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CURSO DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

O PAPEL DO ÓXIDO NÍTRICO NAS ALTERAÇÕES COMPORTAMENTAIS ELETROENCEFALOGRÁFICAS E NEUROQUÍMICAS INDUZIDAS PELO METILMALONATO EM ESTRIADO DE RATOS

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Esse trabalho é dedicado ao nosso grupo de pesquisa, a minha maior descoberta.

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ABREVIATURAS

EIM	Erros Inatos do Metabolismo
SDH	Sucinato desidrogenase
NMDA	*N*-methyl-$_D$-aspartato
$NO^{\cdot}$	Óxido Nítrico
RL	Radical Livre
EAO	Espécie Ativa de Oxigênio
EAN	Espécie Ativa de Nitrogênio
SOD	Superóxido Desmutase
$ONOO^{-}$	Peroxinitrito
7-NI	7-nitroindazol
AM	Azul de Metileno
CAT	Catase
PTZ	Pentiletetrazol
GM1	Gangleosídeo GM1
NOSn	Óxido Nítrico Sintase Neuronal
NOSe	Óxido Nítrico Sintase Endotelial
NOSi	Óxido Nítrico Sintase Induzida
NOSmit	Óxido Nítrico Sintase mitocondrial
L-NAME	Metil éster de N^{w} –nitro-L-arginina

RESUMO

A acidemia metilmalônica é um erro inato do metabolismo caracterizado pelo acúmulo tecidual de ácido metilmalônico (MMA), dano oxidativo e alterações neurológicas, como degeneração estriatal e convulsões. Considerando que o óxido nítrico é um mensageiro químico trans-sináptico que está envolvido em diversos eventos fisiopatologógicos e seu papel na toxicidade induzida pelo MMA é pouco conhecido, nós decidimos investigar a participação deste radical livre nas alterações comportamentais e neuroquímicas induzidas pela administração intraestriatal de MMA. No primeiro trabalho, foi evidenciado que a administração intraestriatal de um inibidor não-seletivo da enzima óxido nítrico sintase, metil éster de N^{ϖ}-nitro-L-arginina (L-NAME: 10^{-4} - 10^{0} nmol/0,5 μl), exerceu efeito bifásico nas convulsões e na carbonilação proteíca induzidas pela injeção de MMA (4,5 μmol/1,5 μl; 30 minutos após a injeção de L-NAME) no estriado de ratos. Estes resultados sugeriram um envolvimento do óxido nítrico nas convulsões e no dano oxidativo induzido pelo MMA. Em um segundo momento, confirmamos o envolvimento do óxido nítrico nas convulsões induzidas por MMA, uma vez que a injeção intraestriatal de MMA causou um aumento na concentração de nitrito e nitrato (NO_2 e NO_3) estriatal. Além disso, os episódios convulsivos induzidos por MMA apresentaram uma correlação significativa com a inibição da atividade da Na^+,K^+-ATPase no estriado injetado, mas não com os níveis de carbonilação protéica, um marcador de dano oxidativo protéico. Neste estudo também foi avaliado o efeito da administração intraestriatal do azul de metileno (AM; 0,015 a 1,5 nmol/ 0,5 μl), que possui atividade antioxidante e é um inibidor da guanilato ciclase, nas convulsões e dano oxidativo induzidos pelo MMA. O AM (1,5 nmol/0,5 μl) diminuiu a formação de NO_2 e NO_3 estriatal e preveniu as convulsões, a carbonilação protéica e a inibição da atividade da Na^+,K^+-ATPase induzidos pelo MMA. Esses dados sugerem que a atividade da Na^+,K^+-ATPase pode ser de grande importância para a gênese das convulsões induzidas pelo MMA e que o AM exerce efeito neuroprotetor contra as alterações comportamentais e neuroquímicas induzidas pelo MMA. Além disso, se essas alterações ocorerem nos pacientes com acidemia metilmalônica, é possível propor que o AM poderia ser considerado como uma terapia adjuvante para o tratamento desta acidemia. O efeito da administração do 7-nitroindazol (7-NI; 3-60 mg/kg, i.p.), um inibidor da enzima NOSn, nas convulsões, carbonilação protéica, produção de NO_2 e NO_3 e na atividade da Na^+,K^+-ATPase após trinta minutos da administração intraestriatal de MMA (6 μmol/ 2 μl) também foi avaliado. O tratamento com 7-NI (60 mg/kg, i.p.) potencializou as convulsões, aumentou a carbonilação protéica e reduziu a produção de NO_2 e NO_3 induzidos pelo MMA, entretanto, este tratamento não alterou a atividade da Na^+,K^+-ATPase. A adminstração intraestriatal de L-arginina (50 nmol/ 0.5 μl), mas não de D-arginina (5 and 50 nmol/ 0.5 μl), aumentou a produção de NO_2 e NO_3 e preveniu as convulsões, a carbonilação protéica e a inibição da atividade da Na^+,K^+-ATPase induzidos pelo MMA. Esses resultados sugerem que o óxido nítrico neuronal pode exercer um efeito protetor sobre as convulsões bem como nas alterações neuroquímicas evidenciadas neste modelo de acidemia orgânica. Concluindo, estes resultados ampliam o papel dos radicais livres nas alterações comportamentais e neuroquímicas induzidas pela administração intra-estriatal de MMA.

ABSTRACT

Methylmalonic acidemias comprehends a group of inborn erros of the metabolims characterized by the accumulation of methylmalonic acid (MMA), oxidative damage and neurological alterations, such as basal ganglia degeneration ans seizures. Since nitric oxide (NO) is involved in the pathogenesis of different neurodegeneative diseases, in this study we investigated the role of NO in the behavioral and neurochemical alterations induced by the intrastriatal injection of MMA. We show that the intrastriatal injection of L-NAME (10^{-4} – 10^{0} nmol/ 0,5 μl), a nonspecific inhibitor of nitric oxide synthase (NOS) caused a biphase effect on MMA-induced convulsions (4,5 μmol/1,5 μl) and total on total protein carbonylation. These results suggested that NO is involved in the convulsion induced by MMA. In the following set of experiments, we showed that MMA increased total nitrite and nitrate production in the striatum and that the convulsions, protein carbonylation, nitrate production and Na^{+},K^{+}-ATPase induced by MMA were preventes by methylene blue, an inhibitor of NOS and guanilate cyclase that is also an antioxidant. In this study we also showed that Na^{+},K^{+}-ATPase correlated with time spend convulsioning. In the last group of experiments we tested the effect of 7-NI, a compound that has been regarded as a selective NOSn inhibitor, on the seizures, protein carbonylation, production of nitrates and nitrites and Na^{+},K^{+}-ATPase activity inhibition induced by MMA (6 μmol/2 μl). 7-NI paradoxically increased convulsions and oxidative damage but decreased nitrites livels. 7-NI administration, however, had no effect on MMA-induced Na^{+},K^{+}-ATPase inhibition. L-Arginine, but not D-Arginine, decreased the convulsions, Na^{+},K^{+}-ATPase inhibition and protein carbonylation induced by MMA. Our results suggest thet NO may have some protective effect against MMA-induced convulsions, but these data may be interpreted also as a possible lack of selectivity of 7-NI against NOSn. According to this view, the current described protective effect of L-Arginine against MMA-induced convulsion may be related to its ability to cause vasodilation, increased perfusion and consequent better metabolic satate. In conclusion this study shows that the NO cascade is activated during MMA-induced seizures. However, further studies, with specific inhibitors for NOS isoforms, are necessary to clarify if NO is involved in the genesis of the convulsions induced by MMA.

Sumário

I. INTRODUÇÃO

I.1. Erros Inatos do Metabolismo

Erros inatos do metabolismo (EIM) são distúrbios hereditários, resultado de deficiências em atividades enzimáticas, o que ocasiona um bloqueio de diversas rotas metabólicas. Esse bloqueio, além de induzir um acúmulo de substâncias tóxicas e/ou a falta de substâncias essenciais, gera distúrbios no desenvolvimento físico e mental (Oberholzer et al, 1967). No início do século XX, Archibald Garrod empregou o termo EIM para se referir as situações clínicas que ele acreditou serem conseqüentes a defeitos em vias metabólicas. A primeira doença estudada foi a alcaptonúria, que leva a artrite e se caracteriza, bioquimicamente, pelo aumento da excreção de ácido homogentísico. Atualmente, o termo EIM se aplica a um grupo de doenças geneticamente determinadas, decorrente de deficiência em alguma via metabólica que está envolvida na síntese (anabolismo), transporte ou na degradação (catabolismo) de uma substância. A medida em que se aprofunda o conhecimento médico, aumenta o número de erros inatos conhecidos, que hoje ultrapassam 500.

Apesar de serem eventos raros, os EIM representam importante problema de saúde e seu diagnóstico, freqüentemente, se constitui em desafio para o clínico. Em algumas populações e grupos étnicos isolados, certos erros do metabolismo, bastante raros na população em geral, podem ser extremamente freqüentes. Como exemplos, temos as doenças de Tay-Sachs e Gaucher entre os judeus originários do Leste europeu, conhecidos como “ashquenazim”, e a tirosinemia tipo I nos canadenses de ascendência francesa. A

consangüinidade é um fator que aumenta consideravelmente o risco de qualquer doença genética de herança recessiva, inclusive os EIM.

Frequência estimada de alguns erros inatos do metabolismo	
Fenilcetonúria (caucasianos)	1/15.000
Acidemia glutárica tipo I	1/30.000
Homocistinúria	1/100.000
Acidemia metilmalônica	1/100.000

Tabela 1. Alguns erros inatos do metabolismo mais freqüentes na população.

Em 1980, Chalmers e colaboradores observaram uma prevalência maior de acidemias orgânicas do que de aminoacidopatias, até então os EIM mais estudados, em pacientes hospitalizados. De fato, em função dos progressos feitos no campo do diagnóstico dos erros inatos do metabolismo, evidenciou-se que as acidemias orgânicas são, realmente, os erros inatos mais freqüentes na população (Scriver, 1995, Wajner et al., 2001).

I.1.2. Acidemia Metilmalônica

A acidemia metilmalônica é um erro inato do metabolismo autossômico recessivo caracterizado por um acúmulo de ácido metilmalônico e de outros metabólitos como propionato, metilcitrato, β-OH- propionato e cetonas de cadeia longas. Esse acúmulo se deve à deficiência na atividade da enzima metilmalonil-CoA mutase (EC 5.4.99.2), responsável pela conversão do L-metilmalonil CoA a sucinil CoA na rota da degradação do propionato (Scriver, 1995).

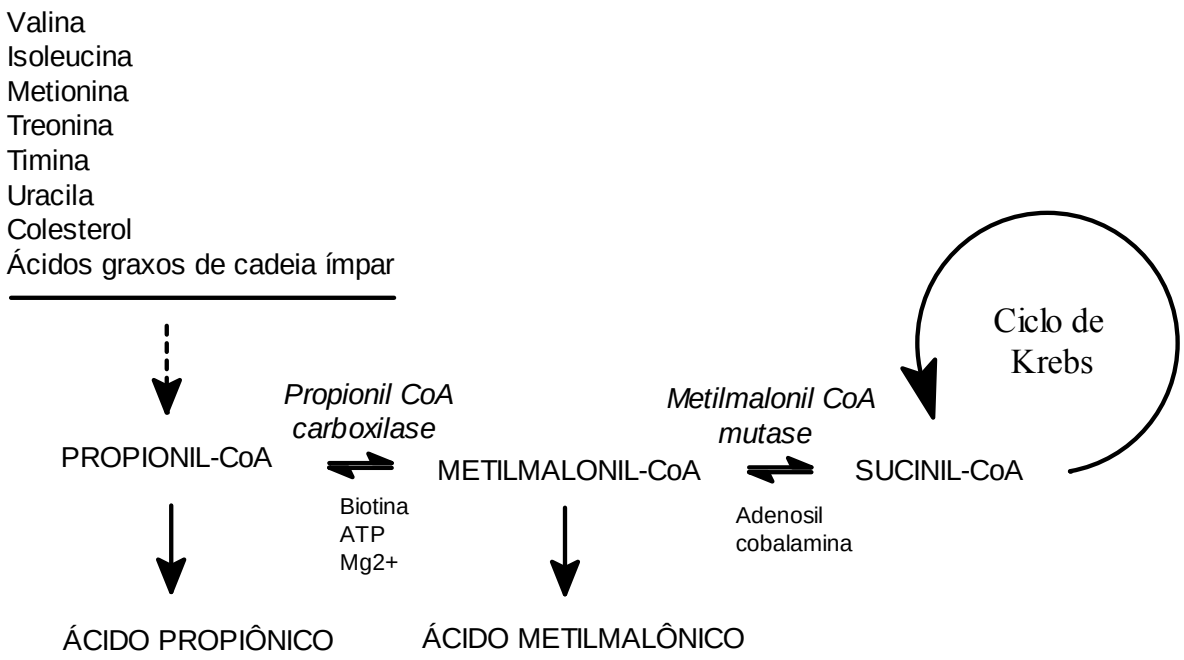

Figura 1 - Metabolismo da enzima metilmalonil CoA mutase: enzima responsável pela conversão do L-metilmalonil CoA a sucinil CoA na rota de degradação de aminoácidos de cadeia ramificada, ácidos graxos de cadeia ímpar e do propionato.

A acidemia metilmalônica foi descrita pela primeira vez por Oberholzer e colaboradores em 1967, que observaram quantidades elevadas de ácido metilmalônico (MMA) no sangue e na urina em crianças que apresentavam cetoacidose. O acúmulo do MMA pode gerar, além de uma acidose metabólica, hipercetonemia, hipoglicemia, hiperamonemia, neutropenia e trombocitopenia (Fenton & Rosenberg, 1995). Os pacientes afetados apresentam alterações neurológicas, como degeneração seletiva dos núcleos da base, convulsões, retardo mental e discinesia. O quadro de cetoacidose se instala nos primeiros dias de vida (21 dias), e os pacientes geralmente vão a óbito (Ogier et al, 1990). Os pacientes que sobrevivem (menos de 15 %) apresentam convulsões, retardo mental,

psicomotor e alterações neuropsiquiátricas, tais como quadro de agressividade, comportamento autista e perda do tônus muscular verificado após crises de descompensação metabólica (Dutra, 1992).

I.1.3. Ações do MMA no Metabolismo Intermediário

O principal metabólito acumulado na acidemia metilmalônica é o MMA. O MMA é gerado por tiólise espontânea a partir L-metilmalonil CoA que se acumula nos tecidos, resultado de uma deficiência na atividade da metilmalonil CoA mutase (Fenton & Rosenberg, 1995). Utter em 1970, demonstrou que a piruvato carboxilase pode ser inibida por metilmalonil CoA, e que altos níveis de MMA comprometem a homeostase da glicemia, particularmente por prejudicar a gliconeogênese hepática (Arinze et al, 1979).

De acordo com Oberholzer et al. (1967), a incapacidade de manutenção da glicemia leva a uma maior utilização dos ácidos graxos e conseqüente formação de corpos cetônicos. Altas concentrações de metilcitrato, um metabólito que se acumula na doença, leva à inibição das enzimas do ciclo de Krebs: citrato sintase, aconitase, isocitrato desidrogenase. Como conseqüência, diminui a utilização de acetil CoA pelos tecidos, o que gera um quadro de hipercetonemia (Ando et al, 1971; Cheema Dhadli et al, 1975).

Experimentos realizados *in vivo* e *in vitro* em cérebro de ratos, revelaram que o MMA é capaz de inibir a atividade da enzima beta-hidróxi-butirato desidrogenase (Patel et al, 1976). Esta enzima é responsável pela conversão de beta-hidróxi-butirato em acetoacetato, substratos importantes para a síntese de ácidos graxos no sistema nervoso central de animais lactentes (Patel & Owen, 1976). Com isso, ocorre um decréscimo na incorporação de beta-hidróxi-butirato em lipídios cerebrais não saponificáveis e em frações de ácidos graxos. Frankel e colaboradores, em 1973, observaram que o acúmulo de

metilmalonil CoA inibe a atividade das enzimas acetil CoA carboxilase e a sintetase de ácidos graxos em fígado e cérebro de ratos deficientes em vitamina B_{12}, ocasionando uma diminuição na síntese de ácidos graxos e um acúmulo de diferentes metabólitos, comuns na acidemia metilmalônica. De fato, o MMA, em concentrações compatíveis com as encontradas em pacientes com acidemia metilmalônica inibe a incorporação de [U-^{14}C]acetato em prismas de córtex cerebral de ratos, confirmando que este metabólito inibe a síntese de lipídios (Mello et al, 1997).

Em 1993 Dutra e colaboradores demonstraram que o MMA é capaz de inibir a sucinato desidrogenase (SDH) e a beta–hidróxi-butirato desidrogenase cerebral *in vitro.* Wajner e colaboradores (1992), observaram que este ácido reduz a produção de CO_2 e aumenta a concentração de lactato no cérebro. A diminuição na atividade das enzimas sucinato desidrogenase e beta–hidróxi-butirato desidrogenase causado pelo acúmulo de MMA parece estar relacionada com uma redução da utilização de glicose e beta–hidróxi-butirato por prismas cerebrais de ratos em jejum.

A diminuição na produção de CO_2, o aumento na produção de lactato e depleção de creatina e fosfocreatina na presença de MMA (Royes et al., 2003; 2006) sugere que este ácido orgânico causa um prejuízo no metabolismo aeróbico, que pode estar relacionada à inibição da SDH inicialmente descrita por Dutra e colaboradores (1989; 1991) e depois confirmada por Toyoshima e colaboradores (1995) e por Fleck e colaboradores (2004). Além disso, pacientes com acidemia metilmalônica ou propiônica em acidose apresentam uma redução de até 70% da atividade da enzima citocromo oxidase no fígado (Hayasaka et al., 1982). A partir destes achados, tem sido postulado que o MMA possa causar interferência no metabolismo aeróbico celular, levando provavelmente a uma diminuição na produção de ATP, e conseqüente excitotoxicidade secundária.

I.2. Excitotoxicidade mediada por receptores glutamatérgicos

Apesar da transmissão glutamatérgica ser fundamental para processos fisiológicos, sabe-se que uma ativação supra-fisiológica de receptores glutamatérgicos pode provocar morte neuronal, também chamada de excitotoxicidade (Olney et al, 1978; Fenton & Rosenberg, 1995).

O termo excitotoxicidade foi inicialmente usado para descrever um aumento na ativação de receptores glutamatérgicos via administração de altas doses de glutamato exógeno (Olney & Ho, 1970). Após a descoberta de que antagonistas glutamatérgicos poderiam proteger contra a a perda neuronal induzida por diversos insultos neurológicos, como isquemia e hipóxia, passou-se a admitir que o glutamato endógeno poderia ser mediador de processos excitotóxicos quando em altas concentrações na fenda sináptica (Obrenovitch, 1999; Obrenovitch et al, 2000).

A neurotoxicidade induzida pelo glutamato pode ter como causa: i) uma hipersensibilidade dos receptores ao glutamato devido a uma perda na seletividade iônica, aumento na densidade ou anormalidades na modulação; ii) aumento na concentração de glutamato no meio extracelular (aumento na liberação ou redução da captação) e/ou iii) deficiência no metabolismo energético e nas defesas antioxidantes (Greene & Greenamyre, 1996; Olney et al, 1978; Obrenovitcht al, 2000; Rothsthein et al, 1992; Battaglioli et al, 1993; Robinson & Dowd, 1997). Além disso, diversos estudos sugerem a participação de inibidores metabólicos na toxicidade induzida por glutamato, uma vez que eles facilitam a ativação de receptores NMDA. Entre eles podemos citar: rotenona, cianeto, oxiaminoacetato, 3-nitropropionato, malonato e metilmalonato (McDonald & Schoepp, 1993; Brouillet et al, 1994; Zeevalk et al, 1995; Behrens et al, 1995; Pavlakovic et al, 1995;

Mello et al, 1997, Royes et al., 2003; 2006). Neste contexto, uma estimulação excessiva de receptores glutamatérgicos, induz a perda do equilíbrio do Ca^{2+} intracelular (Choi et al, 1992). O aumento da concentração de Ca^{2+} intracelular provoca um aumento na produção de radicais livres, disfunção mitocondrial, e conseqüente diminuição na produção de energia (Gunter et al, 1994; Dugan et al, 1995; Coyle & Puttfarcken, 1993).

Há evidências de que a depleção energética induzida por inibidores da sucinato desidrogenase, como 3NP e MMA, desencadeie despolarização e dano celular por falência na atividade de ATPases de membranas (Nathanson et al, 1995; McLaughlin et al, 1998, Malfatti et al., 2003). De fato, a depleção energética aumenta a liberação de glutamato não só por mecanismos dependentes de cálcio (por despolarização da membrana), mas também pela inibição e/ou reversão de mecanismos de recaptação deste aminoácido excitatório na pré-sinapse e nas células gliais, levando a um aumento de glutamato na fenda sináptica (Madl & Burgesser, 1993).

A despolarização da membrana pós-sináptica causada pela falência na atividade das ATPases leva à saída do íon Mg^{2+}, que bloqueia o canal do receptor NMDA de maneira dependente de voltagem, e permite a entrada de íons Na^{+} e Ca^{2+} para o meio intracelular (McDonald & Schoepp, 1993). O dano excitotóxico primário é desencadeado pela entrada de Na^{+} através de receptores ionotrópicos do subtipo AMPA/Kainato (Chan et al, 1979). O aumento de Ca^{2+}, via ativação de receptores do subtipo NMDA, leva à ativação de enzimas dependentes deste íon, e que estão envolvidas na produção de radicais livres como xantina oxidase (Said et al., 2000), fosfolipase C (Umemura et al., 1992) e ciclooxigenase (Pepicelli et al., 2005), calcineurina (Armstrong, 1989; Snyder & Sabatini, 1995) e óxido nítrico sintetase, que produz óxido nítrico ($NO^{\bullet}$) (Snyder & Bredt, 1992), e a própria disfunção

mitocondrial (Patel, 2000), que estão associadas ao aparecimento de convulsões e morte celular (Figura 2).

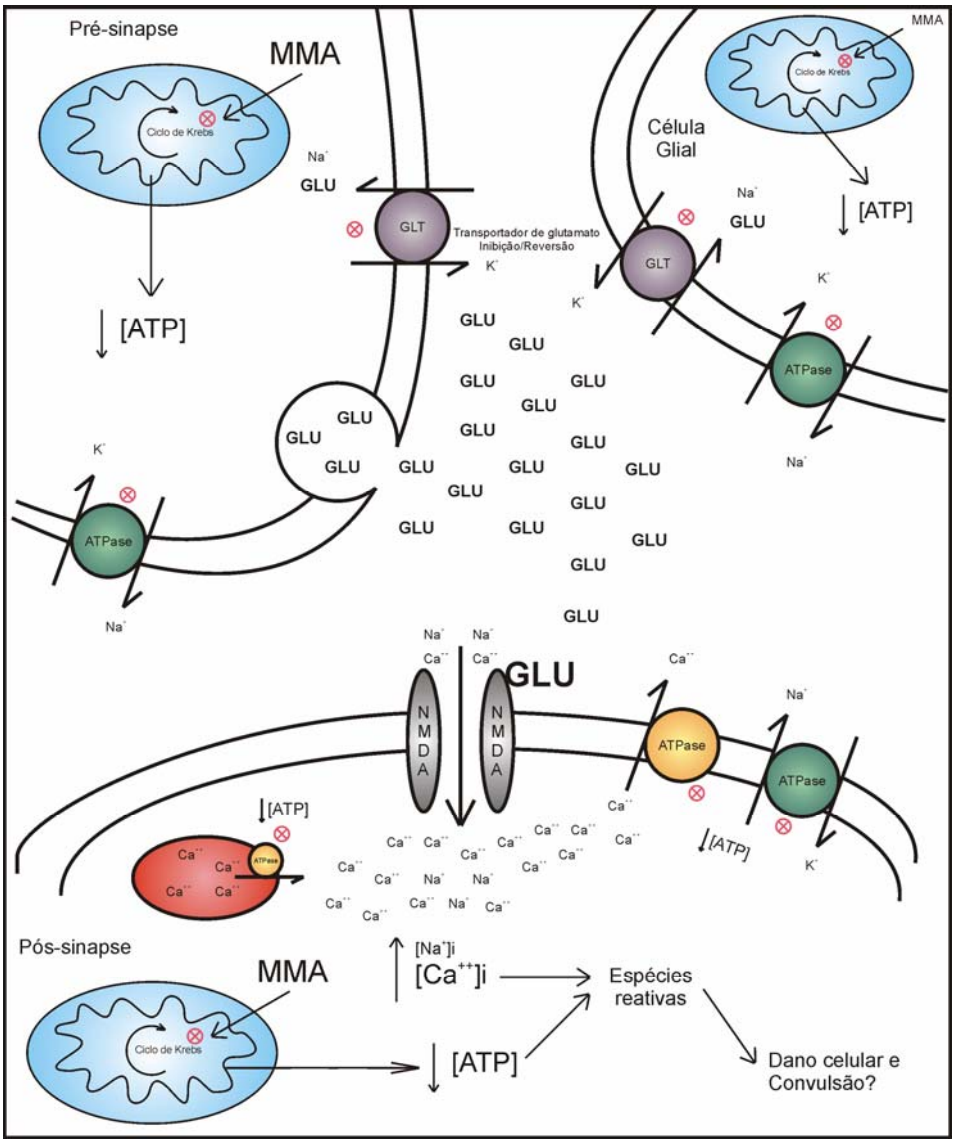

Figura 2. Modelo de falência energética e excitotoxicidade induzida pelo glutamato no modelo de acidemia metilmalônica. A inibição da SDH neuronal e glial induzida pelo MMA causa falência energética e inibição das ATPases, causando a despolarização e alterações nos gradientes iônicos. A despolarização provoca a liberação de glutamato armazenado nas vesículas sináticas e a perda do gradiente leva a inibição e a reversão dos transportadores de glutamato a nível glial e neuronal. O aumento de glutamato na fenda sináptica e a falência energética, que também atinge a membrana pós-sináptica, induz a despolarização, deslocamento de Mg^{2+} presente no canal do receptor NMDA e o influxo de Ca^{2+} para o meio intracelular. O acúmulo de Ca^{2+} intracelular, proveniente do meio extracelular e da inibição de ATPases presentes no retículo endoplasmástico, provavelmente está envolvido na propagação do foco de despolarização, na geração de radicais livres e na gênese das convulsões induzidas pelo MMA.

1.3. Efeito dos Radicais Livres no Sistema Nervoso Central

I.3.1. Conceito e Formação dos Radicais Livres e Espécies Reativas

Do ponto de vista químico, um radical livre (RL) é definido como qualquer átomo, grupo de átomos ou molécula capaz de existir sob forma independente, que contém um ou mais elétrons desemparelhados (Del Maestro, 1980; Southorn & Powis, 1988; Halliwell & Gutteridge, 1999) (Figura 3). Portanto, os radicais livres podem ser formados pela adição ou pela perda de um elétron de uma substância não-radical. Entretanto, existem compostos tão reativos quanto os radicais livres que não possuem elétron não-pareado na última camada e, portanto, não podem ser classificados como radicais livres (Dröge, 2002). Essas substâncias são classificadas de maneira mais ampla como espécies ativas do oxigênio (EAO) ou espécies ativas de nitrogênio (EAN).

As EAO geralmente têm uma meia-vida curta, e sua recombinação química é quase imediata. Estudos mostram que elas têm uma forte tendência para estabilizar sua órbita externa captando um elétron de outro átomo ou molécula, que por sua vez se torna uma molécula radicalar.

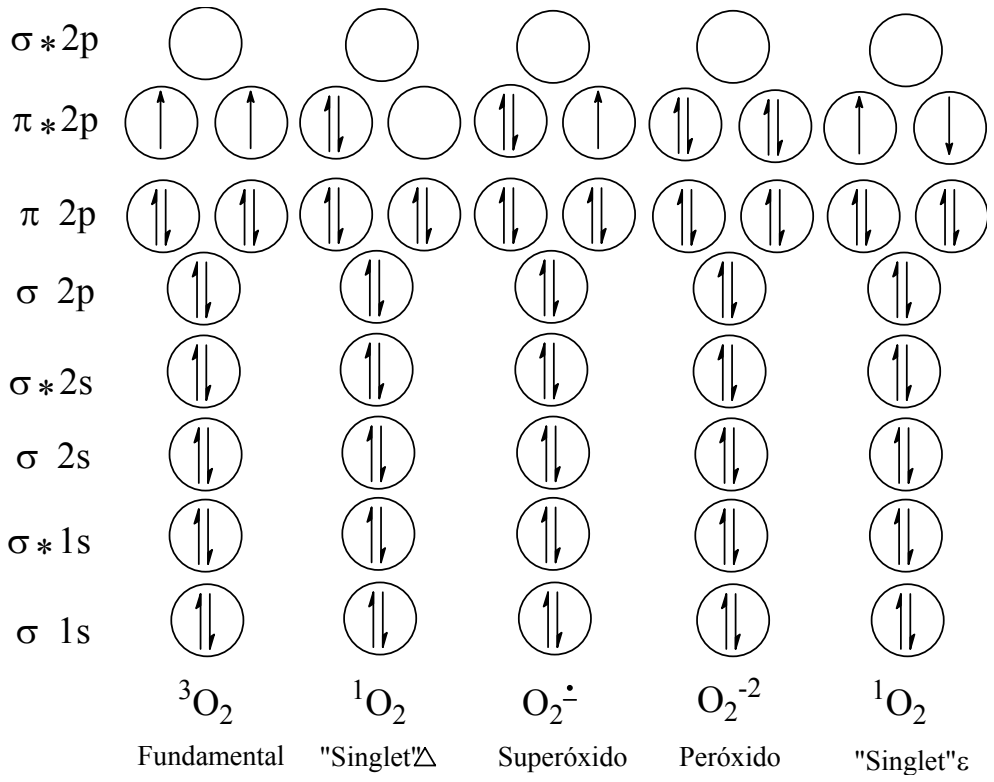

Figura 3. Configuração eletrônica das Espécies Ativas e Oxigênio (EAO) (Haliwell & Gutteridge, 1999)

Uma fonte importante de RLs é o sistema de transporte de elétrons mitocondrial (Del Maestro, 1980; Southorn & Powis, 1988), sendo seu principal sítio de formação complexo citocromo b-ubiquinona (Tyler, 1975). Na mitocôndria, a citocromo oxidase promove a redução completa de uma molécula de O_2 em uma molécula de água e, para isso, são necessários quatro elétrons:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

Contudo, nem sempre o oxigênio se transforma diretamente em água. Como conseqüência de sua configuração eletrônica, a molécula de oxigênio tem forte tendência, durante as reações, em receber um elétron de cada vez, formando uma série de intermediários tóxicos e reativos (Meneghini, 1987), tais como: radical superóxido (O_2^-), o peróxido de hidrogênio (H_2O_2), ambos listados na figura acima, e o radical hidroxil ($^{\cdot}OH^{\bullet}$). O primeiro e o último apresentam elétrons desemparelhados e são classificados como RLs. Já o H_2O_2 não tem elétrons desemparelhados na última camada, e é classificado como uma EAO (Fisher, 1987).

O superóxido é o primeiro intermediário formado a partir da redução incompleta do oxigênio molecular na formação da H_2O (Harris, 1992), e a partir dele podem se formar outras espécies ativas de oxigênio, como o radical hidroxil e o peróxido de hidrogênio (Esterbauer et al., 1986; Pryor, 1984). Em condições fisiológicas, o H_2O_2 é formado na mitocôndria em função da atividade metabólica (Chance et al., 1979). Tem sido mostrado que, em alguns órgãos, a atividade celular leva à formação de H_2O_2 , tanto pelos peroxissomos quanto por enzimas citosólicas (Boveris & Chance, 1973; Chance et al., 1979). O H_2O_2 é gerado a partir do superóxido por meio de dismutação, sendo esta reação catalisada pela enzima superóxido dismutase (SOD) (Figura 4) (Fridovick, 1975; Fridovick, 1978). A isoforma da SOD presente na matriz mitocondrial é dependente de manganês (MnSOD), enquanto isoforma da SOD presente no citosol é a dependente de cobre e zinco (CuZnSOD, Fridovich, 1978). O RL mais reativo é o radical hidroxil, que pode ser formado pela reação do H_2O_2 com íons cobre (I) ou ferro (II). Esta reação é conhecida como reação de Fenton (Figura 5).

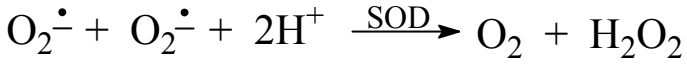

Figura 4 - Dismutação do radical superóxido por ação da superóxido dismutase (SOD).

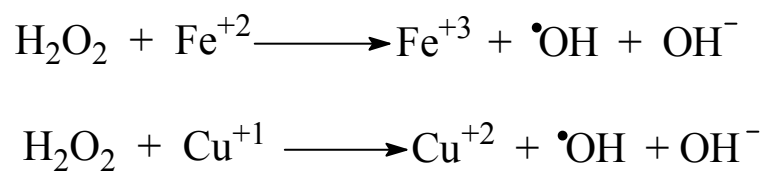

Figura 5 – Reação de Fenton.

Os metais de transição podem catalisar a reação entre o H_2O_2 e $O_2^{\bullet-}$, levando à produção de radical hidroxil. Esta reação é conhecida como reação de Haber-Weiss, e catalisada pelo cobre (II) ou ferro (III) (Figura 5), que pode ser oriundo da ferritina, hemoglobina ou mioglobina (Werns & Luchesi, 1990).

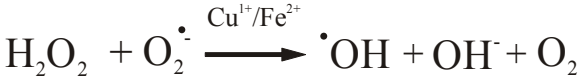

Figura 6 – Reação de Haber-Weiss

As EAN também são formadas tanto no citosol como na mitocôndria, e as mais representativas são o óxido nítrico ($NO^{\bullet}$) e o peroxinitrito ($ONOO^-$). O $NO^{\bullet}$ é gerado apartir da arginina por ação da enzima óxido nítrico sintase (NOS), presente no citosol e na mitocôndria (Riobo et al., 2002). O $NO^{\bullet}$ é essencial para vasorregulação, agregação plaquetária e neurotransmissão, mas em excesso inibe a citocromo-c oxidase levando à consequente formação de $O_2^{\bullet-}$ (Forfia et al., 1999). O $ONOO^-$, entretanto, é formado apartir de uma reação controlada entre $NO^{\bullet}$ e $O_2^{\bullet-}$ (Radi et al., 1994) e ele pode difundir-se para o meio

intra ou extracelular, e então promover a oxidação de lipídios, proteínas e DNA (Beckman et al., 1996). Além disso, o $ONOO^-$ pode inibir a cadeia respiratória, levando a morte neuronal e produzir íons nitrônio, que são capazes de nitrificar resíduos de tirosina (Beckman et al., 1996).

As EAOs são necessárias para o funcionamento normal do organismo e são continuamente produzidas e neutralizadas pelo sistema de defesa antioxidante. Entretanto, quando as espécies ativas são produzidas em altas concentrações, ou quando as defesas antioxidantes são deficientes, elas podem causar dano celular, representando um mecanismo fundamental para as doenças em seres humanos denominado "estresse oxidativo" (Halliwell and Gutteridge, 1999).

Embora todos os tecidos dos organismos aeróbicos possam sofrer dano oxidativo, o sistema nervoso é o mais suscetível às espécies reativas de oxigênio. Uma das razões é o seu alto consumo de O_2, já que o sistema nervoso é responsável por aproximadamente 20% do consumo basal de O_2 corporal, apesar de seu peso corresponder a apenas 2% do peso corporal. Portanto, há uma grande quantidade de O_2 para uma massa de tecido relativamente pequena (Halliwell & Gutteridge, 1999). Além disso, outros fatores também contribuem para tornar o cérebro vulnerável às reações de oxidação, tais como as altas concentrações de lipídios poli-insaturados, que servem como substrato para peroxidação lipídica e a deficiência de mecanismos protetores antoxidantes. A atividade baixa de enzimas antioxidantes e sua localização nas células gliais (Floyd & Hensley, 2002) fazem com que os neurônios sejam menos protegidos contra as espécies reativas geradas no cérebro. Além disso, regiões específicas do cérebro, como a substância negra, caudato, putâmen e globo pálido possuem altas concentrações de ferro (Floor, 2000), que associadas aos níveis cerebrais elevados de vitamina C (Miele et al., 1990), possibilitam a reação de Fenton (Nold &Wennemers, 2000).

Finalmente, o aumento na concentração extracelular de glutamato por causas metabólicas diversas, leva ao excessivo e contínuo aumento de cálcio livre intracelular e sódio em neurônios adjacentes, ocasionando alta produção espécies ativas de oxigênio e de nitrogênio (Resnick & Parker, 1993; Cassarino & Bennet Jr., 1999).

Dentre as diversas reações envolvendo espécies reativas de oxigênio em sistemas biológicos, está a oxidação de proteínas. A determinação de grupos carbonil nas proteínas oxidadas tem se tornado um dos métodos bioquímicos mais utilizados na investigação do dano oxidativo protéico (Pratico e Delanty, 2000). Além disso, as proteínas podem sofrer modificações de grupos tióis e nitrosilação de grupos fenóis acarretando na sua fragmentação. Particularmente, os aminoácidos histidina, arginina e lisina são os principais alvos das espécies reativas para a produção de grupos carbonil (Uchida, 2003), e a oxidação destes resíduos implica perda de função protéica. A adição de grupos carbonil também pode ocorrer pela formação de aductos com a acroleína e com o HNE, dois aldeídos formados na peroxidação lipídica (Uchida, 1999), que também acarretam perda da função protéica (Lovell *et al.*, 2000). O conteúdo de grupos carbonil está aumentado na neurodegeneração relacionada com a idade (Stadtman, 2001), no neurotrauma (Luo et al., 2005) e em modelos experimentais de doenças neurodegenerativas, como nas doenças de Alzheimer (Lovell et al., 2001), Huntington (Tunez et al., 2004), Parkinson (Butterfield & Kanski, 2001), e em modelos de convulsões induzidos por pentilenotetrazol (Oliveira et al., 2004) e MMA (Ribeiro et al., 2005; Royes et al., 2006).

De fato, estudos recentes sugerem o envolvimento das espécies reativas nas convulsões induzidas pelo ácido metilmalônico, na medida em que as convulsões induzidas pela administração intra-estriatal de metilmalonato são atenuadas pela administração de antioxidantes, como o gangliosídeo GM1 e o α-tocoferol (Fighera et al., 1999; 2003). Da

mesma forma, a administração de amônia (um agente pró-oxidante) potencializa as convulsões induzidas por este ácido orgânico (Marisco et al., 2003). Além disto, estudos *in vitro* e *in vivo* mostraram que o MMA inibe a atividade da enzima Na^+,K^+-ATPase (Wyse et al., 2000; Malfatti et l., 2003), responsável pela manutenção do gradiente de Na^+ e K^+ através das membranas celulares, o que é essencial para a mantenção do potencial de membrana (Jorgensen, 1986; Stryer, 1996).

A inibição da Na^+, K^+-ATPase induz um aumento na excitabilidade neuronal, e facilita o aparecimento e a propagação de convulsões em diversos modelos de epilepsia (Mori et al., 1986; Grisar et al., 1992; Oliveira et al., 2004; Ribeiro et al., 2005). De fato, estudos com pacientes epilépticos, observa-se um decréscimo da atividade da Na^+-K^+-ATPase no tecido cerebral (Rapport et al., 1975). De acordo com vários autores, a inibição da atividade da Na^+-K^+-ATPase se deve à sua suceptibilidade aos radicais livres, visto que a mesma é uma proteína de membrana e grupos sulfidrila e resíduos de aminoácidos particularmente sensíveis ao dano oxidativo (Jamme et al., 1995; Morel et al., 1998; Siems et al., 1998). Contudo, é importante enfatizar que a atividade da Na^+-K^+-ATPase pode ser regulada por outros agentes ou mecanismos, como os glicosídeos cardíacos ouabaína e a digoxina, que inibem a enzima (Rose & Valdes, 1994), bem como por fosforilação mediada pela proteína quinase C (PKC) e pela proteína quinase G (PKG) (Boldyrey et al, 1997; Muriel and Sandoval, 2000; Muriel et al., 2003; Barriviera et al., 2005).

I.4. Características, síntese e metabolismo do óxido nítrico

O óxido nítrico é uma molécula gasosa simples, habitualmente encontrada no ar atmosférico em pequenas quantidades, e altamente tóxica por ser um radical livre. Além disto, por possuir elétrons desemparelhados na última camada e existir também na forma de

íon nitroso (NO^+), esta molécula é extremamente instável e tende a reagir com outras moléculas (Guix et al., 2005). Quando em solução, o óxido nítrico tem uma meia-vida de menos de 10 segundos, devido à sua rápida oxidação a nitrito (NO_2^-), nitrato (NO_3^-) e peroxinitrito ($ONOO^-$) (Beckman, 1996).

O óxido nítrico liga-se à hemoglobina e a outras proteínas que contém núcleo heme, levando ao término de sua atividade biológica (Snyder et al., 1992). No processo de formação de NO (Figura 7), o aminoácido não essencial L-arginina, é transformado em um intermediário (N^G-hidróxi-L-arginina) na presença de nicotinamida-adenina-dinucleotídeo-fosfato reduzida (NADPH) e Ca^{2+}. Esta reação ocorre na presença de oxigênio molecular e o resíduo guanidino da L-arginina produz o radical livre gasoso, óxido nítrico, e L-citrulina, em um processo catalisado pela enzima óxido nítrico sintase (Kuo et al., 1992).

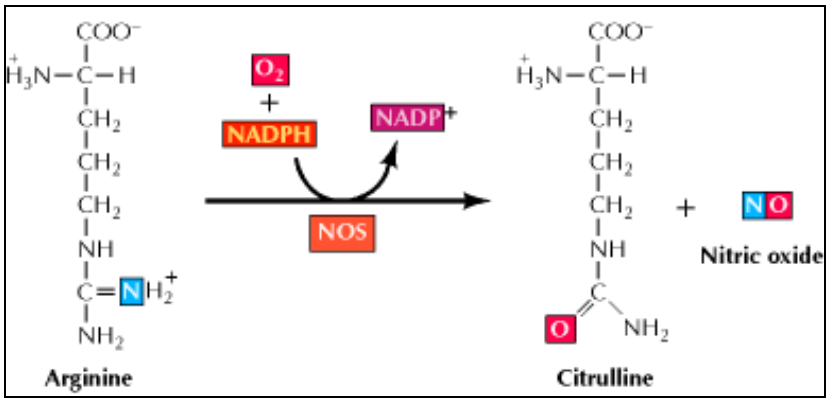

Figura 8. Reação da enzima óxido nítrico sintase (NOS). Extraído de The Cell: A Molecular Approach (Cooper, 2000).

Muitas células são capazes de sintetizar óxido nítrico por hemeproteínas da família citocromo P450-semelhantes, também chamadas de NOS, como mencionado

anteriormente. Estas enzimas são dependentes de oxigênio, NADPH, flavinas e biopterina para exercer suas atividades. Até o presente momento, foram isoladas e clonadas 4 isoenzimas: a óxido nítrico sintase endotelial, neuronal, induzida e mitocondrial. Estas isoenzimas são semelhantes estruturalmente, porém reguladas de modo diverso e induzidas a partir de genes localizados nos cromossomos 7, 12, 17 e 27, respectivamente. A óxido nítrico sintase neuronal, também chamada de NOSn está presente em neurônios e foi inicialmente purificada em cerebelo de camundongos e porcos (Schmidt et al., 1992). Além disto, esta proteína foi clonada de cérebro de humanos e mantém uma seqüência de aminoácidos altamente conservada entre as espécies, ocorrendo 93% de identidade entre a óxido nítrico sintase neuronal de humanos e de camundongos (Konturek et al., 1995). Sua presença é evidenciada em neurônios, células epiteliais, sistema nervoso central e periférico, medula adrenal, músculo esquelético células β do pâncreas. Além de regular a transmissão sináptica no SNC, esta enzima atua na regulação central da pressão sanguínea, no relaxamento do músculo liso e na vasodilatação via nervos periféricos (Förstermann et al., 1994). A enzima óxido nítrico sintase induzida (NOSi) não é expressa constituitivamente, e é induzida, em macrófagos e outras células, por lipopolisacarídeos bacterianos e/ou citocinas (Galea et al., 1992; Lee et al., 1993). Esta enzima é capaz de produzir óxido nítrico por um longo tempo, e está envolvida em diversos processos patológicos, mas particularmente no processo inflamatório (Guix et al., 2005). De fato, o NO produzido pelos macrófagos para induzir a morte de micróbios, parasitas ou células tumorais, pode também lesar células saudáveis vizinhas, sendo este um dos mecanismos responsáveis pela maioria dos processos inflamatórios patológicos e doenças auto-imunes (Stweart anf Heales, 2003).

A enzima óxido nítrico sintase endotelial (NOSe) é expressa constitutivamente nas células endoteliais, podendo também ser chamada NOSc ou EC-NOS (*endothelial constitutive NO synthase*). O gene da enzima óxido nítrico sintase endotelial foi clonado a partir de células endoteliais de bovinos e humanos, confirmando o local preferencial de produção e ação do NO. Apesar da sua expressão ser restrita a células endoteliais vasculares (Marsden et al., 1993), tem sido evidenciado a expressão desta enzima em outros tipos de células, como neurônios (Abe et al., 1997) e astrócitos (Iwase et al., 2000) sendo sua expressão em humanos significantemente suprimida em situações de hipóxia (Wong et al., 1996). O NO é sintetizado pela ativação da NOSc basal (em células endoteliais vasculares e neurônios) segundos a minutos após o aumento na concentração de cálcio, em resposta à ativação de receptores da superfície celular e mecanismos de transdução de sinal (Esplugues, 2002). Além disto, esta enzima apresenta forma monomérica e peso molecular de 133 kd com síntese basal de NO na faixa de concentração picomolar (Kuo & Schroeder, 1995). Já a enzima óxido nítrico sintase mitocondrial (NOSmit) é uma genuína enzima mitocondrial, diferente das outras 3 isoenzimas. A enzima NOSmit é regulada por mecanismos de fosforilação e modificações pós-traducionais ainda não bem elucidados. Além disto, a disponibilidade de substratos (L-arginina) e cofatores (NADPH, Ca^{2+}, oxigênio, FAD, FMN) envolvidos na atividade desta enzima ainda é alvo de discussão. Enquanto que alguns autores sugerem a participação efetiva do Ca^{2+} na ativação enzima NOSmit (Ghafourrifar & Riechter,1997), outros suportam que sua atividade está relacionada com a disponibilidade de L-arginina na mitocôndria (Steffen et al., 2001). Entretanto, a maioria dos estudos sugere que a NOSmit atinge a velocidade máxima quando a mitocôndria possui todos os elementos necessários para a reação

(substratos e cofatores). Assim, o aumento de óxido nítrico inibiria a respiração celular quando houvesse aumento no metabolismo mitocondrial (Brookes et al., 2004).

As isoenzimas óxido nítrico sintase também podem ser caracterizadas pela capacidade de produção de óxido nítrico, bem como a duração de suas atividades enzimáticas. As isoformas NOSn e NOSe são de baixo débito, estando envolvidas em processos homeostáticos como neurotransmissão, peristaltismo, e controle da pressão arterial. Já a isoenzima iNOS permanece em atividade por horas, e apresenta um mecanismo sinérgico de indução, inclusive pelo próprio óxido nítrico produzido.

Outra classificação das enzimas óxido nítrico sintases é determinada pela sua dependência de cálcio para ativação. A concentração de Ca^{2+} livre no citoplasma exerce uma grande influência na astividade das enzimas óxido nítrico sintase neuronal e endotelial. Além de representar um importante sinalizador citoplasmático, o Ca^{2+} se liga a proteínas intracelulares, como a calmodulina e modula outras atividades enzimáticas, como fosfatases e proteínas quinases (Prast & Philippu, 2001). Desta forma, a chegada de um potencial de ação ativa canais de Ca^{2+} dependentes de voltagem na membrana e permite o influxo de Ca^{2+} para o meio intracelular. Concentrações elevadas de Ca^{2+} intracelular (400 nM) são requeridas para que a calmodulina se ligue à NOS, e a ative. Por outro lado, uma diminuição de Ca^{2+} intracelular é responsável pela dissociação do complexo Ca^{2+}-calmodulina, fazendo com que a enzima diminua sua atividade (Esplugues, 2002).

Um outro importante mecanismo de regulação, evidenciado tanto no SNC quanto no SNP, é a fosforilação das enzimas óxido nítrico sintase. Diversos estudos têm evidenciado inibições da atividade da enzima NOSn causados por fosforilação via proteina-

quinases dependente de AMPc (Bredt et al., 1992; Brune & Lapetina, 1991), proteína quinase C (dependente de diacilglicerol e Ca^{2+}) ou proteína quinase II, dependente do complexo Ca^{2+} calmodulina (Nakane, et al., 1991; Bredt et al., 1992; Hayashi et al., 1999; Komeima et al., 2000).

Entretanto, no SNC a produção de óxido nítrico é predominantemente regulada pelo influxo de Ca^{2+}, via ativação de receptores glutamatérgicos do subtipo NMDA (Esplugues, 2002). Receptor este, que está ligado à enzima NOSn através de uma proteína de membrana, PSD-95 que aproxima a enzima do receptor. Esta aproximação se deve a interação entre resíduos de aminoácidos da enzima com a proteína PSD-95 e a grande afinidade dos resíduos de aminoácidos serina, treonina e valina do receptor NMDA com esta proteína de membrana. Este mecanismo, além de aproximar o receptor com a enzima óxido nítrico sintase neuronal, facilita a interação da enzima com o Ca^{2+} proveniente do meio extracelular via ativação do receptor NMDA (Kornau et al., 1995; Tomita et al., 2001). Além disto, estudos tem evidenciado que a estimulação do receptor NMDA induz a ativação da enzima calcineurina, que defosforila a NOSn. Este processo de defosforilação ativa a NOSn e aumenta a produção de NO, que está envolvido na liberação de neurotransmissores como glutamato (Kaminska et al., 2004). Neste contexto, tem sido sugerida a participação desta enzima, calcineurina, na viabilidade neuronal, uma vez que achados experimentais sugerem uma efetiva proteção exercida pela administração prévia de inibidores da calcineurina e do complexo Ca^{2+} calmodulina, ciclosporina (CsA) e FK506, contra a morte de neurônios corticais induzida por NMDA. Esta proteção estaria associada ao aumento nos níveis de fosforilação da enzima NOSn e diminuição nos níveis de cGMP e da produção de espécies reativas (Dawson et al., 1993).

I. 4.1. Papel Fisiológico e Patológico do Óxido Nítrico no SNC

Desde que foi descoberto como um mensageiro químico neuronal, o NO tem exercido um importante papel na "plasticidade" sináptica (capacidade de modulação e adaptação das sinapses para transmissão do impulso nervoso) (Prast & Philippu, 2001). Além disto, o NO induz a mudanças neuronais, sinalizando funções através de diversas vias. A ativação da enzima guanilato ciclase (sGC), aumento na formação de 3'-5' guanosina monofosfato cíclico (cGMP) bem como a ativação de proteínas quinases dependentes de cGMP tem sido sugerido como a principal via de transdução de sinal exercido pelo NO no SNC (Wang & Robinson, 1997; Smolenski et al., 1998).

Muitos estudos têm evidenciado que a síntese de óxido nítrico é estimulada pelo glutamato. O glutamato liberado em função de um estímulo difunde-se na fenda sináptica, ligando-se aos receptores do tipo NMDA no terminal pós-sináptico. Estes receptores estão acoplados a canais de íons cálcio e sua ativação permite o influxo de Ca^{2+} para o interior do terminal pós-sináptico, onde o Ca^{2+} se associa à calmodulina e ativa a enzima óxido nítrico sintase neuronal, promovendo a formação de óxido nítrico. Uma proteína acoplada ao receptor NMDA, denominada PSD95, e citada anteriormente, também realiza o acoplamento entre este receptor e a NOSn, ativando-a de uma maneira independente do complexo cálcio/camodulina como descrito anteriormente (Aarts et al., 2002; Aarts & Tymianski, 2004). Tal como outros mensageiros químicos (CO e metabólitos do ácido araquidônico), o NO não atua especificamente no neurônio onde é formado, mas opera em outras células do organismo (Prast & Philippu, 2001). Dependendo do tipo de neurônio e da sua localização no SNC, o aumento na concentração de cGMP dependente de NO exerce diferentes funções como mostrado no Quadro1, abaixo.

Quadro 1. Efeitos celulares do aumento de GMP_c dependente de NO em diferentes estruturas cerebrais.

Área do Cérebro	Função celular	Transmissão sináptica	Referência
Hipocampo (slice)	Fosforilação de canais de Ca^2 voltagem dependente	Facilitação	Erdemli & Krnjevis, 1995
Cultura de neurônios piramidais (hipocampo)	Aumento no potencial pós-sinaptico excitatório	Facilitação	Arancio et al., 1995
Neurônios talamocorticais	Aumento no firing neuronal	Facilitação	Paper & Mager, 1992
Estudo *in vivo* e *in vitro* de neurônios talâmicos	Despolarização na membrana neuronal	Facilitação	Shaw, 1999
Núcleo Acumbis	Aumento no potencial pós-sinaptico excitatório	Facilitação	Prast, 1997
Cerebelo (slice)	Diminuição na atividade de receptores AMPA	Redução	Dev & Morris, 1994
Cortex cerebral	Diminuição na atividade dos receptores $GABA_A$	Facilitação	Zarri et al., 1994; Robello et al., 1996
Cultura de neurônios cerebelares	Diminuição na atividade dos receptores $GABA_A$	Facilitação	Zarri et al., 1994; Robello et al., 1996
Medula espinhal (slice)	Diminuição no firing neuronal	Redução	Pehl & Schmid, 1997
Núcleo Tracto Sólitário (slices)	Aumento no firing neuronal	Facilitação	Pehl & Schmid, 1997
Núcleo Supraóptico (slices)	Despolarização de membrana e aumento na condutância iônica	Facilitação	Yang & Hatton, 1999
Hipocampo (slices)	Ativação de receptores D1/D5 e aumento no potencial pós-sinaptico excitatório	Facilitação	Yang, 1999

Apesar do NO modificar atividade neuronal em diferentes regiões do cérebro via ativação de enzimas quinases dependentes de cGMP, este mensageiro químico também modula a função neuronal de forma independente do cGMP. Este fenômeno ocorre através de reações diretas sobre proteínas (nitrosilação) e/ou reações com o radical superóxido

formando peroxinitrito e subseqüente nitrosilação e oxidação de proteínas (Fredstrom, 2002). De fato, estudos em cultura de neurônios corticais demonstram que o aumento na geração de NO reduz o influxo de Cl^- mediado por receptores GABA independente da ativação de cGMP (Robello et la., 1996). Alem disto, a geração de NO aumenta a ligação de AMPA em slices de cérebro de ratos por aumentar a afinidade com o receptor (Dev & Morris, 1994). Este mecanismo é, obviamente, diferente do mecanismo proposto anteriormente (Quadro 1), pelo qual o NO modula os receptores AMPA e $GABA_A$ via ativação da cascata do GMP_c em slices de cerebelo (Dev & Morris, 1994). A partir destes estudos, sugere-se que, em diferentes regiões do cérebro, o mesmo receptor pode ser modulado por NO através de diferentes mecanismos. Neste contexto, a modulação de receptores corresponde em um importante papel do NO na plasticidade neuronal. De acordo com esta afirmação, uma inibição transitória de receptores $GABA_A$ induzidos pelo NO poderia elevar a um aumento na excitabilidade neuronal, enquanto que uma nitrosilação nos sítios modulatórios do receptor NMDA poderia prover um mecanismo de proteção contra uma excessiva estimulação deste receptor (Prast & Philippu, 2001).

Quando o NO é produzido em grandes quantidades, este radical livre deixa de exercer seus efeitos como neuromodulador fisiológico e passa a ser um agente neurotóxico (Guix et al., 20005). Tem sido observado que exagerada produção de NO neuronal é resultado de uma ativação da enzima NOSn seguido de uma persistente estimulação de receptores, principalmente glutamatérgicos bem como uma excessiva estimulação da enzima NOSi por diversos agentes, como endotoxinas e citocinas (Charbier et al., 1999). O excesso de NO produzido pela NOSi induz lipoperoxidação e reage com grupos tióis da glutationa formando os grupos S-nitrosotióis (Fredstom, 2002). A formação destes grupos

induz a inibição de diversas enzimas responsáveis pela manutenção da viabilidade celular, como a Na^+,K^+-ATPase, caspases, gliceraldeído-3-fosfato desidrogenae e creatina quinase (Beckamn, 1996; Clancy & Abramson, 1995; Kang et al., 2000). Esta inibição se deve a presença nestas enzimas de grupos sulfidril que são, particularmente, sensíveis à formação dos grupos S-nitrosotióis induzidos pela geração de NO (Barriviera et al., 2005).

Entretanto, a principal reação responsável pela toxicidade induzida pelo NO é a combinação do mesmo com o radical $O_2^{\bullet-}$. O produto formado desta reação, peroxinitrito ($ONOO^-$) apesar de possuir uma meia vida relativamente curta, é capaz de se difundir através das membranas plasmáticas. Dependendo do pH e da presença de ferro no local, o $ONOO^-$ pode induzir a depleção de grupos tióis e a nitrosilação de resíduos de tirosina e fenóis de proteínas envolvidas no metabolismo energético como SDH e aconitase (Clancy & Abramson, 1995; Beckman & Koppenol, 1996). Além disto, a capacidade de nitrosilação (adição de NO) e nitração (adição de NO_2) exercida pelo $ONOO^-$ sobre o aminoácido tirosina leva a formação de nitrotirosina (Lehnig, 1999) protéicos. O aumento nos níveis de 3-nitrotirosina, um marcador de estresse oxidativo, tem sido evidenciado em diversas doenças neurodegenerativas como Alzheimer (Good et al, 1996; Smith et al., 1997), Parkinson (Good et al, 1998) e esclerose lateral amiotrófica (Cookson & Shaw, 1999).

Existe, contudo, uma relação paradoxal entre os níveis de NO e o dano oxidativo tanto no cérebro como em outros sistemas biológicos. Estudos recentes têm mostrado que o tratamento de astrócitos sobrecarregados de ferro com citocinas aumenta os níveis de NO e diminui o dano oxidativo causado pela reação de Fenton (Robb et al., 1999). Além disto, têm sido observado que o tratamento com NO diminui o dano oxidativo causado pelo tetra-butil-hidroperóxido em mitocôndrias purificadas de astrócitos (Robb & Connor, 2002). No

mesmo estudo foi demonstrado que o NO reduz a suceptibilidade a espécies reativas de oxigênio, usualmente relacionadas com a sobrecarga de ferro em astrócitos, sugerindo um papel antioxidante do NO em situações de estresse oxidativo.

I. 4.2. Participação do NO em diversos modelos de convulsão.

Apesar de existirem evidências convincentes da participação do óxido nítrico na toxicidade induzida por diversos agentes convulsivantes como cainato, pentilenotetrazol e NMDA, o papel do óxido nítrico na patofisiologia das convulsões e no dano oxidativo induzido por estes compostos ainda não está totalmente compreendido. Por considerarem o NO um mensageiro retrógrado, alguns autores sugerem que o mesmo inicie uma cascata de reações que possa prevenir a propagação da atividade convulsiva (Yamamoto et al., 1992; Buisson et al., 1994). Por outro lado, um considerável grupo de autores acredita que um aumento na produção de NO pode estar envolvido na gênese ou propagação da atividade convulsiva induzida por diversos agentes convulsivantes (Maggio et al., 1995; Van Leeuven et al., 1995; Hayashi et al., 2002; Gupta & Dettbarn, 2003). Diversos fatores são determinantes para esta discussão, tais como: variados modelos de convulsão, diversos tipos de inibidores da NOS, espécies de animais, região cerebral estudada e diferentes métodos de determinação de NO.

Neste contexto, foi mostrado que a inibição da NOS neuronal potencializa as convulsões induzidas por ácido caínico e pilocarpina em ratos (Przegalinski et al., 1994; Maggio et al., 1995; Montecot et al., 1997). Outros estudos demonstram que a supressão na produção de NO no cérebro de ratos leva à potencialização das convulsões e do dano oxidativo induzido pelo NMDA (Buisson et al., 1993; Przegalinski et al., 1996). Já a

administração de compostos que aumentam a produção de NO, como nitroprussiato (SNP) e L-arginina, protegem das convulsões induzidas por cainato e por penicilina (De Sarro et al., 1993; Marangos et al.,1994), apesar de exercerem efeitos contrários em outros modelos de convulsão (Mollace et al., 1991; Rajasekaram et al., 2001), sugerindo um efeito convulsivo do NO.

De fato, há evidências de que a diminuição na produção cerebral de óxido nítrico resulta na supressão das convulsões induzidas pelo PTZ e picrotoxina (Oseone et al., 1994, Rajasekaram et al., 2005). Além disto, Yasuda e colaboradores (2001) evidenciaram uma correlação significativa entre o aumento nos níveis de NO com as convulsões induzidas pela injeção intra-hipocacampal de cainato em ratos. Da mesma forma, estudos recentes sugerem que administração de inibidores da NOS, como 7-NI, protegem das convulsões e da formação de espécies reativas induzidas por oxigênio hiperbárico (Chavko et al., 2003) bem como pela administração de agonistas de receptores glutamatérgicos e antagonistas de receptores GABAérgicos (Hayashi et al., 2002; Gupta & Dettbarn, 2003; Rajasekaram et al., 2005).

Contudo, apesar de diversos estudos postularem uma efetiva participação do NO em diversos modelos convulsivos, ainda são escassos estudos evidenciado o papel do óxido nítrico na toxiciddade induzida pelo MMA. Portanto, o presente estudo teve como objetivo determinar o papel deste radical livre nas convulsões e no dano oxidativo induzido pelo MMA.

I. 5. Objetivos e Organização dos Trabalhos desta Tese

Os objetivos deste trabalho foram:

1. Avaliar se a administração do MMA induz carbonilação protéica;
2. Avaliar se um inibidor não seletivo da enzima óxido nítrico sintase (L-NAME) altera as convulsões e carbonilação protéica induzida pelo MMA;
3. Identificar se há correlação entre carbonilação protéica induzida e inibição da Na^+, K^+- ATPase e convulsões induzidas por MMA;
4. Verificar se há variação nos níveis teciduais de nitrito e nitrato após a administração intra-estriatal de MMA;
5. Avaliar o efeito de inibidores da NOS sobre o conteúdo de nitrito e nitrato estriatal basal e sobre o aumento dos níveis teciduais de nitrito e nitrato induzido por MMA;
6. Avaliar se outros inibidores da NOS, como o azul de metileno e 7-NI, atenuam as convulsões induzidas por MMA;
7. Avaliar se outros inibidores da NOS, como o azul de metileno e 7-NI, atenuam a carbonilação de proteínas e a inibição da Na^+, K^+- ATPase induzidas por MMA;
8. Investigar se a administração de L-arginina potencializa as convulsões e aumento de nitratos induzidos por MMA.

Sabendo-se da participação deste radical livre (óxido nítrico) nas convulsões induzidas pelo MMA, o objetivo do estudo que perfaz o capítulo II consistiu em verificar uma possível correlação entre o tempo de convulsão induzido pelo MMA com alterações no conteúdo estriatal de grupos carbonil protéicos e a atividade na enzima Na^+, K^+-

ATPase. Em um segundo momento, foi verificado o efeito da administração de azul de metileno, um composto com propriedades anti-oxidantes e que afeta a produção de óxido nítrico neuronal, sobre as alterações comportamentais, eletroencefalográficas e neuroquímicas induzidas pelo MMA.

O Capítulo III teve como objetivo verificar o efeito da administração prévia de 7-NI (inibidor da enzima óxido nítrico sintase neuronal) e L-arginina (substrato desta enzima) sobre as alterações comportamentais, eletroencefalográficas e neuroquímicas induzidas pelo MMA.

CAPÍTULO II

Involvement of NO in the convulsive behavior and oxidative damage induced by the intrastriatal injection of methylmalonate

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Involvement of NO in the convulsive behavior and oxidative damage induced by the intrastriatal injection of methylmalonate

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Abstract

Acute intrastriatal administration of methylmalonic acid (MMA) induces convulsions through NMDA receptor-mediated mechanisms and increases production of end products of oxidative damage. Although it has been demonstrated that nitric oxide (NO) production increases with NMDA receptor stimulation and contributes to the oxidative damage observed in several neurodegenerative disorders, the role of NO in MMA-induced convulsions has not been investigated to date. In the present study we investigated the effects of the intrastriatal injection of N^{ω}-nitro-L-arginine methyl ester (L-NAME: 10^{-4} to 10^{0} nmol/0.5 μl) on the convulsions and striatal protein carbonylation induced by the intrastriatal injection of MMA (4.5 μmol/1.5 μl). L-NAME (10^{-3} to 10^{-1} nmol) protected against MMA-induced convulsions and protein carbonylation ex vivo. These results suggest the involvement of NO in the convulsive behavior and protein carbonylation elicited by MMA.

Keywords: Convulsion; Protein carbonylation; L-methylmalonic acid; Nitric oxide; Methylmalonic acidemia; L-NAME

Methylmalonic acidurias comprise a group of inherited metabolic disorders caused by either a deficiency of the mitochondrial enzyme methylmalonyl CoA mutase (MCM, EC 5.4.99.2), or defects in the synthesis of 5′-deoxyadenosylcobalamin, the cofactor of MCM. Deficient MCM activity, which physiologically catalyses the reaction of methylmalonyl CoA to succinyl CoA, leads to the primary accumulation of methylmalonyl CoA, and a secondary accumulation of other metabolites, such as succinate, propionate, 3-hydroxypropionate, and 2-methylcitrate [10,25,17]. A large percentage of affected infants die during the first weeks or month of life, and those who survive longer present a variable degree of mental retardation and other neurological abnormalities, like delayed development and seizures [29,4].

Experimental findings in vitro and ex vivo have shown that methylmalonic acid (MMA) inhibits succinate dehydrogenase (SDH) and β-hydroxybutyrate dehydrogenase [9,36,13] impairs mitochondrial function [6,25,36], increases lactate production [31,38], and decreases ATP levels [23], CO_2 production [38] and O_2 utilization [36]. In addition, we have demonstrated that intrastriatal injection of MMA induces convulsive behavior and excitotoxicity though NMDA receptor-mediated mechanisms [8,31].

A significant amount of work has suggested that NMDA receptors and reactive oxygen species (ROS) generation may underlie the neurotoxic effects of SDH inhibitors, such as 3-nitropropionate (3-NP) and malonate [14,33]. In this context, we have demonstrated that intrastriatal MMA administration, besides causing convulsive behavior, increases local thiobarbituric acid reactive substances (TBARS) and protein carbonylation [21,30]. Moreover, while the systemic administration of antioxidants, such GM1, ascorbic acid and α-

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tocopherol attenuate MMA-induced convulsions [11,12], the administration of ammonia, a substance that increases reactive species production, potentiates the convulsive behavior elicited by MMA [22]. These results suggest the involvement of reactive species, in addition to NMDA receptors [8], in the convulsive phenomenon elicited by MMA.

It has been suggested that a great deal of NMDA receptor activation-induced excitotoxicity is due to NO generation [18]. Accordingly, excessive production of nitric oxide (NO), a highly reactive species produced in tissues from L-arginine by the enzyme NO synthase (NOS), inhibits the mitochondrial respiratory chain, leading to mitochondrial damage [35]. In fact, several proteins involved in the energetic metabolism such as aconitase, SDH and creatine kinase (CK), are particularly sensitive to reactive species-induced damage [5,35] and to MMA [6].

Although there is convincing evidence of the participation of NO in the toxicity of glutamate, the role of NO in the pathophysiology of convulsive disorders are not completely defined. While some authors suggest an anticonvulsant role for NO [20], others suggest that it may be a proconvulsant agent [37]. The determining factor for such a discrepancy is not known, but one might argue that methodological differences may account for it. One interesting possibility is that the effect of NO on convulsions may vary with the model of seizure employed. As a matter of fact, it has been demonstrated that inhibition of NOS potentiates seizures induced by kainic acid and pilocarpine in rats [20,24,27]. Furthermore, the inhibition of NOS increases the convulsive behavior and susceptibility to seizures induced by NMDA [7,28]. Conversely, a decrease in tissue NO levels results in suppression of convulsions induced by pentylenetetrazol (PTZ) in rats [26]. Since the role of NO in the toxicity induced by MMA has been almost neglected in the literature, we decided to investigate the effect of N^{ω}-nitro-L-arginine methyl ester (L-NAME) administration, an inhibitor of NOS, on the MMA-induced convulsive behavior and striatal protein carbonylation ex vivo.

Adult male Wistar rats (270–300 g; $n = 8$–10 in each group) maintained under controlled light and environment (12 h light/12 h dark cycle, $24 \pm 1\,^{\circ}C$, 55% relative humidity) with free access to food (Guabi, Santa Maria, Brazil) and water were used. Animal utilization protocols followed the Official Government Ethics guidelines and were approved by the Ethics Committee of the Federal University of Santa Maria. All reagents were purchased from Sigma (St. Louis, MO, USA) and all solutions were prepared with type I ultra pure water. L-NAME was prepared in 100 mM phosphate buffered saline (pH 7.4, PBS).

The animals were anesthetized with Equitesin (1% phenobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol; 3 ml/kg, i.p.) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a cannula was inserted unilaterally into the striatum (coordinates relative to bregma: AP 0 mm, ML 3.0 mm, V 3.0 mm from the dura). Chloramphenicol (200 mg/kg, i.p.) was administrated immediately before the surgical procedure. Three days after the cannula placement, the animals received an intrastriatal injection of L-NAME (10^{-4} to 1 nmol/0.5 μl) or vehicle (PBS 100 mM/ pH 7.4) 30 min before administration of MMA (4.5 μmol/1.5 μl) or saline (6.7 μmol/1.5 μl).

After the injections, the animals were transferred to a round open field (54.7 cm in diameter) with a floor divided into 11 equal areas. The open field sessions lasted 15 min, and during this time the animals were observed for the appearance of convulsive behavior (myoclonic jerks and clonic movements involving hindlimbs and forelimbs contralateral to the injected striatum). The latency for the first convulsive episode and the total time spent convulsing were recorded [8].

Immediately after the behavioral evaluation, the animals were killed by decapitation and had their brain exposed by the removal of the parietal bone. A punch of the injected and noninjected striatum was rapidly obtained using a stainless steel puncher (5 mm in internal diameter) around the site of cannula placing. Tissues were homogenized in 10 volumes (w/v) of 10 mM Tris–HCl buffer pH 7.4 using a glass homogenizer and its carbonyl protein content was determined by the method described by [39], adapted for brain tissue, as below. Briefly, homogenates were diluted to 750–800 (g/ml of protein in each sample, and 1 ml aliquots were mixed with 0.2 ml of 2,4-dinitrophenylhydrazine (DNPH, 10 mM) or 0.2 ml HCl (2 M). After incubation at room temperature for 1 h in a dark ambient, 0.6 ml of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing 3% SDS), 1.8 ml of heptane (99.5%) and 1.8 ml of ethanol (99.8%) were added sequentially, and mixed with vortex agitation for 40 s and centrifuged for 15 min. Next, the protein isolated from the interface was washed two times with 1 ml of ethyl acetate/ethanol 1:1 (v/v) and suspended in 1 ml of denaturing buffer. Each DNPH sample was read at 370 nm in a Hitachi U-2001 spectrophotometer against the corresponding HCl sample (blank), and total carbonylation calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1}, as described by Levine et al. [19]. Protein content for biochemical experiments was measured colorimetrically by the method of Bradford [3] by using bovine serum albumin (1 mg/ml) as standard.

Data from ex vivo total carbonyl determinations were analyzed by a 2 (PBS or L-NAME) × 2 (saline or MMA) × 2 (injected or non-injected hemisphere) factorial ANOVA, with the hemisphere factor treated as a within-subject factor. Post hoc analyses were carried out by the F test for simple effect or the Student–Newman–Keuls test, when appropriate. $P < 0.05$ was considered significant.

The latency for the first convulsive episode and total time spent convulsioning induced by MMA were analyzed by one-way ANOVA, followed by a Student–Newman–Keuls test. All data are expressed as mean + S.E.M.

The involvement of NO in the convulsant effects of MMA was investigated by intrastriatally injecting the animals with L-NAME (10^{-4} to 10^{0} nmol/0.5 μl). The striatal injection of L-NAME had no effect per se on behavioral parame-

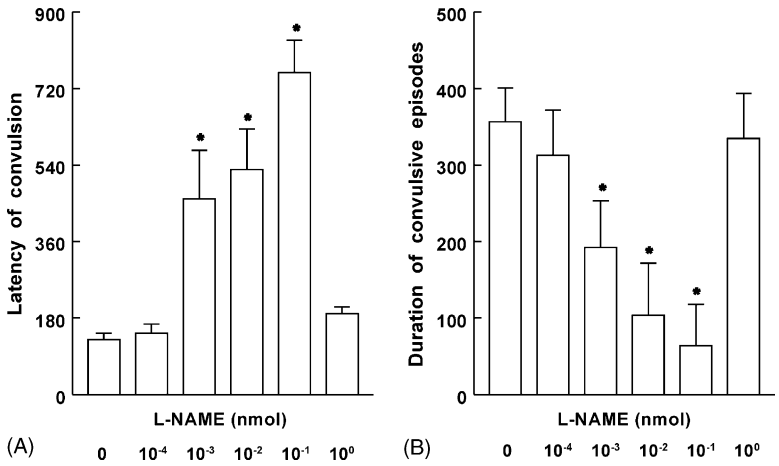

Fig. 1. L-NAME (10^{-3}–10^{-1} nmol/0.5 μl) increases the latency for the first convulsive episode (A) and decreases the duration (B) of convulsive episodes induced by MMA (4.5 μmol/1.5 μl). Data mean + S.E.M. for $n = 8$–10 in each group. *$P < 0.05$ compared with saline-MMA group (F test for simple effect).

ters and striatal protein carbonylation (data not shown). Statistical analysis revealed that the intrastriatal injection of L-NAME (10^{-3} to 10^{-1} nmol/0.5 μl) retarded the appearance [$F(5,102) = 14.13$; $P < 0.05$; Fig. 1A] and decreased the duration [$F(5,102) = 6.08$; $P < 0.05$; Fig. 1B] of convulsive episodes induced by MMA. Post hoc analysis revealed that 1 nmol L-NAME did not protect against the convulsive episodes induced by MMA. These data indicate a biphasic effect of L-NAME on convulsive behavior. The increase of total protein carbonylation induced by striatal MMA administration was prevented by 10^{-3}, 10^{-2} and 10^{-1} nmol L-NAME [$F(11,109) = 16.05$; $P < 0.05$; Fig. 2].

In the present study, we confirm and extend our previous findings that MMA induces convulsions, increases the formation of reactive oxygen species (ROS) ex vivo and it shows, for the first time, that the striatal injection of L-NAME protects against the convulsions and protein carbonylation elicited by MMA.

L-NAME prevented MMA-induced convulsions (Fig. 1A and B), suggesting involvement of NO in the convulsant effect of MMA. Because many doses of L-NAME were administered into the striatum, it is possible that NO exerted a proconvulsant effect in this seizure model. Moreover, considering previous results obtained by our group and by others showing that SDH inhibitors produce at least part of their actions by activation of NMDA glutamate receptors [8,31,41], it is feasible to propose that striatal NO production may induce or exacerbate excitotoxicity induced by MMA through NMDA receptors activation. In fact, it has been suggested that a hyperactivation of glutamate receptors, especially the NMDA subtype, leads to increased intracellular free Ca^{2+}, NOS activation, free radicals generation and mitochondrial dysfunction in several experimental models [1,32,35].

In the present study, we also showed that intrastriatally administrated MMA induces protein carbonylation besides convulsive episodes (Fig. 2). These data replicate previous results [30] and are presumptive evidence that MMA accumulation may cause protein damage. Other studies have also demonstrated that protein modifications induced by oxidative attack on amino acids can lead to the formation of protein carbonyl in several diseases such as Huntington and Alzheimer diseases [2].

In the present study, we demonstrated that small, but not large doses of L-NAME protected against convulsive behavior and protein carbonylation induced by MMA. This biphasic dose-response induced by L-NAME may be another important factor explaining the discrepancies noticed in

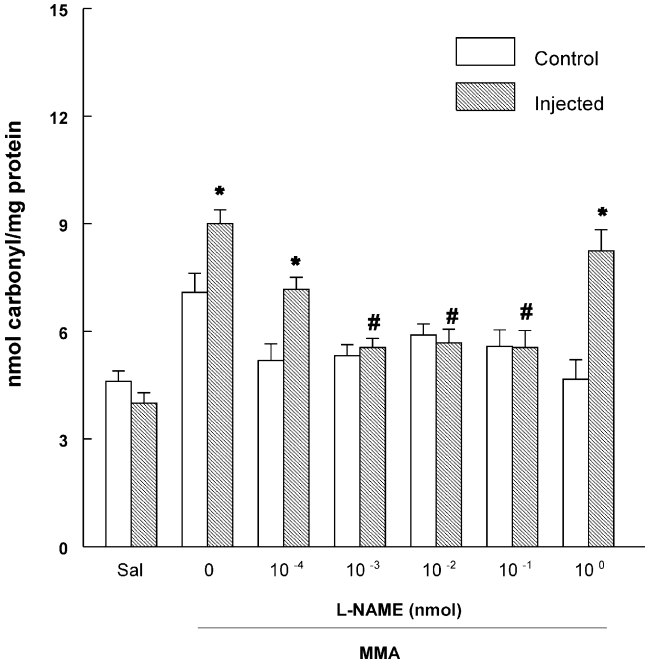

Fig. 2. L-NAME (10^{-3} to 10^{-1} nmol/0.5 μl) prevents the striatal MMA-induced protein carbonylation increase (4.5 μmol/1.5 μl). Data are mean + S.E.M. for $n = 8$–10 in each group. *$P < 0.05$ compared with Saline group. #$P < 0.05$ compared with Saline-MMA group (Student–Newman–Keuls test).

the literature concerning the neuroprotection effects of this drug in several seizures models [16,27,28,34]. It is worth noting that L-NAME is nonspecific inhibitor of NOS, which presents a lower K_i for neuronal (15 nM), than for endothelial NOS (39 nM) [15]. Therefore, it is possible that the presently reported biphasic effect of L-NAME on convulsive behavior and protein carbonylation may be due to a differential effect of increasing amounts of L-NAME on nNOS and eNOS activity. It seems reasonable that low concentrations of L-NAME would preferentially inhibit nNOS activity, while high concentrations of L-NAME would also affect eNOS activity, resulting in an adverse vascular effect (i.e., vasoconstriction and consequent aggravation of tissue metabolic compromise). Accordingly, in models of focal ischemia the selective inhibition of nNOS reduces infarct volume, while the non-selective NOS inhibition results in deleterious alterations of cerebral blood flow and subsequent increases in infarction volume [40,42].

In summary, although the mechanisms underlying brain damage in methylmalonic acidemia are poorly known, the present study suggests the participation of NO in the excitotoxicity elicited by MMA. These results may be of value in understanding the pathophysiology of the neurological features observed in methylmalonic acidemia.

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CAPÍTULO III

Methylene Blue prevents MMA-induced seizures and oxidative damage in rat striatum

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Abstract:

Methylene blue (MB) is a thiazine dye with cationic and lipophilic properties that acts as an electron transfer mediator in the mitochondria. Due to this metabolic improving activity and free radicals scavenging effects, MB has been used in the treatment of methemoglobinemia and ifosfamide-induced encephalopathy. Considering that methylmalonic acidemia consists of a group of inherited metabolic disorders biochemically characterized by impaired mitochondrial oxidative metabolism and reactive species production, we decided to investigate whether MB, protects against the behavioral and neurochemical alterations elicited by the intrastriatal injection of MMA. In the present study we investigated whether intrastriatal injection of MB (0.015 to 1.5 nmol/ 0.5 μl) protects against seizures (evidenced by electrographic recording), protein carbonylation and Na^+,K^+-ATPase inhibition *ex vivo* induced by MMA (4.5 μmol/ 1.5μl). In addition, we investigated whether convulsions elicited by intrastriatal MMA administration are accompanied by striatal protein carbonyl content increase and changes in Na^+,K^+-ATPase activity in rat striatum. The effect of MB (0.015 to 1.5 nmol/ 0.5 μl) and MMA (4.5 μmol/ 0.5μl) on striatal NOx (NO2 plus NO3) content was also evaluated. In the present study we demonstrated that MB treatment affords significant protection against protein carbonylation, Na^+,K^+-ATPase inhibition and electrographic convulsions induced by MMA. Furthermore, statistical analysis revealed that the MMA-induced NOx content increase was attenuated by intrastriatal injection of MB and the duration of convulsive

episodes correlated with Na^+, K^+-ATPase inhibition, but not with MMA-induced total protein carbonylation. In view of that MB decreases MMA-induced neurotoxicity assessed by behavioral and neurochemical parameters, the authors suggest that MB may be of value to attenuate neurological deficits of methylmalonic acidemic patients.

Key words: Convulsion - Protein Carbonylation – Methylmalonate - Na^+,K^+- ATPase - Methylene Blue

1. Introduction

Methylene blue (MB) is a redox compound (thiazine dye) with cationic and lipophilic properties that acts as an electron transfer mediator, stimulating of mitochondrial respiration (Visarius et al.,1997; 1999). Experimental findings have demonstrated that low concentrations of MB improve mitochondrial respiration by shuttling electrons to oxygen in the electron transport chain, thereby increasing cellular oxygen consumption (Visarius et al., 1997; Lindahl et al., 1961). Due to these metabolic improving activities and radical scavenging, MB has been used prophylactically or an antidote for encephalopathy induced by ifosfamide (IFO) in chemotherapeutic treatment (Küpfer et al., 1994; 1999; Pelgrims et al., 2000; Turner et al., 2003; Raj et al., 2003; Pavarana et al., 2005).

Methylene blue also acts as a potent inhibitor of superoxide and hydroxyl radical production by serving as an artificial electron acceptor, diverting electron flow away from the enzyme sites of various oxidases, where molecular oxygen is converted to superoxide radicals (Fridovich et al., 1997; Aeschlimann et al., 1998; Kelner et al., 1998). In this context, a significant amount of work has suggested that MB represents a free radical scavenger which competitively inhibit the reduction of molecular oxygen to superoxide, acting as an alternative electron acceptor for enzymes either in heme or non-heme forms

such as xantine oxidase and nitric oxide synthase (NOS) (Salaris et al., 1991; Mayer et al., 1992; 1993; Volke et al., 1999).

Methylmalonic acidemia is biochemically characterized by a primary accumulation of methylmalonate (MMA) and a secondary accumulation of other metabolites, such as succinate, propionate, 3-hydroxypropionate, and 2- methylcitrate (Fenton et al., 2001; Okun et al., 2002; Kölker et al., 2003) in tissue and body fluids. They are caused by an inherited deficiency of the mitochondrial enzyme methylmalonil-CoA mutase (MCM, EC 5.4.99.2) or by defects in the synthesis of 5'-deoxyadenosylcobalamin, the cofactor of MCM (Fenton et al., 2001). Although the mechanisms of brain damage caused by MMA accumulation are not yet fully established, experimental findings *in vitro* and *ex vivo* support that MMA causes mitochondrial dysfunction (Dutra et al., 1993; Toyoshima et al., 1995; Brusque et al., 2002; Okun et al., 2002; Marsico et al., 2003; Fleck et al., 2004; Schuck et al., 2004; Maciel et al., 2004), leading to increased lactate production (Wajner et al., 1992; Royes et al., 2003), ATP depletion (McLaughlin et al., 1998), decreased CO2 production (Wajner et al., 1992) and O2 utilization (Toyoshima et al., 1995).

Furthermore, a growing body of evidence has suggested that organic acids, which accumulate in some organic acidurias cause reactive species generation (Wajner et al., 2004) and behavioral manifestations, such as seizures (de Mello et al., 1996; Lima et al., 1998; Coitinho et al., 2001). In this context, it has been shown that the intrastriatal administration of MMA induces convulsions, reactive species generation and inhibits Na^+,K^+-ATPase activity in rat striatum (de Mello et al., 1996; Fighera et al., 1999; 2003; Marisco et al., 2003; Wyse et al., 2000, Malfatti et al., 2003; Royes et al., 2005). Although it is difficult at present to determine a cause effect relationship between reactive species and seizures induced by MMA, recent studies from our laboratory have shown that while the

convulsions induced by MMA are attenuated by antioxidant treatment (ascorbate, -tocopherol and GM1 ganglioside) (Fighera et al., 1999; 2003), ammonia, a pro-oxidant agent, increases MMA-induced convulsions (Marisco et al., 2003), suggesting that reactive species are involved in the genesis of convulsions elicited by MMA. Therefore, considering that reactive species generation have been implicated in the convulsive behavior elicited by MMA it is rather possible that drugs, such as MB, which increase brain mitochondrial respiration and act as scavenger of superoxide radical may protect against the acute behavioral and neurochemical effects induced by MMA. In this study we investigate whether the intrastriatal injection of MB attenuates MMA-induced behavioral, electrographic and neurochemical deleterious effects.

2. Materials and methods

2.1 Animals and reagents

Adult male Wistar rats (270-300 g, n= 6 - 8 for each group) maintained under controlled light and environment (12:12 h light-dark cycle, 24 ± 1oC, 55% relative humidity) with free access to food (Guabi, Santa Maria, Brazil) and water were used. Animal utilization protocols followed the Official Government Ethics guidelines and were approved by the University Ethics Committee. All reagents were purchased from Sigma (St. Louis, MO, USA) and all solutions were prepared with type I ultra pure water. MB was prepared in 100 mM phosphate buffered saline (pH 7.4, PBS). Methylmalonate (methylmalonic acid) solutions had their pH set to 7.4 with NaOH.

2.2 Behavioral evaluation and surgical procedure

The animals were anesthetized with Equitesin (1% phenobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol; 3ml/kg, i.p.) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a cannula was inserted unilaterally into the striatum (coordinates relative to bregma: AP 0 mm, ML 3.0 mm, V 3.0 mm from the dura). Chloramphenicol (200 mg/kg, i.p.) was administrated immediately before the surgical procedure. Three days after the cannula placement, the animals received an intrastriatal microinjection of methylene blue (0.015, 0.15 and 1.5 nmol/ 0.5 μl) or vehicle (PBS 100 mM/ pH 7.4) 30 min before the administration of MMA (4.5 μmol/ 1.5 μl) or NaCl (4.5 μmol/ 1.5 μl). After the injections, the animals were transferred to a round open field (54.7 cm in diameter) with a floor divided into 11 equal areas. The open field sessions lasted 15 min, and during this time the animals were observed for the appearance of convulsive behavior (myoclonic jerks and clonic movements involving hindlimbs and forelimbs contralateral to the injected striatum). The latency for the first convulsive episode and the total time spent convulsioning was recorded (de Mello et al., 1996).

2.3. Placement of cannula and electrodes for EEG recordings

A subset of animals was surgically implanted with a cannula and electrodes under stereotaxic guidance. In brief, rats were anesthetized with Equitesin and two screw electrodes were placed bilaterally over the parietal cortex along with a ground lead positioned over the nasal sinus. Bipolar nichrome wire Teflon insulated depth electrodes (100 μm) were implanted ipisilaterally into striatum. For intrastriatal infusion of drugs, a guide cannula (27 gauge) was glued to a multipin socket and inserted through a previously opened skull orifice. The coordinates from bregma for implantation of the electrodes were (in mm): AP, -4.5; L, 2.5; and DV, 2 for the cortex and AP, 0; L, 3; DV, 3.5 for the

striatum (Paxinos, 1986). The electrodes were connected to a multipin socket and, together with the injection cannula, were fixed to the skull with dental acrylic cement. The experiments were performed 7-9 days after surgery.

2.4. EEG recordings and intrastriatal injection of drugs

The procedures for EEG recording and intracerebral injection of drugs were previously described (Cavalheiro et al., 1992). Briefly, the animals were allowed to habituate to Plexiglas cage (25 x 25 x 60 cm) for at least 10 min before the EEG recording.

The rats were then connected to the lead socket in a swivel inside a Faraday's cage. Routinely, a 10 min baseline recording was obtained to establish an adequate control period. The drug injection protocol used in this set of experiments was the same used in those experiments that evaluated the possible protective effect of MB on MMA-induced behavioral convulsions, except that EEG was concomitantly recorded. EEG signals were amplified, filtered (0.1 to 50.0 Hz, bandpass) and recorded using an analogical encephalographer (Berger TP 119). Several epochs were selected from preinfusion and postinfusion periods to determine significant EEG changes. The preinfusion segment was defined as ending immediately before the beginning of infusion. The postinfusion epochs started immediately after the end of MB (1.5 nmol/ 0.5 μl) or vehicle (PBS 100 mM/ pH 7.4) and MMA (4.5 μmol/ 1.5 μl) or NaCl (4.5 μmol/ 1.5 μl) infusion. All intrastriatal injections were made in unanesthetized rats by using a needle (30 gauge) protruding 1 mm below the guide cannula. All drugs were injected over 1 min period by using a Hamilton syringe, and an additional minute was allowed to elapse before removal of needle to avoid backflow of drug through the cannula.

2.5. Colorimetric determination of the protein carbonyl content ex vivo

Immediately after the behavioral evaluation, the animals were sacrificed by decapitation and had their brain exposed by the removal of the parietal bone. A punch of the injected and of the noninjected striatum was rapidly removed using a stainless steel puncher (5 mm in internal diameter) around the site of cannula placing. Striatal tissues were homogenized in 10 volumes (w/v) of 10 mM Tris-HCl buffer pH 7.4 using a glass homogenizer and its carbonyl protein content was determined by the method described by Yan et al. (1995), adapted for brain tissue by Oliveira et al. (2004).

2.6. Na^+,K^+-ATPase activity measurements

The measurement of Na+,K+-ATPase activity was performed in the same fresh, diluted, non-centrifuged homogenates used for determination of the striatal protein carbonyl content. Assay of enzyme activity was performed according by Wyse et al (2000). Briefly, the incubation medium consisted of 30 mM Tris-HCl buffer, pH 7.4; 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM MgCl2, and 50 μg of protein in the presence or absence of ouabain (2 mM), in a final volume of 350 μl. The reaction was started by the addition of adenosine triphosphate (ATP) to a final concentration of 5 mM. After 30 min at 37ºC, the reaction was stopped by the addition of 70 μl of trichloroacetic acid (TCA, 50%). Saturating substrate concentrations were used, and reaction was linear with protein and time. Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate released was quantified by the colorimetric method described by Fiske and Subbarow (1925), and Na^+,K^+-ATPase activity was

calculated by subtracting the ouabain-sensitive activity from the overall activity (in the absence of ouabain).

2.7. Assay of NOx as a marker of NO synthesis

For NOx (NO_2 plus NO_3) determination, striatal tissues were dissected on ice-cold Petri dishes and homogenized in $(NH_4)_2SO_4$ and acetonitrile (96%). The homogenates were centrifuged at 3000 x g for 20 min at 4°C and supernatant was separated for analysis of the NO_2 plus NO_3 as described by Miranda et al (2001). The resulting pellet was suspended in NaOH (3M) for protein determination.

2.8. Protein determination

Protein content was measured colorimetrically by the method of Bradford (1976) and bovine serum albumin (1 mg/ml) was used as standard.

3. Statistical analysis

The latency for convulsion and total time spent convulsioning data were analyzed by one or two-way ANOVA (analysis of variance), depending on the experimental design. *Post hoc* analysis was carried out by the Student Newman Keuls test, when appropriate. Biochemical data were analyzed by a two (PBS or MB) x two (saline or MMA) x two (injected or non-injected hemisphere) factorial ANOVA, with the hemisphere factor treated as a within-subject factor. *Post hoc* analyses were carried out by the F test for simple effect or the Student Newman Keuls test, when appropriate. $P< 0.05$ was considered significant. All data are expressed as mean + S.E.M. Correlation analyses were carried out using the Pearson's correlation coefficient.

4. RESULTS

4.1. Effect of MB on MMA-induced convulsive behavior

The intrastriatal injection of MMA (4.5 μmol/ 1.5 μl) caused the appearance of clonic convulsions (Fig. 1), which were confirmed by electrographic recording (Fig. 2C). Electrographically recorded seizures were accompanied by clonus of the left forelimb and/or hind limb and head, rotational behavior and full lateralization toward the left side of the body. Furthermore, electrographic recording of the ipsilateral striatum revealed that the striatal MMA administration caused the appearance of an epileptogenic focus in the injected striatum, which spread to the ipsi and contralateral cerebral cortex, 5 min after MMA infusion (Fig. 2C), confirming previous results from our group (Malfatti et al., 2003). The effect of the striatal administration of MB on MMA-induced convulsive behavior and electroencephalographic recording is shown in Figures 1 and 3 respectively. Statistical analysis revealed that the intrastriatal injection of MB (1.5 nmol/0.5 μl) protected against the MMA-induced convulsive behavior, measured by latency for the first convulsion [$F(3,65)= 3.37$; $P<0.05$; Fig. 1A], duration [$F(3,65)= 6.26$; $P<0.05$; Fig. 1B] of convulsive episodes and electrographic recording (Fig. 3C).

4.2. Correlation analyses of the MMA-induced convulsion with neurochemical measures (Na^+,K^+-ATPase activity and protein carbonylation)

Considering that reactive species have been implicated in MMA-induced convulsive behavior and that selected targets such as Na^+, K^+-ATPase, seem to be sensitive to free radical-induced damage we decided to investigate whether the duration of convulsive

episodes correlated with total protein carbonylation and with changes in Na^+, K^+-ATPase activity induced by the intrastriatal administration of MMA. Statistical analysis demonstrated that a highly significant correlation between duration of MMA-induced convulsive behavior and Na^+, K^+-ATPase activity inhibition (r=0.994; $P< 0.05$; Fig. 4A). On the other hand, the duration of convulsive episodes induced by MMA did not correlate with total protein carbonyl content in the striatum (Fig. 4B).

4.3. Effect of MB on MMA-induced protein carbonylation

The effects of the administration of MMA and MB injection on the total protein carbonylation of the injected and of the non-injected striata were also determined. Statistical analysis of total protein carbonyl content data considering the hemispheres as a within subject factor revealed a significant pretreatment (PBS or 0.015 to 1.5 nmol/ 0.5 μl; MB) by drug (saline or MMA) by hemisphere (injected or control) interaction [$F(3,72)=11.87$; $P<0.05$; Fig. 5). *Post hoc* analysis showed that the intrastriatal administration of MMA increased the total protein carbonyl content of the injected striatum, and that intrastriatal injection of MB (1.5 nmol/ 0.5 μl) protected against MMA-induced protein carbonylation.

4.4. Effect of MB on MMA-induced Na^+,K^+-ATPase activity inhibition

Figure 6 shows the effect of MB (0.015 to 1.5 nmol/ 0.5 μl) on MMA induced Na^+,K^+-ATPase activity inhibition. Statistical analysis revealed that the decrease of Na^+,K^+-ATPase activity [$F(1,57)=26.83$; $P<0.05$) induced by MMA (4.5 μmol/ 1.5 μl) was prevented by the

intrastriatal injection of MB (1.5 nmol/ 0.5 μl), but not by doses of MB lower than 1.5 nmol.

4.5. Effect of MB and MMA administration on striatal NOx content

The effect of striatal MB (1.5 nmol/ 0.5 μl), and MMA (4.5 μmol/ 1.5 μl) administration on NOx content of the injected and non-injected striata was also determined and is shown in Figure 7. Statistical analysis of the NOx content data revealed a significant pretreatment (PBS or MB) by treatment (saline or MMA) by hemisphere (injected or non-injected) interaction [$F(1,32)=4.69$; $P<0.05$] indicating that the increase of NOx content induced by MMA was attenuated by MB (1.5 nmol/ 0.5 μl).

5. DISCUSSION

In the current work we studied the MB intrastriatal injection in the behavioral, eletrographic and neurochemical alterations elicited by MMA administration. Therefore, we confirm and extend our previous findings that MMA elicits behavioral and eletrographic seizures and rises reactive species generation *ex vivo* and show, for the first time, that: a) MB affords significant protection against MMA-induced protein carbonylation, Na^+,K^+-ATPase inhibition and electrographic convulsions; b) the MMA-induced NOx content increase was prevented by intrastriatal injection of MB and c) the duration of convulsive episodes correlated with Na^+,K^+-ATPase inhibition, but not with MMA-induced total protein carbonylation.

Over the last ten years, MB has been prophylactically or acutely used as an antidote for ifosfamide (IFO)-induced encephalopathy (Küpfer et al., 1994; Ferreiro et al., 1995;

Haluzik et al., 1995; Zulian et al., 1995; Alonzo et al., 1996; Demandt and Wandt, 1996; Visarius et al., 1997), acting as an electron transfer mediator to stimulate mitochondrial respiration (Küpfer et al., 1994). In addition, experimental findings have demonstrated that doses of MB that increase brain oxygen consumption also facilitate memory retention in rats (Cada et al., 1995; Callaway et al., 2002; 2004; Riha et al., 2005). Accumulating evidence suggests that MB may represent a scavenger of radical superoxide by acting as an alternative electron acceptor for tissues oxidases, which competitively inhibits the reduction of molecular oxygen to superoxide (McCord et al., 1970; Salaris et al., 1991; Kelner et al., 1998). In the line of this view, recent studies have demonstrated that the inhibition of oxygen radical formation by MB prevents lipopolysaccharide-induced fever (Riedel et al., 2003).

In the present study, we demonstrated that intrastriatal administration of MB attenuated the MMA-induced convulsive behavior (evidenced by electrographic changes) (Fig. 1; 3C) and protected against protein carbonylation induced by this organic acid (Fig. 5). These results reinforce the view that in some inborn errors of metabolism, including methylmalonic acidemia, the mitochondrial dysfunction and oxidative stress may be the cause underlying several pathological conditions, such as seizures (Wajner et al., 2004). Therefore, one might suggest that the MB related antioxidant effects in this model of organic acidemia might underlie its anticonvulsant action.

However, MB at doses capable of fully preventing MMA-induced protein carbonyl formation (Fig. 5) did not prevent MMA-induced convulsions (Fig. 1), and in consequence a cause-effect relationship between MMA-induced oxidative damage and convulsive activity is difficult to demonstrate. In fact, the results presented in this report indicate that the duration of convulsive episodes induced by MMA did not correlate with total protein

carbonyl content in the striatum (Fig. 4B). One might consider the possibility that total protein carbonylation may not reveal oxidative damage of selective vulnerable targets which would predispose to the development of convulsions and might correspond to a small fraction of total carbonyl content. In agreement to this view and based in the positive-correlation between duration of MMA-induced convulsive behavior and inhibition of enzyme activity Na^+, K^+-ATPase (Fig. 4A), we suggest that oxidative attack of selected target such as Na^+, K^+-ATPase enzyme might represent a important role in the development and/or propagation of MMA-induced convulsive behavior. In this context, it has been showed that protein modifications induced by direct oxidative attack on lysine, arginine, proline or threonine or by secondary reaction of cysteine, histidine or lysine can lead to the formation of protein carbonyl in several diseases (Berlett et al., 1997; Beal, 2002; Dalle-Donne et al., 2003), but, in some cases, only selected proteins show an increase in the levels of carbonylation (Castegna et al., 2002).

It has long been known that selected targets of free radical as Na^+,K^+-ATPase seems to be particularly sensitive to oxidative damage since its inhibition has been associated with alterations in the membrane lipid composition (Jamme et al., 1995) and in the redox state of regulatory sulfhydryl groups (Morel et al, 1998). Regarding this point, it is possible that more sensitive targets to oxidative damage, such as Na^+,K^+-ATPase would require additional antioxidant protection and therefore, this interpretation might explain why high MB doses protected against MMA-induced Na^+,K^+-ATPase inhibition in our experimental conditions (Fig. 6).

In the present study we also demonstrated that intrastriatal MMA administration induces NOx increase (Fig. 7). This observed increase in the local content of NOx agrees with our recent study and others, which have proposed the involvement of neuronal $NO^{\cdot}$

generation in the neurotoxicity generated by SDH inhibitors (Schulz et al., 1996; Matthews et al., 1997; Royes et al., 2005). In addition, since MB afforded significant protection against MMA-induced NOx increase, we propose that a direct inhibition of neuronal NOS caused by MB (Mayer et al., 1992; Volke et al., 1999) interferes in the $NO^{\cdot}$ transduction pathway, protecting therefore against the MMA-induced excitotoxicity. In agreement with this view a substantial body of evidence suggests that the intracellular $NO^{\cdot}$ increases caused by impairment mitochondrial function may lead to stimulation of several cascade events causing therefore, a dysfunction of excitatory neurotransmission (Dawson et al., 1991; Zarri et al., 1994; Robello et al., 1996) and consequently oxidative damage (Esplugues et al., 2002).

In summary, although further in-depth studies are necessary to definitely establish a role for reactive species in the MMA-induced convulsive behavior, we suggest that inhibition of selected targets to free radicals as Na^{+}, K^{+}-ATPase, plays an important role in the hyperexcitability and concomitant oxidative damage induced by MMA. Furthermore, in the present study we evidenced an effective protection exerted by MB treatment against behavioral, electrographic and neurochemical deleterious effects induced by MMA. Considering that MB is a Food and Drug Administration (FDA)-approved drug used as a neuroprotective redox agent to prevent drug-induced encephalopathy in cancer treatment (Pelgrims et al., 2000; Turner et al., 2003; Raj et al., 2003; Pavarana et al., 2005) it is plausible to propose that MB may be valuable to attenuate neurological deficits of methylmalonic acidemic patients as well as to introduce a new class of drugs in the management of methylmalonic acidemia.

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FIGURES AND LEGENDS

Figure 1

Intrastriatal injection of MB (1.5 nmol/0.5 μl) increases the latency for the first convulsive episode (A) and decreases the duration (B) of convulsive episodes induced by MMA (4.5 μmol/1.5 μl). Data mean + S.E.M. for n= 8 in each group. **P*<0.05 compared with Saline - MMA group (*F* test for simple effect).

Figure 2

Basal electroencephalographic recording (preinfusion) (A); saline postinfusion (B)and typical seizure sequences observed after MMA intrastriatal injection (4.5μmol/ 1.5 μl) during 15 min (postinfusion) (C) which were accompanied by thebehavioral alterations

described in the Results section. ISTR, ipsilateral striatum; ICTX, ipsilateral cortex and CCTX, contralateral cortex. Calibration bars, 100 μV
and 1s.

Figure 3

Basal electroencephalographic recording (preinfusion) (A); MB (1.5 nmol/ 0.5 μl) postinfusion (B) and the effect of MB (1.5 nmol/ 0.5 μl) on seizure sequences observed after MMA intrastriatal injection (4.5 μmol/ 1.5 μl) during 15 min (postinfusion) (C). ISTR, ipsilateral striatum; ICTX, ipsilateral cortex and CCTX, contralateral cortex. Calibration bars, 100 μV and 1s.

Figure 4 A

Significant correlation between duration of MMA-induced convulsive behavior and striatal Na+, K+-ATPase activity inhibition (r=0.994; Pearson's correlation coefficient)

Figure 4 B

The duration of convulsive episodes induced by MMA (4.5 μmol/ 1.5 μl) does not correlate with total carbonyl content of the injected striatum in each animal (Pearson's correlation coefficient).

Figure 5

MB (0.015 to 1.5 nmol/0.5 μl) prevents the striatal MMA-induced protein carbonylation increase (4.5 μmol/1.5 μl) *ex vivo*. Data are mean + S.E.M. for n= 6 - 8 in each group. **P*

<0.05 compared with Saline group. #*P*<0.05 compared with Saline-MMA group. ‡*P*<0.05 compared with MB (0.015 nmol/0.5 μl)-MMA group (Student-Newman-Keuls test).

Figure 6

The effect of MB (0.015 to 1.5 nmol/ 0.5 μl) on the MMA-induced (4.5 μmol/1.5 μl) $Na^{+,}$ K^{+}-ATPase activity inhibition. Data are mean + S.E.M. for n = 6 - 8 in each group. **P* <0.05 compared to control striatum. #*P*<0.05 compared with Saline- MMA group.

Figure 7

The effect of MB (1.5 nmol/ 0.5 μl) on the MMA-induced (4.5 μmol/1.5 μl) NOx (NO_2 plus NO_3) increase. Data are mean + S.E.M. for n = 6 - 8 in each group. **P* <0.05 compared to control striatum. #P<0.05 compared with Saline-MMA group.

Figure 1

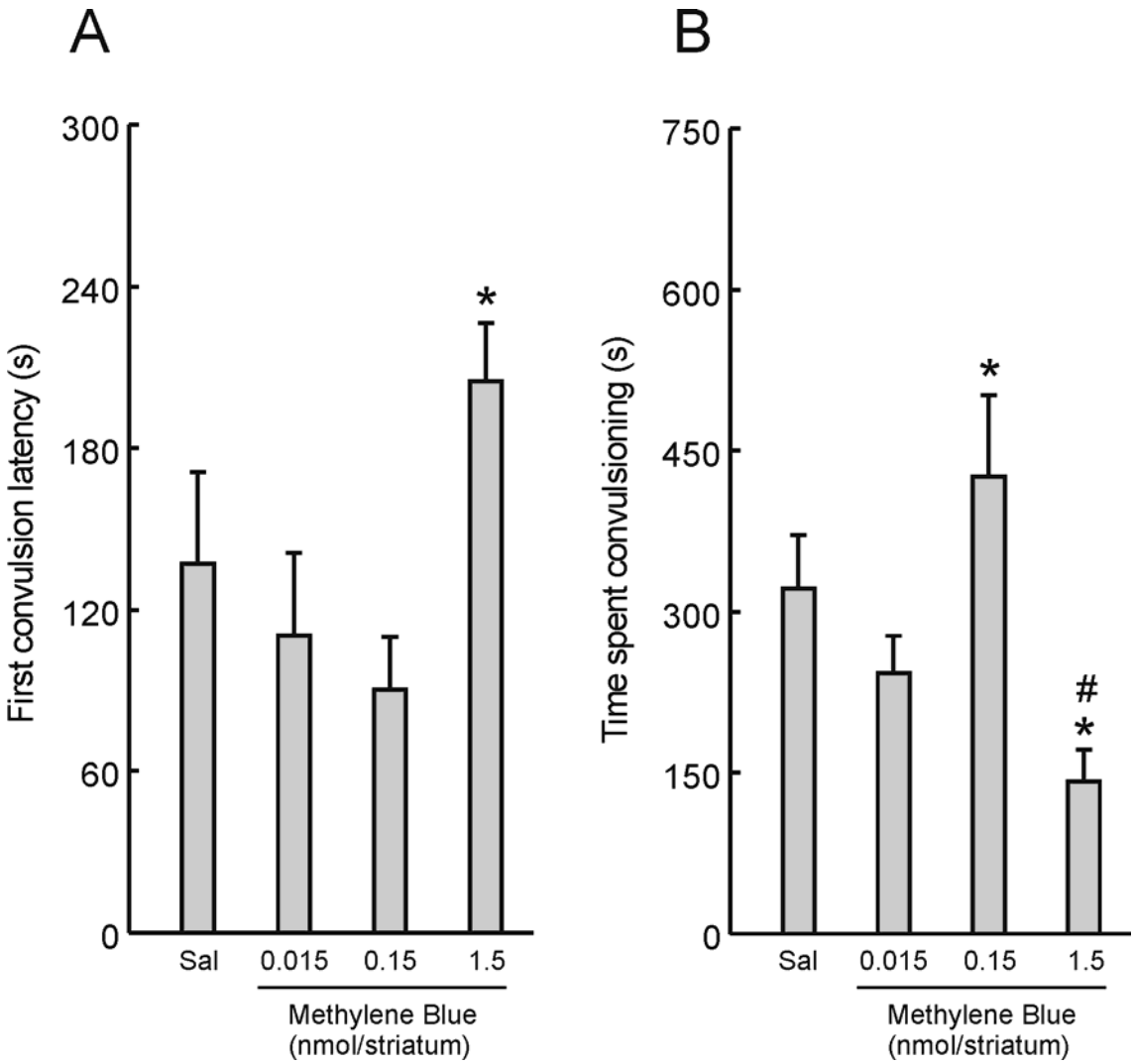

A
First convulsion latency (s)
300
240
180
120
60
0
*
Sal
0.015
0.15
1.5
Methylene Blue
(nmol/striatum)
B
Time spent convulsioning (s)
750
600
450
300
150
0
*
#
*
Sal
0.015
0.15
1.5
Methylene Blue
(nmol/striatum)

Figure 2

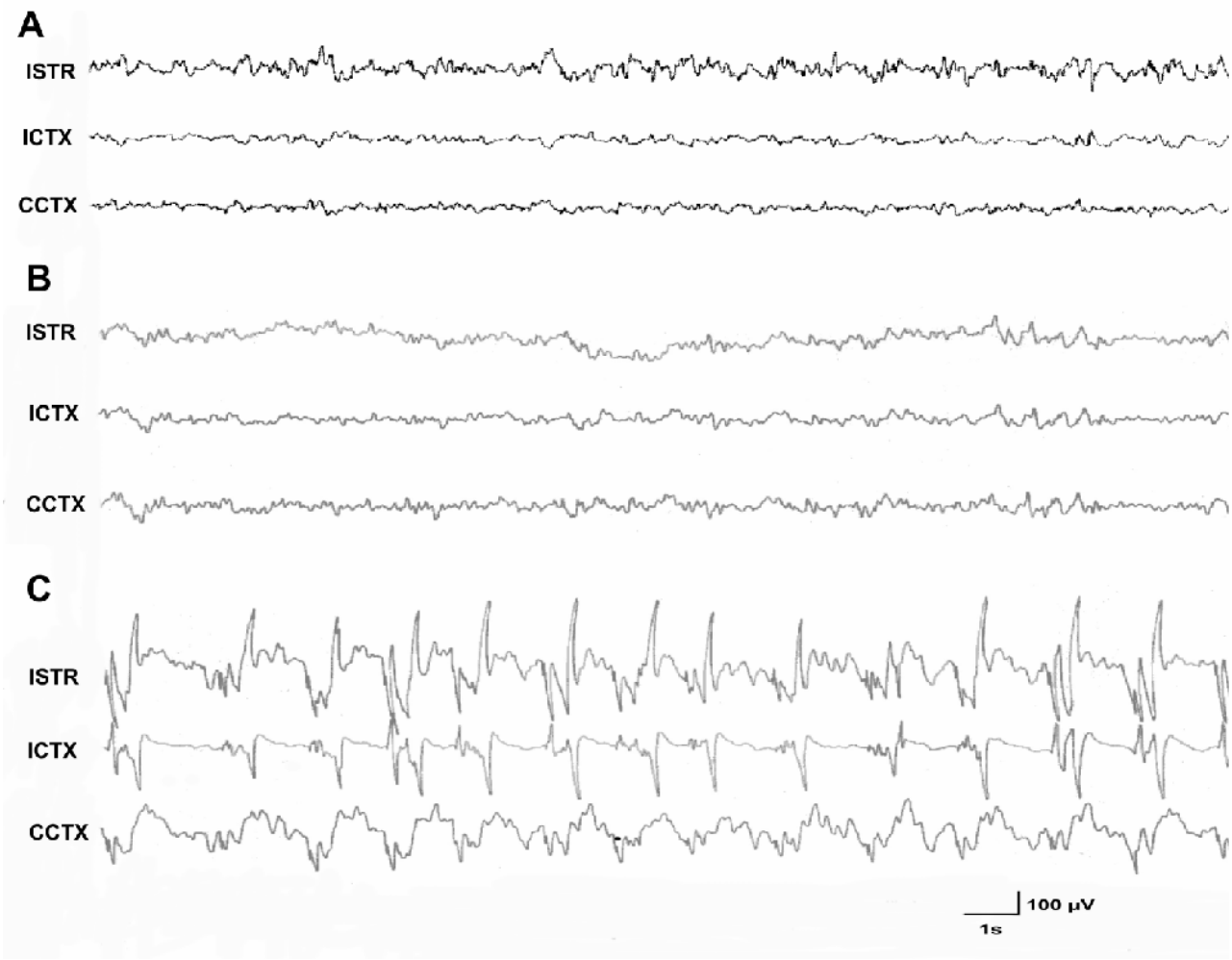


Figure 3

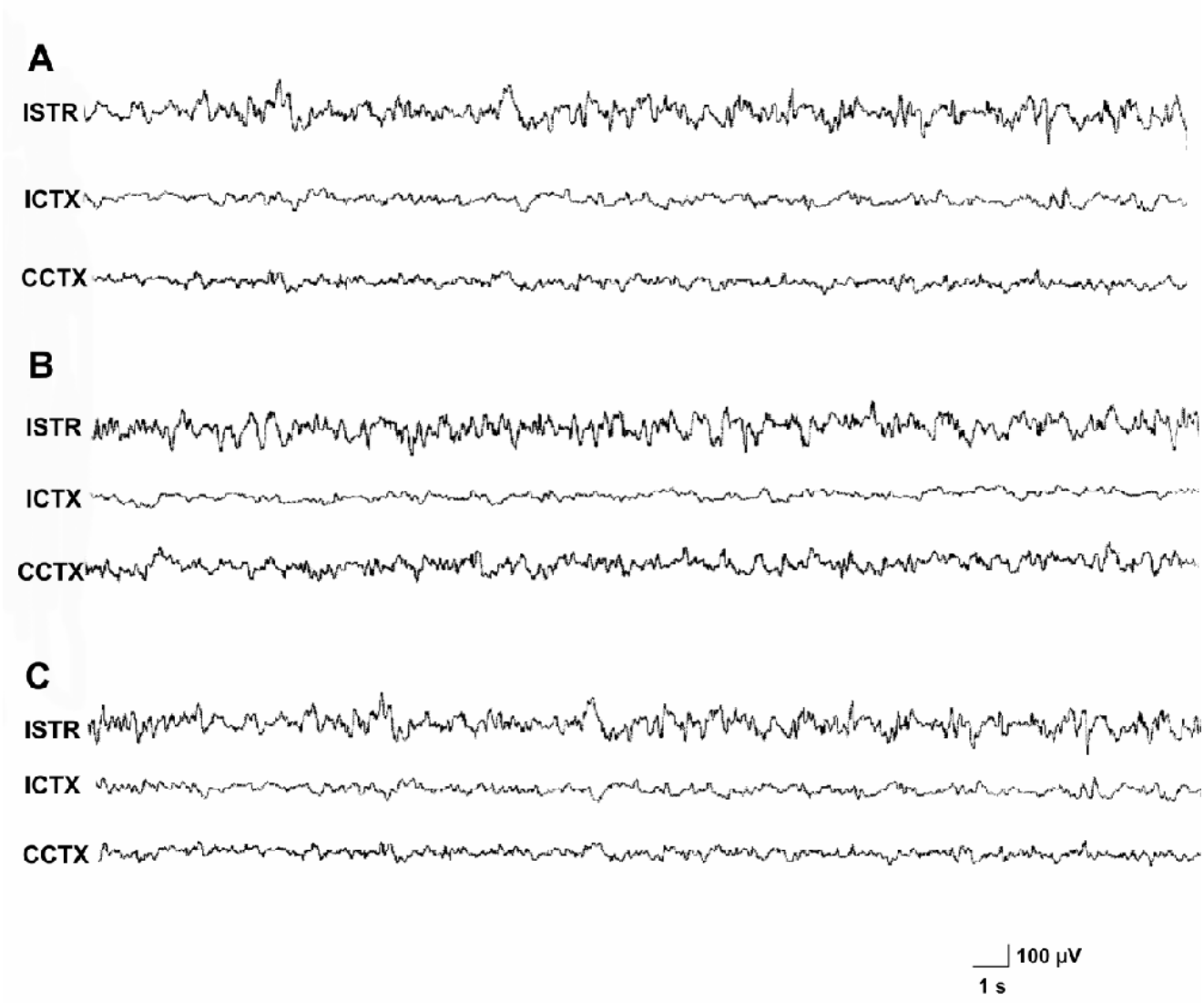


Figure 4A

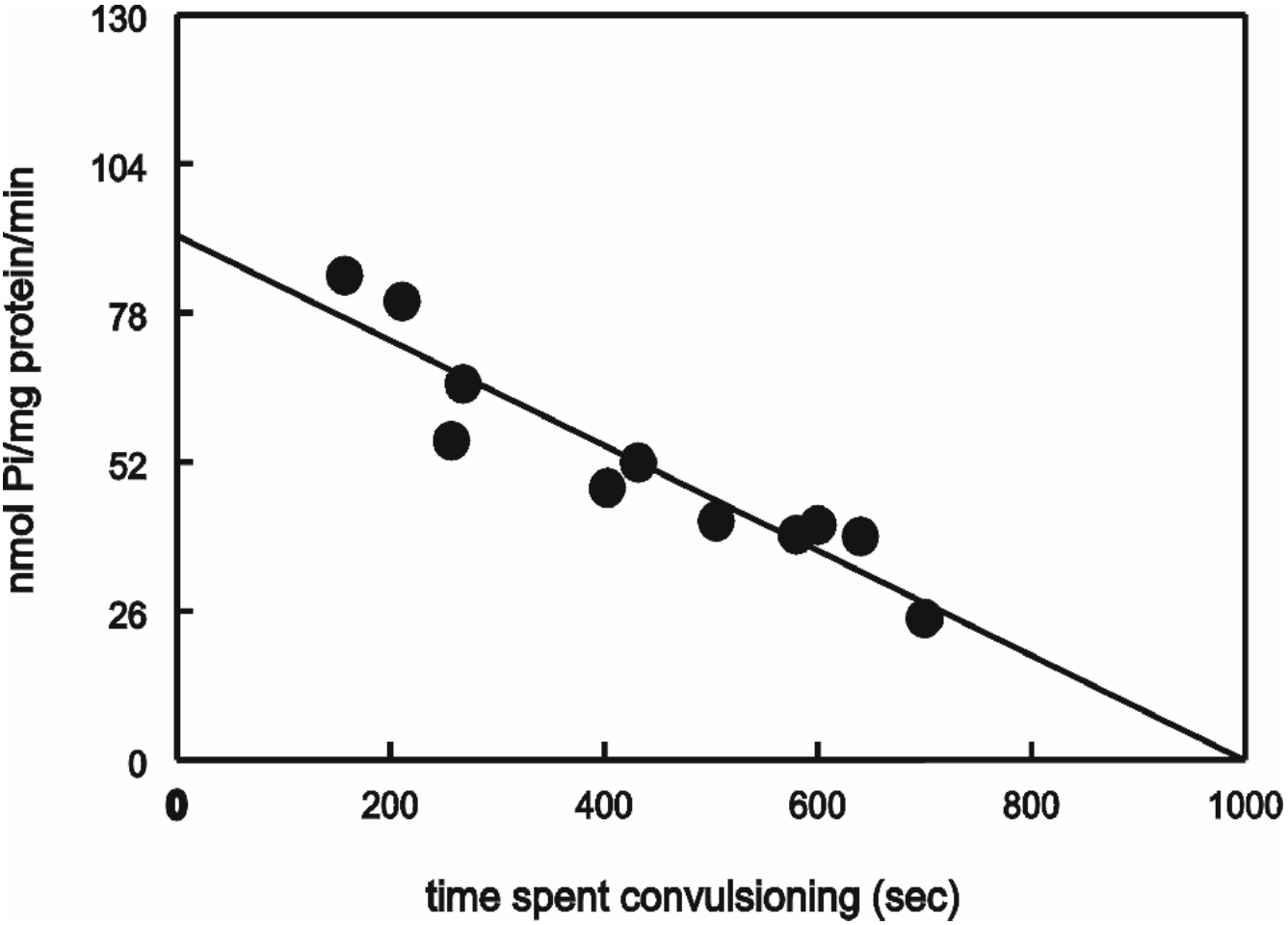

130
104
78
52
26
0
nmol Pi/mg protein/min
0
200
400
600
800
1000
time spent convulsioning (sec)

Figure 4B

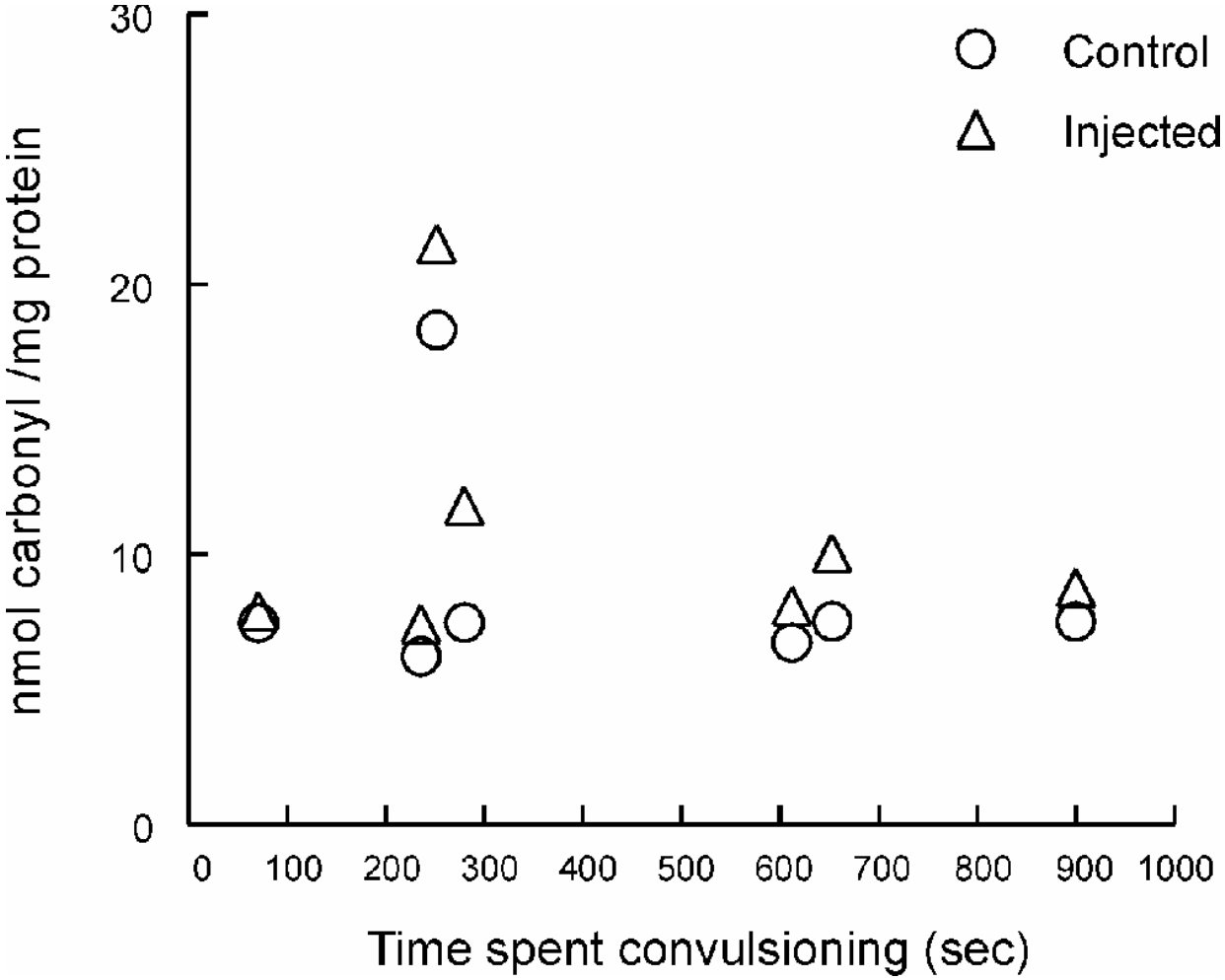

30
20
10
0
nmol carbonyl /mg protein
Control
Injected
0
100
200
300
400
500
600
700
800
900
1000
Time spent convulsioning (sec)

Figure 5

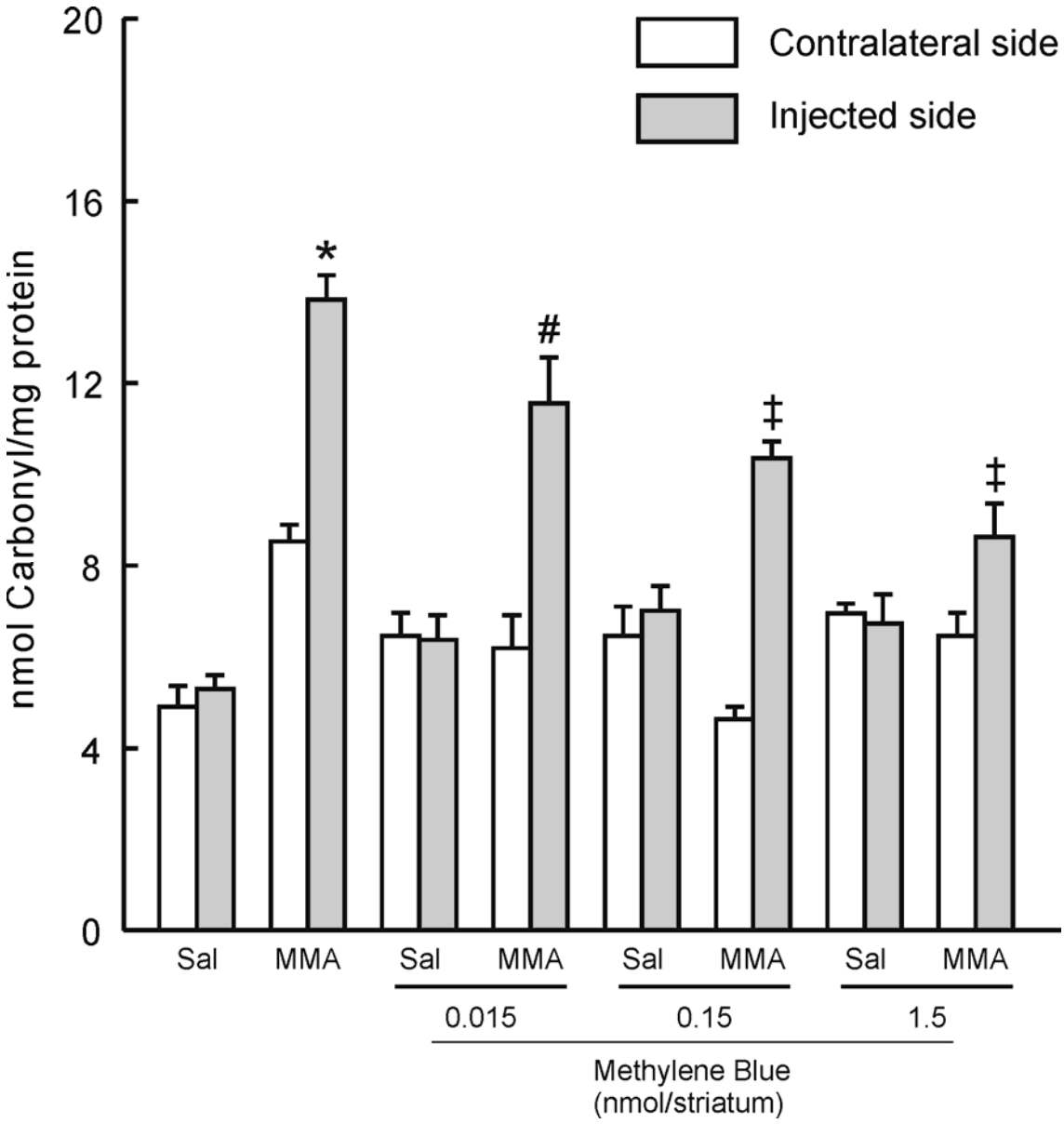

Contralateral side
Injected side
nmol Carbonyl/mg protein
20
16
12
8
4
0
*
#
‡
‡
Sal
MMA
Sal
MMA
Sal
MMA
Sal
MMA
0.015
0.15
1.5
Methylene Blue
(nmol/striatum)

Figure 6

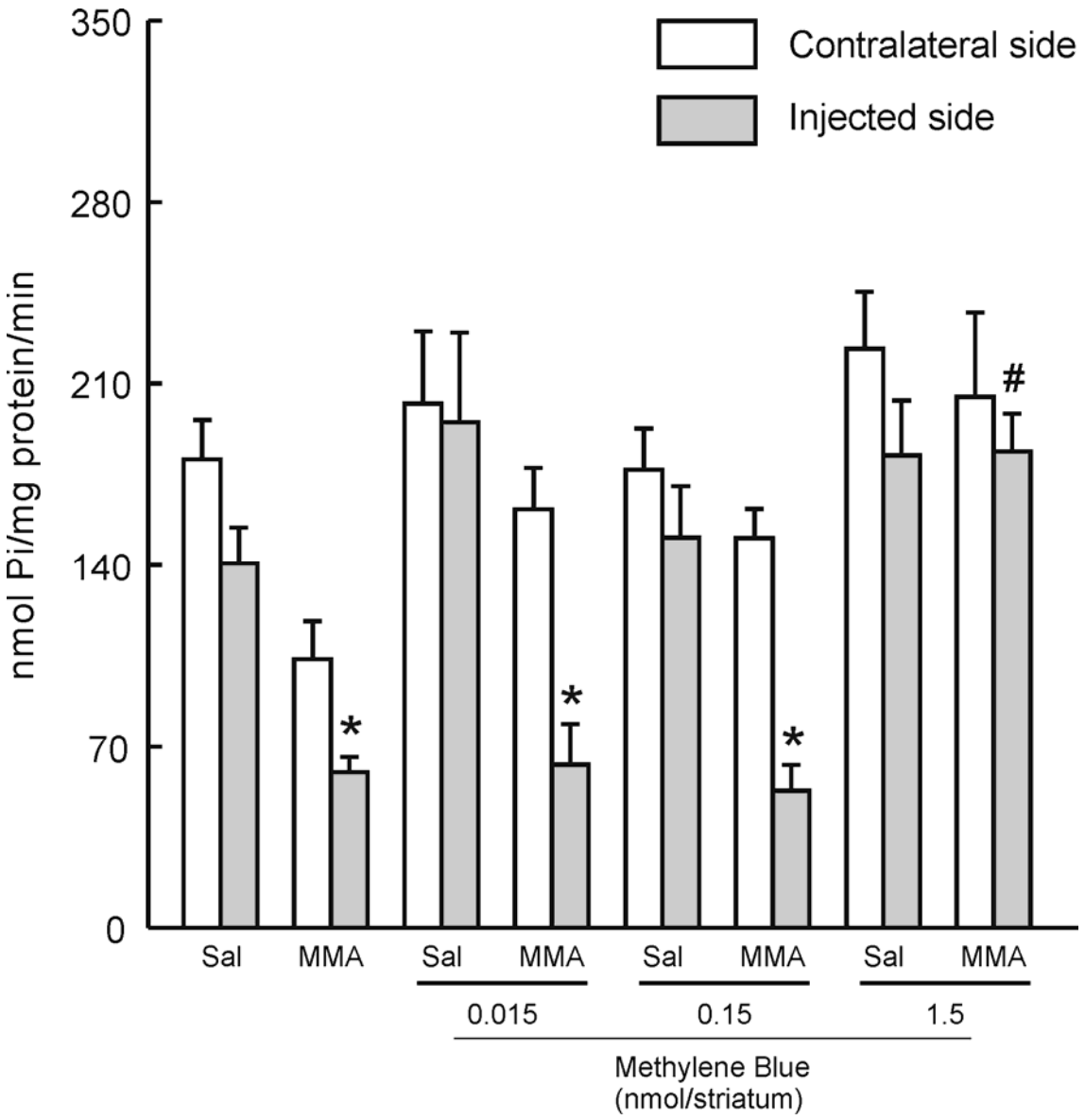
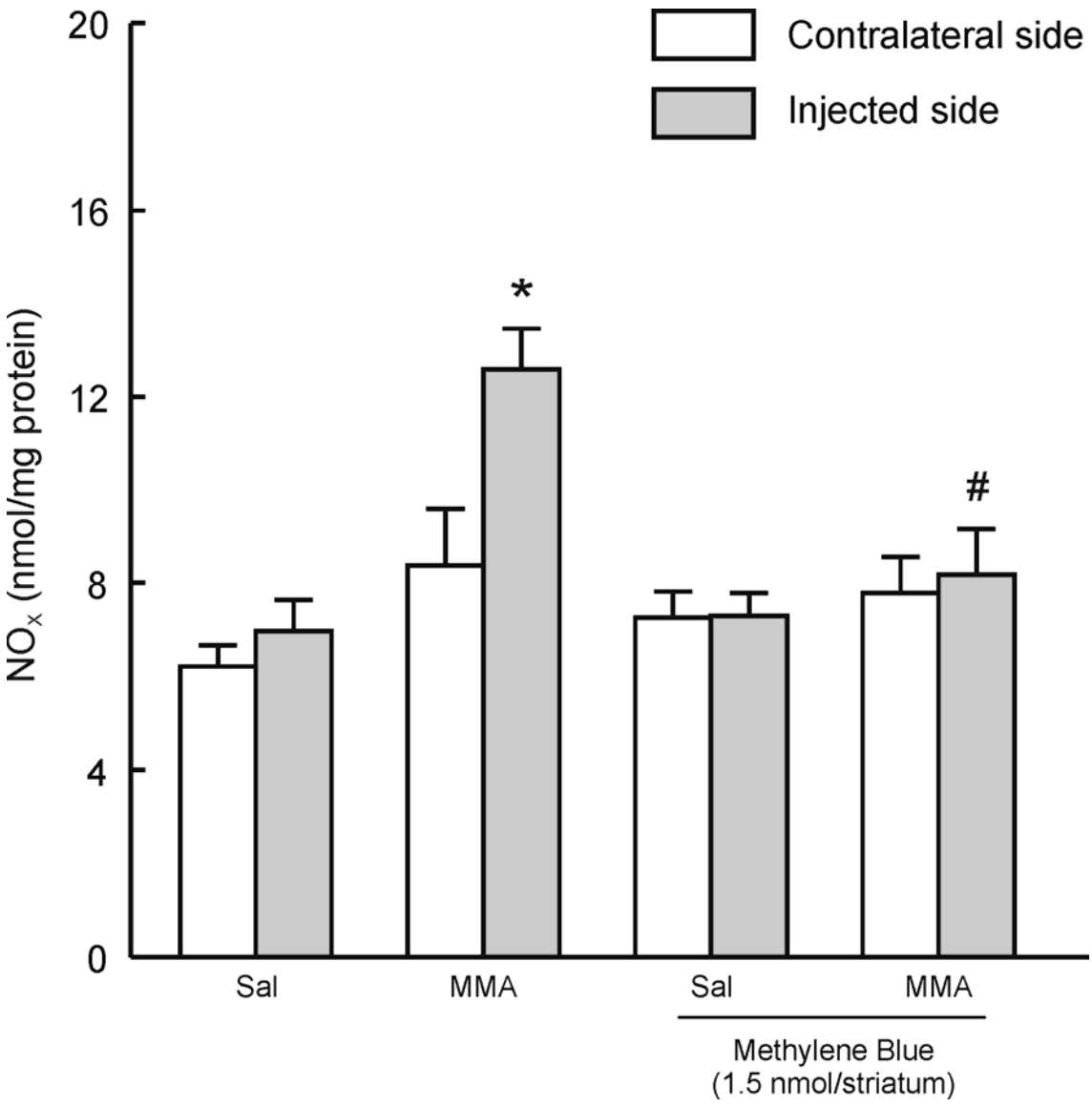

Contralateral side
Injected side
nmol Pi/mg protein/min
350
280
210
140
70
0
Sal
MMA
Sal
MMA
Sal
MMA
Sal
MMA
0.015
0.15
1.5
Methylene Blue
(nmol/striatum)
*
*
*
#

Figure 7


Contralateral side
Injected side
20
16
12
8
4
0
NOx (nmol/mg protein)
*
#
Sal
MMA
Sal
MMA
Methylene Blue
(1.5 nmol/striatum)

CAPÍTULO IV

7-Nitroindazole increases MMA-induced convulsions and oxidative damage. NO: Villain or hero?

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Abstract

Methylmalonic acidemias consist of a group of inherited metabolic disorders caused by deficiency of methylmalonyl-CoA mutase activity and biochemically characterized by

methylmalonate (MMA) accumulation, impairment mitochondrial oxidative metabolism and reactive species production. Preliminary studies with NO synthase inhibitors have suggested that NO plays a role in the convulsant effect of MMA. However, definitive biochemical and electrophysiological evidence of the involvement of NO in the convulsions induced by MMA are lacking. In this study we investigated whether the inhibition of nitric oxide synthase (NOS) by 7-nitroindazol (7-NI, 3-60 mg/kg, i.p.) altered the convulsions, protein oxidative damage, NO_x (NO_2 plus NO_3) production and Na^+,K^+-ATPase activity inhibition induced by MMA. 7-NI decreased striatal NO_x content, but increased the seizures and protein carbonylation induced by MMA (6 μmol/ striatum). The intrastriatal injection of L-arginine (50 nmol/ 0.5 μl), but not of D-arginine (50 nmol/ 0.5 μl), increased striatal NO_x content and protected against MMA-induced electrographic seizures, striatal protein carbonylation and Na^+,K^+-ATPase inhibition. Furthermore, L-arginine (50 nmol/ 0.5 μl) and MMA had no additive effect on NO_x increase. These results are experimental evidence that endogenous NO plays a protective role in the convulsions and acute neurochemical alterations induced by this organic acid.

Keywords: *Methylmalonic acid; seizure; protein carbonylation; 7-nitroindazole; Na^+,K^+-ATPase; striatum, convulsions, succinate dehydrogenase, nitric oxide, nitric oxide synthase.*

Introduction

Methylmalonic acidemias comprise a group of inherited metabolic disorders caused by a deficiency of the mitochondrial enzyme methylmalonyl CoA mutase (MCM, EC 5.4.99.2) or by defects in the synthesis of 5'-deoxyadenosylcobalamin, the cofactor of MCM. Deficient MCM, which physiologically catalyses the reaction of methylmalonyl CoA to succinyl CoA, leads to the primary accumulation of methylmalonyl CoA, and to the secondary accumulation of other metabolites, such as propionate, 3-hydroxypropionate, and 2-methylcitrate (Fenton et al., 2001). Furthermore, it has been shown that patients with methylmalonic acidemia, during acute metabolic crises, present elevated amounts of lactate in globus pallidus suggesting inhibition of mitochondrial energy metabolism *in vivo* (Trinh et al., 2001). In this context, there is a considerable body of evidence suggesting that MMA impairs mitochondrial function, since it increases lactate production *ex vivo* and *in vitro* (Wajner et al., 1992; Greenamyre et al., 1994; Royes et al., 2003) and decreases ATP levels (McLaughlin et al., 1998), CO_2 production (Wajner et al., 1992) and O_2 utilization (Toyoshima et al., 1995).

Methylmalonate causes convulsive behavior through glutamatergic mechanisms (de Mello et al., 1996) and striatal degeneration (Narasimhan et al., 1996). The depolarizing effect of MMA on isolated neurons and astrocytes was confirmed by electrophysiological methods (McLaughlin et al., 1998), and the intrastriatal injection of MMA causes electrographically recorded convulsive activity (Malfatti et al., 2003).

A substantial body of evidence has been gathered suggesting that excessive glutamate receptor stimulation, in particular the NMDA receptor, is implicated as a major pathway that leads to convulsions and oxidative damage elicited by MMA (de Mello et al., 1996;

Royes et al., 2003; 2005). Accordingly, it has been shown that the intrastriatal administration of MMA, besides causing convulsive behavior, increases protein carbonylation and thiobarbituric acid reacting substances (Malfatti et al., 2003; Royes et al., 2005). Furthermore, it has been demonstrated that while the administration of antioxidants, such GM1, ascorbic acid and α-tocopherol (Fighera et al., 1999; 2003) attenuate, the pretreatment with ammonia (a pro-oxidant agent) increases the MMA-induced convulsions (Marisco et al., 2003), further suggesting that reactive oxygen species play a role in MMA-induced convulsions. Recently, it has been proposed that NO, a reactive nitrogen species, may play a role in the convulsions induced by MMA since they are attenuated by the nonspecific inhibitor of NOS, L-NAME.

One of the most significant achievements in biology during the last decade has been the polyfunctional role of nitric oxide (NO) in several physiological functions of animals that include synaptic plasticity, smooth muscle relaxation, macrophage-mediated cytotoxicity, cellular differentiation and apoptosis (Prast and Philippu, 2001). According to present conceptions, NO is a gaseous chemical free radical messenger synthetized from $_{L}$-arginine by four NO synthase (NOS) isoforms: neuronal NOS (nNOS), macrophage NOS (iNOS, inducible), endothelial NOS (eNOS), and mitochondrial (mitNOS), which when produced in an excessive amount changes from a physiological neuromodulator to a neurotoxic agent. (Giulivi, 2003; Guix et al., 2005). In line of this view, it has been claimed that NO overproduction can be due nNOS activation following persistent stimulation of excitatory amino acids receptors mediating glutamate toxicity and/or to iNOS induction by diverse stimuli, such as endotoxin or cytokines (Chabrier et al., 1999). In agree with this view, a substantial body of evidence have demonstrated that a

hyperactivation of glutamate receptors, especially the NMDA subtype, leads to increased free Ca^{2+}, NOS activation, ROS generation and mitochondrial dysfunction in several model of neurodegenerative diseases (Stewart and Heales, 2003).

Several reports have evidenced the involvement of NO in various models of epilepsy (de Sarro et al., 1991; Paoletti et al., 1998; Borowicz et al., 2000; Itoh et al. 2004; de Vasconcelos et al., 2004; Kato et al., 2005), however, the mechanisms underlying the regulation exerted by NO during seizures has never been clearly understood. The relation between amino acids and NOS, their localization in brain as well as activation of specific NOS isoforms in several models of epilepsy has led to numerous studies of NO to evidence contradictory results. Considering that NO is an retrograde messenger, some authors believe that NO may be an endogenous anticonvulsant by to initiate a cascade of reactions which prevent the expansion of convulsive activity induced by several convulsive agents such pilocarpine and PTZ (Maggio et al., 1995; Przegalinski et al., 1996). On the other hand, other authors believe that NO may be involved in the development of epileptiform seizures and increase the production of free radicals which dramatically increase the neurotoxic effect induced by agonist of glutamate receptors and antagonist GABA receptors, such as kainic acid and picrotoxin respectively (Hayashi et al., 2002; Gupta and Dettbarn, 2003; Rajasekaran, 2005). Furthermore, previous experimental findings from our group have demonstrated that intrastiatal injection of N^{ϖ}-nitro-L–arginine methyl ester (L-NAME), a non-selective NOS inhibitor that attenuates both eNOS and nNOS activity, exerts a biphasic effect on MMA-induced convulsive behavior and protein carbonylation (Royes et al., 2005). These results suggest a differential involvement of nNOS and eNOS enzymes on MMA-induced convulsive behavior and oxidative damage. In this context,

considering that the participation of neuronal NO in the excitotoxicity induced by MMA is poorly know, we decided to investigate the effect of the selective nNOS inhibitor, 7-nitroindazole (7-NI) and L-arginine (substrate for NOS) administration on MMA-induced behavioral, electrographic and neurochemical deleterious effects.

METHODS

Animals and reagents

Adult male Wistar rats (270-300 g, n= 8-10 for each group) maintained under controlled light and environment (12:12 h light-dark cycle, 24 ± 1^{o}C, 55% relative humidity) with free access to food (Guabi, Santa Maria, Brazil) and water were used. Animal utilization protocols followed the Official Government Ethics guidelines and were approved by the University Ethics Committee. All reagents were purchased from Sigma (St. Louis, MO, USA) and respective solutions were prepared in type I ultra pure water. The 7-NI was sonicated in peanut oil and $_{L}$-arginine was prepared in 100 mM phosphate buffered saline pH 7.4, PBS. Methylmalonate (methylmalonic acid) solutions had their pH set to 7.4 with NaOH.

Behavioral evaluation and surgical procedure

The animals were anesthetized with Equitesin (1% phenobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol; 3 ml/kg, i.p.) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a cannula was inserted unilaterally into the striatum (coordinates relative to bregma: AP 0 mm, ML 3.0 mm, V 3.0

mm from the dura). Chloramphenicol (200 mg/kg, i.p.) was administrated immediately before the surgical procedure. Three days after the cannula placement, the animals were injected with 7-NI (3, 10, 30 and 60 mg/kg; i.p.) or NaCl (0.9%, i.p.) 30 min before the administration of MMA (6 μmol/ 2 μl) or NaCl (9 μmol/ 2 μl). The effect of the administration of $_{L}$-arginine and $_{D}$-arginine on the MMA-induced convulsion and striatal biochemical parameters was evaluated 3 days after the surgery, as described above, excepted that the animals were injected with $_{L}$-arginine (5 and 50 nmol/ 0.5 μl), $_{D}$-arginine (5 and 50 nmol/ 0.5 μl) or vehicle (PBS 100 mM/ pH 7.4) 30 min before the intrastriatal administration of MMA (4.5 μmol/ 1.5 μl) or NaCl (6.7 μmol/ 1.5 μl).

Immediately after the injections, the animals were transferred to a round open field (54.7 cm in diameter) with a floor divided into 11 equal areas. The open field sessions lasted 15 min, and during this time the animals were observed for the appearance of convulsive behavior (myoclonic jerks and clonic movements involving hindlimbs and forelimbs contralateral to the injected striatum). The latency for the first convulsive episode and the total time spent convulsioning was recorded during fifteen minutes (de Mello et al., 1996).

Placement of cannula and electrodes for EEG recordings

A subset of animals was surgically implanted with a cannula and electrodes under stereotaxic guidance. In brief, rats were anesthetized with Equitesin and two screw electrodes were placed bilaterally over the parietal cortex along with a ground lead positioned over the nasal sinus. Bipolar nichrome wire Teflon-insulated depth electrodes (100 μm) were implanted ipisilaterally into striatum. For intrastriatal infusion of drugs, a guide cannula (27 gauge) was glued to a multipin socket and inserted through a previously

opened skull orifice. The coordinates from bregma for implantation of the electrodes were (in mm): AP, -4.5; L, 2.5; and V, 2 for the cortex and AP, 0; L, 3; V, 3.5 for the striatum (Paxinos, 1986). The electrodes were connected to a multipin socket and, together with the injection cannula, were fixed to the skull with dental acrylic cement. The experiments were performed 7-9 days after surgery.

EEG recordings and intrastriatal injection of drugs

The procedures for EEG recording and intracerebral injection of drugs were previously described (Cavalheiro et al., 1992). Briefly, the animals were allowed to habituate to a Plexiglas cage (25 x 25 x 60 cm) for at least 10 min before the EEG recording. The rats were then connected to the lead socket in a swivel inside a Faraday's cage. Routinely, a 10 min baseline recording was obtained to establish an adequate control period. The drug injection protocol used in this set of experiments was the same used in those experiments that evaluated the effect of 7-NI, $_{L}$-arginine and $_{D}$-arginine on MMA-induced behavioral seizures, except that EEG was concomitantly recorded. EEG signals were amplified, filtered (0.1 to 50.0 Hz, bandpass) and recorded using an analogical encephalographer (Berger TP 119).

Several epochs were selected from preinfusion and postinfusion periods to determine significant EEG changes. The preinfusion segment was defined as ending immediately before the beginning of infusion. The postinfusion epochs started immediately after the end of 7-NI (60 mg/kg, i.p.), $_{L}$- arginine (50 nmol/ 0.5 μl), $_{D}$-arginine (50 nmol/ 0.5 μl) and MMA (6 μmol/ 2 μl) or NaCl (9 μmol/ 2 μl) infusion.

All intrastriatal injections were made in unanesthetized rats by using a needle (30 gauge) protruding 1 mm below the guide cannula. All drugs were injected over 1 min period by using a Hamilton syringe, and an additional minute was allowed to elapse before removal of needle to avoid backflow of drug through the cannula.

Colorimetric determination of the protein carbonyl content ex vivo

Immediately after the behavioral evaluation, the animals were sacrificed by decapitation and had their brain exposed by the removal of the parietal bone. A punch of the injected and of the noninjected striatum was rapidly removed using a stainless steel puncher (5 mm in internal diameter) around the site of cannula placing.

Striatal tissues were homogenized in 10 volumes (w/v) of 10 mM Tris-HCl buffer pH 7.4 using a glass homogenizer and its carbonyl protein content was determined by the method described by Yan et al (1995), adapted for brain tissue by Oliveira et al (2004).

Na^+,K^+-ATPase activity measurements

The measurement of Na^+,K^+-ATPase activity was performed in the same fresh, diluted, non-centrifuged homogenates used for determination of the striatal protein carbonyl content. Assay of enzyme activity was performed according by Wyse et al (2000). Briefly, the incubation medium consisted of 30 mM Tris-HCl buffer, pH 7.4; 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$, and 50 µg of protein in the presence or absence of ouabain (2 mM), in a final volume of 350 µl. The reaction was started by the addition of adenosine triphosphate (ATP) to a final concentration of 5 mM. After 30 min at 37ºC, the reaction was stopped by the addition of 70 µl of trichloroacetic acid (TCA, 50%). Saturating substrate concentrations were used, and reaction was linear with protein and

time. Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate released was quantified by the colorimetric method described by Fiske and Subbarow (1925), and Na^+,K^+-ATPase activity was calculated by subtracting the ouabain-sensitive activity from the overall activity (in the absence of ouabain).

Assay of NOx as a marker of NO synthesis

For NOx (NO_2 plus NO_3) determination, striatal tissues were dissected on ice-cold Petri dishes and homogenized in $(NH4)_2SO_4$ and acetonitrile (96%). The homogenates were centrifuged at 3000 x g for 20 min at 4°C and supernatant was separated for analysis of the NO_2 plus NO_3 as described by Miranda et al (2001). The resulting pellet was suspended in NaOH (3M) for protein determination.

Protein determination

Protein content was measured colorimetrically by the method of Bradford (1976) and bovine serum albumin (1 mg/ml) was used as standard.

Statistical analysis

The latency for convulsion and total time spent convulsioning data were analyzed by one or two-way ANOVA (analysis of variance), depending on the experimental design, followed by the Student Newman Keuls test. Biochemical data were analyzed by a two (Saline or 7-NI) x two (PBS or $_L$-arginine) x two (PBS or $_D$-arginine) x two (saline or MMA) x two (injected or non-injected hemisphere) factorial ANOVA, with the hemisphere factor treated as a within-subject factor. *Post hoc* analyses were carried out by the F test for simple effect or the Student Newman Keuls test, when appropriate. $P< 0.05$ was considered significant. All data are expressed as mean + S.E.M.

Results

In the present study we confirmed our previous results, since statistical analysis revealed that intrastriatal injection of MMA (6 μmol/ 2 μl) caused the appearance of clonic convulsions (Fig. 1), which were confirmed by electrographic recording (Fig. 2C). Electrographically recorded seizures were accompanied by clonus of the left forelimb and/or hind limb and head, rotational behavior and full lateralization toward the left side of the body. In addition, the seizures induced by MMA were defined by occurrence of discrete episodes consisting of the simultaneous occurrence of at least two of the following alterations in all four recording leads: high-frequency and/or polyspike complexes and/or high-voltage synchronized spike activity that sometimes appeared almost continuously.

The involvement of neuronal NO on MMA-induced convulsive behavior and electrographically recorded seizures was investigated by injection the animals with 7-NI (a selective nNOS inhibitor), $_{L}$-arginine (a precursor of NO) and $_{D}$-arginine (inactive enantiomer of $_{L}$-arginine). Statistical analysis revealed that the pretreatment with 7-NI (60 mg/kg, i.p.) decreased the latency for the first convulsion [$F(5,92)=11.38$; $P<0.05$; Fig. 1A] and increased the duration of convulsive episodes induced by MMA [$F(5,92)=12.45$; $P<0.05$; Fig. 1B]. Furthermore, the electrographic recording of the ipsilateral striatum and cortex ipsi and contraleral revealed that the abnormal discharger appearance after striatal MMA administration was increased by the 7-NI (60 mg/kg, i.p.) pretreatment. Furthermore, the electrographic recording of the ipsilateral striatum of this group revealed that MMA-induced epileptogenic focus in the injected striatum, spread to the ipsi and contralateral cerebral cortex 5 min after MMA infusion (Fig. 3C).

The effect of $_{L}$-arginine and $_{D}$-arginine on MMA-induced behavioral and electroencephalographic seizures is shown in Figures 4, 5 and 6 respectively. Statistical analysis revealed that the intrastriatal injection of $_{L}$-arginine (50 nmol/ 0.5 μl) but not $_{D}$-arginine (50 nmol/ 0.5 μl) protected against the MMA-induced behavior, measured by latency for the first convulsion [$F(2,45)= 10.37$; $P<0.05$; Fig. 4A], duration [$F(2,45)= 8.06$; $P<0.05$; Fig. 4B] of convulsive episodes and electrographic recording (Figs. 5C and 6C).

Considering that reactive species have been implicated in MMA-induced convulsive behavior and that selected targets such as Na^+, K^+-ATPase, seem to be sensitive to free radical-induced damage (Fighera et al., 1999; 2003; Malfatti et al., 2003; Royes et al., 2005) we decided to investigate the role of neuronal NO on changes in striatal protein carbonylation and Na^+, K^+-ATPase activity after MMA administration. Statistical analysis of the carbonyl content data revealed a significant pretreatment (saline or 7-NI) by treatment (saline or MMA) by hemisphere (injected or non-injected) interaction [F (5,90)= 3.80; $P<0.05$]. Post hoc analysis revealed that 7-NI, at the dose of 60 mg/kg, significantly increased MMA-induced carbonylation (Fig. 7). Furthermore, statistical analysis revealed that the intrastriatal administration of MMA induced a significant inhibition of Na^+, K^+-ATPase activity [$F(1,93)=26.83$; $P<0.05$] and that the pretreatment with 7-NI (all doses) was ineffective against this inhibition (Fig. 8). On the other hand, the intrastriatal injection of $_{L}$-arginine (50 nmol/ 0.5 μl) significantly attenuated MMA-induced protein carbonylation [$F(2,40)=4.56$; $P< 0.05$; Fig. 9] and prevented the inhibitory effect exerted by intrastriatal injection of MMA (4.5 μmol/ 1.5 μl) on Na^+,K^+-ATPase activity [$F(2,40)=4.69$; $P< 0.05$; Fig. 10]. In addition, the figures 9 and 10 show that same amount

of $_{D}$-arginine (50 nmol/ 0.5 μl) did not prevents the MMA-induced protein carbonylation and Na^{+}, K^{+}-ATPase inhibition, respectively.

If NO acts as an endogenous neuroprotector agent, it is plausible to propose that the severity of experimentally induced seizure are linked to a significant decrease in the synthesis of NO in this model of organic acidemia. In support of this suggestion, we decided to investigate the effect of 7-NI, $_{L}$-arginine, $_{D}$-arginine and MMA administration on NOx content of the injected and non-injected striata.

Statistical analysis (three-way ANOVA, with the hemispheres treated as a within-subjected factor) revealed a significant pretreatment (saline or 7-NI) by treatment (saline or MMA) by hemisphere (control or injected) interaction [$F(3,57)=10.68$; $P<0.05$] and a significant effect of pretreatment (saline or 7-NI) [$F(3,57)=17.27$; $P<0.05$] indicating that the intrastriatal administration of MMA (6 μmol/ 2 μl) induced a significant increase in the striatal NOx content of the injected striatum, and that 7-NI pretreatment (60 mg/kg, i.p.) attenuated this striatal NOx content increase (Fig. 11). Furthermore, post hoc analysis (Student-Newman-Keuls test) showed that 7-NI (60 mg/kg, i.p.) caused a general decrease in the striatal NOx content *per se* (both hemispheres). In contrast, the direct application of $_{L}$-arginine (50 nmol/ 0.5 μl) but not $_{D}$-arginine (50 nmol/ 0.5 μl) besides to exert an effectively protection against behavioral and neurochemical deleterious effects induced by MMA, increased of striatal NOx content *per se* [$F(2,53)=9.49$; $P<0.05$, Fig. 12).

Discussion

In the present study we demonstrated that intrastriatal injection of MMA causes the appearance of behavioral and electrographic seizures, which are accompanied by an

increase in total protein carbonylation and striatal Na^+,K^+-ATPase activity inhibition. These results are agreement with previous findings from our group (Fighera et al., 1999; 2003; Malfatti et al., 2003; Royes et al., 2005), which suggest the involvement of reactive species generation in MMA-induced convulsive behavior. In addition, the present results confirm the participation of neuronal NO in the excitotoxicity elicited by MMA, since the administration of selective nNOS inhibitor (7-NI), besides to increase of polyspike-and-wave dischargers in the striatum, cortex ipsi and contraleral, decreased the latency for the first convulsion and increased the duration of convulsive episodes induced by MMA. The increase of MMA-induced behavioral and electroencephalographic seizures induced by 7-NI pretreatment, was accompany by striatal protein carbonylation increase and Na^+,K^+-ATPase activity inhibition. On the other hand, the intrastriatal injection of substrate for NOS enzyme ($_L$-arginine) decreased the appearance of MMA-induced seizures episodes and attenuated the protein carbonylation and Na^+, K^+-ATPase activity inhibition induced by this organic acid.

The results presented in this report also revealed that the 7-NI pretreatment besides to decrease the striatal NOx content *per se*, it increased of MMA-induced behavioral and electrographic seizures. Considering that the injection of $_L$-arginine but not $_D$- arginine increased of striatal NOx content *per se* and protected against MMA-induced behavioral and electrographic seizures, it is tempting to propose that neuronal NO production exerts an anticonvulsant effect in this model of seizure.

The effects of NOS inhibitors have been extensively studied in an effort to elicit the role of NO in various seizures models (de Sarro et al., 1991; Paoletti et al., 1998; Itoh et al., 2004; de Vasconcelos et al., 2000; Borowicz et al., 2000; Kato et al., 2005), however, there is still some debate whether NO is pro- or anti-convulsant. The determining factor for such a

discrepancy is not known, but one might argue that methodological differences may account for it. In this context, it has been shown that the effects of NOS inhibitor on seizures activity may differ depending on dose, administration route, animal species and differences in NO production between the specific tissues (Wojtal et al., 2003). Previously, we demonstrated that low doses of $_{L}$-NAME (a non-selective inhibitors NOS) attenuated the MMA-induced convulsive behavior and protein carbonylation (Royes et al., 2005), suggesting a proconvulsant effect of neuronal NO in this model of seizure. Nevertheless, the results presented in this report are in apparent conflict with our previous findings, since an extensive inhibition of striatal NO content caused by 7-NI pretreatment, enhanced the seizures severity induced by MMA.

Considering that NMDA receptor-mediated activation of striatal NO system is involved in the feedback inhibition of NMDA receptor function in numerous physiological and pathophysiological events (Guix et al., 2005), it is plausible to propose that a reaction between MMA-induced NO production with thiol groups of this receptor might lead to downregulation of NMDA-receptor activity. In this context, the striatal NO production may initiate a cascade of reaction at the attempt to prevent the expansion of convulsive activity induced by intrastriatal injection of MMA. In agree with this view, a substantial body of evidence has been suggested that endogenous NO release in response to NMDA receptor activation may play an important role in the depression of NMDA receptor activation and, therefore, arresting seizure activity in several models of epilepsy (Guix et al., 2005).

Furthermore, since the activation of the NO/guanylate cyclase pathway elicited by NMDA receptor stimulation induces γ- aminobutyric acid (GABA) release in the striatum (Hanania and Johnson, 1998; Calabrese et al., 2000), the authors suggest that an inhibition of nNOS

caused by 7-NI might lead a striatal GABA release decrease and, therefore, potentiate the phasic excitations induced by glutamate in this model of organic acidemia. In support of this view, it has been demonstrated that acute intrastriatal administration of MMA induces seizures through glutamatergic mechanism (de Mello et al., 1996) in rats, probably involving Krebs cycle inhibition, ATP depletion, and free-radical generation (Fighera et al., 1999; 2003; Marisco et al., 2003; Fleck et al., 2004; Royes et al., 2003; 2005). Moreover, it is well known that most of striatal spiny neurons are constituted by spiny GABAergic projecting cells with abundant cortical and subcortical connections, strongly activated in several model of seizures (Folbergrova et al., 2000; Kovács et al., 2003; Oliveira et al., 2004).

Another interesting possibility to explain the neuroprotective effect exerted by neuronal NO in this model of excitotoxicity may be related to the redox status of the cell. Lipton et al. (1993) demonstrated that a reducing intracellular environment, NO exerts neurotoxic actions, however, an intracellular oxidizing environment favor formation of the nitrosonium ion, which may downregulation the NMDA receptor by S-nitrosylation. Therefore, it is plausible that MMA-induced intracellular oxidizing environment may influence in the NO-mediated neuroprotetor effect in this model of organic acidemia. However, further studies are needed to clarify this point.

In recent years, a significant amount of work have demonstrated that metabolic disorders, particularly those due to respiratory chain defects as methylmalonic acidemia, are often associated with increased free radical production (Wajner et al., 2004). Although, there is evidence that oxidative stress and mitochondrial dysfunction may occur as a consequence of prolonged epileptic seizures (Patel, 2003), little is know about participation of free radicals in the genesis of seizures. In this context, we have showed that *ex vivo*

intrastriatal injection of MMA, besides causing convulsive behavior, reduces brain phosphocreatine levels (Royes, et al., 2003), increases local TBARS and protein carbonylation (Fighera et al., 2003; Marisco et al., 2003; Royes et al., 2005) suggesting that the brain damage in this organic acidemia may occur via interaction of oxidative stress, metabolic failure and excitotoxicity. The currently reported MMA-induced increase in protein carbonyl content and Na^+, K^+-ATPase activity inhibition *ex vivo* replicates previous studies from our group that have suggested a role for ROS in the convulsive behavior induced by MMA (Malfatti et al., 2003; Royes et al., 2005).

Although any cellular constituent may be a target for free radical damage (Dawson and Dawson, 1996) the inhibition of the some selected target such as Na^+, K^+-ATPase may play an important role in the hyperexcitability induced by MMA. It has long been known that Na^+, K^+-ATPase enzyme is particularly sensitive to free radical induced-damage since its inhibition has been associated with alterations in plasma membrane lipid composition (Jamme et al., 1995), in the redox state of regulatory sulfhydryl groups (Morel et al., 1996) and other amino acid residues caused by free radicals and lipid peroxidation (Siems et al., 1996). Moreover, experimental findings have demonstrated that the oxidative and nitrosative stress induce protein carbonylation (Dean et al., 1997) increasing the susceptibility to proteolysis and marked impairment of protein functionally (Dalle-Donne et al., 2003). Therefore, it is tempting to propose that oxidative attack of selected target such as Na^+, K^+-ATPase enzyme might represent an important role in the development and/or propagation of MMA-induced convulsive behavior. In agree with this view, recent experimental finding demonstrated a positive correlation between duration of convulsive episodes induced by MMA with striatal Na^+, K^+-ATPase activity inhibition (Royes et al., unpublished data).

It has long been known that NO, when produced in an excessive amount, may change from a physiological neuromodulator to a neurotoxic agent leading to mitochondrial damage in several neurodegenerative disorders (Stewart and Heales, 2003). Moreover, a growing body of evidence has suggested that reactive nitrogen species inhibit the activity of Na^+, K^+-ATPase by oxidation of SH groups and consequently alteration of the membrane fluidity (Boldyrey et al, 1997; Muriel and Sandoval, 2000; Muriel et al., 2003; Barriviera et al., 2005). Nevertheless, although it is believed that inhibition of the mitochondrial respiratory chain and Na^+, K^+-ATPase activity induced by NO and its toxic metabolic peroxynnitrite ($ONOO^-$) may be an important factor in several acute and chronic neurological disorders such epileptic seizures (Stewart and Heales, 2003; Patel, 2004), there is paradoxical relationship between the levels of NO and oxidative stress in brain and other biological systems.

In this context, the increase of protein carbonylation and Na^+,K^+-ATPase inhibition in rat striatum induced by 7-NI pretreatment, as well as, the effective protection exerted by $_L$-arginine, suggest that striatal NO generation increase induced by MMA, seems to be a physiological response attempting to counteract the oxidative insult elicited by this organic acid. Accordingly, Sharpe et al (2003) evidenced from *in vitro* experiments that in a range of concentrations from 25 nM to 100 nM, NO act as an antioxidant agent, protecting a number of organic molecules from oxidation from Fenton chemistry. Furthermore, it has been found that NO is able to protect animal and plant cell types from oxidative damage resulting from superoxide, hydrogen peroxide and alkyl peroxides by acting as terminator of free radical chain reaction (Wink et al., 1995; 1996; Yalowich et al., 1999; Beligni and Lamattina, 2002), suggesting that NO has as an important role antioxidant in protecting biological tissues.

In summary, the present study described that a striatal NO decrease caused by 7-NI treatment exacerbated the MMA-induced behavioral and electrographic seizures, increased total protein carbonylation and inhibited the Na^+, K^+-ATPase activity. Furthermore, we demonstrated that the injection of $_L$-arginine but not $_D$- arginine increased of striatal NOx content *per se* and protected against MMA-induced electrographic seizures, protein carbonylation and Na^+, K^+-ATPase inhibition. These results indicate a significant participation of NO in the excitotoxicity elicited by this organic acid. In addition, suggest that the adverse effect exerted 7-NI on MMA-induced electrographic seizures and neurochemical parameters may be due to a neuronal NO content depletion exacerbating the convulsant and oxidative damage in this model of organic acidemia.

Thus, considering that methylmalonic acidemia, together with others amino acidophathies constitute the most prevalent groups of inherited metabolic disorders in high-risk populations(Orgier de Baulny et al., 2005), the demonstration that a neuronal NO content depletion potentates MMA-induced toxicity may be of value in understating the physiopathology of the neurological signs observed in this organic acidemia.

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FIGURES AND LEGENDS

Figure 1

Effect of 7 NI (3, 10, 30 and 60 mg/Kg; i.p.) on latency for the first convulsive episode (A) and increases the duration (B) of convulsive episodes induced by MMA (6 μmol/ 2 μl). Data mean + S.E.M. for n= 8-10 in each group. * $P<0.05$ compared with Saline - MMA group (*F* test for simple effect).

Figure 2

Basal electroencephalographic recording (preinfusion) **(A)**; saline postinfusion **(B)** and typical seizure sequences observed after MMA intrastriatal injection (6 μmol/ 2 μl) during 15 min (postinfusion) **(C)** which were accompanied by the behavioral alterations described in the Results section. ISTR, ipsilateral striatum; ICTX, ipsilateral cortex and CCTX, contralateral cortex. Calibration bars, 100 μV and 1s.

Figure 3

Basal electroencephalographic recording (preinfusion) **(A)**; 7-NI (60 mg/Kg; i.p.) postinfusion **(B)** and the effect of 7-NI (60 mg/Kg; i.p.) on seizure sequences observed after MMA intrastriatal injection (6 μmol/ 2 μl) during 15 min (posinfusion) **(C)**. ISTR, ipsilateral striatum; ICTX, ipsilateral cortex and CCTX, contralateral cortex. Calibration bars, 100 μV and 1s.

Figure 4

Effect of $_{L}$-arginine (5 and 50 nmol/ 0.5 μl) and $_{D}$-arginine (50 nmol/ 0.5 μl) on the latency for the first convulsive episode (A) and duration (B) of convulsive episodes induced by MMA (4.5 μmol/ 1.5 μl). Data mean + S.E.M. for n= 8-10 in each group. * $P<0.05$ compared with Saline - MMA group (*F* test for simple effect).

Figure 5

Basal electroencephalographic recording (preinfusion) **(A)**; $_{L}$-arginine (50 nmol/ 0.5 μl) postinfusion **(B)** and the effect of $_{L}$-arginine (50 nmol/ 0.5 μl) on seizure sequences observed after MMA intrastriatal injection (4.5 μmol/ 1.5 μl) during 15 min (posinfusion) **(C)**. ISTR, ipsilateral striatum; ICTX, ipsilateral cortex and CCTX, contralateral cortex. Calibration bars, 100 μV and 1s.

Figure 6

Basal electroencephalographic recording (preinfusion) **(A)**; $_{D}$-arginine (50 nmol/ 0.5 μl) postinfusion **(B)** and the effect of $_{D}$-arginine (50 nmol/ 0.5 μl) on seizure sequences observed after MMA intrastriatal injection (4.5 μmol/ 1.5 μl) during 15 min (posinfusion) **(C)**. ISTR, ipsilateral striatum; ICTX, ipsilateral cortex and CCTX, contralateral cortex. Calibration bars, 100 μV and 1s.

Figure 7

Effect of 7-NI (3,10,30 and 60 mg/Kg, i.p.) on the MMA-induced (6 μmol/ 2 μl) protein carbonylation ex vivo. Data are mean + S.E.M. for n = 8- 10 in each group. *P <0.05

compared to contralateral side. $^{\#}P<0.05$ compared with Saline-MMA group (Student-Newman-Keuls test).

Figure 8

Effect of 7-NI (3,10,30 and 60 mg/Kg, i.p.) on the MMA-induced (6 μmol/ 2 μl) Na^+,K^+-ATPase activity inhibition ex vivo. Data are mean + S.E.M. for n = 8- 10 in each group. * Indicates a significant difference ($P<0.05$) compared with Saline group (Student-Newman-Keuls test).

Figure 9

Effect of $_L$-arginine (5 and 50 nmol/ 0.5 μl) and $_D$-arginine (50 nmol/ 0.5 μl) on MMA-induced (4.5 μmol/ 1.5 μl) protein carbonylation ex vivo. Data mean + S.E.M. for n= 8-10 in each group. * $P<0.05$ compared with Saline group; $^{\#}$ $P<0.05$ compared with Saline-MMA group; ‡ $P<0.05$ compared with $_L$-arginine (5 nmol/ 0.5 μl) –MMA group (Student-Newman-Keuls test).

Figure 10

Effect of $_L$-arginine (5 and 50 nmol/ 0.5 μl) and $_D$-arginine (50 nmol/ 0.5 μl) on the MMA-induced (4.5 μmol/ 1.5 μl) Na^+,K^+-ATPase activity inhibition ex vivo. Data are mean + S.E.M. for n = 8- 10 in each group. * Indicates a significant difference ($P<0.05$) compared with Saline group; $^{\#}$ $P<0.05$ compared with Saline-MMA group (Student-Newman-Keuls test).

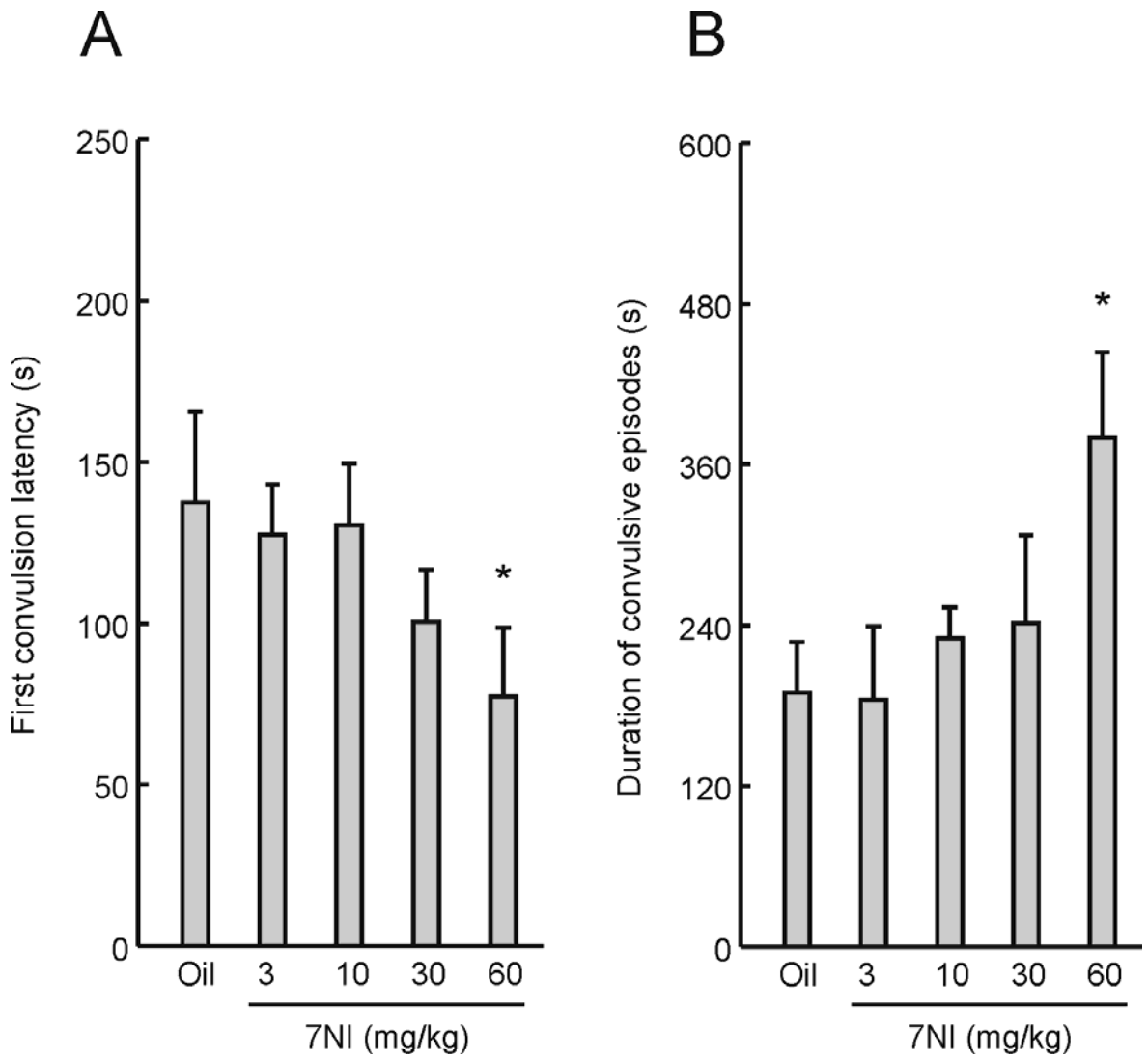
Figure 11

The effect of 7- NI (60 mg/Kg, i.p.) on the MMA-induced (6 μmol/ 2 μl) NOx (NO_2 plus NO_3) increase. Data are mean + S.E.M. for n = 8- 10 in each group. **P* <0.05 compared with Saline group; $^{\#}P$<0.05 compared with Saline-MMA group (Student-Newman-Keuls test).

Figure 12

The effect of $_{L}$-arginine (5 and 50 nmol/ 0.5 μl) and $_{D}$-arginine (50 nmol/ 0.5 μl) on the MMA-induced (4.5 μmol/ 1.5 μl) NOx (NO_2 plus NO_3) increase. Data are mean + S.E.M. for n = 8- 10 in each group. **P* <0.05 compared with Saline group (Student-Newman-Keuls test).

Figure 1


A
First convulsion latency (s)
250
200
150
100
50
0
Oil
3
10
30
60
7NI (mg/kg)
*
B
Duration of convulsive episodes (s)
600
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240
120
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Oil
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60
7NI (mg/kg)
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Figure 2

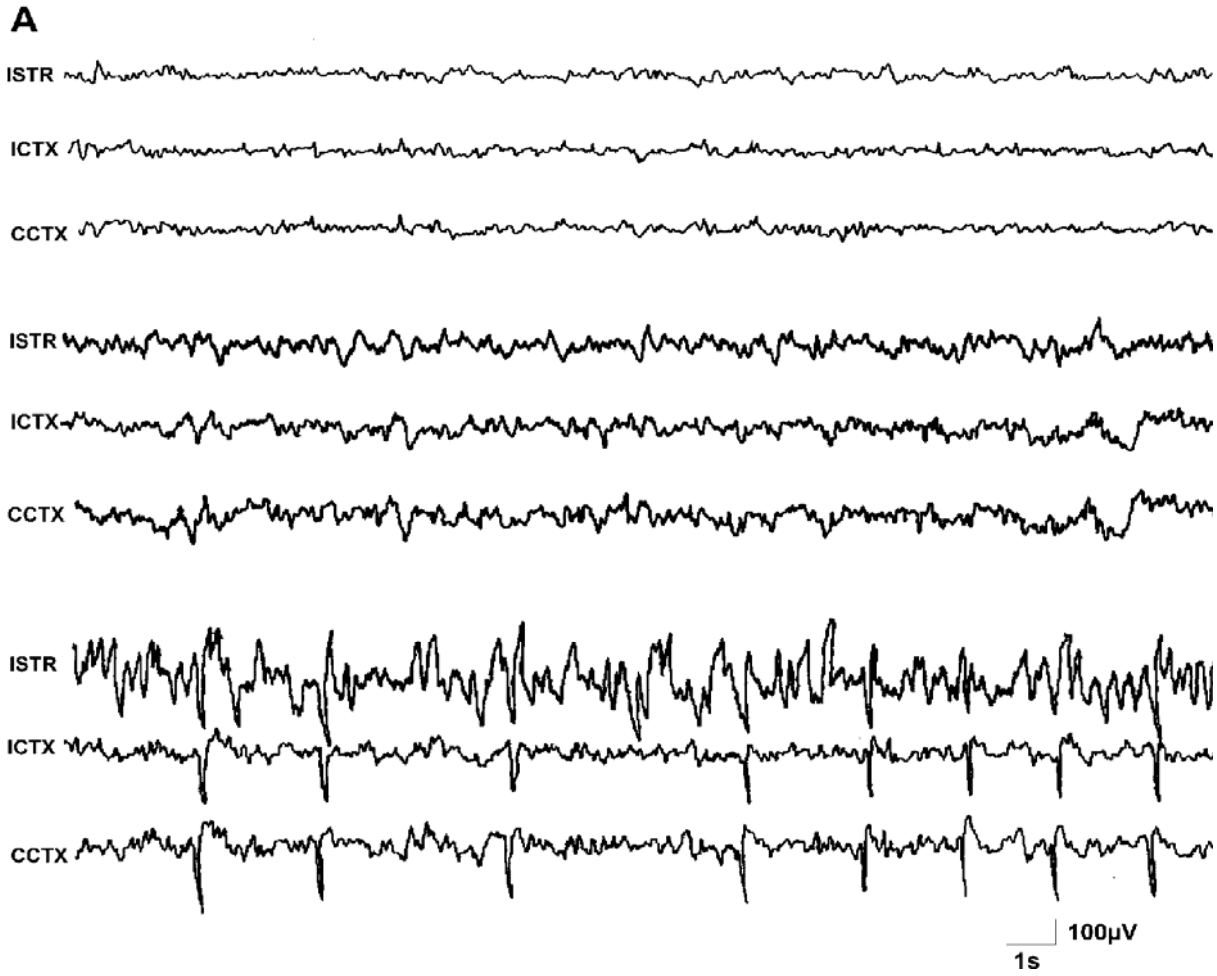


Figure 3

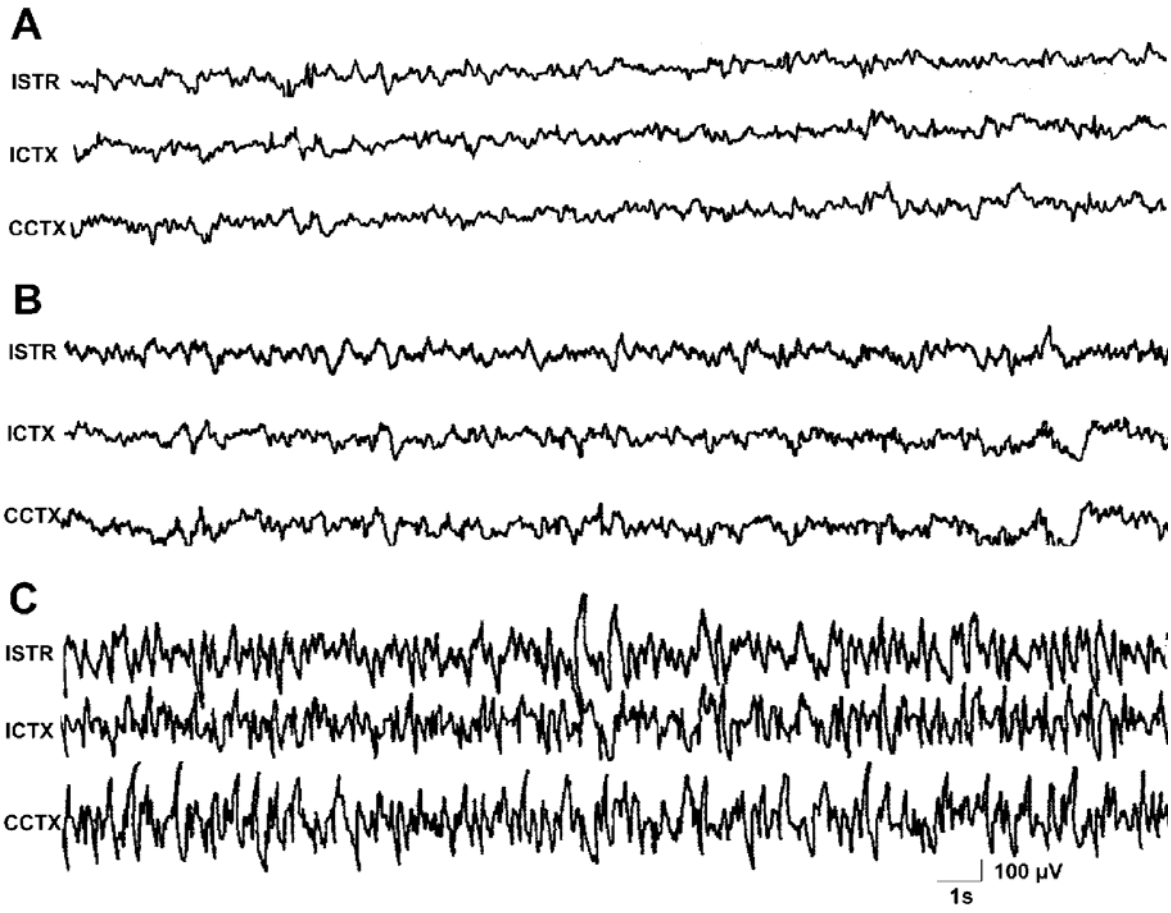
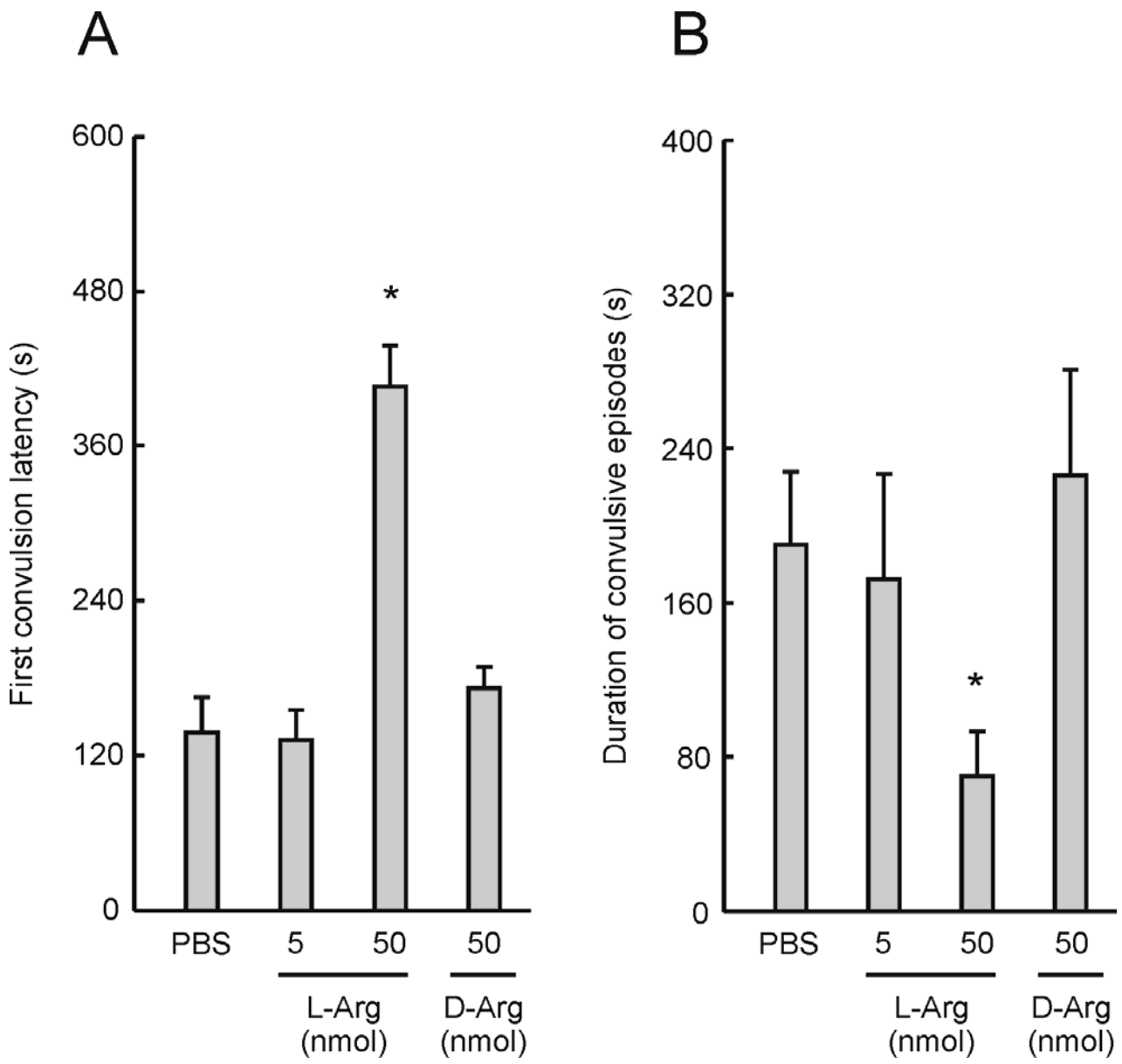


Figure 4


A
600
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240
120
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First convulsion latency (s)
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PBS
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50
50
L-Arg
(nmol)
D-Arg
(nmol)
B
400
320
240
160
80
0
Duration of convulsive episodes (s)
*
PBS
5
50
50
L-Arg
(nmol)
D-Arg
(nmol)

Figure 5

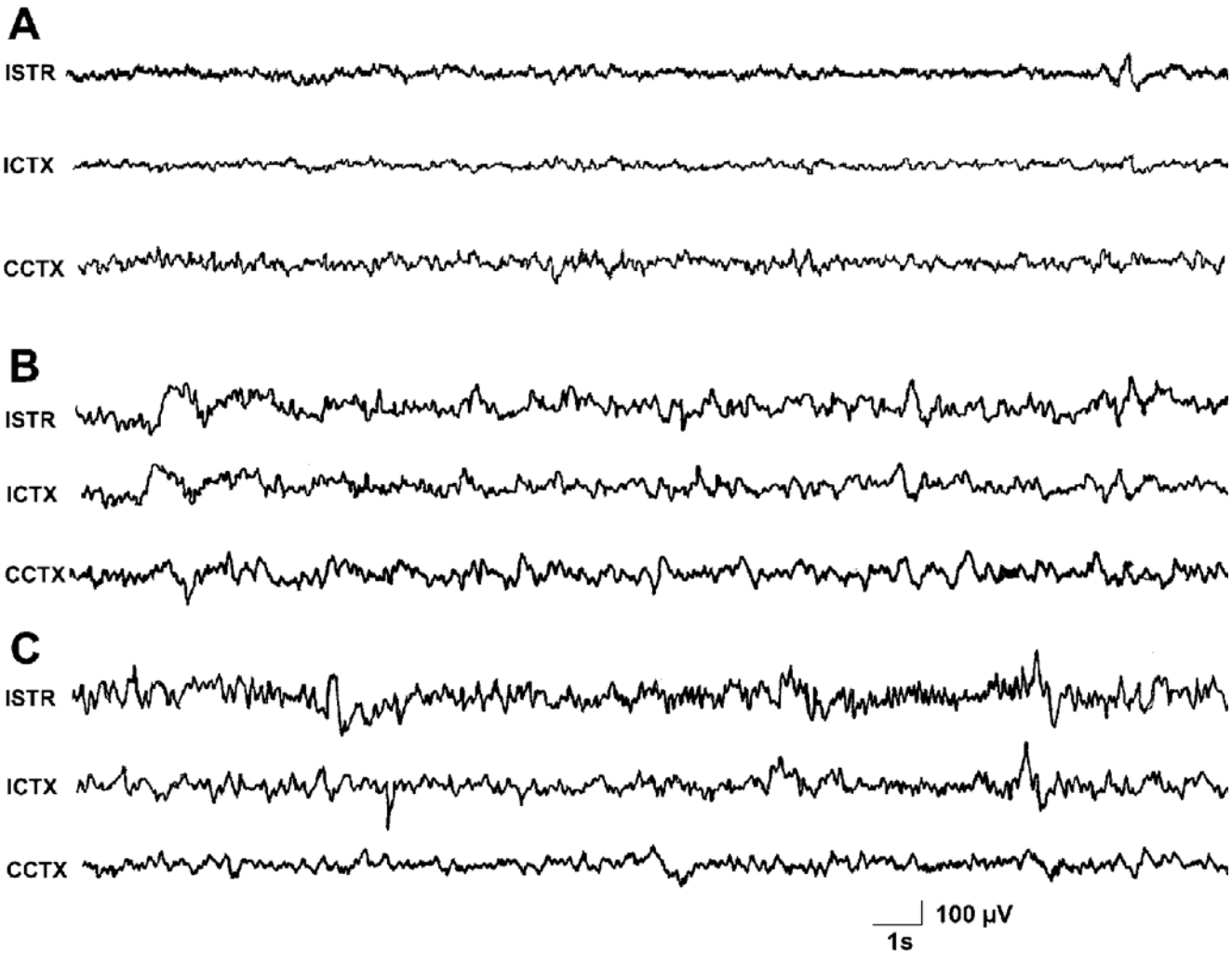


Figure 6

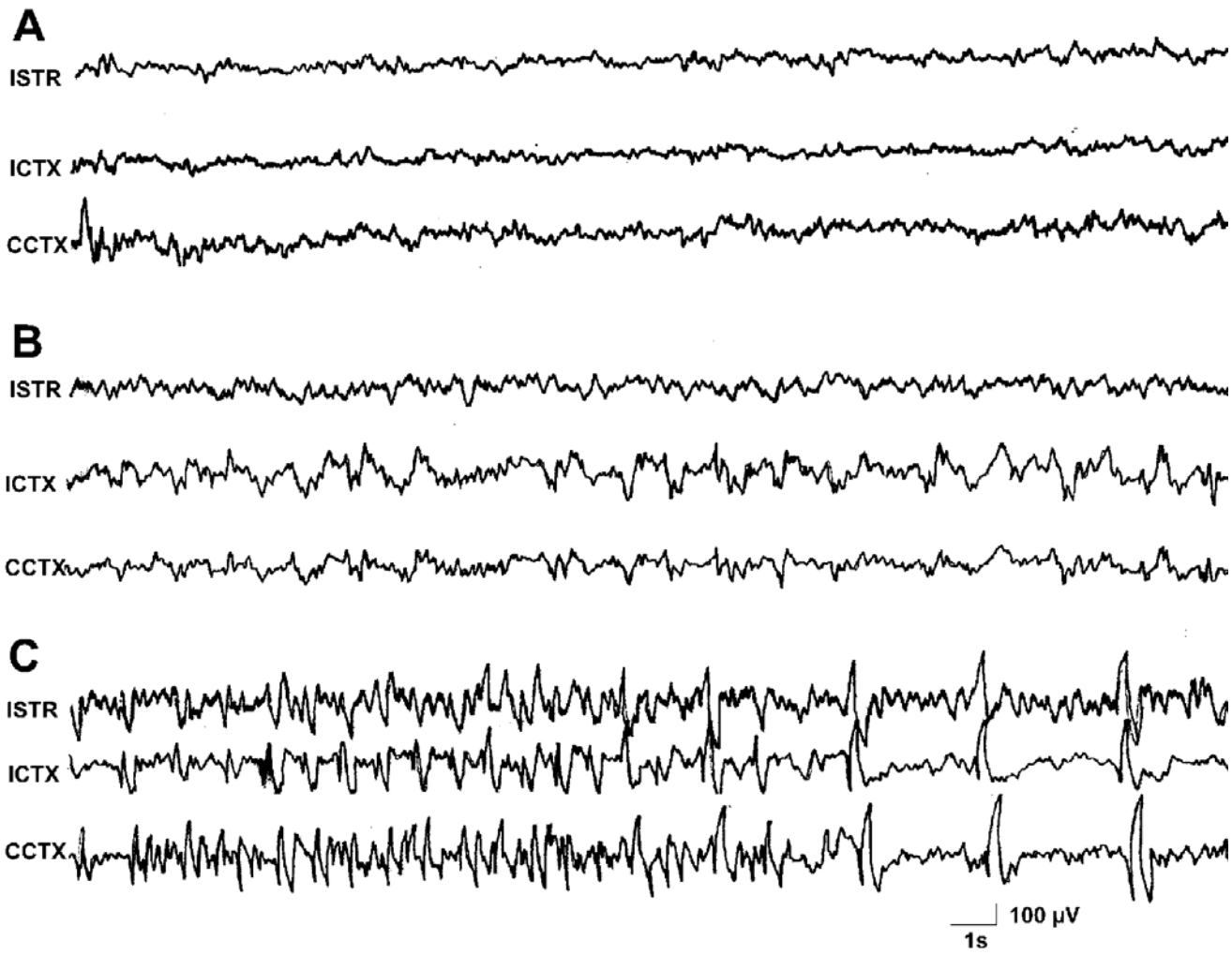
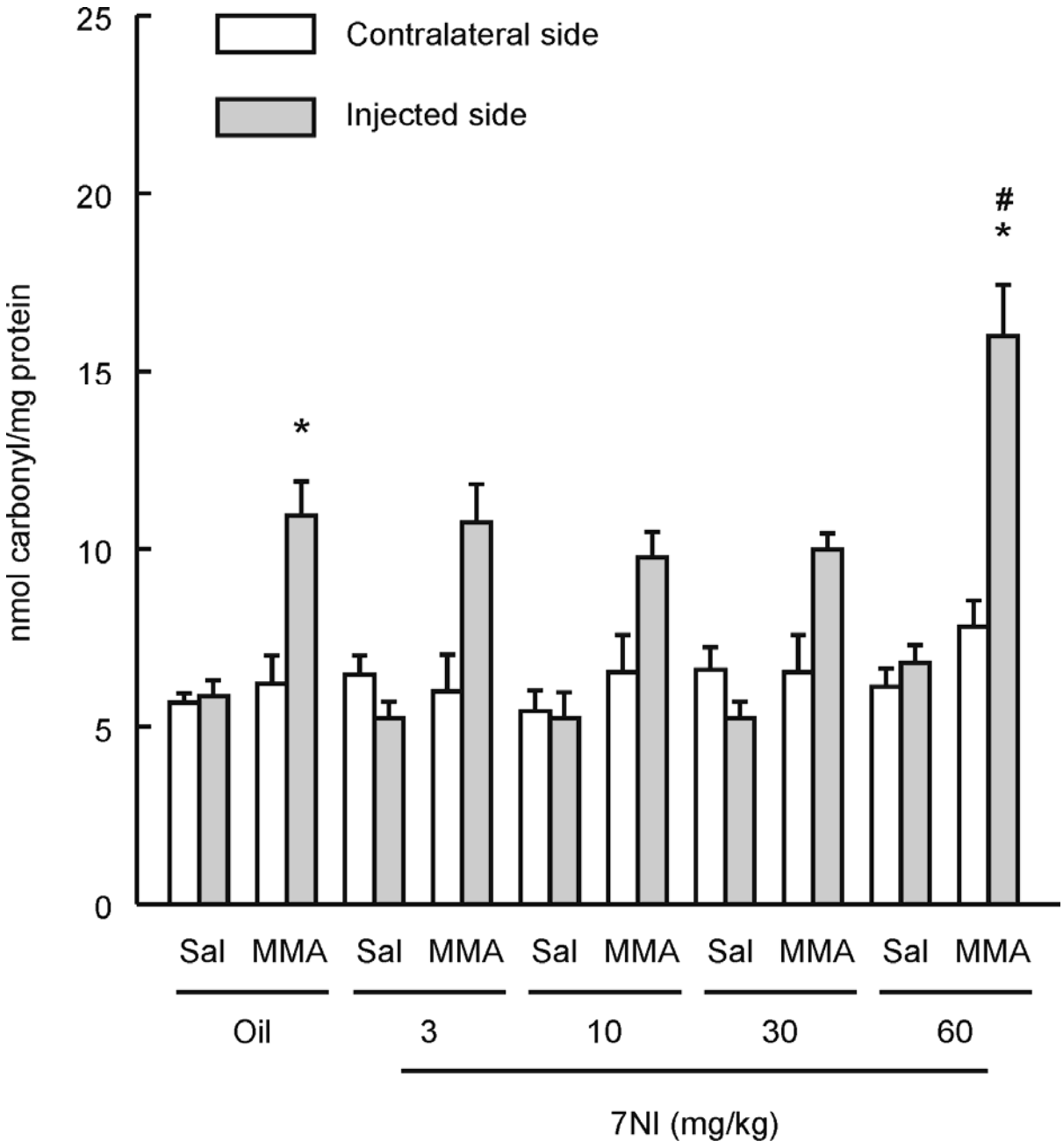


Figure 7


Contralateral side
Injected side
nmol carbonyl/mg protein
25
20
15
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5
0
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*
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MMA
Sal
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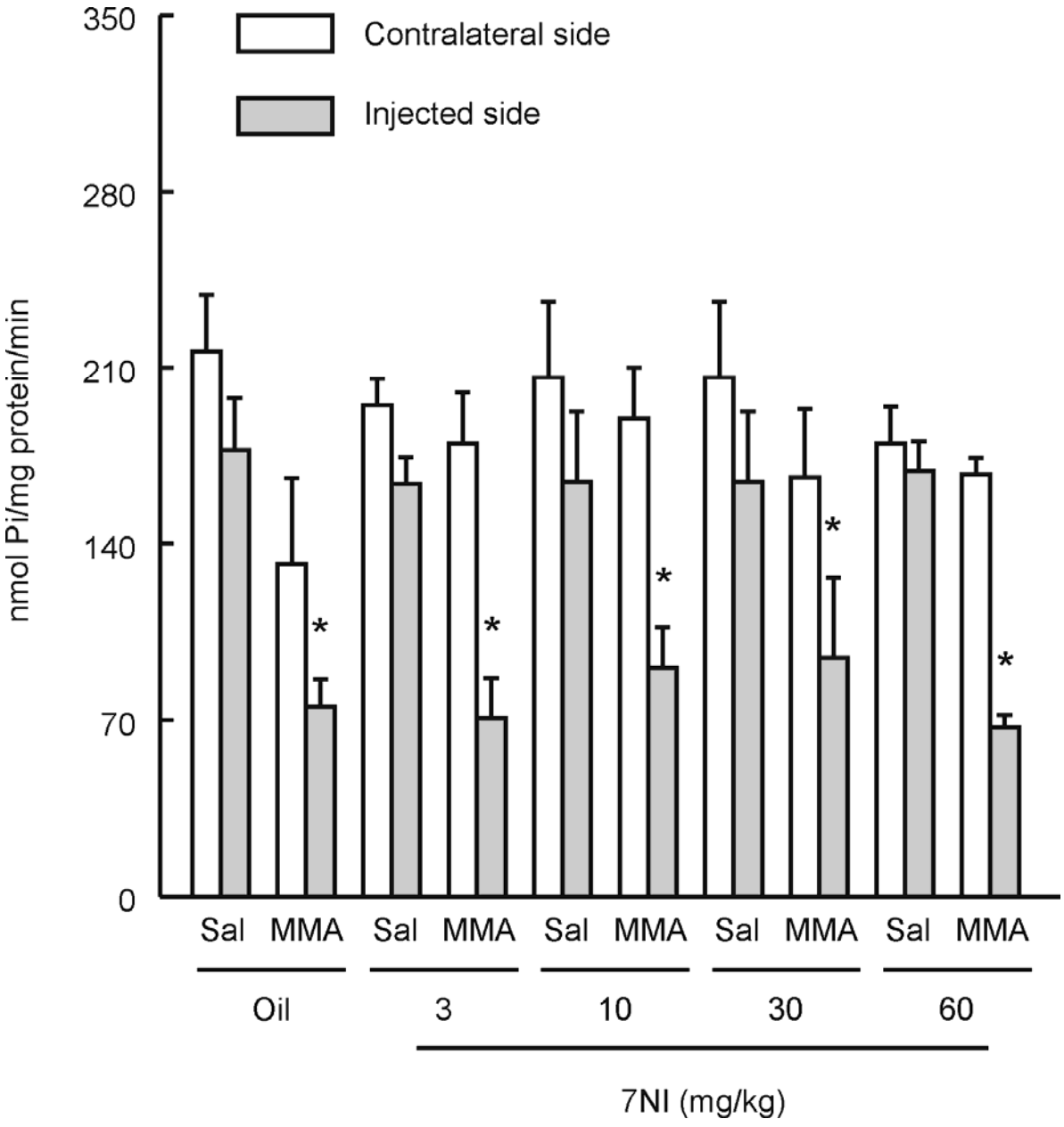
Sal
MMA
Oil
3
10
30
60
7NI (mg/kg)

Figure 8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Injected side
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210
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nmol Pi/mg protein/min
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Sal
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Oil
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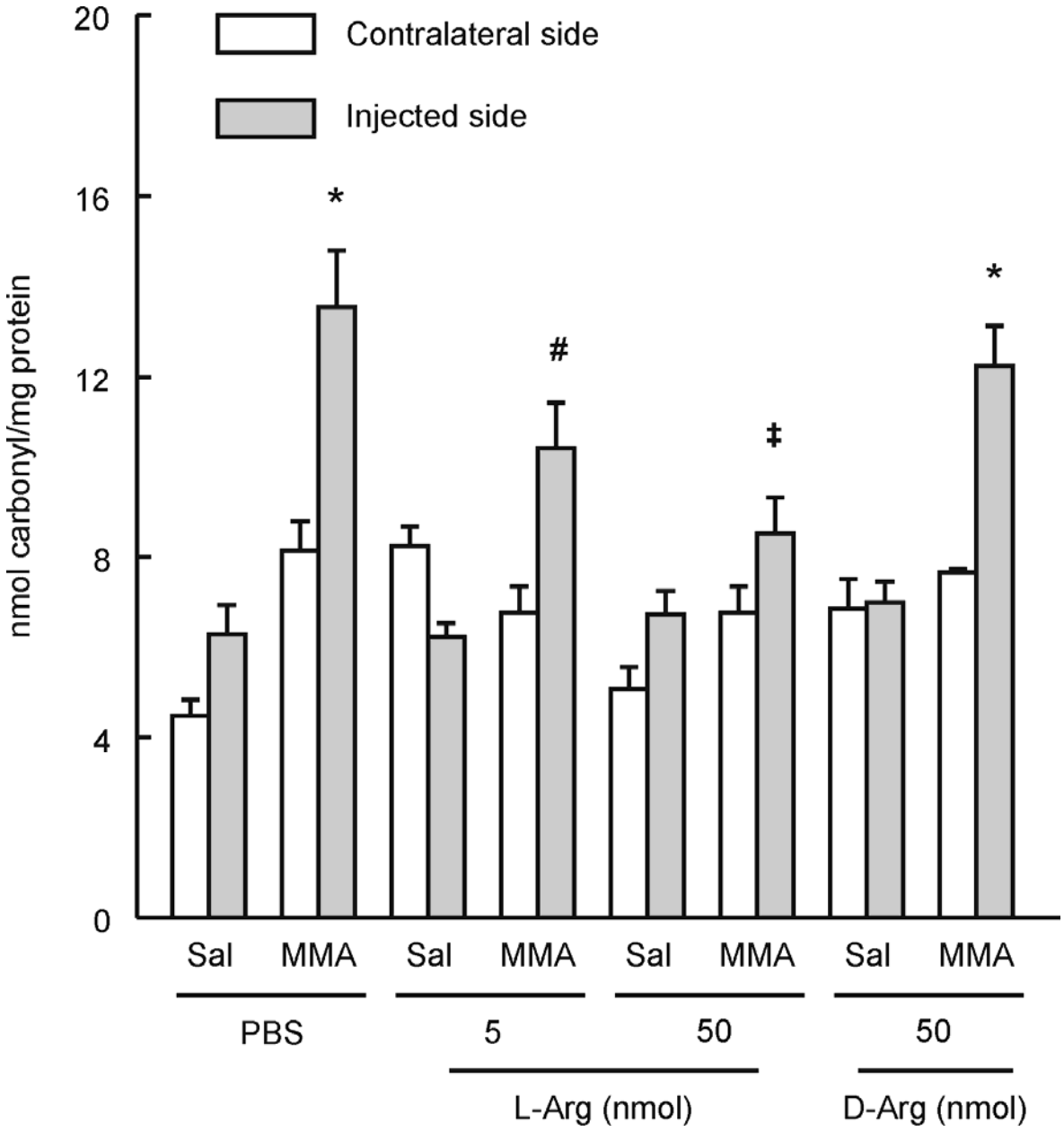
30
60
7NI (mg/kg)

Figure 9


Contralateral side
Injected side
nmol carbonyl/mg protein
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Sal
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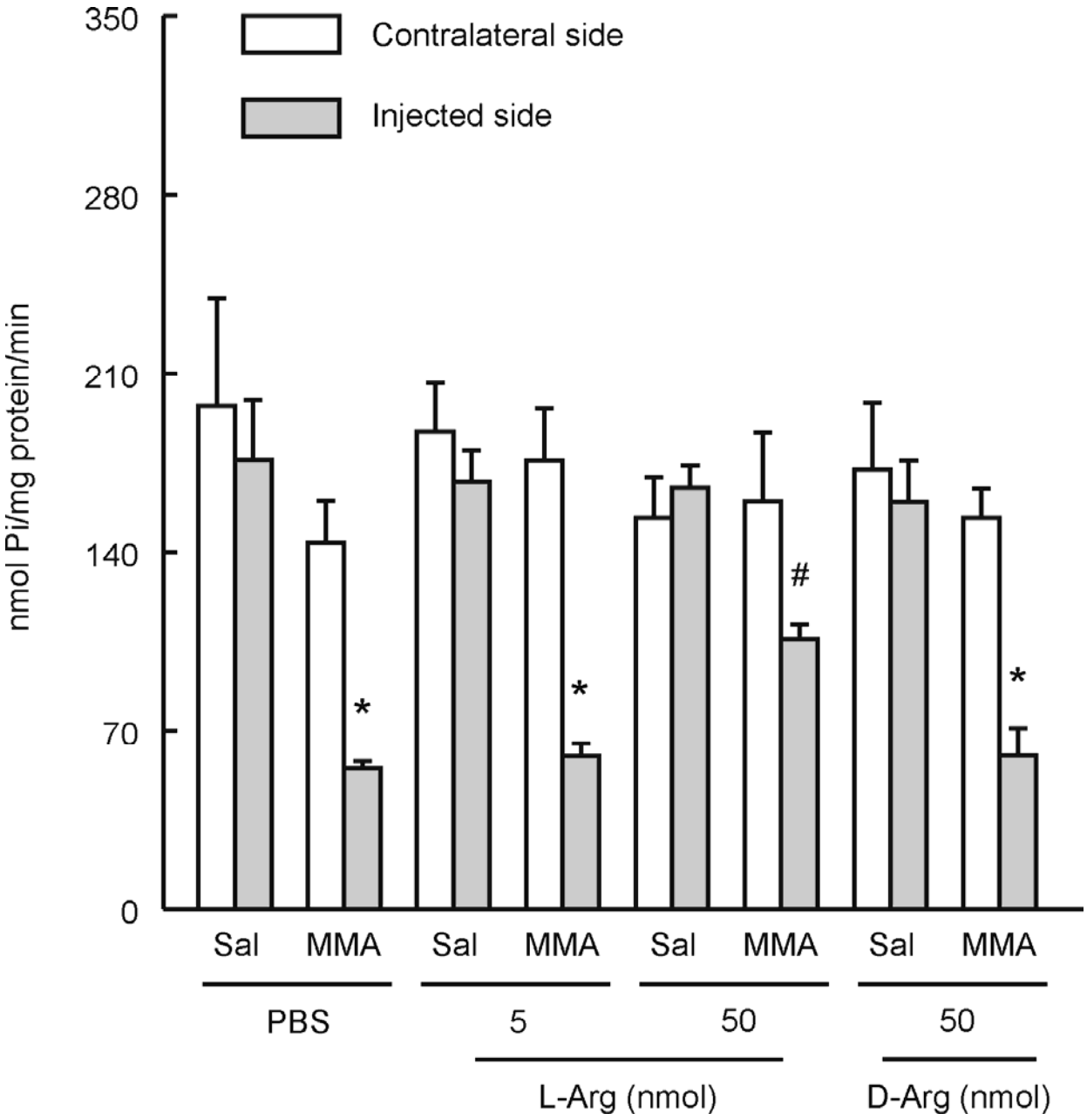
Sal
MMA
PBS
5
50
50
L-Arg (nmol)
D-Arg (nmol)

Figure 10


Contralateral side
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nmol Pi/mg protein/min
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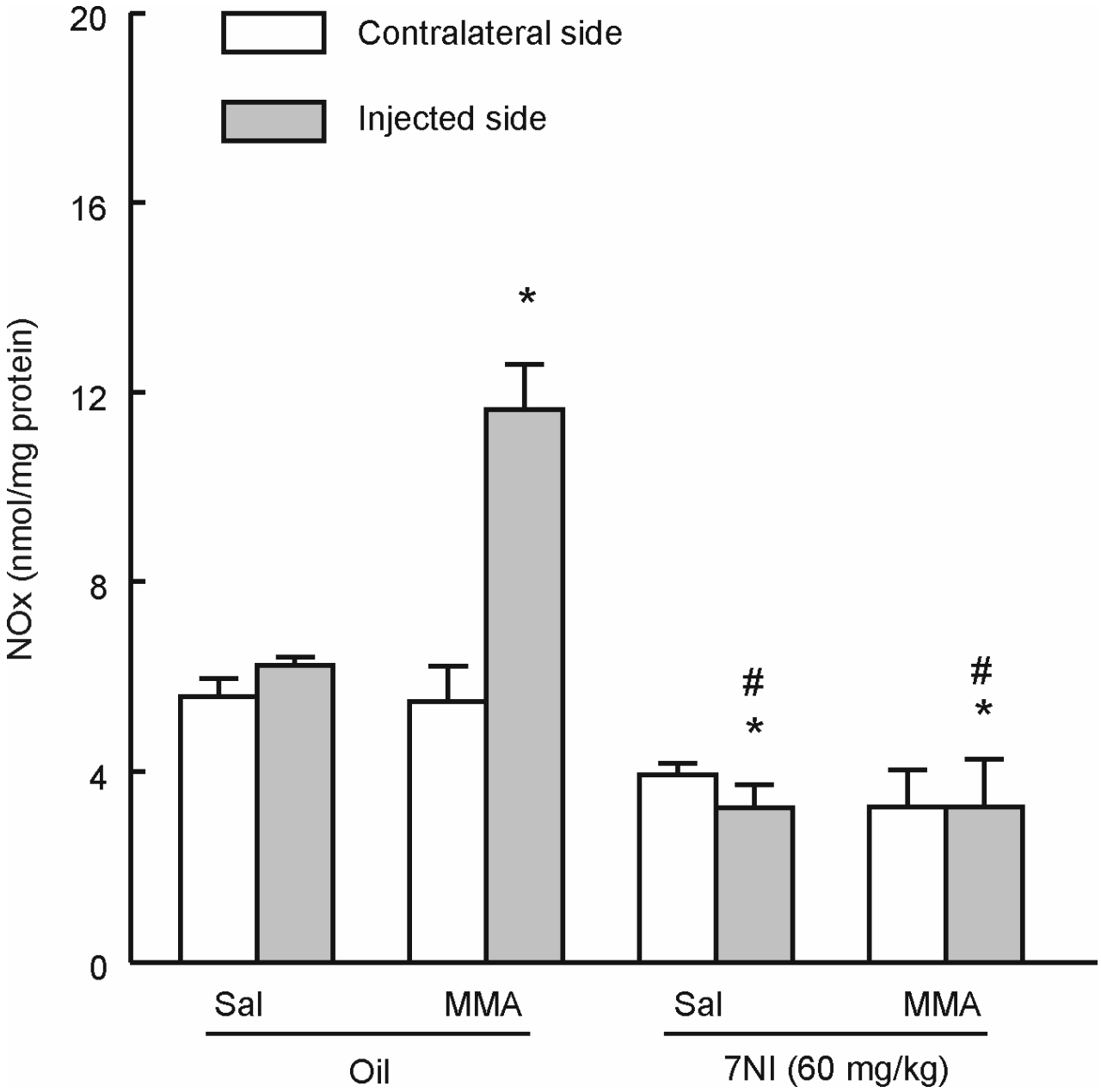
Sal
MMA
Sal
MMA
PBS
5
50
50
L-Arg (nmol)
D-Arg (nmol)

Figure 11


20
16
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8
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NOx (nmol/mg protein)
Contralateral side
Injected side
*
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*
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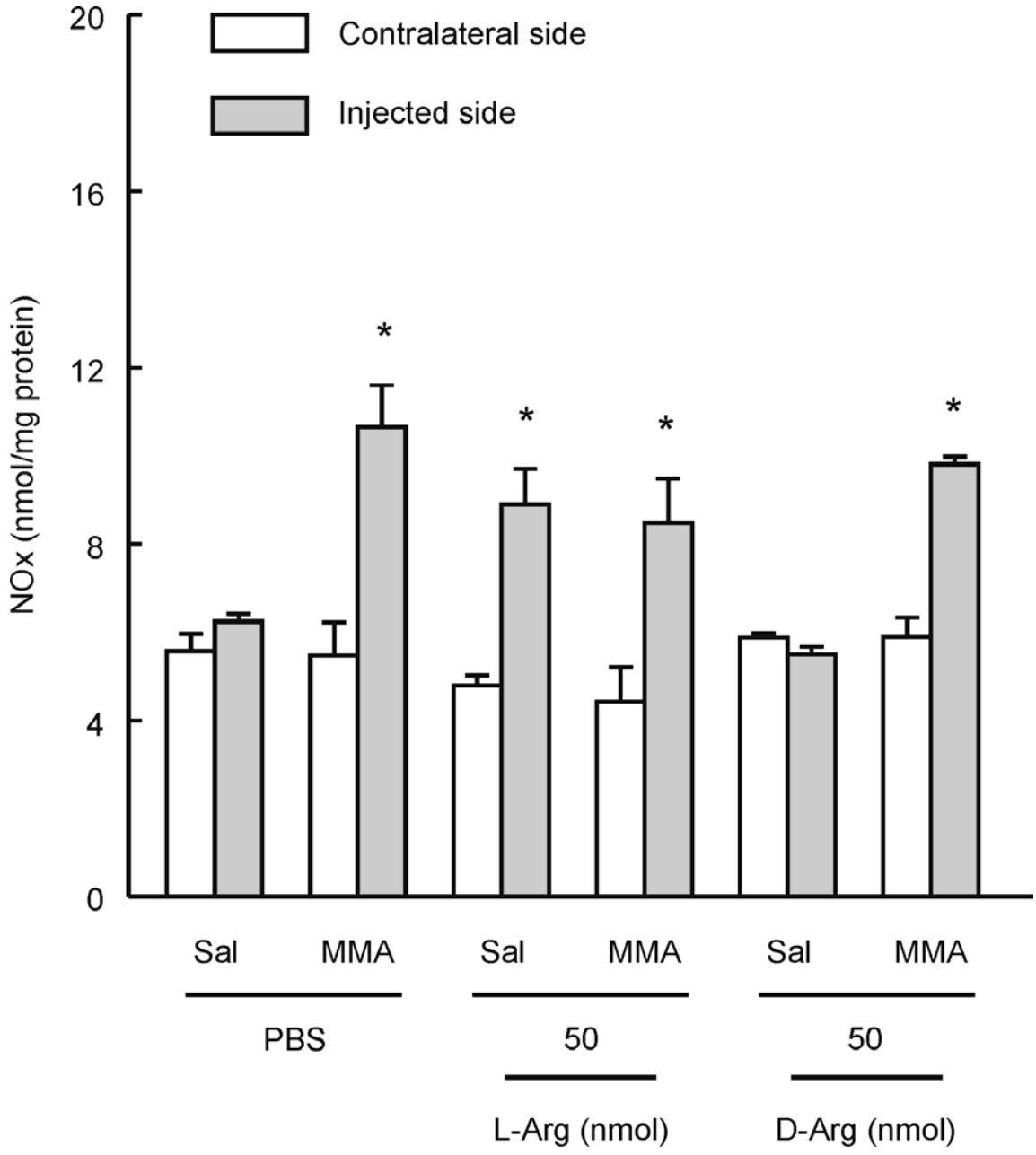
*
Sal
MMA
Sal
MMA
Oil
7NI (60 mg/kg)

Figure 12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NOx (nmol/mg protein)
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L-Arg (nmol)
D-Arg (nmol)

CAPÍTULO V

DISCUSSÃO GERAL

V - Discussão Geral

A acidemia metilmalônica é um erro inato do metabolismo caracterizado pelo acúmulo tecidual de metilmalonil CoA e propionil CoA que, por tiólise espontânea, geram metabólitos secundários como metilmalonato, propionato, β-OH-propionato e cetonas de cadeia longa (Scriver, 1995). Como descrito anteriormente na introdução deste trabalho, a acidemia metilmalônica é causada pela deficiência ou falta de atividade da enzima metilmalonil-CoA mutase, enzima envolvida na rota de degradação de aminoácidos de cadeia ramificada, ácidos graxos de cadeia ímpar, cadeia lateral do colesterol e algumas bases nitrogenadas, além de ser responsável pela conversão do propionil CoA em metilmalonil CoA (Fenton & Rosenberg, 1995).

O acúmulo de MMA parece ser o principal responsável pelas alterações neurológicas características desta doença, que incluem retardo mental e motor, degeneração dos núcleos da base e convulsões. Contudo, pouco se sabe sobre os mecanismos de neurotoxicidade envolvidos nesta patologia.

A hipótese mais aceita para a neurotoxicidade induzida pelo MMA é a inibição metabólica. Estudos *in vitro* indicam que o MMA é capaz de inibir competitivamente a sucinato desidrogenase, (Dutra et al, 1993; Toyoshima et al, 1995), diminuir a produção de CO_2 e aumentar os níveis de lactato em fatias de tecido cerebral (Wajner et al, 1992). Além disso, Hayasaka e colaboradores, em 1982, observaram que pacientes com acidemia metilmalônica ou propiônica, em acidose, apresentavam inibição da citocromo oxidase. A partir destes relatos, tem sido postulado que o acúmulo de MMA prejudica o metabolismo aeróbico celular. Há evidências de que o MMA induz convulsões por inibir a SDH e ativar

secundariamente receptores NMDA (Mello et al., 1996; Royes et al., 2003), uma vez que as convulsões induzidas por MMA são inibidas por MK-801 e atenuadas por sucinato. Além disso, sabe-se que o MMA não interage diretamente com receptores do glutamato (Mello et al., 1997), sugerindo, portanto, que a depleção de ATP devido à falência do complexo II seja o evento que desencadeia a excitação que se traduz nas convulsões observadas nos animais injetados com este ácido e, talvez, nos pacientes com acidemia metilmalônica. Recentemente foi mostrado que a injeção aguda de creatina aumenta o conteúdo de fosfocreatina cerebral e atenua as convulsões e o dano oxidativo induzidos pela administração intra-estriatal de MMA, constituindo evidência adicional de que as convulsões induzidas por MMA são provavelmente secundárias à depleção primária de ATP, fosfocreatina e ativação de mecanismos excitotóxicos secundários, que inclui a produção de espécies reativas (Royes et al., 2006).

A participação de espécies reativas está relativamente bem estabelecida na neurotoxicidade induzida por inibidores da SDH, como malonato e 3-nitropropionato (3-NPA) (Greenamyre, et al., 1994; Schulz et al. 1996), uma vez que ela é atenuada pela administração de coenzima Q (Beal et al., 1994) e pela super-expressão da Cu/Zn-superóxido dismutase (SOD) (Beal et al., 1995). A super-expressão da SOD também reduz marcadores de dano oxidativo induzido por 3-NPA (Beal et al., 1995). Da mesma forma, o nocaute do gene da enzima óxido nítrico sintase neuronal e a administração de inibidores da enzima óxido nítrico sintase inibem a produção de radicais livres e a morte celular induzida por malonato (Schulz et al., 1996). Além disso, há evidências de que os radicias livres formados durante os estados de depleção energética e por outras causas, estão envolvidos na gênese de convulsões. Os efeitos anti-epiléticos do alopurinol em camundongos espontaneamente epiléticos estão associados com aumento na atividade da

Cu^{+2}/Zn^{+2} SOD (Murashima et al., 1998). Neste mesmo sentido, a administração intracortical de ferro induz descargas epilépticas e, concomitantemente, aumenta os níveis de radicais livres no córtex, sendo que ambos fenômenos são inibidos por antioxidantes (Hiramatsu et al., 1996).

O envolvimento das espécies reativas nas convulsões induzidas pela injeção intra-estriatal de MMA foi recentemente caracterizado por estudos que evidenciaram uma diminuição da atividade da Na^{+},K^{+}-ATPase, um aumento na produção de espécies reativas ao ácido tiobarbitúrico (indicativo de lipoperoxidação) e carbonilação protéica após a administração deste ácido orgânico no estriado (Malfatti et al., 2003; Royes et al., 2006). Também foi demonstrado que as convulsões induzidas por MMA são atenuadas por ácido ascórbico, α-tocoferol e por gangliosídeo GM1, sendo que os dois últimos também inibem a produção de TBARS induzida por MMA intra-estriatal. (Fighera et al., 1999; 2003). Além disso, a injeção sistêmica de amônia (um agente pró-oxidante), em concentrações que não têm efeito *per se* sobre o comportamento e que tampouco alteram a inibição da SDH induzida por MMA *in vitro*, é capaz de aumentar a produção de TBARS no estriado e potencializar as convulsões induzidas por MMA (Marisco et al., 2003). Entretanto, ainda são escassos estudos que definam o papel das espécies reativas, particularmente do óxido nítrico, no comportamento convulsivo induzido pelo MMA.

No intuito de avaliar o papel deste radical livre no modelo de toxicidade induzida pelo MMA, o presente estudo investigou, primeiramente, se a administração intra-estriatal de L-NAME (um inibidor não-especifico da enzima óxido nítrico sintase) atenuaria as convulsões e a carbonilação protéica induzidas pelo MMA. A injeção intra-estriatal de L-NAME não teve efeito *per se* sobre os parâmetros comportamentais dos ratos, nem sobre o

conteúdo estriatal de grupos carbonil protéicos. Entretanto, a administração de pequenas doses L-NAME (10^{-3} a 10^{-1} nmol/0.5 μl) aumentou a latência para o aparecimento das convulsões, atenuou a duração dos episódios convulsivos e protegeu contra a carbonilação protéica induzida pelo MMA, enquanto que a maior dose de L-NAME (1 nmol/0.5 μl) não teve efeito protetor. O aumento no conteúdo de grupos carbonil protéicos induzidos pela injeção de MMA observado no presente estudo é mais um indício do envolvimento das espécies reativas neste modelo de convulsões induzidas pelo MMA. Neste estudo propomos que o efeito bifásico do L-NAME sobre o comportamento convulsivo e a carbonilação protéica no estriado pode ser devido ao seu efeito diferencial na atividade das enzimas óxido nítrico sintase endotelial (K_i 39 nM), e neuronal (K_i 15 nM). Desta forma, baixas doses de L-NAME inibiriam, preferencialmente, a enzima NOSn, enquanto que altas doses deste composto afetariam a atividade da NOSe, levando a um efeito vascular adverso (vasoconstrição) e conseqüente agravamento do estresse metabólico. De fato, estudos com modelos de isquemia cerebral mostram que uma inibição seletiva da enzima NOSn diminui o volume da lesão induzida pela isquemia, enquanto que a inibição não-seletiva resulta em diminuição no fluxo sanguíneo cerebral e consequente aumento do volume do infarto (Yoshida et al., 1994; Zhang et al., 1996).

Apesar do envolvimento de espécies reativas na neurotoxicidade induzida pelo MMA (Fighera et al., 1999; 2003; Marisco et al., 2003; Malfatti et al., 2003; Ribeiro et al., 2005; Royes et al., 2006) estar relativamente bem estabelecido, é difícil determinar se há uma relação causa-efeito entre as convulsões e o dano oxidativo evidenciado neste modelo de acidemia orgânica. Neste sentido, o objetivo do estudo mostrado no Capítulo III foi determinar se existia uma correlação entre convulsões, aumento conteúdo de grupos

carbonil protéicos e inibição da atividade da Na^+,K^+-ATPase induzidas pela administração intra-estriatal de MMA. Considerando que a disfunção metabólica primária causada pelo acúmulo de MMA leva a um prejuízo no metabolismo aeróbico e excitotoxicidade secudária através da ativação de receptores glutamatérgicos, nós decidimos investigar se a administração prévia de azul de metileno, um composto com propriedades anti-oxidantes e que também aumenta o consumo de oxigênio pela cadeia respiratória, teria efeito neuroprotetor contra as convulsões (evidenciadas também pelas alterações eletroencefalográficas), carbonilação protéica e inibição da enzima Na^+, K^+-ATPase. Foi evidenciado, neste estudo, uma correlação significativa entre duração das convulsões e diminuição na atividade da enzima Na^+, K^+-ATPase no estriado. Entretanto, não foi evidenciada correlação entre convulsões e o aumento do conteúdo de grupos carbonil protéicos induzidos pelo MMA.

Estes resultados sugerem que, apesar da injeção intra-estriatal de MMA induzir convulsões e um aumento no conteúdo de grupos carbonil protéicos no estriado, é difícil postular que haja uma relação causa-efeito entre estes eventos porque: I) o conteúdo de grupos carbonil protéicos representa o total de proteínas carboniladas no estriado, o que não afasta a hipótese de que proteínas específicas, que não representem uma fração significativa dentro do conteúdo de proteínas totais, sejam oxidadas e, seletivamente, se relacionem ao fenômeno convulsivo. Deste modo, a inibição de uma enzima, como a Na^+,K^+-ATPase por exemplo, poderia estar envolvida na gênese e propagação das convulsões induzidas pelo MMA por um mecanismo oxidativo, sem repercurtir um aumento na carbonilação protéica total. II) a administração prévia de pequenas doses de azul de metileno diminuiu o conteúdo de grupos carbonil protéicos, mas não protegeu das

convulsões induzidas pelo MMA, sugerindo que estes eventos podem ser dissociados, o que sugere que eles não são interdependentes.

Diversos estudos têm mostrado que o azul de metileno, além de agir como um potente inibidor da produção de radical superóxido e radical hidroxil por servir como aceptor artificial de elétrons (Salaris *et al.*, 1991), inibe a óxido nítrico sintase (Mayer et al., 1993, Volke et al., 1999) e a guanilato ciclase (Gruetter *et al.*, 1981). Desta forma, é plausível supor que azul de metileno possa exercer o efeito neuroprotetor aqui descrito por interferir na resposta nitrérgica, o que foi confirmado pela capacidade do azul de metileno de inibir o aumento de nitratos induzido por MMA; seja porque ele inibe a enzima óxido nítrico sintase (Mayer et al., 1993; Volke et al., 1999) e a produção de óxido nítrico, ou porque ele diminui a produção de GMP_c pela guanilato ciclase. O resultado esperado, em ambos os casos, seria a proteção contra a excitotoxicidade induzida pelo MMA. Contudo, não podem ser afastados outros dois mecanismos igualmente importantes para o azul de metileno, que são o aumento da eficiência dos processos de transporte de elétrons na cadeia respiratória, o que facilita a produção de ATP, e a sua ação antioxidante. Como há evidência experimental de que o MMA causa inibição dos processos celulares de obtenção de energia e geração de espécies reativas, estes mecanismos também poderiam ser eficientes para diminuir a toxicidade do MMA, independente da ação do azul de metileno sobre o metabolismo do óxido nítrico. Assim, o azul de metileno, devido aos seus múltiplos mecanismos de ação putativos, é de pouco valor como ferramenta farmacológica para verificar o envolvimento da guanilato ciclase ou do óxido nítrico em qualquer evento, inclusive o aqui descrito. Contudo, o seu uso aprovado na clínica para o tratamento de condições como metahemoglobinemia (Maimo & Redick, 2004) e choque séptico (Donati *et al.*, 2002), torna-o um composto interessante para um ensaio clínico em pacientes com acidemia metilmalônica. Enfim, talvez seja esta, a aplicabilidade

clínica imediata, a maior contribuição deste estudo sobre a ação protetora do azul de metileno contra as convulsões induzidas por metilmalonato.

Os efeitos de inibidores da enzima óxido nítrico sintase têm sido objeto de estudo de diversos autores na tentativa de elucidar o papel do óxido nítrico em diversos modelos de convulsão (de Sarro et al., 1991; Paoletti et al., 1998; Itoh et al., 2004; de Vasconcelos et al., 2000; Borowicz et al., 2000; Kato et al., 2005). Entretanto, as discrepâncias existentes entre os resultados nos levam a acreditar que a metodologia aplicada, como rota de administração, o inibidor da NOS utilizado, a dose administrada e produção diferenciada de óxido nítrico nas diferentes regiões do cérebro, sejam alguns dos fatores de discordância entre os autores sobre o real papel do óxido nítrico nos modelos de convulsão. Neste contexto, a fato da administração de doses baixas de L-NAME (um inibidor inespecífico da enzima óxido nítrico sintase) atenuar o efeito convulsivo e o dano oxidativo induzido pelo MMA, pode sugerir o envolvimento da óxido nítrico sintase neuronal nas convulsões e oxidação induzidas por MMA, uma vez que o K_i do L-NAME para a NOSn é menor do que o K_i para a isoforma endotelial, como citado anteriormente. Assim, segundo esta interpretação, doses maiores do inibidor deveriam causar vasoconstrição e agravar o quadro metabólico, como observado experimentalmente.

Segundo a interpretação acima, os achados experimentais apresentados no estudo com 7-NI, um composto que é considerado um inibidor mais seletivo da NOSn por não causar alterações na pressão arterial sistêmica, seriam conflitantes com os resultados do primeiro estudo com L-NAME, uma vez que o 7-NI paradoxalmente aumentou as convulsões induzidas por MMA. Devido a este efeito paradoxal, no estudo com 7-NI foram realizados controles adicionais e experimentos mais detalhados para evidenciar se o MMA induz a formação de óxido nítrico (na forma de nitratos) e se este, de fato, estaria envolvido

nas convulsões induzidas por MMA. Assim, a depleção de nitratos induzida por 7-NI, assim como prevenção do 7-NI contra o aumento de nitratos induzido por MMA foram confirmados por determinações bioquímicas, indicando que a dose de 7-NI empregada neste estudo foi efetiva. Mais do que isso, a L-arginina, mas não a D-arginina, um isômero que não serve como substrato da NOS (Alderton *et al.*, 2001), re-estabeleceu o aumento de nitratos induzidos por MMA na presença de 7-NI, se constituindo em evidência adicional de que o MMA induz a formação de óxido nítrico via NOS. Por fim, a última evidência experimental de que, de fato, o MMA aumenta a produção de nitratos via NOS, é que os efeitos do MMA e da L-arginina sobre os níveis de nitratos não são aditivos, sugerindo que tanto o aumento de nitratos induzido por MMA, como por L-arginina, ocorra por uma mesma via metabólica.

Embora todos os experimentos de determinação de nitratos realizados no estudo com 7-NI suportem que o MMA aumenta a produção de óxido nítrico, os mesmos estudos indicam, de forma contundente, que a inibição da enzima óxido nítrico sintase por 7-NI potencializa as convulsões, a carbonilação de proteínas e a inibição da Na^+,K^+-ATPase induzidos por MMA. Se assumirmos, portanto, que o 7-NI é realmente um inibidor seletivo da NOSn, tal corpo de resultados sugere um papel protetor para o óxido nítrico de origem neuronal nas convulsões induzidas por MMA.

De fato, existe uma relação paradoxal no que diz respeito aos níveis de óxido nítrico e stress oxidativo, tanto no cérebro quanto em outros tecidos. Por exemplo, Sharpe e colaboradores (2003) evidenciaram, em estudos *in vitro*, que concentrações de óxido nítrico entre 25 nM a 100 nM, exercem efeito antioxidante na reação de Fenton. Além disto, diversos estudos têm demonstrado uma ação antioxidante do óxido nítrico contra formação de radical superóxido e peróxido de hidrogênio em diversos tecidos biológicos (Wink et al., 1995; 1996;

Yalowich et al., 1999; Beligni and Lamattina, 2002). Neste contexto, o aumento na carbonilação protéica e uma inibição da enzima Na^+,K^+-ATPase induzido pela administração prévia de 7-NI, bem como uma efetiva proteção exercida pela administração de L-arginina sugere que o produção de óxido nítrico no estriado após a injeção intra-estriatal de MMA seja uma resposta protetora para controlar o dano oxidativo induzido pelo acúmulo deste ácido orgânico. Além disso, o estado redox da célula pode influenciar no efeito neuroprotetor do óxido nítrico neste modelo de toxicidade. Lipton e colaboradores (1993) mostraram que a produção de óxido nítrico em um ambiente intracelular reduzido exerce efeito neurotóxico, enquanto que a produção deste radical livre em um ambiente intracelular oxidante favorece a nitrosilação de receptores NMDA, e a inibição da sua condutância. Desta forma, é possível que um ambiente intracelular mais oxidado induzido pelo MMA influencie na ação neuroprotetora do óxido nítrico neste modelo de acidemia orgânica, mas estudos adicionais serão necessários para elucidar esta questão.

Em contraponto à discussão acima, se considerarmos a possibilidade de que o 7-NI possa não ser um verdadeiro inibidor seletivo da NOSn e inibir também a isoforma endotelial, a discussão acima se torna praticamente uma extensão daquela do trabalho com L-NAME, em que os efeitos do MMA seriam potencializados por uma vasoconstrição induzida pelo 7-NI. Na verdade diversos estudos descrevem o 7-NI como sendo um inibidor seletivo da nNOS (Chavko *et al.*, 2001; Benamar *et al.*, 2003; Gil *et al.*, 2003; Urch & Dickenson, 2003; Brown *et al.*, 2004; Talukder *et al.*, 2004; Wangensteen *et al.*, 2004; Sjakste *et al.*, 2005; Mishra & Delivoria-Papadopoulos, 2006; Rawls *et al.*, 2006), uma vez que o IC_{50} deste composto determinado para a NOSi, NOSn e NOSe é 9,7; 8,3 e 11,3; respectivamente (Alderton *et al.*, 2001). Embora no presente estudo não tenhamos avaliado o estado da perfusão cerebral na presença de 7-NI, a administração sistêmica deste composto na dose de 50 mg/kg causa uma

diminuição no fluxo sanguíneo cerebral de quinze a vinte e oito por cento (Zagvazdin *et al.*, 1998), o que confirma a ação do 7-NI sobre a isoforma endotelial da NOS.

Embora pacientes com acidemia metilmalônica apresentem alterações comportamentais e bioquímicas, como convulsões e dano oxidativo (Fenton, & Rosenberg, 1995) compatíveis com o presente modelo experimental, é difícil extrapolar resultados obtidos com animais para o ser humano. Contudo, o papel do óxido nítrico na excitotoxicidade induzida pelo MMA, evidenciado no presente estudo, pode ser de grande valia para o entendimento da fisiopatologia desta doença bem como no estabelecimento novas condutas para tratamento dos pacientes com acidemia metilmalônica.

CAPÍTULO VI

CONCLUSÕES

VI- CONCLUSÕES

VI. 1. Conclusões Específicas

- O MMA induz carbonilação protéica;
- A administração de um inibidor não seletivo da enzima óxido nítrico sintase (L-NAME) exerce efeito bifásico sobre as convulsões e carbonilação protéica induzida pelo MMA.
- Há correlação significativa entre duração dos episódios convulsivos e inibição da enzima Na^+,K^+-ATPase, mas não entre duração dos episódios convulsivos e carbonilação protéica total induzida por MMA.
- Verificou-se um aumento na quantidade de nitrito e nitrato após a administração de MMA
- A administração de azul de metileno e 7-NI diminuiu os níveis de nitrito e nitrato, e preveniram o aumento de nitratos e nitritos induzidos por MMA.
- Enquanto a administração de azul de metileno atenuou, a administração de 7-NI potencializou as convulsões induzidas por MMA.
- Enquanto a administração de azul de metileno atenuou, a administração de 7-NI a carbonilação de proteínas e a inibição da Na^+, K^+- ATPase induzidas por MMA.
- A administração de L-arginina não potencializa as convulsões e aumento de nitratos induzidos por MMA.

VI. 2. Conclusão Geral

Este trabalho mostra, pela primeira vez, que o ácido metilmalônico aumenta a produção de óxido nítrico no cérebro, e que a administração de compostos que atuem no sistema nitrérgico, como o azul de metileno, pode ser de valia no tratamento de pacientes com acidemia metilmalônica, uma vez que inibe as convulsões e o dano oxidativo induzido por este ácido orgânico. Contudo, os resultados obtidos com inibidores da NOS foram controversos, provavelmente porque estes compostos não tinham a seletividade necessária para bloquear as respostas específicas ao subtipo de NOS estimulada pelo MMA. Embora o corpo de resultados obtido nesta tese mostra que a cascata do óxido nítrico é estimulada pelo MMA, estudos complementares com inibidores mais específicos das isoformas da NOS devem ser realizados para elucidar em que extensão o óxido nítrico medeia as convulsões induzidas por MMA, ou atua como um sistema contrarregulatório protetor.

CAPÍTULO VII

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