction in mild to moderate hypoxia. **Am. J. Med.**, 70, 1247–1254, 1981.

GOLDBERG, N. D., PASSONNEAU, J. V., LOWRY, O. H. Effects of Changes in Brain Metabolism on the Levels of Citric Acid Cycle Intermediates. **J. Biol. Chem.**, 241, 3997 - 4003, 1966.

GOTTLIEB, M., LEAL-CAMPANARIO, R., CAMPOS-ESPARZA, M. R., SÁNCHEZ-GÓMEZ, M. V., ALBERDI, E., ARRANZ, A., DELGADO-GARCIÍA, J. M., GRUART, A., MATUTE, C. Neuroprotection by two polyphenols following excitotoxicity and experimental ischemia. **Neurobiol. Dis.,** 23, 374 – 386, 2006.

GREENE, J. G., GREENAMYRE, J. T. Characterization of the excitotoxic potential of the reversible succinate dehydrogenase inhibitor malonate. **J. Neurochem.**, 64, 430-436, 1995.

GREENE, J. G., GREENAMYRE, J. T. Manipulation of membrane potential modulates malonate-induced striatal excitotoxicity in vivo. **J. Neurochem.**, 66, 637-643, 1996.

GREENE, J. G., PORTER, R. H. P., ELLER, R. V., GREENAMYRE, J. T. Inhibition of succinate dehydrogenase by malonic acid produces an excitotoxic lesion in rat striatum. **J. Neurochem.**, 61, 1151-1154, 1993.

GROTTO, D., SANTA MARIA, L. D., BOEIRA, S., VALENTINI, J., CHARÃO, M. F., MORO, A. M., NASCIMENTO, P. C., POMBLUM, V. J., GARCIA, S. C. Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography – visible detection. **J. Pharm. Bio. Anal.,** 43, 619-624, 2006.

GUPTA, S., SHARMA, S. S. Neuroprotective Effects of Trolox in Global Cerebral Ischemia in Gerbils**. Biol. Pharm. Bull**., 29, 957—961, 2006.

GUTTERIDGE J. M. Hydroxyl radicals, iron, oxidative stress, and neurodegeneration. **Ann. NY Acad. Sci.**, 738, 201-213, 1994.

GUTTERIDGE, J. M. C. Hydroxyl radical formation from the auto-reduction of a ferric citrate complex. **Free Radic. Biol. Med.**, 11, 401-406, 1991.

HALLIWELL, B. Reactive oxygen species and the central nervous system. **J. Neurochem.**, 59, 1609–1623, 1992.

HALLIWELL, B., GUTTERIDGE, J. M. C. **Free radicals in Biology and Medicine**, 2ª ed, New York: Clerendon Press, 1989.

HENSHAW. R., JENKINS, B. G., SCHULZ, J. B., FERRANTE, R. J., KOWALL, N. W., ROSEN, B. R., BEAL, M. F. Malonate produces striatal lesions by indirect NMDA receptor activation. **Brain Res.**, 647, 161-166, 1994.

HOYER, S., KRIER, C. Ischemia and the aging brain. Studies on glucose and energy metabolism in rat cerebral cortex. **Neurobiol. Aging**, 7, 23-29, 1986.

HUANG, X., DAI, J., FOURNIER, J., ALI, A. M., ZHANG, Q., FRENKEL, K. Ferrous ion autoxidation and its chelation in iron-loaded human liver HepG2 cells. **Free Radic. Biol. Med.**, 32, 84-92, 2002.

JOSEPHY, P. D. **Molecular Toxicology**, New York: Oxford University Press, 1997.

KAYASHIMA, T., KATAYAMA, T. Oxalic acid is available as a natural antioxidant in some systems, **Biochim. et Biophys. Acta**, 1573, 1-3, 2002.

KHAND, F. D., GORDGE, M. P., ROBERTSON, W. G., NORONHA-DUTRA, A. A., HOTHERSALL, J. S. Mitochondrial superoxide production during oxalate-mediated oxidative stress in renal epithelial cells. **Free Rad. Biol. & Med.**, 32, 1339–1350, 2002.

KIKUGAWA, K., KOJIMA, T., YAMAKI, S., KOSUGI, H. Interpretation of the thiobarbituric acid reactivity of rat liver and brain homogenates in the presence of ferric ion and ethylenediamine-tetra-acetic acid. **Anal. Biochem.,** 202, 249-255, 1992.

KOHJIMOTO, Y., KENNINGTON, L., SCHEID, C. R., HONEYMAN, T. W. Role of phospholipase A(2) in the cytotoxic effects of oxalate in cultured renal epithelial cells. **Kidney Int.**, 56, 1432–1441, 1999.

KOSUGI, H., KOJIMA, T., KIKUGAWA, K. Thiobarbituric acid-reactive substances from peroxidized lipids. **Lipids**, 24, 873-881, 1989.

KOWALTOWSKI, A., CASTILHO, R. F., VERCESI, A. E. Mitochondrial permeability transition and oxidative stress. **FEBS Letters**, 495, 12-15, 2001.

LACHILI, B., HININGER, I., FAURE, H., ARNAUD, J., RICHARD, M. J., FAVIER, A., ROUSSEL, A. M. Increased lipid peroxidation in pregnant women after iron and vitamin C supplementation. **Biol. Trace Element Res.**, 83, 103-110, 2001.

LEHNINGER, A. L. **Bioquímica.** Edgard Blücher, 2ª edição, São Paulo, v. 2, 1976.

LEPAGE, G., MUNOZ, G., CHAMPAGNE, J., ROY, C. C. Preparative steps necessary for the accurate measurements of malondialdehyde by high-performance liquid chromatography. **Anal. Biochem.,** 197, 277-283, 1991.

LIBERT, B., FRANCESCHI, V. R. Oxalate in crop plants, **J. Agric. Food Chem**., 35, 926–938, 1987.

LIU, T. H., BECKMAN, J. S., FREEMAN, B. A., HOGAN, E. L., HSU, C. Y. Polyethylene glycol-conjugated superoxide dismutase and catalase reduce ischemic brain injury. **Am. J. Physiol.**, 256, H589– H593, 1989.

LUNDGREN, J., SMITH, M. L., BLENNOW, G., SIESJO, B. K. Hyperthermia aggravates and hypothermia ameliorates epileptic brain-damage. **Exp. Brain Res.**, 99, 43-55, 1994.

MAILLY, F., MARIN, P., ISRAEL, M., GLOWINSKI, J., PRÉMONT, J. Increase in external glutamate and NMDA receptor activation contribute to H_2O_2 – induced neuronal apoptosis. **J. Neurochem.**, 736, 1181-1188, 1999.

MALLET, R. T., SUN, J. Antioxidant properties of myocardial fuels. **Mol. Cell. Biochem.**, 253, 103- 111, 2003.

MARAGOS, W. F., SILVERSTAIN, F. S. The mitochondrial inhibitor malonate enhances NMDA toxicity in the neonatal rat striatum. **Develop. Brain Res.**, 88, 117-121, 1995.

MASSEY, L.K., PALMER, R.G., HORNER, H.T. Oxalate content of soybean seeds (Glycine max : Leguminosae), soyfoods, and other edible legumes. **J. Agric. Food Chem**. 49, 4262–4266, 2001.

MEDVEDEVA, L. V., POPOVA, T. N., ARTYUKHOV, V. G., MATASOVA, L. V., AKATOVA, R. V. Oxidative status and distribution of NADP-dependent isocitrate dehydrogenase and aconitate hydratase in rat cardiomyocytes under normal conditions and during ischemia. **Bul. Exp. Biol. Med.**, 134, 130-134, 2002.

MINOTTI, G., AUST, S. D. An investigation into the mechanism of citrate-Fe^{2+}-dependent lipid peroxidation. **Free Rad. Biol. Med.**, 3, 379- 387, 1987.

MINOTTI, G., AUST, S. D. Redox cycling of iron and lipid peroxidation. **Lipids**, 27, 219-226, 1992.

MORIMOTO, T., KIDA, K., NAGAO, H., YOSHIDA, K., FUKUDA, M., TAKASHIMA, S. The pathogenic role of the NMDA receptor in hyperthermia-induced seizures in developing rats. **Develop. Brain Res.**, 84, 204-207, 1995.

MORONI, F. Tryptophan metabolism and brain function: focus on kynurenine and other indole metabolites. **Eur. J. Pharmacol.,** 375, 87-100, 1999.

MORONI, F., LOMBARDI, G., CARLA, V., PELLEGRINI, D., CARASSALE, G. L., CORTESINI, C. Content of quinolinic acid and of other tryptophan metabolites increase in brain regions of rats used as experimental models of hepatic encephalopathy. **J. Neurochem.**, 46, 869-874, 1986.

MUTHUKUMAR, A., SELVAM, R. Role of glutathione on renal mitochondrial status in hyperoxaluria. **Mol. Cell Biochem.**, 185, 77–84, 1998.

NELSON, D. L., COX, M. M. **Lehninger, Princípios de Bioquímica**, Sarvier, 3ª edição, São Paulo, 2002.

NOWAK, G., CLIFTON, G. L., BAKAJSOVA, D. Succinate Ameliorates Energy Deficits and Prevents Dysfunction of Complex I in Injured Renal Proximal Tubular Cells. **J. Pharmacol. Exp. Ther.**, 324, 1155- 1162, 2008.

OH, T. J., KIM, I. G., PARK, S. Y., KIM, K. C., SHIM, H. W. NAD-dependent malate dehydrogenase protects against oxidative damage in Escherichia coli K-12 through the action of oxaloacetate. **Environ. Toxicol. Pharmacol.,** 11, 9-14, 2002.

OHKAWA, H., OHISHI, N., YAGI, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. **Anal. Biochem.,** 95, 351- 358, 1979.

OLIVER, C., STARKE-REED, P., STADTMAN, E., LIU, G., CARNEY, J., FLOYD, R. Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/ reperfusion-induced injury to gerbil brain. **Proc. Natl. Acad. Sci. USA**, 87, 5144–5147, 1990.

OUBIDAR, M., BOQUILLON, M., CHRISTINE M., CHRISTINE B., BELEY, A. BRALET, J. Effect of intracellular iron loading on lipid peroxidation of brain slices. **Free Radic. Biol. Med.**, 21, 763-769, 1996.

OUBIDAR, M., BOQUILLON, M., MARIE, C., SCHREIBER, L. BRALET, J. Ischemia-induced brain iron delocalization: effect of iron chelators. **Free Radic. Biol. Med.**, 16, 861-867, 1994.

PATEL, R., GARG, R., ERANDE, S., MARU, G. B. Chemopreventive herbal anti-oxidants: Current status and future perspectives. **J. Clin. Biochem. Nutr.,** 40, 82-91, 2007.

PIANTADOSI, C. A., ZHANG, J. Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. **Stroke**, 27, 327–332, 1996.

PORCIÚNCULA, L. O., ROCHA, J. B. T., CIMAROSTI, H., VINADE, L., GHISLENI, G., SALBEGO, C. G., SOUZA, D. O. Neuroprotective effect of ebselen on rat hippocampal slices submitted to oxygen-glucose deprivation: correlation with immunocontent of inducible nitric oxide synthase. **Neurosci. Lett.,** 346, 101-104, 2003.

REHNCRONA, S. HAUGE, H. N. SIESJÖ, B. K. Enhancement of iron-catalyzed free radical formation by acidosis in brain homogenates: difference in effect by lactic acid and CO_2. **J. Cereb.Blood Flow Metab.**, 9, 65–70, 1989.

RODRÍGUEZ- MARTÍNEZ, E., CAMACHO, A., MALDONADO, P. D., PEDRAZA-CHAVERRÍ, J., SANTAMARÍA, D., GALVÁN- ARZATE, S., SANTAMARÍA, A. Effect of quinolinic acid on endogenous antioxidants in rat corpus striatum. **Brain Res.**, 858, 436-439, 2000.

SAKAMOTO, A., OHNISHI, S. T., OHNISHI, T., OGAWA, R. Protective effect of a new anti-oxidant on the rat brain exposed to ischemia-reperfusion injury: inhibition of free radical formation and lipid peroxidation. **Free Radic. Biol. Med.**, 11, 385-391, 1991.

SAKAMOTO, M., TAKESHIGE, K., YASUI, H., TOKUNAGA, K. Cardioprotective effects of Succinate against Ischemia/Reperfusion injury. **Surg. Today. Jpn. J. Surg.**, 28, 522-528, 1998.

SANTAMARÍA, A., RÍOS, C. MK-801, an N-methyl-D-aspartate receptor antagonist, blocks quinolinic acid-induced lipid peroxidation in rat corpus striatum. **Neurosci. Lett.**, 159, 51-54, 1993.

SCHEID, C., KOUL, H., HILL, A., LUBER-NAROD, J., KENNINGTON, L., JONASSEN, J., MENON, M. Oxalate toxicity in LLC-PK1 cells: Role of free radicals. **Kidney Int**., 49, 413-419, 1996.

SCHULZ, J. B., HUANG, P. L., MATTHEWS, R. T., PASSOV, D., FISHMAN, M. C., BEAL, M. F. Striatal malonate lesions are attenuated in neuronal nitric oxide synthase knockout mice. **J. Neurochem.**, 67, 430-433, 1996.

SCHWARZ, R., OKUNO, E., WHITE, R. J., BIRD, E. D., WHETSELL, W. O. JR. 3-Hydroxyantranilat oxygenase activity is increased in the brain of Huntington disease victims. **Proc. Natl. Acad. Sci. USA**, 85, 4079- 4081, 1988.

SELVAN, R., KURIEN, T. B. Induction of lipid-peroxidation by oxalate in experimental rat urolithiasis. **J. Biosci.,** 12, 367-373, 1987.

SIESJÖ, B. K., BENDEK, G., KOIDE, T., WESTERBERG, E., WIELOCH, T. Influence of acidosis on lipid peroxidation in brain tissues in vitro. **J. Cereb. Blood Flow Metab.**, 5, 253–258, 1985.

SILVA, C. G., HERDEIRO, R. S., MATHIAS, C. J., PANEK, A. D., SILVEIRA, C. S., RODRIGUES, V. P., RENNÓ, M. N., FALCÃO, D. Q., CERQUEIRA, D. M., MINTO, A. B. M. Evaluation of antioxidant activity of Brazilian plants. **Pharm. Res.**, 52, 229-233, 2005.

SOKOLOWSKA, M., OLESZEK, A., WODEK, L. Protective effect of alpha-ketoacids on the oxidative hemolysis. **Pol. J. Pharmacol.**, 51, 429-434, 1999.

STÍPEK, S., STASTNÝ, F., PLÁTENÍK, J., CRKOVSKÁ, J. ZIMA, T. The effect of quinolinate on rat brain lipid peroxidation is dependent on iron. **J. Neurochem.**, 30, 233-237, 1997.

STRYER, L. **Bioquimica**. 3ª ed., Rio de Janeiro, Guanabara Koogan, 1988.

SULEIMAN, M. S., HALESTRAP, A. P., GRIFFITHS, E. J. Mitochondria: a target for myocardial protection. **Pharmacol Ther**, 89, 29–46, 2001.

TAKAYANAGI, R., TAKESHIGE, K., MINAKAMI, S. NADH- and NADPH-dependent lipid peroxidation in bovine heart submitochondrial particles. Dependence on the rate of electron flow in the respiratory chain and an antioxidant role. **Biochem. J.**, 192, 853-860, 1980.

TANG, L. X., YANG, J. L., SHEN, X. Effects of additional iron-chelators on Fe^{2+}-initiated lipid peroxidation: evidence to support the Fe^{2+}…Fe^{3+} complex as the initiator. **J. Inorg. Biochem.**, 68, 265-272, 1997.

TATUM, V. L., CHANGCHIT, C., CHOW, C. K. Measurement of malondialdehyde by high-performance liquid chromatography with fluorescence detection. **Lipids**, 25, 226-229, 1990.

TIMBRELL, J. **Principles of Biochemical Toxicology**, 3ª ed, London: Taylor & Francis, 2000.

VALKO, M., IZAKOVIC, M., MAZUR, M., RHODES, C. J., TELSER, J. Role of oxygen radicals in DNA damage and cancer incidence. **Mol. Cell. Biochem.,** 266, 37-56, 2004.

VALKO, M., RHODES, C. J., MONCOL, J., IZAKOVIC, M., MAZUR, M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. **Chem. Biol. Interact**., 160, 1-40, 2006.

VALKO, M., LEIBFRITZ, D., MONCOL, J., CRONIN, M. T. D., MAZUR, M., TELSER, J. Free radicals and antioxidants in normal physiological functions and human disease. **Int. J. Biochem. Cell Biol**., 39, 44-84, 2007.

VEENA, C. K., JOSEPHINE, A., PREETHA, S. P., RAJESH, N. G., VARALAKSHMI, P. Mitochondrial dysfunction in an animal model of hyperoxaluria: A prophylactic approach with fucoidan. **Eur. J. Pharmacol.**, 579, 330-336, 2008.

VELVIZHI, S., DAKSHAYANI, K, B., SUBRAMANIAN, P. Effects of α-ketoglutarate on antioxidants and lipid peroxidation products in rats treated with ammonium acetate. **Nutrition,** 18, 747-750, 2002 a.

VELVIZHI, S., NAGALASHMI, T., MOHAMED ESSA, M., DAKSHAYANI, K, B., SUBRAMANIAN, P. Effects of α-ketoglutarate on lipid peroxidation and antioxidant status during chronic ethanol administration in Wistar rats. **Pol. J. Pharmacol.**, 54, 231-236, 2002 b.

VIANELLO, A., MACRI, F., CAVALLINI, L., BINDOLI, A. Induction of lipid peroxidation in Soybean mitochondria and protection by respiratory substrates. **J. Plant Physiol.**, 125, 217-224, 1986.

WAGNER, C., FACHINETTO, R., DALLA CORTE, C. L., BRITO, V. B., SEVERO, D., DIAS, G. O. C., MOREL, A. F., NOGUEIRA, C. W., ROCHA, J. B. T. Quercitrin, a glycoside form of quercetin, prevents lipid peroxidation in vitro. **Brain Res.**, 1107, 192-198, 2006.

WANG, X., LO, E. H. Triggers and mediators of hemorrhagic transformation in cerebral ischemia. **Mol. Neurobiol.**, 28, 229–244, 2003.

WEBER, G. F. Final common pathways in the neurodegenerative diseases: regulatory role of the glutathione cycle. **Neurosci. and Biobeh. Rev.**, 23, 1079-1089, 1999.

WILLIAMS, R. J., SPENCER, J. P. E., RICE-EVANS, C. Flavonoids: antioxidants or signalling molecules? **Free Radical Biol. Med.**, 36, 838–849, 2004.

YAMAMOTO, H., MOHANAN, P. V. Effect of α-ketoglutarate and oxaloacetate on brain mitochondrial DNA damage and seizures induced by kainic acid in mice. **Toxicol. Lett.**, 143, 115-122, 2003.

YANG, C. S., LIN, N. N., TSAI, P. J., LIU, L., KUO, J. S. In vivo evidence of hydroxyl radical formation induced by elevation of extracellular glutamate after cerebral ischemia in the cortex of anesthetized rats. **Free Radic. Biol. Med.**, 20, 245–250, 1996.

YOUNES, M., TREPKAU, H. D., SIEGERS, C. P. Enhancement by dietary iron of lipid-peroxidation in mouse colon. **Res. Com. Chem. Pathol. Pharmacol**., 70, 349-354, 1990.

YU, L., LATRIANO, L., DUNCAN, S., HARTWICK, R. A., WITZ, G. High-performance liquid chromatography analysis of the thiobarbituric acid adducts os malonaldehyde and trans, trans-muconaldehyde. **Anal. Biochem**., 156, 326-333, 1986.

ZEEVALK, G. D., BERNARD, L. P., NICKLAS. W. J. Oxidative stress during energy impairment in mesencephalic cultures is not a downstream consequence of a secondary excitotoxicity. **Neurosci.**, 96, 309–316, 2000.

ZORATTI, M., SZABO, I. The mitochondrial permeability transition. **Biochim Biophys Acta**, 1241, 139–176, 1995.