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Oxidative stress induction by *cis*-4-decenoic acid: Relevance for MCAD deficiency

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Abstract

Patients affected by medium-chain acyl-CoA dehydrogenase deficiency (MCADD) suffer from acute episodes of encephalopathy whose underlying mechanisms are poorly known. The present work investigated the *in vitro* effect of *cis*-4-decenoic acid (cDA), which accumulates in MCADD, on important parameters of oxidative stress in cerebral cortex of young rats. cDA markedly induced lipid peroxidation, as verified by the increased levels of spontaneous chemiluminescence and thiobarbituric acid-reactive substances. Furthermore, cDA significantly increased carbonyl formation and sulphydryl oxidation, which is indicative of protein oxidative damage, and promoted 2′,7′-dihydrodichlorofluorescein oxidation. It was also observed that the non-enzymatic tissue antioxidant defenses were decreased by cDA, whereas the antioxidant enzyme activities catalase, superoxide dismutase and glutathione peroxidase were not altered. Moreover, cDA-induced lipid peroxidation and GSH reduction was totally blocked by free radical scavengers, suggesting that reactive species were involved in these effects. The data indicate that oxidative stress is induced by cDA in rat brain *in vitro* and that oxidative damage might be involved in the pathophysiology of the encephalopathy in MCADD.

Keywords: *Reactive oxygen species, cis-4-decenoic acid, MCAD deficiency, lipid oxidation, protein oxidation, oxidative stress*

Introduction

Medium chain acyl-CoA dehydrogenase (MCAD; E.C. 1.3.99.3) deficiency (MCADD) is the most common inherited defect of fatty acid β-oxidation, with an approximate prevalence of 1:10 000 newborns [1,2]. The MCAD enzyme is responsible for the first step in mitochondrial β-oxidation of CoA esters of medium-chain fatty acids. A defect of this enzyme activity leads to predominant tissue accumulation of the medium-chain fatty acids octanoic (OA), decanoic (DA) and *cis*-4-decenoic (cDA), as well as their glycine and L-carnitine derivatives [3]. Clinical presentation of MCADD is related to fasting and other conditions with increased metabolic stress, that precipitate acute symptoms such as seizures, drowsiness or lethargy that may develop into coma or even sudden death [4]. Progressive encephalopathy with brain abnormalities is also found in this disorder [4–6]. Although most patients present during early infancy, some case reports have described neonatal [7–9] and adult presentations [10–12]. Treatment for ill patients consists of high amounts of glucose and L-carnitine administration during the acute episodes, as

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well as fat restriction, fasting avoidance and L-carnitine supplementation after recovery [2,13]. MCADD is often misdiagnosed as Reye syndrome because the neurological manifestations of these disorders are very similar [14]. Furthermore, OA accumulates in both MCADD and Reye syndrome, but the presence of cDA is pathognomonic of MCADD [15].

There are only few studies showing neurotoxic effects of OA, DA and cDA *in vitro* and *in vivo*. It was previously demonstrated that OA *in vivo* administration alter organic acid transport in rat choroid plexus, leading to impairment of the transchoroidal clearance of OA and similar compounds [16]. It was postulated that this effect may contribute to accumulation of medium-chain fatty acids (MCFA) in the brain and cerebral spinal fluid and possibly to the encephalopathy of patients affected by Reye syndrome. Other *in vitro* studies demonstrated that OA, DA and cDA were able to impair several parameters of energy metabolism in cerebral cortex of rats including the respiratory chain, mitochondrial creatine kinase and Na^{+},K^{+}-ATPase activities [17–19], these effects being more pronounced with cDA. It was then presumed that this fatty acid may be the main neurotoxin in MCADD. Furthermore, since cDA was also able to elicit lipid peroxidation, it was suggested that reactive species generation could be responsible for the inhibitory effects of this fatty acid on the activities of these critical enzymes [19].

These observations led us to investigate in more details the *in vitro* effects of cDA on a wide spectrum of oxidative stress parameters, namely thiobarbituric acid-reactive substances (TBA-RS), spontaneous chemiluminescence, carbonyl content, sulphydryl oxidation, oxidation of mitochondrial membrane sulphydryl-bound protein groups (SBPG), 2′,7′-dihydrodichlorofluorescein (DCFH) oxidation, total-radical trapping antioxidant potential (TRAP), total antioxidant reactivity (TAR), reduced glutathione (GSH) levels, as well as on the activities of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in homogenates from cerebral cortex of young rats in the hope to contribute to a better understanding of the mechanisms underlying the neurological dysfunction found in MCAD deficient patients. We also tested the role of antioxidants on some effects elicited by cDA.

Material and methods

Reagents

All chemicals were purchased from Sigma (St. Louis, MO), except for *cis*-4-decenoic acid (cDA) which was prepared by Dr Ernesto Brunet, Madrid, Spain, with 99% purity. cDA, decanoic acid (DA) and oleic acid (OLA) were dissolved on the day of the experiments in the incubation medium used for each technique with pH adjusted to 7.4. The final concentrations of the acids in the medium ranged from 0.1–1.0 mM.

Animals

A total of 92 30-day-old Wistar rats obtained from the Central Animal House of the Departamento de Bioquímica, ICBS, UFRGS, were used. Rats were kept with dams until weaning at 21 days of age. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature ($22 \pm 1^{\circ}$C) colony room. The 'Principles of Laboratory Animal Care' (NIH publication no. 80-23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were made to minimize the number of animals used and their suffering.

Tissue preparation and incubation

On the day of the experiments the animals were sacrificed by decapitation without anaesthesia and the brain was rapidly excised on a Petri dish placed on ice. The olfactory bulbs, pons, medulla, cerebellum and striatum were discarded and the cerebral cortex was peeled away from the subcortical structures, weighed and homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at $750 \times g$ for 10 min at 4°C to discard nuclei and cell debris [20]. The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl at 37°C for 1 h with cDA at concentrations of 0.1, 0.5 or 1 mM, 1.0 mM DA or 1 mM OLA. Controls did not contain this metabolite in the incubation medium. Immediately after incubation, aliquots were taken to measure the values of TRAP, TAR, GSH, TBA-RS, chemiluminescence, carbonyl and sulphydryl content, DCFH oxidation, as well as the activities of CAT, SOD and GPx.

Oxidation of protein-bound sulphydryl (thiol) groups (PBSG) was studied in purified mitochondrial membrane preparations. The dissected cerebral cortex was homogenized (1:5 w/v) in 10 mM Tris, pH 7.4 containing 0.25 m sucrose. The homogenates were centrifuged at $650 \times g$ for 10 min to discard nuclei and cell debris. After a new centrifugation at $25\,000 \times g$ for 15 min, the resulting pellet containing the mitochondria was homogenized in 0.1 m Tris, pH 8.0 and the aliquots were stored at -70°C. On the day of the experiment, the aliquots were frozen/thawed three times and centrifuged at $15\ 000 \times g$

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for 2 min to collect the mitochondrial membranes. The resulting pellet was washed three times with 6.5% TCA with centrifugation at 15 000 × *g* for 2 min. The final pellet was resuspended in 0.5 m Tris buffer, pH 8.3, containing 0.5 mM EDTA and used to measure PBSG content.

Preparation of mitochondrial fractions

Mitochondrial fractions were prepared from cerebrum (total brain excluding cerebellum, olfactory bulbs, pons and medulla) of 30-day-old rats. The cerebrum was homogenized in 10 volumes of 5 mM potassium phosphate buffer, pH 7.4, containing 0.3 m sucrose, 5 mM MOPS, 1 mM EGTA and 0.1% bovine serum albumin. The homogenate was centrifuged at 1 500 × *g* for 10 min at 4°C and the pellet was discarded. The supernatant was then centrifuged for a further 10 min at 4°C at 15 000 × *g* in order to isolate the mitochondria present in the pellet, which was finally suspended in the same buffer. Disrupted mitochondrial fractions obtained by freezing/thawing three times were incubated at 37°C for 1 h with cDA at concentrations of 1.0 mM. Immediately after incubation, aliquots were used to measure TBA-RS.

Isolation of sub-mitochondrial particles

Sub-mitochondrial particles were prepared at 4°C from frozen and thawed mitochondria (20 mg protein/ml) according to Poderoso et al. [21]. The obtained sub-mitochondrial particles were washed twice with 140 mM KCl, 20 mM Tris-HCl, pH 7.4, and suspended in the same medium. The suspended particles were then incubated at 37°C for 1 h with 1 mM cDA. Immediately after incubation, aliquots from this preparation were used to measure superoxide formation.

Thiobarbituric acid-reactive substances (TBA-RS) levels

TBA-RS was determined according to the method of Esterbauer and Cheeseman [22]. Briefly, 300 μL of cold 10% trichloroacetic acid were added to 150 μL of cDA pre-treated cerebral cortex supernatants and centrifuged at 300 × *g* for 10 min. Three hundred microlitres of the supernatant were transferred to a pyrex tube and incubated with 300 μL of 0.67% TBA in 7.1% sodium sulphate in a boiling water bath for 25 min. The tubes containing the mixture were allowed to cool in running tap water for 5 min. The resulting pink-stained TBA-RS was determined in a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane and each curve point was subjected to the same treatment as supernatants. Some experiments were performed in the presence or absence of reduced glutathione (GSH; 100 μm or 1 mM), melatonin (MEL; 200 μm or 1 mM), the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 500 μm or 1 mM), trolox (TRO; 1.5 μM or 10 μM), desferoxamine (DFO; 250 nM), L-carnitine (CAR; 1 mM), catalase (CAT; 2.5 mU/mL[1] or 10 mU/mL) plus superoxide dismutase (SOD; 2.5 mU/mL or 10 mU/mL), GM1 (10 μM), taurine (TAU; 1 mM) or creatine (Cr; 3 mM). Doses of these antioxidants were chosen according to previous works [22–24]. TBA-RS values were calculated as nmol of TBA-RS/mg protein and expressed as percentage of control.

Chemiluminescence

Samples were assayed for spontaneous chemiluminescence in a dark room by the method of Gonzalez et al. [23]. Incubation flasks contained 3.5 mL of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl were counted for background chemiluminescence during 5 min. An aliquot of 500 μL of cortical supernatant was immediately added and chemiluminescence was measured for 30 min at room temperature. The background chemiluminescence was subtracted from the total value and the results were calculated as cpm/mg protein and expressed as percentage of control.

Determination of protein carbonyl formation content

PCF (protein carbonyl content formation), a marker of oxidized proteins, was measured spectrophotometrically according to Reznick and Packer [24]. One hundred microlitres of the aliquots from the incubation were treated with 400 μL of 10 mM 2,4-dinitrophenylhidrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank control) and left in the dark for 1 h. Samples were then precipitated with 500 μL 20% TCA and centrifuged for 5 min at 10 000 × *g*. The pellet was then washed with 1 mL ethanol:ethyl acetate (1:1, v/v) and re-dissolved in 550 μL 6 m guanidine prepared in 2.5 N HCl Then, the tubes were incubated at 37°C for 5 min to assure the complete dissolution of the pellet and the resulting sample was determined at 365 nm. The difference between the DNPH-treated and HCl-treated samples was used to calculate the carbonyl content. The results were calculated as nmol of carbonyls groups/mg of protein, using the extinction coefficient of 22 000 × 106 nmol/mL for aliphatic hydrazones.

2′,7′-dihydrodichlorofluorescein (DCFH) oxidation

Reactive species production was assessed according to LeBel et al. [25] by using 2′,7′-dihydrodichlorofluorescein diacetate (DCF-DA). DCF-DA prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl was incubated with the pre-treated cerebral cortex supernatants during 30 min at 37°C.

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DCF-DA is enzymatically hydrolysed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of reactive species (RS). The DCF fluorescence intensity parallels to the amount of RS formed. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. Calibration curve was performed with standard DCF (0.25–10 μM) and the levels of RS were calculated as pmol DCF formed/mg protein.

Superoxide content

Superoxide production was determined spectrophotometrically according to Poderoso et al. [21] after exposition of sub-mitochondrial particles to 1 mM cDA. The assay is based on superoxide-dependent oxidation of epinephrine to adenochrome at 37°C (E_{480nm} = 4.0 mM/cm). The reaction medium consisted of 230 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, pH 7.4, 0.1 mM catalase, 1 mM epinephrine and 7 mM succinate. Superoxide dismutase was used at 0.1–0.3 mM final concentrations as a negative control to confirm assay specificity.

Total radical-trapping antioxidant potential (TRAP)

TRAP, representing the total non-enzymatic antioxidant capacity of the tissue, was determined by measuring the chemiluminescence intensity of luminol induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) according to the method of Lissi et al. [26]. The reaction mixture containing 4 mL 10 mM ABAP dissolved in 0.1 m glycine buffer, pH 8.6 and 10 μL of luminol (4 mM) generates, at room temperature, an almost constant light intensity corresponding to free radical formation, which was measured in a Wallac 1409 liquid scintillation counter. This was considered to be the initial chemiluminescence values. Then, 30 μL of 300 μM trolox (soluble α-tocopherol analogue) or 30 μL of cortical supernatants were added to the reaction medium. The addition of trolox or supernatants provokes a marked reduction of the light intensity, which is maintained for a certain period after which light intensity rapidly increase. This period corresponds to induction time (IT) and represents TRAP measurement. IT is directly proportional to the antioxidant capacity of the tissue and the IT of each sample was compared with the IT of trolox. TRAP values were expressed as nmol trolox/mg of protein.

Total antioxidant reactivity (TAR)

TAR, which represents the quality of the tissue antioxidants, was determined by measuring the luminol chemiluminescence intensity induced by ABAP according to the method of Lissi et al. [27]. The chemiluminescence value was measured after 1 min after adding 4 mL 2 mM ABAP (in 0.1 M glycine buffer, pH 8.6) and 10 μL of luminol into a glass scintillation vial (initial chemiluminescence). Ten microlitres of 10–100 μM trolox (calibration curve) or brain supernatants, which decrease light intensity, were then added and chemiluminescence was measured after 60 s (final chemiluminescence). The ratio between the initial and the final chemiluminescence values is used to calculate TAR measurement. TAR values were expressed as nmol trolox/mg of protein.

Reduced glutathione (GSH) content

Reduced glutathione (GSH) concentrations were measured according to Browne and Armstrong [28]. cDA pre-treated cerebral cortex supernatants were diluted in 20 volumes of (1:20, v/v) 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. One hundred microlitres of this preparation were incubated with an equal volume of *o*-phthaldialdehyde (1 mg/mL methanol) at room temperature during 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. Some experiments were performed in the presence or absence of melatonin (MEL; 1 mM), the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 1 mM), trolox (TRO; 10 μM) or catalase (CAT; 2.5 mU/mL or 10 mU/mL) plus superoxide dismutase (SOD; 2.5 mU/mL or 10 mU/mL). Calibration curve was prepared with standard GSH (0.01–1 mM) and the concentrations were calculated as nmol/mg protein.

The oxidation of a commercial solution of GSH (1 mM) was also tested by exposing this solution to 1.0 mM cDA for 60 min in a medium devoid of brain supernatants. After cDA exposition, 7.4 mM *o*-phthaldialdehyde was added to the vials and the mixture was incubated at room temperature during 15 min.

Sulphydryl (thiol) group oxidation

This assay is based on the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm [29]. Briefly, 40 μL of mitochondrial membrane suspension or 120 μL of cortical supernatant were incubated at 37°C for 1 h with cDA at concentrations of 0.1, 0.5 and 1 mM. Then, 1 mL of 0.1 mM DTNB was added. This was followed by 30 min incubation at room temperature in a dark room. Absorption was measured at 412 nm. Some experiments were carried out in the presence of 1 mM decanoic (DA) or 1 mM oleic (OLA) acids. The protein-bound sulphydryl content is inversely correlated to oxidative damage to

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proteins. Results were reported as nmol TNB/mg protein.

Catalase (CAT) activity

CAT activity was assayed according to Aebi [30] by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and the supernatants containing 0.1–0.3 mg protein/mL. One unit (U) of the enzyme is defined as 1 mol of H_2O_2 consumed per minute. The specific activity was expressed as U/mg protein.

Superoxide dismutase (SOD) activity

SOD activity was determined according to Bannister and Calabrese [31] using a spectrophotometric assay based on superoxide-dependent oxidation of epinephrine to adrenochrome at 32°C. Absorption was measured at 480 nm (4.0/mMcm). The reaction medium consisted of 50 mM glycine buffer pH 10.2, 0.1 mM catalase and 1 mM epinephrine. SOD specific activity is represented as U/mg protein.

Glutathione peroxidase (GPx) activity

GPx activity was measured according to Wendel [32] using tert-butylhydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium containing 100 mM potassium phosphate buffer/ethylenediaminetetraacetic acid 1 mM, pH 7.7, 2 mM, glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, 0.1 mM NADPH and the supernatant containing 0.2–0.3 mg protein/mL. One GPx unit (U) is defined as 1 mol of NADPH consumed per minute. The specific activity was calculated as mU/mg protein.

Protein determination

Protein was measured by the method of Lowry et al. [33] using bovine serum albumin as standard.

Statistical analysis

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

Results

cDA increased lipid peroxidation in rat cerebral cortex

We initially tested the influence of cDA on the lipid peroxidation parameters TBA-RS levels and chemiluminescence. We used a total of 12 animals for these experiments. Figure 1 shows that when cortical homogenates were exposed for 60 min to cDA, at 0.5 mM and 1 mM concentrations, TBA-RS levels significantly increased up to 58% ($F_{(3,20)} = 32.8$; $p < 0.001$) in a dose-dependent manner ($\beta = 0.87$; $p < 0.001$). Similarly, we found that cDA significantly increased the chemiluminescence values up to 39% ($F_{(3,20)} = 5.26$; $p < 0.01$). These results indicate that *in vitro* lipid peroxidation was increased in cerebral cortex exposed to cDA.

Next, we incubated cerebral cortex homogenates from four rats during 15, 30, 45 or 60 min in the presence or absence of cDA (0.1–1.0 mM) and observed a significant stimulatory effect of 1.0 mM cDA on lipid peroxidation (TBA-RS) at 30, 45 and 60 min incubation and at 60 min incubation with 0.5 mM cDA (Figure 2).

We also pre-incubated brain cortical homogenates for 15 min with reduced glutathione (GSH; 100 μM), melatonin (MEL; 200 μM), the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 500 μM), trolox (TRO; 1.5 μM), desferoxamine (DFO; 250 nM), L-carnitine (CAR; 1 mM), catalase (CAT; 2.5 mU/mL) plus superoxide dismutase (SOD; 2.5 mU/mL), GM1 (10 μM), taurine (TAU; 1 mM) or creatine (Cr; 3 mM). cDA (1.0 mM) was then added and incubation proceeded

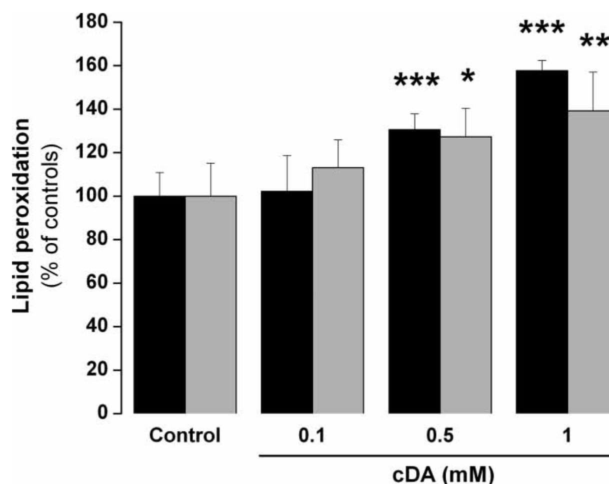

Figure 1. *In vitro* effect of *cis*-4-decenoic acid (cDA) on the lipid peroxidation parameters thiobarbituric acid-reactive substances (TBA-RS; black bars) and spontaneous chemiluminescence (gray bars) in rat cerebral cortex. Values are means ± standard deviation for six independent experiments performed in duplicate or triplicate and are expressed as % of controls (Controls: Chemiluminescence 1019 ± 154 cpm/mg protein; TBA-RS levels 3.42 ± 0.37 nmol/mg protein). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to controls (Duncan multiple range test).

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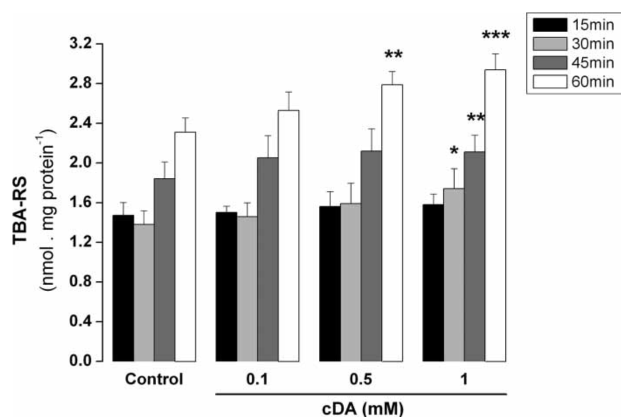

Figure 2. *In vitro* effect of exposition time on *cis*-4-decenoic acid (cDA) stimulatory effect on thiobarbituric-acid reactive substances (TBA-RS) in rat cerebral cortex. Cortical homogenates were incubated for 15, 30, 45 or 60 min with cDA (0.1–1.0 mM) and TBA-RS levels measured afterwards. Values are means ± standard deviation for four independent experiments performed in triplicate and are expressed as nmol/mg protein. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to controls (Duncan multiple range test).

for 60 min, after which TBA-RS levels were measured. For these experiments a total of 15 rats were used. We can observe in Figure 3A that TAU, Cr and GM1, at the common doses used as antioxidants, prevented cDA-induced increased lipid peroxidation ($F_{(11,62)} = 3.35$; $p < 0.01$). In contrast, GSH, TRO, CAT plus SOD, MEL, L-NAME, CAR and DFO were not able to prevent cDA increase of TBA-RS values (results not shown). However, higher doses of GSH (1 mM), TRO (10 μM) and CAT plus SOD (10 mU/mL of each enzyme), but not MEL (1 mM) and L-NAME (1 mM), also prevented cDA-induced increase of TBA-RS ($F_{(11,55)} = 25.6$; $p < 0.001$) (Figure 3B).

We also verified that concentrations as high as 1.0 mM of cDA did not change TBA-RS levels in mitochondrial preparations, indicating that generation of oxidants by this organic acid causing lipid damage occurred via cytosolic rather than mitochondrial mechanisms (results not shown).

cDA induces protein oxidative damage in rat cerebral cortex

We also evaluated the effect of cDA on carbonyl formation and sulphydryl oxidation in cortical homogenates (protein oxidation). Brains from 11 animals were used in these experiments. We found that protein carbonyl groups were increased (up to 67%) in cortical homogenates in the presence of cDA ($F_{(3,16)} = 4.72$; $p < 0.05$) in a dose-dependent manner ($\beta = 0.68$; $p < 0.001$) (Figure 4A). cDA also induced significant sulphydryl oxidation (31%) at 1 mM ($F_{(3,18)} = 6.76$; $p < 0.01$) (Figure 4B), similarly to OLA which decreased the sulphydryl groups by ~54%. In contrast, DA did not alter this parameter.

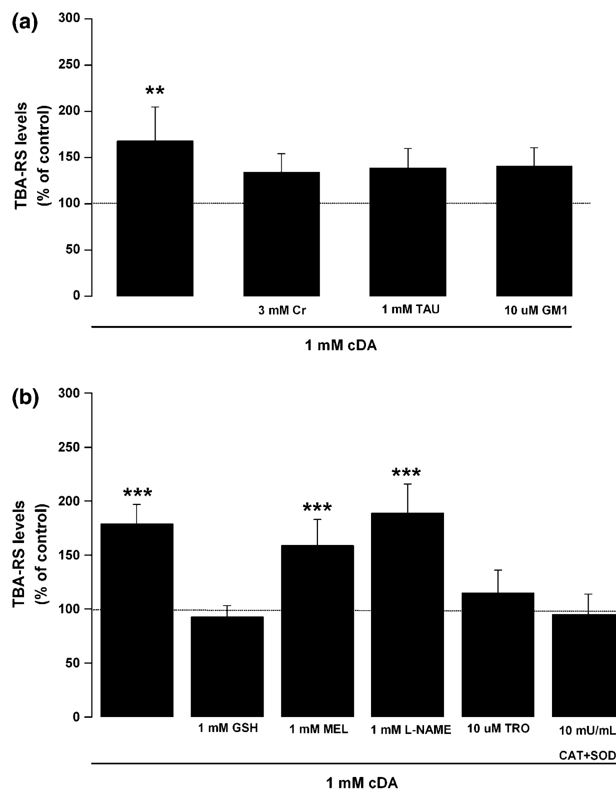

Figure 3. *In vitro* effect of creatine (Cr), taurine (TAU) and GM1 (A) and of the antioxidants GSH, MEL, L-NAME, TRO and CAT+SOD (B) on *cis*-4-decenoic acid (cDA)-induced increase of thiobarbituric-acid reactive substances (TBA-RS) in rat cerebral cortex. Cortical homogenates were pre-incubated for 15 min with the antioxidants before the addition of 1 mM cDA. Values are means ± standard deviation for four independent experiments performed in triplicate and are expressed as % of control (Controls: 3.99 ± 0.67 nmol/mg protein). **$p < 0.01$; ***$p < 0.001$ compared to controls (Duncan multiple range test).

cDA provokes oxidation of DCFH

Next, we assessed the influence of cDA on DCFH oxidation in cerebral cortex from six rats. We observed that cDA significantly increased (up to 28%) DCFH oxidation ($F_{(3,20)} = 7.86$; $p < 0.001$) in a dose-dependent manner ($\beta = 0.73$; $p < 0.001$) (Figure 5).

cDA does not affect superoxide generation

We also evaluated superoxide generation in submitochondrial particles from cerebral cortex supernatants exposed for 1 h to 1.0 mM cDA and observed that this fatty acid did not increase superoxide content (results not shown).

Reduction of brain non-enzymatic antioxidant defenses by cDA

We also investigated the *in vitro* effect of cDA on the non-enzymatic antioxidant defenses, by measuring TRAP, TAR and GSH levels. For these experiments a total of 22 rats were used. cDA, at concentrations of 0.5 mM and higher, significantly diminished TRAP,

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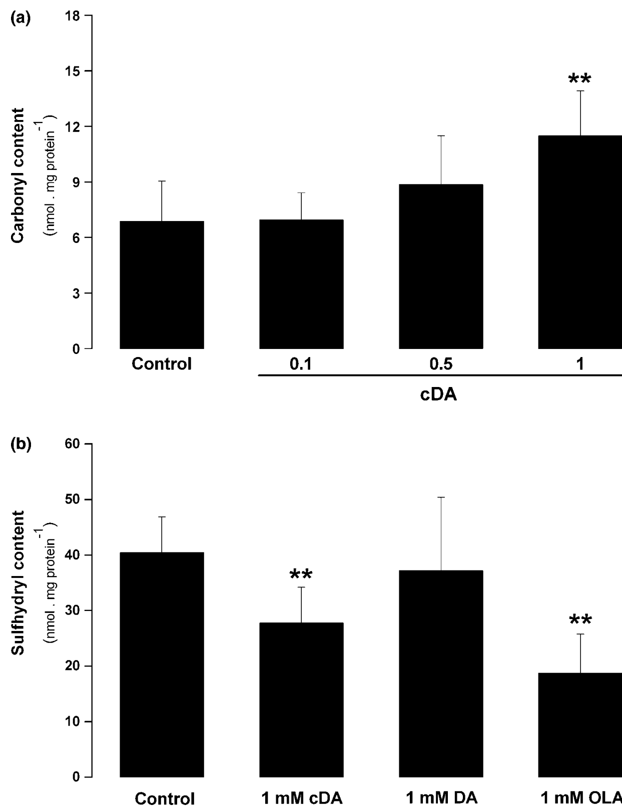

Figure 4. *In vitro* effect of *cis*-4-decenoic acid (cDA) on carbonyl (A) and sulphydryl content (B) in rat cerebral cortex. Values are means ± standard deviation for five independent experiments performed in triplicate and are expressed as nmol/mg protein. $^{**}p < 0.01$ compared to control (Duncan multiple range test).

TAR and GSH values (up to 35%) in rat cortical homogenates in a dose-dependent manner (TRAP: ($F_{(3,16)} = 8.87$; $p < 0.001$) ($\beta = 0.77$; $p < 0.001$); TAR: ($F_{(3,28)} = 10.7$; $p < 0.001$) ($\beta = 0.71$; $p < 0.001$); GSH: ($F_{(3,32)} = 14.2$; $p < 0.001$) ($\beta = 0.75$;

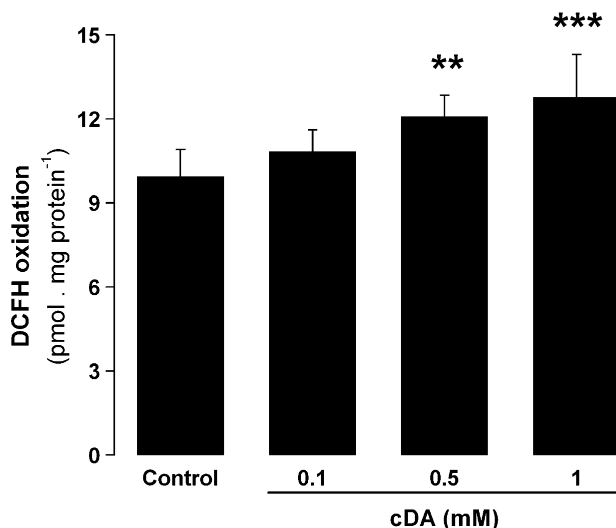

Figure 5. *In vitro* effect of *cis*-4-decenoic acid (cDA) on DCFH oxidation in rat cerebral cortex. Values are means ± standard deviation for six independent experiments performed in triplicate and are expressed as pmol/mg protein. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control (Duncan multiple range test).

$p < 0.001$)) (Figure 6). It was also observed that cDA-induced GSH reduction was totally prevented by the scavengers MEL and TRO, but not by CAT plus SOD ($F_{(4,29)} = 13.8$; $p < 0.001$). Taken together, the data indicate a marked reduction of the brain non-enzymatic antioxidant defenses by cDA. Furthermore, cDA-induced reduction of TRAP (total tissue non-enzymatic antioxidant defenses) was inversely correlated with TBA-RS values (a lipid peroxidation parameter) ($\beta = -0.69$; $p < 0.001$), suggesting that free radicals inducing lipid peroxidation were probably responsible for the decrease of the non-enzymatic antioxidant defenses (Figure 7).

The next set of experiments was designed to investigate whether the cDA-induced decrease of brain GSH levels was due to a direct oxidative attack, rather than due to promotion of free radicals. We therefore exposed commercial GSH, as well as membrane protein-bound sulphydryl groups (PBSG) purified from mitochondrial fractions to 0.1–1.0 mM cDA in the absence of tissue supernatants. It was observed that cDA by itself did not oxidize free GSH or PBSG, suggesting that cDA *per se* is not a direct oxidant agent, but elicits oxidative damage probably via free radical generation (Table I).

Antioxidant enzyme activities were not affected by cDA

Finally, the *in vitro* effect of cDA on catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were examined by pre-incubating cortical homogenates for 60 min with cDA (0.1, 0.5 and 1.0 mM cDA). For these experiments we used 12 animals. We did not verify any alteration on CAT, SOD and GPx activities, suggesting that cDA did not affect these brain antioxidant enzymes (Table II).

Discussion

Patients with medium-chain acyl-CoA dehydrogenase (MCAD) deficiency (MCADD) present acute episodes of decompensation characterized by severe hypoketotic hypoglycaemia, acidosis, hyperammonaemia, vomiting, hypotonia, coma, seizures or even death, which occurs in about 20–25% of the affected individuals during the first episode [4,34–37]. Furthermore, a considerable number of those who survive present a variable degree of neurological dysfunction with cerebral abnormalities [5,6,10,38–40], whose pathophysiology is not yet defined. In this context, although high ammonia levels and hypoglycaemia could be tentatively related to the neurological dysfunction in these patients, the moderate degree of these laboratorial alterations usually do not lead to disturbances of neural function. Therefore, it could be presumed that the increased tissue concentrations of the medium-chain fatty acid octanoate, decanoate

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Figure 6. *In vitro* effect of *cis*-4-decenoic acid (cDA) on the non-enzymatic antioxidant parameters total-radical trapping antioxidant potential (TRAP) (A), total antioxidant reactivity (TAR) (B) and reduced glutathione (GSH) levels (C) in rat cerebral cortex. The effects of cDA on GSH levels in the presence of the antioxidants MEL, TRO and CAT plus SOD were also tested (D). Values are means ± standard deviation for five-to-nine independent experiments performed in duplicate or triplicate and are expressed as nmol/mg protein. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ compared to controls (Duncan multiple range test).

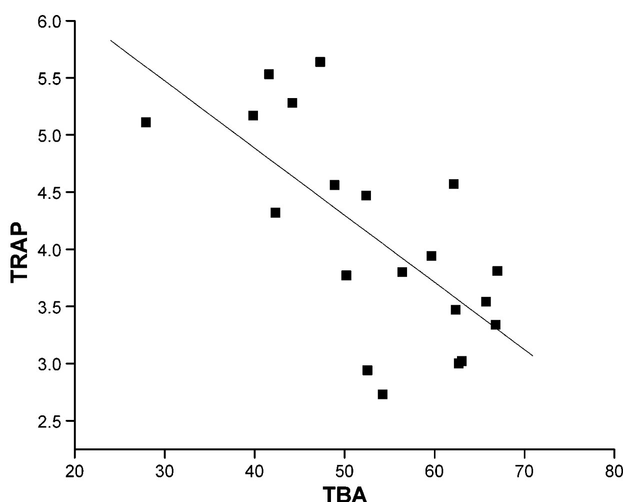

Figure 7. Correlation between thiobarbituric acid-reactive substances (TBA-RS) and total-radical trapping antioxidant potential (TRAP) in rat cerebral cortex exposed to 0.1–1.0 mM *cis*-4-decenoic acid (cDA). Values are means ± standard deviation for five-to-six independent experiments performed in triplicate and are expressed as nmol/mg protein for TBA-RS and TRAP.

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Table I. Effect of *cis*-4decenoic acid (cDA) on membrane protein-bound sulphydryl groups (PBSG) purified from mitochondrial fractions of rat cerebral cortex and on commercial reduced glutathione (GSH).

Mitochondrial PBSG oxidation	
Control	63.0 ± 8.72
0.1 mM cDA	63.9 ± 6.16
0.5 mM cDA	59.8 ± 8.33
1.0 mM cDA	54.9 ± 5.76
GSH oxidation	
Control	100 ± 4.04
1.0 mM cDA	116 ± 7.93

Values are means ± standard deviation for three-to-six independent experiments performed in triplicate and are expressed as nmol TNB/mg protein (mitochondrial PBSG oxidation) and percentage of control (GSH oxidation; controls: 1125 ± 47.4 unidades de fluorescência). No significant differences were detected (One-way ANOVA).

(DA) and *cis*-4-decenoate (cDA) that accumulate in MCADD may be responsible, at least in part, for the neurological symptoms in this disorder.

Considering that oxidative stress is involved in the pathophysiology of common neurodegenerative disorders [41–46] and of some inborn errors of metabolism [47–52], in the present study we evaluated the *in vitro* influence of cDA, the pathognomonic compound in MCADD, on several parameters of oxidative stress in cerebral cortex of young rats in order to evaluate whether this pathomechanism could be acting in this disorder. We first observed that cDA markedly increased TBA-RS (60%) and spontaneous chemiluminescence (40%). Furthermore, TBA-RS values gradually increased when cerebral cortex homogenates were exposed for increasing periods to cDA, suggesting that free radical synthesis was probably involved in this effect. Light emitted in the chemiluminescence assay usually arises from peroxidizing lipids due to an increase in reactive oxygen or nitrogen species production and TBA-RS reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation [53]. Therefore, our data strongly indicate that cDA induced lipid peroxidation in cerebral cortex *in vitro*.

We also observed that the strong *in vitro* lipid peroxidation caused by cDA was totally prevented by high doses of the free radical scavengers GSH, TRO and the combination of CAT plus SOD, but not by smaller doses of these antioxidants. Furthermore, Cr, TAU and GM1, but not DFO, MEL, L-NAME and CAR, also prevented cDA-induced increase of TBA-RS. These data suggest that cDA provoked a strong pro-oxidant effect on membrane lipids from cerebral cortex, indicating that the toxic effect exerted by cDA is mediated by reactive oxygen species generation. In contrast, cDA did not change TBA-RS levels measured in purified mitochondrial preparations (post-mitochondrial supernatants), suggesting that the generation of oxidants by this organic acid causing lipid oxidative damage occurred via cytosolic rather than mitochondrial mechanisms.

Protein carbonyl formation and sulphydryl oxidation, useful markers for assessing oxidative protein damage, were also markedly increased by cDA, implying that this unsaturated carboxylic acid also provokes protein oxidation [24,55]. Similarly, the unsaturated oleic acid (OLA), but not decanoic acid (DA), a saturated fatty acid with the same carbon chain as cDA, significantly enhanced sulphydryl oxidation, suggesting that this effect may be related to the unsaturated bond. Previous findings showing that the polyunsaturated arachidonic acid induces reactive oxygen species reinforces this assumption [54]. On the other hand, the observations that cDA was not able to oxidize purified thiol groups from purified mitochondrial membranes as well as commercial reduced glutathione in the absence of brain supernatants in the incubation medium suggest that this fatty acid is not *per se* an oxidant agent and that it probably provoked lipid and protein oxidative damage via free radical induction.

With regard to the antioxidant defenses, cDA markedly reduced the total content of non-enzymatic antioxidants (TRAP values) and GSH, the main natural-occurring antioxidant, as well as the capacity to rapidly handle increased free radical generation (TAR measurement) in cerebral cortex. Since these parameters are used to evaluate the capacity of a tissue to prevent and respond to the damage associated to free radical processes, it can be concluded that the rat cortical antioxidant defenses were severely compromised by cDA [20,27,53]. We verified that MEL and TRO, but not SOD plus CAT, were able to prevent cDA-decrease of GSH levels in cortical supernatants, suggesting that the highly toxic hydroxyl radical, which is the principal species scavenged by MEL [56], and peroxyl radicals, which are scavenged by TRO, were probably involved in the reduction of GSH provoked by cDA.

We also observed that cerebral cortex homogenates exposed to cDA resulted in an enhanced DCFH oxidation, which is predominantly dependent on hydrogen peroxide [57]. These results allied to those showing that GSH, TRO and SOD plus CAT fully

Table II. Effect of *cis*-4decenoic acid (cDA) on the antioxidant enzyme activities catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in rat cortical supernatants.

	CAT	SOD	GPx
Control	2.74 ± 0.09	2.08 ± 0.19	13.6 ± 2.83
0.1 mM cDA	2.48 ± 0.49	1.71 ± 0.68	13.9 ± 1.25
0.5 mM cDA	2.40 ± 0.52	2.35 ± 0.62	13.0 ± 1.94
1.0 mM cDA	2.58 ± 0.54	1.81 ± 0.35	11.4 ± 4.26

Values are means ± standard deviation for four independent experiments performed in duplicate and are expressed as: CAT and SOD: U/mg protein and GPx: mU/mg protein. No significant differences were detected (One-way ANOVA).

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prevented the stimulation of *in vitro* lipid peroxidation elicited by cDA suggest that the free radicals hydroxyl, peroxyl and hydrogen peroxide, which are scavenged by these antioxidants [58–61], are involved in the pro-oxidant effects of cDA. In contrast, we found that cDA did not affect superoxide generation in sub-mitochondrial particles obtained from cerebral cortex supernatants, ruling out an important involvement of this free radical in cDA pro-oxidant effects.

A significant inverse correlation between TBA-RS increase and TRAP decrease caused by cDA was also verified in the present study, reinforcing the view that the decrease of the brain antioxidant defenses and the induction of lipid peroxidation were mediated by increased reactive species generation. Therefore, it is feasible that the marked decline of the cerebral non-enzymatic antioxidant defenses (TRAP, GSH and TAR) probably reflects the rapid consume of highly reactive antioxidants due to the generation of free radicals elicited by cDA.

Although we cannot at the present establish the exact signal transduction cascades by which cDA induced lipid and protein oxidative damage in cerebral cortex, it may be presumed that free radicals elicited by this fatty acid could initiate the classical cascades leading to these pro-oxidant effects [62].

We also observed that the activities of the antioxidant enzymes CAT, GPx and SOD were not altered by exposing cortical supernatants for 60 min to cDA at concentrations as high as 1.0 mM. However, we cannot exclude the possibility that sustained high concentrations of cDA, as occurs in MCADD, could lead to up-regulation of these enzymes via gene transcription in order to counterbalance increased free radical generation, as occurs in other pathological conditions involving oxidative stress [63–65].

Since oxidative stress results from imbalance between the total antioxidant defense of the tissue and the reactive species generated, our present data strongly indicate that cDA provokes a significant *in vitro* stimulation of oxidative stress in cerebral cortex, a deleterious cell condition, which induces oxidation of lipids and proteins and reduces antioxidant defenses [53]. At this point, it should be emphasized that the brain has low cerebral antioxidant defenses compared with other tissues [66], a fact that makes this tissue more vulnerable to increased reactive species. In fact, oxidative stress has been implicated in the pathophysiology of common neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, as well as in epileptic seizures and demyelination [24,63,66,67].

We cannot establish whether our data have a pathophysiological significance, since brain concentrations of cDA in MCAD deficient patients are unknown. It should, however, be noted that the significant alterations of the oxidative stress parameters elicited by cDA occurred at high micromolar levels (0.50 mM and higher) and acute encephalopathy occurs in these patients particularly during metabolic crises, in which the concentrations of the accumulating metabolites dramatically increase. Furthermore, it has been proposed that the concentrations of organic acids accumulating in various organic acidemias are higher in the neural cells as compared to the serum or CSF [68].

In conclusion, the present data indicate that oxidative stress is induced by cDA in the brain of young rats. In case these present findings are confirmed *in vivo* in animal experiments and also in tissues of patients affected by MCADD, it is tempting to speculate that excessive reactive species generation might contribute, at least in part, to the neuropathology of this disorder. It seems therefore reasonable to propose that antioxidants may serve as an adjuvant therapy to specific diets or to other pharmacological agents used for these patients, especially during crises, to avoid oxidative damage to the brain.

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