3.2. – MODULAÇÃO DA PRODUÇÃO DE ESPÉCIES REATIVAS AO ÁCIDO TIOBARBITÚRICO (TBARS) EM CÉREBRO DE RATOS *IN VITRO* POR INTERMEDIÁRIOS DO CICLO DE KREBS

Artigo 2

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

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

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

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

INTRODUCTION

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Animals

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

Tissue Preparation

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

Lipid Peroxidation Assay

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

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

Degradation of Deoxyribose

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

Chelating Properties of the Tricarboxilic Acid Intermediates Assay

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

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

Thiol Assay

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

Statistical Analysis

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

RESULTS

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

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

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

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

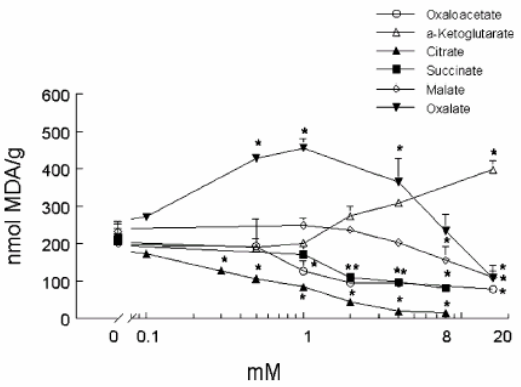

Fig. 1. Effect of citrate, succinate, malate, oxaloacetate, α-ketoglutarate and oxalate in basal- TBARS production on brain S1. Low-speed supernatant (S1) from brain was incubated for 60 min in a medium containing 10 mM Tris–HCl buffer, pH 7.4, in the presence of indicated concentrations of Krebs cycle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

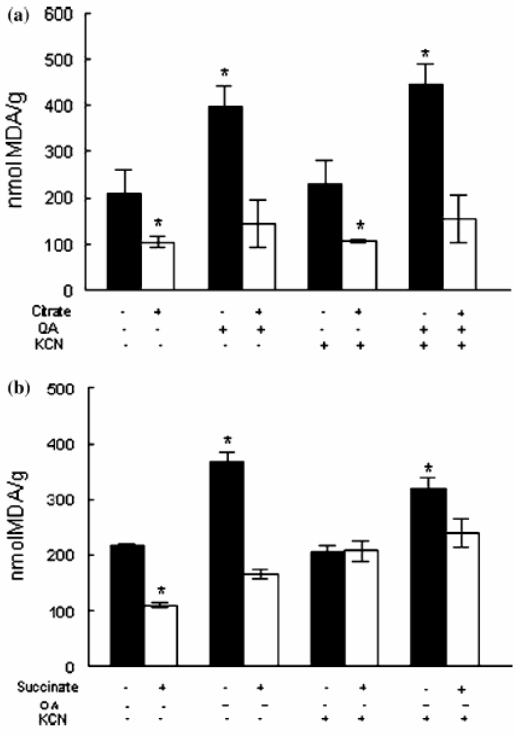

Fig. 2. Antioxidant effect of citrate and succinate against basal or QA induced lipid peroxidation. (a) Antioxidant effect of 0.5 mM citrate against basal TBARS production or 1 mM QA induced TBARS production, in the absence or presence of 1 mM KCN. (b) Antioxidant effect of 4 mM succinate against basal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

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

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

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

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

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

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

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

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

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

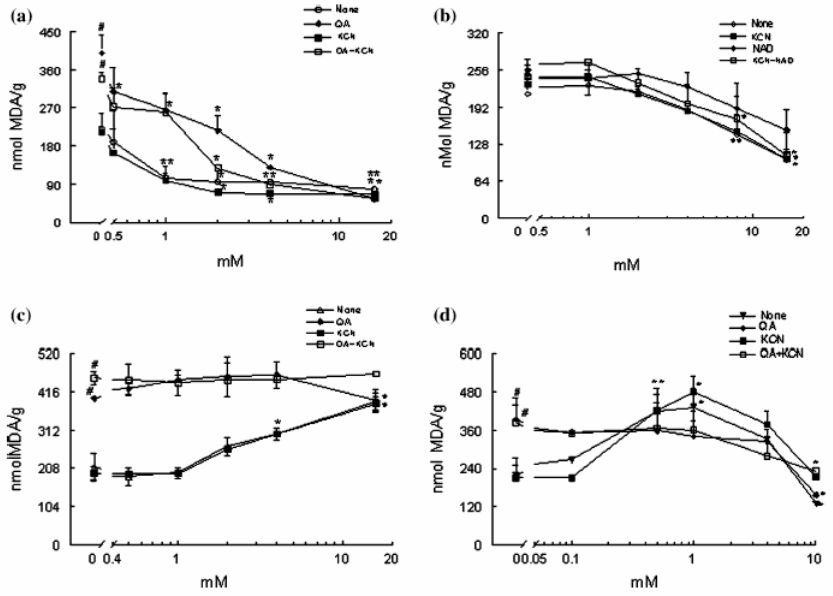

Fig. 3. Antioxidant effect of oxaloacetate and malate, and pro-oxidative effect of α-ketoglutarate and oxalate on rat brain S1. (a) Antioxidant effect of oxaloacetate (0.5 up to 16 mM) against basal or 1 mM QA- induced TBARS production, in the presence or absence of 1 mM KCN. (b) Antioxidant effect of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

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

Iron Chelating Properties of Krebs Cycle Intermediates

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

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

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

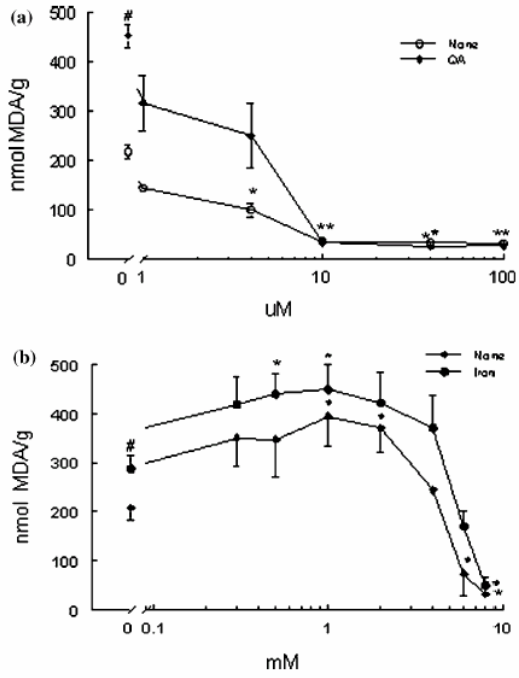

Fig. 4. Role of iron on the pro-oxidant effect QA in rat brain S1. (a) Effect of DFO (1 up to 100 μM) on basal or 3 mM QA-induced TBARS production. (b) Effect of QA (0.3 up to 10 mM) on basal and 0.3 μM iron- induced TBARS production. The values are expressed as nmol of MDA per gram of tissue. Data are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

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

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

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

Effect of Citrate and Succinate on Total–SH Content

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

DISCUSSION

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

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

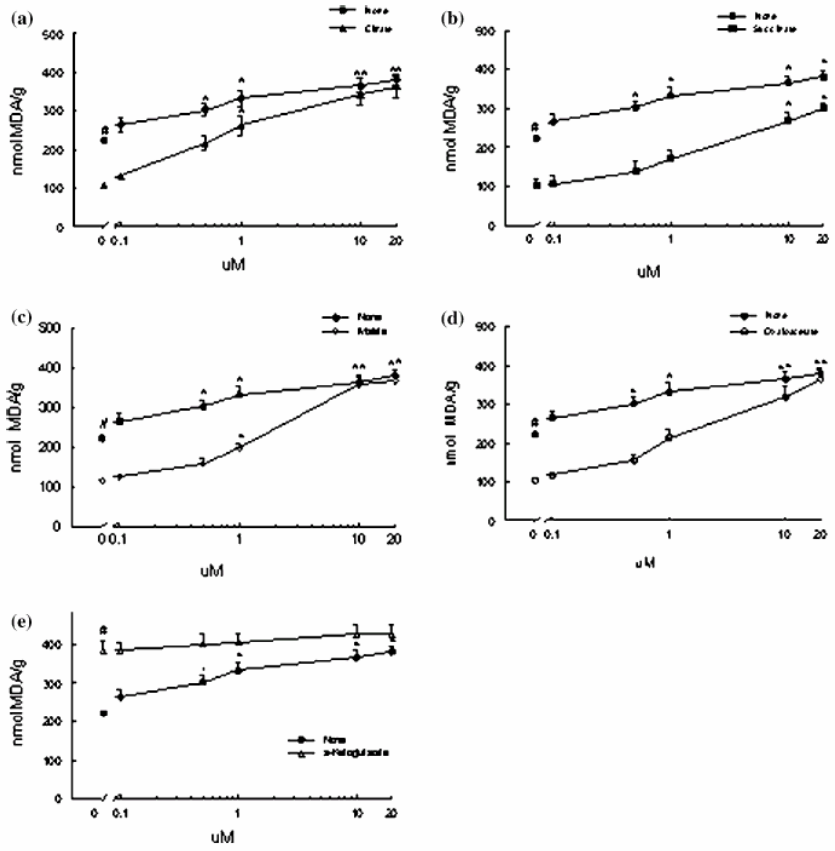

Fig. 5. Antioxidant actions of Krebs cycle intermediates against iron–induced TBARS production. (a) Effect of 0.5 mM citrate on basal or iron (0.1 up to 20 μM) induced TBARS formation. (b) Effect of 4 mM succinate on basal or iron (0.1 up to 20 μM) induced TBARS formation. (c) Effect of 16 mM malate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

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

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

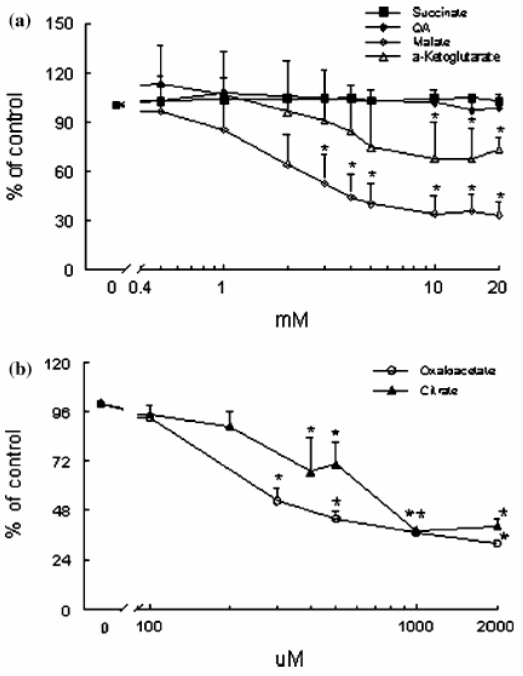

Fig. 6. Iron chelating properties of Krebs cycle intermediates. (a) Effect of QA, succinate, malate and α-ketoglutarate on colored iron–phenantroline complex formation. (b) Effect of oxaloacetate and citrate on colored iron–phenantroline complex formation. The values are expressed as % of control. Absorbance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

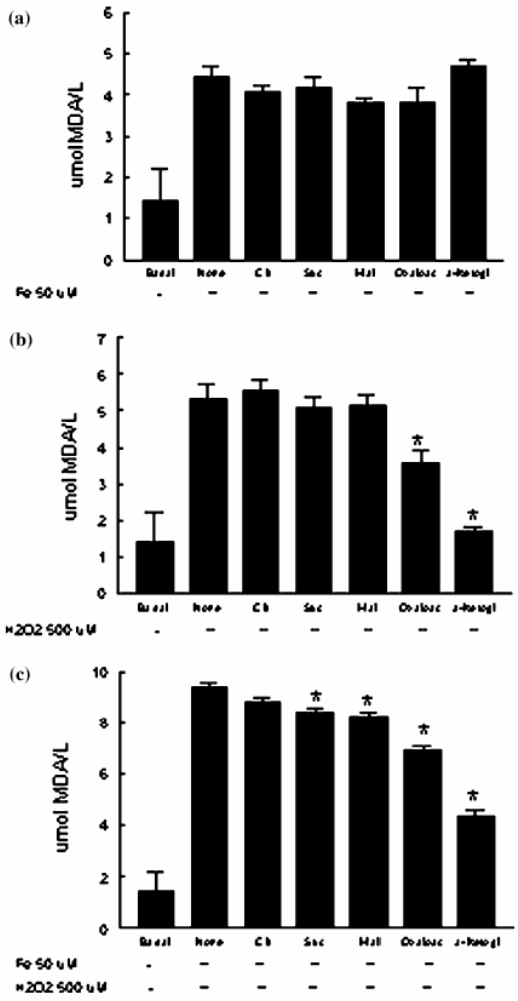

Fig. 7. Effect of Krebs cycle intermediates against deoxyribose degradation. (a) Effect of Krebs cycle intermediates against deoxyribose degradation induced by 50 μM Fe^{2+}. (b) Effect of Krebs cycle intermediates against deoxyribose degradation induced by 500 μM hydrogen peroxide. (c) Effect of Krebs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

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

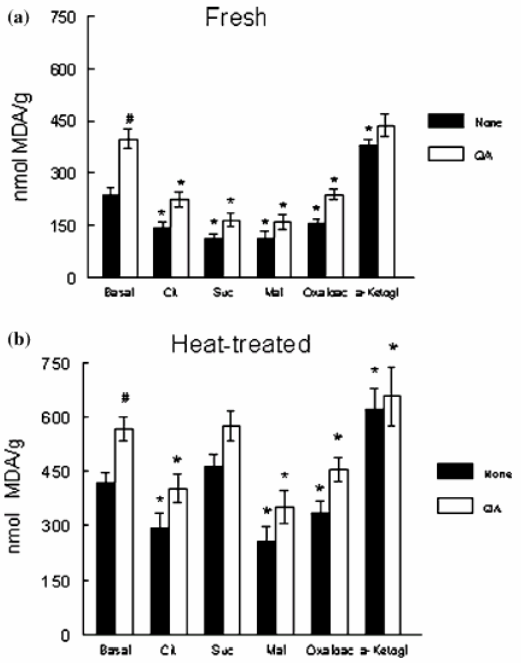

Fig. 8. Effect of Krebs cycle intermediates when heat-treated preparations are used. (a) Effect of Krebs cycle intermediates on basal and 1 mM QA induced TBARS production, using fresh tissue preparations. (b) Effect of Krebs cycle intermediates on basal and 1 mM QA induced TBARS production, using heat-treated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

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

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

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

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

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4. DISCUSSÃO

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

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

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

geração de radicais livres. Essa idéia está de acordo com dados prévios que demonstraram claramente que uma razão adequada Fe^{2+}/ Fe^{3+} é essencial para que o ferro exerça seus efeitos pró-oxidantes (Tang e cols., 1997; Caro e Cederbaum, 2004).

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

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

Os resultados apresentados no **Artigo 2** indicam que o efeito antioxidante dos intermediários do Ciclo e Krebs varia, dependendo da sua estrutura e da sua concentração

(Figura 1, Artigo 2). O citrato, o sucinato, o oxaloacetato e o malato, preveniram significativamente a produção de TBARS basal, ou induzida por AQ ou, ainda, induzida por Fe^{2+} (Figuras 2, 3 e 5, Artigo 2) em S1 de cérebro de ratos. O α-cetoglutarato foi capaz de induzir *per se* um aumento na produção de TBARS (Figura 3, Artigo 2), enquanto que o oxalato, o qual tem semelhança estrutural com os intermediários do Ciclo de Krebs, teve um efeito bifásico (Figura 3, Artigo 2), isto é, em baixas concentrações (0,5 – 4 mM) o oxalato induziu um aumento na produção de TBARS, mas em altas concentrações foi capaz de inibi-la. Porém, o(s) mecanismo(s) pelo(s) qual(is) os intermediários do Ciclo de Krebs exercem seus efeitos antioxidantes é distinto e depende do intermediário usado.

O efeito do sucinato mostrou-se sensível à temperatura, sendo abolido quando o S1 de cérebro de ratos foi pré-tratado por 100ºC por 10 min (Figura 8, Artigo 2). Da mesma forma, o efeito do sucinato foi completamente abolido quando cianeto de potássio foi adicionado ao meio (Figura 2B, Artigo 2). Por outro lado, o efeito do citrato, malato e oxaloacetato não foram alterados pelo pré-tratamento a alevadas temperaturas (Figura 8, Artigo 2). Considerando o efeito do KCN, bem como o efeito de pré-tratamento a 100ºC, supomos que o efeito do sucinato é dependente de alguma atividade enzimática, enquanto que o efeito dos demais intermediários não.

Compostos capazes de quelar ferro podem prevenir a produção de TBARS. Assim a DFO, um quelante clássico de íons férricos (Fe^{3+}), reduziu consideravelmente a produção de TBARS em S1 de cérebro de ratos (Figura 4A, Artigo 2). Da mesma maneira que a DFO, os intermediários do Ciclo de Krebs, exceto o sucinato, exibiram atividade quelante de íons ferrosos (Fe^{2+}) (Figura 6, Artigo 2). Porém, somente o citrato e o oxaloacetato exibiram atividade quelante em concentrações fisiológicas. Sendo assim, parte da atividade antioxidante desses intermediários pode ser atribuída a suas atividades quelantes de íons ferrosos. Contudo, o oxaloacetato foi um agente quelante mais eficiênte que o citrato, enquanto que esse reduziu a produção de TBARS mais eficientemente que o oxaloacetato. Parte dessas discrepâncias pode ser atribuída ao fato que alguns complexos de ferro, tais como os complexos ferro/citrato, apresentam atividade redox (Chiueh, 2001).

Os resultados dos ensaios da degradação da desoxirribose induzida por ferro e peróxido de hidrogênio demonstram que os intermediários do Ciclo de Krebs não são capazes de prevenir a degradação induzida por ferro. Porém, o oxaloacetato e o α-

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

Compostos capazes de interagir com íons ferro (agentes quelantes) podem tanto inibir quanto induzir a produção de TBARS. O efeito pró ou antioxidante dos mesmos depende da habilidade desses em modular uma adequada razão Fe^{2+}/Fe^{3+} no meio (Minotti e Aust, 1987; Oubidar e cols., 1994; Chiueh, 2001; Caro e Cederbaum, 2004). Sendo assim, a adição de um quelante pode alterar a razão Fe^{2+}/Fe^{3+}, a qual tem atividade máxima quando a proporção for 1:1 (Fe^{2+}: Fe^{3+}) (Tang e cols., 1997; Caro e Cederbaum, 2004), fazendo com que a geração de radicais livres aumente (se for razão 1:1) ou diminua. Por exemplo, o quelante 2,2' dipiridil (o qual liga-se firmemente com Fe^{2+} inibindo seu ciclo redox), e a DFO (a qual liga-se fortemente somente a Fe^{3+} inibindo o ciclo redox do ferro e bloqueando sua atividade catalítica) possuem atividade antioxidante por impedir a formação de uma adequada razão Fe^{2+}/ Fe^{3+} (Huang e cols., 2002), impedindo dessa forma a iniciação, bem como a propagação das reações de peroxidação lipídica dependentes de íons ferro. Considerando o que foi exposto, sugerimos que o citrato age como um potente antioxidante quando o S1 de cérebro de ratos é usado, porém não tem efeito quando o Fe^{2+} e/ou peróxido de hidrogênio são usados em um sistema para gerar radicais livres. Essas

observações estão de acordo com os resultados de Gutteridge, os quais mostram que os complexos ferro/citrato foram ativos frente à degradação da desoxirribose induzida por ferro e peróxido de hidrogênio. Gutteridge mostrou ainda que aumentando a razão ferro/citrato ocorre uma redução na degradação da desoxirribose, obtendo degradação máxima quando a razão ferro/citrato aproxima-se de 1:1 (Gutteridge, 1991).

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

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

5. CONCLUSÕES

De acordo com os resultados apresentados nesta dissertação podemos concluir que:

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

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

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

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

- O efeito antioxidante do sucinato está relacionado à atividade da SDH.

6. PERSPECTIVAS

Baseado nos resultados apresentados nessa dissertação faz-se necessário:

- Estudar o efeito dos intermediários do Ciclo de Krebs frente a outros sistemas pró-oxidantes, tais como em presença de malonato, na tentativa de melhor elucidar o(s) mecanismo(s) pelo(s) qual(is) esses exercem seus efeitos *in vitro*.

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

- Estudar os mecanismos envolvidos na atividade do oxalato sobre a produção de TBARS *in vitro*.

- Investigar o efeito do oxalato na produção de TBARS em preparações de fígado e rim de ratos.

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

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

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