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Iron Mobilization by Succinylacetone Methyl Ester in Rats. A Model Study for Hereditary Tyrosinemia and Porphyrias Characterized by 5-Aminolevulinic Acid Overload

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Accumulation of 5-aminolevulinic acid (ALA) is an event characteristic of porphyrias that may contribute to their pathological manifestations. To investigate effects of ALA independent of porphyrin accumulation we treated rats with the methyl ester of succinylacetone, an inhibitor of 5-aminolevulinic acid dehydratase that accumulates in the porphyric-like syndrome hereditary tyrosinemia. Acute 2-day treatment of fasted rats with succinylacetone methyl ester (SAME) promoted a 27% increase in plasma ALA. This increase in plasma ALA was accompanied by augmentation of the level of total nonheme iron in liver (37%) and brain (20%). Mobilization of iron was also indicated by 49% increase in plasma iron and a 77% increase in plasma transferrin saturation. Liver responded with a mild (12%) increase in ferritin. Under these acute conditions, some indications of oxidative stress were evident: a 15% increase in liver reactive protein carbonyls, and a 42% increase in brain subcellular membrane TBARS. Brain also showed a 44% increase in CuZnSOD activity, consistent with observations in treatment with ALA. Overall, the data indicate that SAME promotes ALA-driven changes in iron metabolism that could lead to increased production of free radicals. The findings support other evidence that accumulation of ALA in porphyrias and hereditary tyrosinemia may induce iron-dependent biological damage that contributes to neuropathy and hepatoma.

Keywords: Succinylacetone, 5-aminolevulinic acid, porphyria, ferritin, iron, oxidative stress

Abbreviations: SAME, succinylacetone methyl ester; SOD, superoxide dismutase; HT1, hereditary type 1 tyrosinemia; ALA, 5-aminolevulinic acid; ALAD, 5-aminolevulinic acid dehydratase; ALAS, 5-aminolevulinic acid synthase; AIP, acute intermittent porphyria; DOVA, 4,5-dioxovaleric acid; CNS, central nervous system; OPA, *o*-phthalaldehyde

INTRODUCTION

Hereditary type 1 tyrosinemia (HT1) results from an inherited deficiency in fumarylacetoacetate

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hydrolase, the enzyme involved in the last step in the tyrosine catabolic pathway, resulting in the accumulation of succinylacetone.[1,2] This disease is an autosomal recessive disorder, characterized by progressive liver damage from infancy, a high risk for hepatocellular carcinoma, a reversible renal Fanconi syndrome, porphyria-like abdominal crises and frequent neurological manifestations.[1,3]

Although HT1 has been the subject of many clinical and biological studies during the past years, the pathogenesis of this genetic disease is still not completely understood. In particular, the mechanism that triggers the cellular damage in the liver and leads to hepatoma is still unknown.[2] The HT1 syndrome resembles very closely that of acute intermittent porphyria (AIP),[1] where 5-aminolevulinic acid (ALA) accumulates along with other porphyrin intermediates. Succinylacetone is a potent inhibitor ($K_i = 0.03\,\mu mol/l$)[4] of 5-aminolevulinic acid dehydratase (ALAD)[5] and therefore promotes ALA accumulation, like in AIP and lead poisoning.[6]

Oxidative stress may be a consequence of ALA accumulation. Reactive oxygen species (H_2O_2, $O_2^{\bullet -}$, and $HO^\bullet$ radical) are formed by the metal-catalyzed aerobic oxidation of ALA at physiological pH.[7] ALA has been shown to play prooxidant roles both *in vitro* and *in vivo*, causing oxidative damage for example in proteins, liposomes, DNA and subcellular structures like mitochondria and synaptosomes, and causing iron mobilization.[8]

ALA may contribute to pathological manifestations and carcinogenesis by several mechanisms. Oxidative stress triggered by the Fenton and/or iron-catalyzed Haber–Weiss reactions appear to play a role in iron-induced carcinogenesis.[9] Noteworthy in this regard is that ALA promotes iron release from ferritin, causes iron and ferritin accumulation in brain and liver of ALA-treated rats,[10] and induces activation of the cytosolic iron regulatory protein 1 in M8 cells.[11] That ALA induces oxidative stress and DNA damage *in vivo* was demonstrated by 4.5-fold increased levels of 8-oxo-7,8-deoxyguanine in liver DNA of rats treated with ALA.[12]

However, injection of ALA to animals produces equivocal results due to accumulation of porphyrins, which can catalyze NADPH-dependent radical production[13] and are powerful photosensitizers.[14] This casts uncertainty on the extent to which effects observed in treatment with ALA result from ALA or from porphyrin accumulation. Injection of succinylacetone however can help distinguish these effects. As succinylacetone inhibits ALA dehydratase, located prior to synthesis of porphyrins in the pathway of heme synthesis, the accumulation of ALA can be induced, without inducing the accumulation of porphyrins.

To characterize specific effects of ALA accumulation *in vivo* therefore, we administered succinylacetone methyl ester (SAME) to rats, and examined influences on iron metabolism, protein oxidative damage (reactive protein carbonyls), lipid peroxidation (TBARS) and superoxide dismutase (SOD) activities in the liver, brain and plasma. The methyl ester of succinylacetone was chosen as the administered form owing to its prompt diffusibility across membranes, as demonstrated previously by Carvalho *et al.*[11] The current model involved an acute two-day treatment with SAME to investigate effects of ALA accumulation prior to significant depletion of cellular heme. The main objectives of this work are: (i) to determine the acute effects of SAME administration on ALA accumulation, and (ii) to evaluate the effects generated by SAME on parameters of oxidative stress and iron metabolism observed to change with ALA treatment. The results are hoped to give some indication of the extent to which effects observed with ALA treatment are a consequence of ALA, or of accumulated porphyrins, and to provide insights into the possible roles of ALA in the pathologies of hereditary type 1 tyrosinemia and porphyrias.

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MATERIALS AND METHODS

Reagents

ALA, SOD, horse heart cytochrome *c* (type III), ascorbic acid, 2,2′-bipyridine, guanidine, tritiated borohydride, pepsin, perchloric acid, trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonicacid (HEPES), β-mercaptoethanol, *o*-phthalaldehyde (OPA), albumin (bovine serum), Folin Ciocalteu's reagent, streptomycin, heparin, succinylacetone, tris(hydroxymethyl) aminomethane (Tris), xanthine, and xanthine oxidase were purchased from Sigma Chemical Company, St. Louis, MO. Chloroform, ethanol, ethyl acetate, ethyl ether, hydrochloric acid, potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium bicarbonate, and sodium chloride were from Merck, Darmstadt, Germany. Sodium cyanide was purchased from May & Bayer Laboratory Chemicals LTD., Dagenham, England and trifluoroacetic acid was purchased from Riedel-De Haën AG, Seelze-Hannover. Immunochemical ferritin determination was carried out with the COBAS® Core-Ferritin EIA kit, from F. Hoffmann-La Roche Ltd, Basel, Switzerland. Serum iron, iron binding capacity and transferrin saturation was carried out with the TIBC and Fe kit from Boehringer Mannheim. Organic solvents were of HPLC grade. Other reagents were of analytical grade. Solutions were prepared with distilled, Millipore MilliQ deionized water.

Methods

Succinylacetone Methylation

SAME was prepared by direct reaction of succinylacetone with diazomethane in ethyl ether as solvent. After evaporation of the ethyl ether at room temperature, the SAME residue was dissolved in saline and adjusted to pH 7.0 with sodium bicarbonate to be used in the rat treatments.

Animal Treatment and Sample Preparation

Male Wistar rats (250–300 g) were injected intraperitonially with SAME (40 mg/kg body weight; three doses in 36 h; $n=7$), and the controls were injected with saline pH 7.0 ($n=8$). The amount of succinylacetone injected was as in Puy *et al.*[15] and Tschudy *et al.*,[16] and the maximum volume injected was 0.2 ml. The rats were fasted for 40 h before the first injection and during the treatment. The animals were sacrificed by ether anaesthesia 2 h after the third administration. Blood samples were withdrawn into heparin-containing tubes and immediately centrifuged at 800× *g*. The plasma was kept at −80°C until assayed for ALA, ferritin, and transferrin saturation. Erythrocytes were isolated for further SOD analysis.

Liver and brain homogenates were prepared in 10 mM HEPES pH 7.2 containing 125 mM KCl with a glass-teflon homogenizer. Assays of total nonheme iron were performed in samples of fresh whole homogenate. Samples for other determinations were prepared in appropriate buffers, frozen whole or clarified at 3000× *g*, and kept at −80°C until assayed.

Determination of ALA

Isocratic liquid determinations of the ALA derivative with OPA were performed on an HPLC system with electrochemical detection as described by Costa *et al.*[17] The plasma sample was deproteinized using $HClO_4$ and later neutralized by addition of $NaHCO_3$ crystals to *ca.* 7.4. before use as described by Costa *et al.*[17] OPA reagent was made up in the proportions described by Lindroth and Mopper,[18] and derivatization of the samples was done by mixing 10 µl of calibrator solution or deproteinized plasma, 5 µl of OPA reagent (36 mmol/l), and 35 µl of water.[17]

The HPLC system consisted of a 515 HPLC Pump Waters coupled to a Waters 464 Pulsed electrochemical detector from Waters Corporation. The detector working electrode was maintained at +0.6 V vs Ag/AgCl, and its signal was

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delivered to a Digital computer with data collection handling provided by Millenium Chromatography Manager Software. All analyses were performed with Nova-Pak 15.0 cm × 3.9 mm (i.d.) Waters Associates 4.0-μm C_{18} columns. The mobile phase was 0.05 M phosphate buffer (pH 7.0)/containing 8% acetonitrile and 2.4 mM EDTA. The samples were introduced with a 20-μl external loop injector from Rheodyne, model 7725i-037, and eluted with the mobile phase circulating at 1.0 ml/min.

Determination of Ferritin

Homogenates of liver or brain (330 mg), were prepared for ferritin determination according to Dexter *et al.*[19] Quantification was performed with monoclonal mouse antiferritin antibodies from COBAS Core-Ferritin EIA kit designed for human plasma ferritin and calibrated with ferritins of rat liver and human plasma from Sigma as standards to analyze levels of ferritin in liver and brain respectively.

Determination of Total Nonheme Iron

Total nonheme iron was assayed spectrophotometrically with 2,2′-bipyridine, as described by Bralet *et al.*[20] Fresh tissue samples (50 mg) were homogenized in 2 ml of 0.10 M HCl containing pepsin (10 mg/ml), ascorbic acid (20 mg/ml), 5 mM 2,2′-bipyridine (final pH 2.5) and incubated at 37°C for 24 h. After incubation, the samples were centrifuged at maximum rotation in an Eppendorf microcentrifuge and the absorbance read at 520 nm in the supernatant. Iron concentration was calculated using a calibration curve prepared with $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (0.05–5 μg Fe^{II}/ml).

Determination of Plasma Iron and Transferrin Saturation

Measures in plasma of total iron, iron binding capacity (TIBC), and transferrin saturation were conducted in the Clinical Analysis Laboratory of the Faculty of Pharmaceutical Sciences, University of São Paulo, using colorimetric kits from Boehringer Mannheim.

Determination of Reactive Protein Carbonyls

The reactive carbonyl content in protein of liver and brain samples was measured by the tritiated borohydride method described by Levine *et al.*,[21] without prior protein precipitation. The assay was performed using homogenate, at 1.0 and 0.4 mg protein/ml for liver and brain respectively, that had been centrifuged at 3000× *g*. This preparation was thus devoid of nuclei (and their interfering nucleic acids), and contained soluble protein and the majority of mitochondria and other subcellular organelles. Protein was determined by the Lowry method in the initial homogenates, and by absorbance at 280 nm in the final guanidine · HCl-dissolved assay samples. The number of reactive carbonyls per mg protein was calculated by assuming 10,000 cpm for 1 nmol carbonyl in samples treated with 100 mCi/mmol NaB^3H_4.[21]

Determination of TBARS

Intracellular lipid peroxidation was evaluated by measuring thiobarbituric acid reactive substances (TBARS) in 3000× *g*-centrifuged homogenates containing 0.1 mM EDTA by the method of Fraga *et al.*[22] The assay was calibrated with a malondialdehyde standard solution, and the values expressed as malondialdehyde equivalents per mg protein.

Determination of SODs (CuZnSOD and MnSOD)

SODs were assayed spectrophotometrically using the ferricytochrome *c* reduction assay described by Flohé and Ötting[23] with the modifications suggested by Crapo *et al.*[24] Liver and brain SOD activities were measured in 3000× *g* clarified tissue homogenates containing 0.1 mM EDTA. For erythrocyte SOD, the harvested erythrocytes (1.0 ml) were agitated with cloroform : ethanol

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(3 : 5) (0.45 ml) for 20 min, centrifuged at 3000× g for 20 min, and the supernatant assayed for SOD activity. SOD activities in the erythrocyte extract and in tissue homogenates were measured from inhibition of the reduction of cytochrome c by superoxide radicals produced from xanthine oxidase plus xanthine, monitored at 550 nm. Samples were diluted with buffer (6–30 μg protein in 15–25 μl depending on the origin: erythrocyte, liver or brain) in order to obtain the concentration that inhibits the rate of cytochrome c reduction by 50%. MnSOD in liver and brain homogenates was estimated from the activity measured in the presence of 3 mM cyanide. The activities were expressed in U/mg protein, considering one unit of SOD that which inhibits the rate of cytochrome c reduction, under the conditions specified, by 50%.

Determination of Protein

Protein was measured by the method of Lowry *et al.*[25] using bovine serum albumin as a standard.

Statistical Treatment

Data are expressed as mean ± SD. Comparisons between groups were made following nonparametric analysis of Mann–Whitney. Statistical significance was assigned to values with $p < 0.05$.

RESULTS

SAME Effect on Plasma ALA Concentration

In prior studies, chronic treatment of *ad libitum*-fed rats with succinylacetone for 4 days did not result in any significant plasma ALA differences between control and treated animals (data not shown). With fasted animals submitted to an acute treatment with SAME however, the ALA levels increased 27% (Table I). This result confirms absorption of SAME and inhibition of ALAD. These results are in agreement with those obtained by Carvalho *et al.*[11] showing an increase of the intracellular ALA concentration in M8 cells incubated with SAME and of Tschudy *et al.*[16] showing that 12 h administrations of succinylacetone to rats maintains >92% inhibition of liver ALAD activity.

SAME Effect on Total Nonheme Iron Contents

SAME treatment significantly increased total nonheme iron in rat liver and brain, 37% and 20% respectively (Table II). In this regard, Adams *et al.*[26] reported that administration of succinylacetone to rat reticulocytes increased iron uptake by increasing the number of surface transferrin receptors and the rate of receptor recycling. Also, part of the iron that fuels the free pool might derive from ALA-induced iron release from ferritin.[27]

TABLE I Effect of SAME on the ALA level in rat plasma

Plasma	ALA (μM)
Control	2.8 ± 0.5
Treated	3.5 ± 0.6*

Data are shown as mean ± SD using the average of two independent measurements from each sample. *Significantly different at $p < 0.05$ ($p = 0.037$).

TABLE II Effect of SAME on total nonheme iron levels in rat liver and brain

	Total nonheme iron
Liver homogenate	(μg/g fresh tissue)
Control	48.4 ± 10.5
Treated	66.1 ± 10.7*
Brain homogenate	
Control	12.0 ± 1.4
Treated	14.3 ± 1.4*
Plasma	(μg/dl)
Control	51.60 ± 9.9
Treated	76.83 ± 12.3*

Data are shown as mean ± SD using the average of three independent measurements from each sample. *Significantly different at $p < 0.05$ (liver $p = 0.019$; brain $p = 0.028$, plasma $p = 0.018$).

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SAME Effect on Plasma Iron and Transferrin Saturation

Plasma iron increased 49% (Table II), and iron saturation of plasma transferrin increased 77% (Table III) in the rats treated with SAME. Since these rats were fasted during treatment, this result reflects significant iron mobilization from tissues.

SAME Effect on Ferritin Levels

The level of ferritin in rat liver was increased by 12% upon SAME treatment, but in plasma and brain no significant difference was observed (Table III). According to Cairo *et al.*,[28] changes in iron availability and oxidative stress can regulate ferritin gene expression at several levels, with the translational control being quantitatively more relevant. An increase in the free iron pool, caused both by heme destruction and by ferritin degradation, stimulates ferritin synthesis transcriptionally and post-transcriptionally.[28] The elevation in the total nonheme iron by SAME treatment, which may increase iron-catalyzed generation of reactive oxygen species and ferritin degradation, conceivably triggers a compensatory increase in ferritin synthesis, that is mildly evident in the liver of these SAME-treated rats.

TABLE III Effect of SAME on ferritin levels and plasma transferrin saturation

	Ferritin	*Transferrin saturation
Liver homogenate	(μg/g fresh tissue)	
Control	29.8 ± 3.3	
Treated	33.2 ± 1.4*	
Brain homogenate		
Control	21.2 ± 6.8	
Treated	25.9 ± 5.4	
Plasma	(ng/ml)	(%)
Control	4.1 ± 0.8	28.6 ± 5.2
Treated	3.7 ± 0.6	50.7 ± 10.1**

Data are shown as mean ± SD using the average of three independent measurements from each sample. *Significantly different at $p < 0.05$ ($p = 0.025$). **Significantly different at $p < 0.01$ ($p = 0.0062$).

SAME Effect on TBARS and Protein Reactive Carbonyl Content

Some indications of increased oxidative stress were evident under the current conditions of SAME treatment. Using TBARS as an index of lipid peroxidation, a 42% increase was seen in brain, with no change in liver (Table IV). Metal-catalyzed introduction of carbonyl groups into proteins is a hallmark for oxidative modification of proteins in conditions of oxidative stress. Under the current conditions of treatment with SAME, a mild (15%) increase in protein carbonyls was observable in liver, but not in brain (Table IV).

SAME Effect on SOD Activities

The activity of CuZnSOD increased by 44% in brain of rats treated with SAME; no significant differences were observed in liver and erythrocytes (Table V). No differences were observed in MnSOD activities in brain and liver.

DISCUSSION

Through a comparison of results obtained with treatments using ALA and those using SAME we aimed to distinguish effects of ALA from those of porphyrin accumulation. Several effects of ALA administration have been characterized

TABLE IV Effect of SAME on levels of TBARS and reactive protein carbonyls in rat liver and brain

	TBARS (nmol MDA/mg protein)	Protein carbonyl (nmol/mg protein)
Liver homogenate		
Control	0.018 ± 0.004	2.2 ± 0.3
Treated	0.019 ± 0.004	2.7 ± 0.5*
Brain homogenate		
Control	0.057 ± 0.012	5.5 ± 0.7
Treated	0.081 ± 0.016*	5.1 ± 0.5

Data are shown as mean ± SD using the average of two independent measurements from each sample. *Significantly different at $p < 0.05$.

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TABLE V Effect of SAME on SOD activity in rat liver, brain and erythrocytes

	CuZnSOD (U/mg of protein)	MnSOD (U/mg of protein)
Liver homogenate		
Control	89.6 ± 4.4	4.8 ± 1.0
Treated	87.9 ± 6.8	4.9 ± 0.4
Brain homogenate		
Control	109.7 ± 31.6	5.5 ± 0.7
Treated	157.8 ± 16.1*	4.5 ± 1.7
Erythrocytes		
Control	49.3 ± 6.7	—
Treated	44.9 ± 1.0	—

Data are shown as mean ± SD using the average of three independent measurements for each sample. *Significantly different at $p < 0.05$ ($p = 0.022$).

including: increased nonheme iron in liver and increased nonheme iron and ferritin in brain,[27,29] increased CuZnSOD activities in brain, liver and muscle,[29,30] increased spontaneous chemiluminescence of liver, muscle and brain,[31] and increased oxidative DNA damage.[12] *In vitro*, ALA has been observed to produce metal-catalyzed oxidative damage to DNA,[12,32] lipids[33] and proteins.[7,34] It should be noted that while acute treatment of rats with ALA gave observable changes in iron metabolism[29] and liver chemiluminescence,[31] increases in markers of oxidative stress (protein carbonyls, TBARS) were observed with longer treatments.[29]

Acute SAME Treatment Increases ALA Levels in Fasted Rats

With succinylacetone, although a potent and specific inhibitor of ALAD *in vitro*, it has had variable effectiveness *in vivo*. When in previous analyses we injected succinylacetone twice a day (5 and 40 mg/kg) for four days to *ad libitum*-fed rats, plasma and tissue levels of ALA did not increase significantly (unpublished results). Nevertheless, it was possible to identify a dose dependent increase of total nonheme iron in liver. These results agree also with those described by Puy *et al.*[15] that succinylacetone administration did not increase ALA in rats that were fed during the four day treatment, but increased the levels if combined with a fast. Tschudy *et al.*[16] found that fasting of rats greatly augmented induction of ALAS (thereby increasing ALA synthesis) on treatment with succinylacetone. Results in fasted rats are relevant in that fasting precipitates porphyric attacks in AIP patients.[6]

In the current studies, when the animals were treated with SAME and fasted during the treatment, an increase of the plasma ALA levels relative to controls can be verified (27%, Table I). The results with SAME in rats are consistent with results of Carvalho *et al.*[11] in cultured Chinese hamster pulmonary fibroblasts showing an increase in intracellular and extracellular medium ALA, and an activation of iron responsive element binding protein 1 (IRP1) after SAME treatment. In the cultured cells, intracellular ALA levels increased greatly after 15 min of incubation with 4 mM SAME (92% higher than in control samples), subsequently decreasing and reaching a plateau at 2 h of incubation at levels 40% higher than those of control samples. *N*-acetylcysteine inhibited IRP1 activation, suggesting that the observed effect is mediated by an oxidative process.[11]

Administration of SAME Increased Tissue and Plasma Nonheme Iron Levels

The effects of SAME administration on tissue iron in the current studies were similar to effects found previously with administration of ALA. SAME increased the total nonheme iron in brain (20%) and liver (37%) (Table II), and ferritin (12%) in liver (Table III). Results obtained by Oteiza *et al.*[27] and Demasi *et al.*[29] with ALA-treated rats showed increased nonheme iron in liver and cerebral cortex (20% with acute treatment and 68% with chronic treatment). In the current studies, the 49% increase in plasma iron and 77% increase in plasma transferrin saturation further confirmed substantial liberation of iron with this acute treatment with SAME. An effect not found in the

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current study was an increase in ferritin in cerebral cortex (71% with prolonged ALA treatment), perhaps due to the short duration of this treatment for an adaptive response.

The current results of increased liver ferritin, although mild under the current conditions, agree with those obtained by Eisenstein *et al.*[35] in hepatoma cells and rat fibroblasts showing that succinylacetone does not block the induction of ferritin by heme and heme oxygenase. The authors concluded that: (i) the release of iron by heme oxygenase plays an essential role in the induction of ferritin synthesis by heme and (ii) chelatable iron can regulate ferritin synthesis independently of heme formation. That both treatment of rats with ALA, which does not decrease heme synthesis, and SAME give increases in ferritin, suggests that changes in nonheme iron rather than heme influence liver ferritin synthesis in the current studies.

The current results on iron metabolism are also consistent with an effect of SAME on oxidative stress. Cairo *et al.*[28] showed that ferritin synthesis is stimulated significantly in liver of rats under conditions of oxidative stress. That reactive oxygen species and ALA are able to release iron from ferritin becomes important considering that the oxidation of ALA is catalyzed by complexes of iron, generating reactive species of oxygen[27,36] that could exacerbate these effects. Also, ALA or SAME, in an antioxidant-inhibitable manner, activates IRP1[11] which acts to induce transferrin receptors and thereby enhance iron uptake into cells.

The increase in intracellular iron by SAME has particular implications for oxidative stress to mitochondria, and for carcinogenesis. In a previous study of succinylacetone-treated rat reticulocytes,[26] an increase of iron uptake was observed, which accumulated mainly in the mitochondria, especially (70–75%) in the inner membrane fraction. Under normal conditions, there is no significant intracellular or intramitochondrial free iron pool in reticulocytes. An increase of free iron in the mitochondria is important with regard to ALA because this organelle is the site of ALA synthesis and a source of respiration-derived active oxygen. Other studies have shown that ALA promotes damage to isolated mitochondria of rat liver and brain through metal-catalyzed reaction with oxygen and production of reactive oxygen species.[34,37–40] In hereditary tyrosinemia and porphyrias, extensive damage is seen to mitochondria in liver, e.g. Ref. [41]. Together with the carcinogenicity of iron[9] this damage may contribute to the hepatotoxicity and development of hepatic carcinomas in these syndromes.[1–3,42,43]

Treatment with SAME Increased CuZn Activity in Brain and gave Indications of Oxidative Stress

That the activity of CuZnSOD was notably increased in brain after treatment with SAME, is in concordance with previous studies using ALA treatment.[29,30] Treatment with ALA produced significant increases in the activity of CuZnSOD in brain in both sedentary and endurance-trained rats (61% in the sedentary ones),[30] and as in the current study with SAME, no change in the activity of brain MnSOD. However, with prolonged ALA treatment significant increases in CuZnSOD and MnSOD in liver were also observed.[30] Nevertheless, the current results substantiate an acute effect of ALA accumulation on brain CuZnSOD activity.

Along with an increase in the activity of CuZnSOD in brain homogenates after prolonged treatment with ALA, Demasi *et al.*[29] observed an increase in protein carbonyls in cortex homogenate. We were unable to confirm a significant increase in protein carbonyls in brain with short-term SAME treatment, but did observe a mild (15%) increase in liver protein carbonyls.

In brain, the observed increase in TBARS suggests increased oxidative stress to lipids with short-term SAME treatment. In previous studies of prolonged ALA administration,[29] increased TBARS were only observed in a synaptic membrane fraction, and not in whole brain or

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cerebral cortex homogenate. In the current studies, the brain homogenate was centrifuged at 3000× g to remove unbroken cells, nuclei, and large membrane fragments, so represents mainly synaptosomes and intracellular organelles. These results with SAME treatment would thus be consistent with increased oxidative stress to mitochondria, which has been observed with ALA *in vitro*.[34,37–40]

The selective effect of SAME on CuZnSOD and TBARS in brain may be partially explained by previously observed differential influences of succinylacetone on brain and liver. Wyss *et al.*[44,45] studied the distribution and impact on heme metabolism in brain, liver and kidney from rats treated *in vivo* with succinylacetone and observed a greater retention of succinylacetone in brain and kidney than in liver, by a ratio of approximately 3 : 1. δ-Aminolevulinate dehydratase was decreased to less than 10% of control activity in all the three tissues after three daily injections, but heme biosynthesis in the brain was most adversely affected. Wyss and collaborators[45] suggest that such effects could result in impaired oxidative metabolism in brain, producing the central nervous system (CNS) manifestations of tyrosinemia.

Implications for Hereditary Tyrosinemia

The current results add significance to the accumulation of succinylacetone in hereditary tyrosinemia. Other metabolites that accumulate in hereditary type 1 tyrosinemia due to fumarylacetoacetate hydrolase deficiency, and might contribute are fumarylacetoacetate and maleylacetoacetate.[46] In hepatocytes that lack fumarylacetoacetate hydrolase activity, the accumulation of tyrosine metabolites triggers apoptosis.[47] The results of Muta and Krantz [48] that succinylacetone induces apoptosis of human erythroid progenitor cells support roles for ALA accumulation and/or impaired heme synthesis in triggering apoptosis. In these ways, succinylacetone accumulation may contribute to the progressive liver damage observed in hereditary type 1 tyrosinemia by inducing apoptosis, and, when cells escape apoptosis, to the increased incidence of hepatocellular carcinoma. The current results suggest that disturbed iron metabolism due to succinylacetone-induced ALA accumulation may contribute to these effects. Findings that melatonin confers protection against ALA-induced oxidative toxicity in brain[49] and that *N*-acetylcysteine protects against oxidative stress in brain and liver of lead-exposed mice (where ALA accumulates due to inhibition of ALAD by lead),[50] suggest that these or other antioxidants may help reduce neural and hepatic damage in individuals suffering hereditary type 1 tyrosinemia.

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