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Oxidative Tissue Response Promoted by 5-Aminolevulinic Acid Promptly Induces the Increase of Plasma Antioxidant Capacity

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The heme precursor 5-aminolevulinic acid (ALA), acting as a prooxidant, has been proposed to underlie the clinical manifestations of various porphyric disorders. Accordingly, ALA-generated oxyradicals where shown to cause oxidative lesions in biomolecules and isolated cell organelles and to release iron from ferritin. In rats, administered ALA triggered oxidative stress in liver, brain and red muscles. We now study the correlation between the plasma antioxidant capacity and tissue oxidative damage, after acute (one and two doses) and prolonged (eight doses) ALA treatment of rats (one dose of ALA = 40 mg/kg body weight). The *in situ* spontaneous chemiluminescence intensity increased 5-fold in brain, 50% in liver and 4-fold in soleus muscle upon two dose-treatment, indicating tissue response to oxidative injury by ALA. Chemiluminescence reached the highest intensity after one or two doses of ALA and decreased after eight doses in all tissues. The plasma trapping capacity, evaluated by the luminol/2-amidinopropane system, gave a parallel response: maximum values after two doses and decreased values after prolonged treatment. After eight doses, the ALA concentration was found to be 3-fold above the normal value in plasma, 48% higher in liver and 38% higher in total brain. These data indicate that the plasma antioxidant system responds to ALA treatment and is correlated with tissue chemiluminescence. *In vitro* studies showed that ALA does not interfere with the antioxidant plasma capacity, neither promoting oxidation of plasma elements nor binding to plasma proteins.

Keywords: 5-aminolevulinic acid, reactive oxygen species, porphyrias, lead poisoning, plasma trapping, chemiluminescence, free radicals

Abbreviations used: ALA, aminolevulinic acid; ABAP, 2,2′-azo-bis(2-amidinopropane); DTNB, dithio-bis(2-nitrobenzoic acid); OPA, *ortho*-phthaldehyde; TRAP, plasma antioxidant trapping capacity

INTRODUCTION

5-Aminolevulinic acid (ALA), formed in the rate-limiting step of the heme biosynthetic pathway, has been proposed to act as an endogenous prooxidant in pathological conditions where it accumulates.[1] Indeed, *in vitro* ALA undergoes iron-catalyzed oxidation generating deleterious

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reactive oxygen species (ROS) and ALA carbon-centered radical.[2,3] This chemical behavior may have physiological significance related to cellular iron metabolism as proposed by Minotti[4] and to the neuropsychiatric manifestations of intermittent acute porphyria, lead poisoning and tyrosinosis syndromes where ALA accumulates.[1] The plasma ALA level is 100-fold above normal in the former two diseases.[5,6] Decreased plasma ALA concentration has been associated with amelioration of the symptoms.[7] ALA prooxidant activity is supported by previously reported induction of lipid, protein, DNA and liver mitochondria oxidative damage[8–10] and iron mobilization from ferritin.[11] *In vivo*, ALA was shown to increase production of rat liver 8-hydroxy-2'-deoxyguanosine[12] and to promote iron accumulation in brain and liver.[13,14] Moreover, studies with acute intermittent porphyric carriers revealed increased blood activity of CuZn-superoxide dismutase (CuZnSOD) (32% and 100% in latent and symptomatic patients, respectively) and of glutathione peroxidase (GSH-Px) (*ca.* 2-fold in both cases). Evaluation of erythrocytic CuZnSOD and GSH-Px of lead exposed workers also showed significantly increased activities of both enzymes: 85% and 140%, respectively.[15]

We study here the plasma antioxidant defense response towards ALA overload and oxidative tissue damage. *In situ* chemiluminescence measurements in rat liver, skeletal muscle and brain were used to evaluate the oxidative stress status upon ALA treatment of rats. After acute and prolonged ALA treatment, the plasma trapping capacity was measured by the chemiluminescent ABAP/luminol system[16] as well as plasma, liver and brain ALA levels and the total plasma sulfhydryl content.[17] *In vitro*, ALA interaction with plasma and isolated antioxidants such as Trolox, ascorbate, urate and reduced glutathione was also studied by evaluating their effect on the plasma antioxidant capacity and on oxygen consumption by ALA. The ALA oxidative effect upon plasma proteins was estimated *in vitro* by determination of the carbonyl and total sulfhydryl content.

MATERIALS AND METHODS

Materials

5-Aminolevulinic acid hydrochloride, ascorbic acid, bovine serum albumin, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), luminol, reduced glutathione and uric acid were purchased from Sigma Chemical Co. Chromatographic grade acetonitrile and *o*-phthaldehyde (OPA) were from Merck. Trolox was from Aldrich Chemical Co. and 2,2'-azo-bis(2-amidinopropane) (ABAP) from Polysciences, Warrington, PA, USA. [^{14}C]ALA was obtained from Amersham and NaB[^{3}H]$_4$ from New England Nuclear, USA. All other reagents were analytical grade. Solutions were prepared with Millipore MilliQ purified water.

ALA Treatment of Rats

Male Wistar rats (180–220 g) were injected intraperitoneally with aqueous ALA solution (40 mg/kg body weight, between 8 and 9 *a.m.*). The same volume of water was injected in the control animals. The animals were maintained in a room at 25°C under a 12:12 light-dark cycle and fed a commercial diet *ad libitum* for one week before the beginning of the treatment. The acute treatment consisted of a single dose of ALA and the animals were killed by decapitation 24 h afterwards. In the two-dose and prolonged (eight-dose) protocols, the animals were injected on alternate days and killed by decapitation 24 h after the last dose.

Blood Withdrawal

For blood withdrawal the animals were rapidly anaesthetized in ethyl ether atmosphere, the abdomen opened and the blood taken from the abdominal aorta with a heparinized syringe. The

complete procedure between anaesthesia and blood obtainance took not longer than 2 min. The blood was centrifuged at 900 × g for 10 min and the plasma collected.

Tissue Chemiluminescence

This assay was performed as described by Boveris *et al.*[18] Control and treated rats were anesthetized with pentobarbital (50 mg/kg body weight). The liver and soleus muscle surfaces were exposed by opening the abdominal wall and the skin, respectively. The parietal brain region was opened by incision, partially exposing the two hemispheres. Chemiluminescence was measured with a Johnson Foundation Photocounter (Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA). The photon counting system contained a red sensitive photomultiplier (EMI 9658) cooled with a thermoelectric cooler, therefore able to detect singlet oxygen dimol emission, an amplifier-discriminator connected to a frequency counter and recorder, and a lucite rod as an optical coupler between the studied organ and the photomultiplier. The results were expressed in counts per second (cps) per area of tissue (cps/cm^2).

Plasma Antioxidant (Trapping) Capacity

This assay was performed as described by Lissi *et al.*[16] The method consists of luminol (12.5 μM) luminescence measurement in the presence of ABAP (10 mM) dissolved in 0.10 M phosphate buffer, pH 7.4. Plasma samples (20 μl) were added to this mixture. Determinations were carried out in a LKB Liquid Scintillation Counter at 25°C. The results were calculated as the ratio of the initial intensity (I_o) to the resulted intensity (I) after plasma addition. A plot of I_o/I against increasing Trolox concentrations (10–800 μM) was obtained in parallel. Results of plasma samples are expressed as equivalents of Trolox concentration corresponding to the trapping capacity of the employed plasma (μM of Trolox).

ALA Levels in Plasma and Tissues

ALA was determined in plasma and tissue homogenates by HPLC with electrochemical detection. Ten microliters of sample (standard ALA solution or biological sample) were placed in a small conical vessel, to which 5.0 μl of OPA reagent (36 mM) and mobile phase were added (final volume, 50 μl). This solution was vortex homogenized and a 20 μl aliquot injected into the HPLC system after one min incubation. Chromatographic equipment: Isocratic liquid determinations with ALA-OPA derivatives were performed using a high performance liquid cromatograph equipped with a LC10AD pump coupled to a LECD 6A electrochemical detector from Shimadzu Corp., Kyoto, Japan. The detector working electrode was mantained at 0.6 V *vs* Ag/AgCl and its signal delivered to a 386 ASA computer with data collection and handling provided by Scientific Software, San Ramon, CA, USA. For the ALA assay a RP C-18 Novapak column (15.0 cm × 3.9 mm I.D.) from Waters Corp., Milford, MA, USA and 50 mM phosphate buffer pH 7.0 with 20% acetonitrile and 2.4 mM EDTA as the mobile phase were used. The sample was introduced with a 7125-055 injector equipped with a 20 μl external loop from Rheodyne Incorporated, Cotati, CA, USA and eluted with the mobile phase circulated at 1.0 ml/min.

ALA determinations were performed in plasma and homogenates of total brain and the liver right frontal lobe. Homogenates were prepared in 0.32 M sucrose dissolved in 10 mM Hepes buffer pH 7.4 and centrifuged for 10 min at 1,000 g. Supernatants were collected, frozen at –20°C and assayed within three days.

Plasma Protein Carbonyls

Protein carbonyl groups were ^{3}H-labeled by reduction with NaB[$^3H_4$] and determined as described by Levine et al.[19] Plasma samples were mixed with 10% trichloroacetic acid (TCA)

and centrifuged at 14,000 g. The pellet was washed twice with 10% TCA. The final pellet was resuspended in water and assayed for carbonyl groups. Values are expressed in nmoles of ^{3}H-protein-carbonyl equivalents per mg of protein.

Plasma Sulfhydryls

Sulfhydryl groups were measured according to Koster et al.[17] Plasma (50 µl) was transferred to 0.10 M phosphate buffer (750 µl), pH 7.4 to which 2 mM DTNB (200 µl) were added. This mixture was incubated for 5 min at 37°C. After incubation the absorbance at 412 nm was read. Total sulfhydryl group content was calculated using a molar extinction coefficient of 13,600 $m^{-1}cm^{-1}$. Results are expressed in mM sulfhydryl groups.

Oxygen Consumption

Oxygen consumption was followed on a Yellow Spring model YSI 53 oxygen monitor with a standard ISI 5331 electrode. The reaction was started by addition of 2.5 mM ALA in 0.10 M phosphate buffer, pH 7.4 at 37°C in the presence or absence of plasma, and the oxygen uptake followed for 30 min.

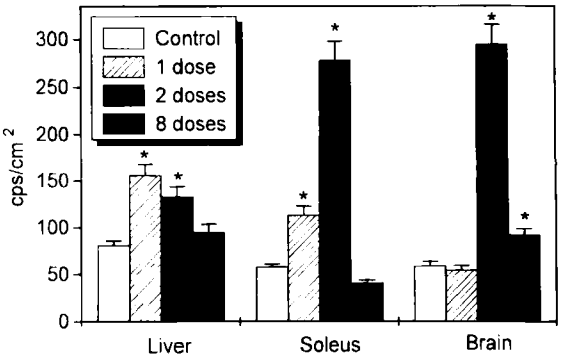

FIGURE 1 Spontaneous chemiluminescence of exposed rat tissues after ALA treatment. Results are expressed as mean SD of one experiment for each scheme of treatment, (n = 6 in each group). Values for the control group represent the average obtained for the control animals in the three schemes of treatment used (n = 18). Two and eight ALA doses (40 mg/kg body weight) were administered on alternate days. *$p < 0.001$ (two way ANOVA).

RESULTS

In Vivo Studies

The intensity of spontaneous chemiluminescence from exposed tissues of ALA-treated rats was markedly increased after two doses (Fig. 1). This alteration was coincident with a high elevation in plasma trapping capacity (Table I). The chemiluminescence intensity from all tissues analyzed as

TABLE I Plasma trapping capacity (TRAP) and ALA levels measurement after ALA treatment of rats

		ALA		TRAP	
Tissue	N° doses	Two	Eight	Two	Eight
Plasma					
	Control	0.25 ± 0.06	0.21 ± 0.07	130 ± 5	120 ± 7
	Treated	0.32 ± 0.07	0.67 ± 0.14[a]	362 ± 8[b]	196 ± 6[b]
Liver					
	Control	3.8 ± 0.6	3.9 ± 0.3	ND	ND
	Treated	5.2 ± 1.6	5.8 ± 1.3[c]		
Brain					
	Control	0.92 ± 0.08	1.3 ± 0.1	ND	ND
	Treated	1.24 ± 0.40	1.8 ± 0.4[d]		

Results are expressed as mean ± SD of two independent experiments (n = 6 in each group). Trapping capacity (TRAP) is referred as µM Trolox as described in Materials and Methods, plasma ALA concentration as µM and, in the liver and brain as nmol/mg protein. ND: not determined. [a]$p < 0.04$, [b]$p < 0.00$, [c]$p < 0.03$, [d]$p < 0.055$ (Student's t test).

well as the plasma trapping capacity showed lower values after prolonged ALA treatment. The augmented plasma trapping capacity detected after two doses of ALA may represent a prompt response to the oxidative process triggered by ALA in tissues. In turn, the decreased plasma trapping capacity after prolonged treatment may be due to increased antioxidant tissue uptake from plasma by the organs as a protective response. *In vitro*, ALA (30 mM) does not modify the plasma antioxidant capacity (Table II).

The ALA levels in plasma, liver and brain after two and eight *i.p.* ALA administrations are shown in Table I. After two doses, no significant ALA increase was observed in plasma or liver and brain homogenates. After eight doses, the ALA level increased *ca.* 3-fold in plasma, 48% in liver and 38% in brain. Recently, we observed that the treatment of either fibroblast, hepatocyte, erythroleukemic or glial cell culture with ALA or succynilacetone, a potent ALA dehydratase inhibitor,[7] does not overload the cells with ALA (E. Bechara and coworkers, unpublished). Maximum intracellular levels achieved were only 40% above the control whereas extracellular ALA increased 8-fold, when hamster lung fibroblast (M8) cells were incubated for 1 h in the presence of 4 mM succinylacetone. These data indicate that the fate of intracellular overproduced ALA is excretion. In contrast, McGillion *et al.*[20] reported that 3 h after *i.p.* ^{14}C-ALA-spiked ALA injection in rats (40 mg/kg body weight), its concentration raises *ca.* 15 times in blood and is largely uptaken by liver (1,300-fold), brain (10-fold) and heart (5-fold). At 72 h it had returned to normal in plasma but continued much greater than normal in those organs. According to these data, ALA is promptly uptaken by the tissues but is also intensely excreted by the kidneys. In any case, the data reported here shows that ALA increases in the organs is accompanied by intensification of the organ chemiluminescence, an index of oxidative insult according to Boveris et al.[18] This is in agreement with our previous findings of biochemical changes in plasma, liver, red muscles and brain of ALA-treated rats towards a glycolytic metabolism.[21]

The total sulfhydryl group content in the plasma of rats with prolonged ALA treatment was not altered (control samples = 0.27 ± 0.07 and treated samples = 0.28 ± 0.05 mM).

TABLE II ALA effect on ABAP/luminol system in the presence of isolated and plasma containing antioxidants

Addition, μM	I_o/I
ALA, 30	1.0
60	1.1
Trolox, 12.5	1.9
plus ALA, 30	1.8
Uric acid, 125	1.0
plus ALA, 30	1.5
Ascorbic acid, 62	1.3
plus ALA, 30	1.1
GSH, 125	1.4
plus ALA, 30	1.5
Plasma	3.3
plus ALA, 30	3.4
plus ALA, 30 mM	3.4

Antioxidants plus ALA were incubated for 24 h at specified concentrations in 50 mM phosphate buffer, pH 7.4 at room temperature. Human plasma (20 μl) was added to the same buffer and emission measurement recorded immediately after plasma or plasma/ALA addition. Results shown represent mean of three determinations which agreed within 5%.

Effect of ALA on Isolated and Plasma-Containing Antioxidants

To further ascertain whether ALA affects the plasma antioxidant capacity, the effect of ALA on the luminol/ABAP luminescent system[16] was measured. As a control, ALA was incubated at increasing concentrations in 0.10 M glycine buffer, pH 8.6 as well as in 0.10 M phosphate buffer, pH 7.4, to which luminol (50 μM) and ABAP (25 mM) were added. At concentrations as high as 30 mM, ALA does not affect the luminol luminescence intensity. That ALA does not affect the antioxidant capacity of isolated plasma components such as ascorbate, urate, reduced glutathione and the water soluble vitamin E analogue Trolox was demonstrated by the lack of any effect on trapping capacity of overnight ALA incubation with any of these plasma antioxidants

(Table II). These results might be important physiologically since ALA could be protected from oxidation by antioxidants dissolved in plasma and therefore be transported and distributed among tissues without being destroyed or generating deleterious free radicals. Accordingly, human plasma (1:30 and 1:15 dilutions) was found to significantly inhibit the ALA (2.5 mM) oxygen uptake (Fig. 2): 3-fold inhibition at 1:15 plasma dilution. At 500 μM ALA, 1:15 diluted plasma almost completely abolished the oxygen uptake (not shown).

The ALA effect on plasma reduced glutathione was also evaluated according to Koster *et al.*[17] ALA (30 mM, 60 mM and 125 mM) was incubated with 5 mM GSH in 0.10 M phosphate buffer, pH 7.4 at room temperature for 4 h. ALA was found not to modify the GSH thiol content in these conditions. Protein carbonyl groups resulting from protein oxidation were investigated *in vitro* by incubating human blood plasma with 20 mM ALA at 37°C for 24 h and measuring tritium incorporation from [^{3}H]$NaBH_4$ in carbonyl protein groups. No difference between ALA treated and untreated plasma was found. Thus, it seems that ALA is not capable of promoting oxidative damage to plasma components; contrarily, its oxidation is inhibited by these antioxidants.

ALA Binding to Plasma Proteins

In order to find out whether or not ALA circulates bound to blood proteins, freshly dialyzed human blood plasma and human albumin (40 mg/ml) were incubated with ^{14}C-ALA at 37°C for 24 h. After incubation, radiolabelled ALA was searched in the protein pellet obtained by precipitation with 10% TCA, followed by centrifugation. This pellet was resuspended, TCA precipitated and the ^{14}C-ALA determined in both supernatant and pellet. That ALA is weakly bound to the protein plasma fraction was suggested by the fact that the ^{14}C-counts in the first supernatant corresponded to 50% of the total plasma and, after protein resuspension and re-precipitation, the ^{14}C-ALA was totally recovered in this second

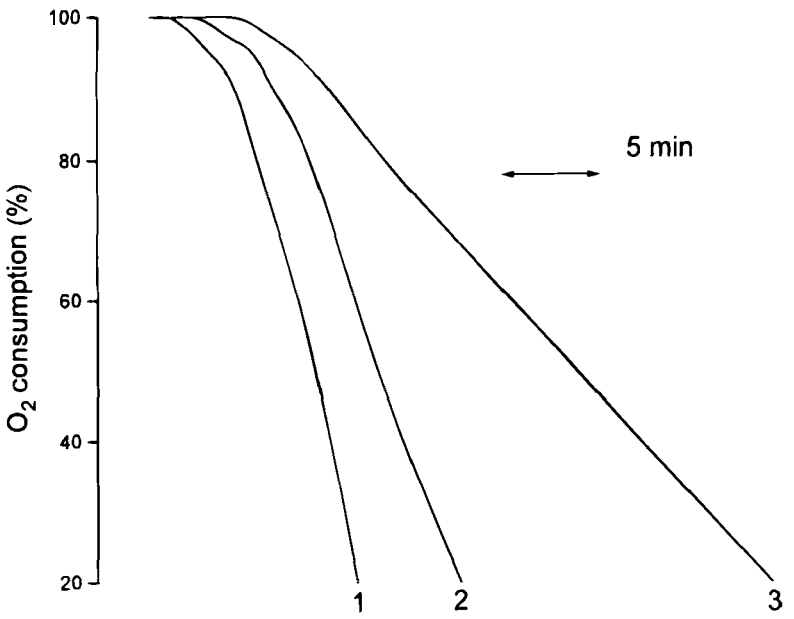

FIGURE 2 Oxygen consumption by ALA in the presence of human plasma. Oxygen uptake recording was started by the addition of 2.5 mM ALA to 0.1 M phosphate buffer, pH 7.4, at 37°C, in the absence (1) or presence of diluted plasma: 1:30 (2) and 1:15 (3). The plasma was diluted in the same buffer.

supernatant. The ^{14}C-ALA determined was less than 0.4% in the last plasma protein pellet and 1% in the albumin precipitate. ^{14}C-ALA was almost completely recovered in the first supernatant. Thus, it seems that ALA barely binds to plasma proteins, including albumin. In parallel, ^{14}C-ALA was incubated with human blood plasma for 2 h and submitted to protein electrophoresis. No ^{14}C-ALA-containing protein fractions was found (not shown).

DISCUSSION

The present results indicate that ALA-treated animals underwent tissue oxidative stress as indicated by *in situ* chemiluminescence measurements (Fig. 1). Tissue chemiluminescence has been widely employed as an indicator of lipid peroxidation and protein oxidation reactions as suggested by Boveris *et al.*[18] and Barnard *et al.*,[22] respectively. Both the plasma antioxidant system and the organ chemiluminescence responded to the ALA treatment (Table I). Our data show that ALA does not (i) interfere with the plasma antioxidant capacity, (ii) promote oxidation of plasma elements and (iii) bind to plasma proteins. A connection between tissue light emission and plasma trapping capacity was thus verified. As reported by Gutteridge and Quinlan,[23] the plasma antioxidant mechanisms are primarily identified with iron-binding and iron-oxidizing proteins. Although the plasma pH may favor ALA enolization, which precedes ALA oxidation,[3] there are no "free" iron complexes in plasma capable of catalyzing ALA autoxidation. In addition, plasma antioxidants would prevent the propagation of ALA oxidation (Fig. 2). One may conclude that the observed increase in the ALA-induced plasma antioxidant capacity may represent a response to tissue oxidative damage due to tissue ALA uptake. Upon two doses, the ALA concentration was not significantly altered in plasma, brain or liver, although the plasma trapping capacity and chemiluminescence emission in tissues were greatly increased. Since the ABAP/luminol chemiluminescence generation is not dependent on metal catalysis, the plasma trapping capacity measured by this method must be related to plasma soluble molecules with reducing properties. Thus, increased plasma trapping capacity induced by ALA treatment may be due to increased plasma antioxidant response.

The data here reported (Table I) show increased ALA levels in liver and brain only after prolonged administration, higher in liver than in brain. In the latter organ, the chemiluminescence levels are higher than in the former after two doses of ALA and do not decrease to the control value after prolonged treatment, whereas in liver it increased after only one dose (Fig. 1). This level is maintained after two doses and decreased with prolonged treatment. The soleus muscle showed a similar behavior to that of brain, which also agrees with biochemical alterations previously observed in these two tissues by Pereira *et al.*[21] It seems that brain is very sensitive to ALA effects since its levels did not increase in brain after two doses, but chemiluminescence response was very much increased. According to McGillion *et al.*[20] ALA is rapidly uptaken by brain and other tissues but, as mentioned above, cells in culture do not accumulate ALA. Instead, they release ALA to the extracellular medium. We might thus suggest a similar mechanism for brain where ALA did not accumulate.

The data presented here show that ALA behaves differently in plasma and tissues. There was no indication of oxidative alterations in the plasma components although the studied tissues seemed to be oxidatively injured as indicated by enhanced direct organ chemiluminescence (Fig. 1). Indeed, previous studies with ALA-treated rats had already revealed alterations of anti-oxidant enzyme activities in brain, soleus, and liver as well increases of lactate and free fatty acid levels in the blood[21] and iron mobilization to and γ-aminobutyric binding alterations in brain.[14] It is possible that the plasma antioxidants and the lack of

plasma "free" iron completely protect ALA from oxidation and consequently halt its potential oxidative effects. The plasma antioxidant system seems here to reflect both the ALA-induced oxidative tissue damage and the plasma trapping capacity. Interestingly, the tissue injury seems to decrease after prolonged ALA treatment as suggested by the organ chemiluminescence data. This observation is of great importance concerning physiological adaptation to chronic oxidative injury.

Porphyrias are characterized by acute episodes accompanied by plasma ALA overload, probably related to tissue accumulation, which could lead to an intense antioxidant response, partly diminishing ALA toxicity. Recently, Juknat et al.[24] proposed that ALA toxicity could result from both its accumulation in the cells and deficient heme biosynthesis, with an additional effect on glucose metabolism. Our free radical hypothesis linked to ALA overload in pophyrias[25] has been evoked by Härtel *et al.*[26] to explain the detection of hydroxyl and carbon-centered radicals directly formed from ALA during photosensitization of green leaves of wheat and lettuce pretreated with this compound. In addition, Hiraku and Kawanishi[27] recently reported the mechanism of Cu(II)-catalyzed oxidative damage of DNA fragments obtained from c-Ha-*ras* proto-oncogene induced by ALA autoxidation. As in our previous studies with DNA/ALA/Fe(II)[28,29], those authors concluded that ALA-generated reactive oxygen species may be related to carcinogenesis of lead compounds and the increased frequency of hepatoma in acute intermittent porphyria.

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