N-Acetyl-L-cysteine protects against δ -aminolevulinic acid-induced 8-hydroxydeoxyguanosine formation

Mozow Yusof, Deniz Yildiz, Nuran Ercal *

Department of Chemistry, University of Missouri-Rolla, Rolla, MO 65401, USA

Received 18 September 1998; received in revised form 14 January 1999; accepted 15 January 1999

Abstract

5-Aminolevulinic acid (ALA) is a heme precursor that accumulates in acute intermittent porphyria and lead poisoning. It has been shown that ALA induces free radical generation and may cause damage to proteins and DNA. In the present study, the effects of ALA on DNA damage and its prevention by *N*-acetyl-L-cysteine (NAC) and the antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) are investigated. Oxidative damage to DNA was quantitated by measuring the increase in 8-hydroxy-2'-deoxyguanosine (oh⁸dG) formation. The time-course study demonstrated that ALA causes a linear increase in oh⁸dG levels in Chinese hamster ovary (CHO) cells. However, direct lead exposure did not cause any measurable increase in oh⁸dG levels. In the presence of either NAC (1 mM) or antioxidant enzymes (10 u/ml SOD and 10 u/ml CAT), oh⁸dG levels returned to the corresponding control levels. This suggests a protective role for NAC and the antioxidant enzymes. To determine the effect of ALA on cell proliferation, cell numbers were counted at the end of 24 h of incubation in the presence and absence of ALA at different concentrations. Results showed that levels of ALA up to 5 mM do not inhibit cell proliferation. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *N*-Acetyl-L-cysteine; δ -Aminolevulinic acid; Cell proliferation; DNA damage

1. Introduction

Lead exposure has been shown to increase levels of intracellular δ -aminolevulinic acid (ALA) by inhibiting the enzyme δ -aminolevulinic acid

dehydratase (ALAD), which is involved in the limiting step of the heme biosynthetic pathway (Haeger-Aronsen et al., 1971; Ribarov and Bochev, 1982; Gibbs et al., 1991). The mechanisms for lead toxicity have yet to be elucidated; however, it has been proposed that lead-induced cellular damage may originate, in part, from ALA-induced oxidative stress. ALA is a heme precursor that accumulates in several disorders,

* Corresponding author. Tel.: +1-573-341-6950; fax: +1-573-341-6033.

E-mail address: nercal@umr.edu (N. Ercal)

including acute intermittent porphyria and tyrosinosis (Hindmarsh, 1986; Demasi et al., 1996). It has been shown that increased intracellular levels of ALA lead to generation of reactive oxygen intermediates (ROIs) and induce oxidative stress (Monteiro et al., 1989; Hermes-Lima et al., 1991). This has been demonstrated in previous studies by an increase in the generation of lipid peroxidation byproduct, malondialdehyde, and a decrease in intracellular free radical scavengers (Neal et al., 1997). Recent *in vivo* and *in vitro* studies have shown that exposure to ALA results in an increased generation of 8-hydroxy-2'-deoxyguanosine (oh⁸dG), a marker for oxidative damage to DNA (Fraga et al., 1994; Hiraku and Kawanishi, 1996). Although lead has been shown to induce oxidative stress and to increase intracellular ALA levels, its effect on oh⁸dG formation has not been investigated. The present study investigated whether ALA enhances oh⁸dG formation, indicating oxidative DNA damage. In addition, the study evaluated the role of *N*-acetyl-L-cysteine (NAC) and antioxidant enzymes in the prevention of ALA-induced generation of oh⁸dG.

2. Materials and methods

Chinese hamster ovary (CHO) K1 cells were obtained from the American Type Culture Collection (Rockville, MD). The items required for the maintenance of cell culture, Ham's F-12 media, fetal calf serum and glutamine were obtained from Sigma Chemical Company (St. Louis, MO). Lead acetate, superoxide dismutase (SOD), catalase (CAT), ALA, NAC, deoxyguanosine, oh⁸dG, phenol, phenol-chloroform, nuclease P1 and alkaline phosphatase were also purchased from Sigma (St. Louis, MO).

2.1. Cells and culture conditions

CHO cells were propagated in Ham's F-12 culture media supplemented with 10% fetal calf serum (FCS) and maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

2.2. Cell treatment

Cells (10 × 10⁶) from exponentially growing cultures were established in culture flasks. In order to establish cell attachment prior to treatment, the cells were incubated in the treatment media for 4 h without retrypsinization before addition of various concentrations of ALA (1–5 mM), lead acetate (100 µg/ml), and co-addition of ALA and lead acetate. Cells were then further incubated for the indicated time intervals in the presence of ALA and lead acetate. NAC (1 mM) and antioxidant enzymes (SOD; 10 units/ml) and CAT (10 units/ml) were added along with ALA to assess the protection. At the end of incubation, the cells were trypsinized and collected for DNA isolation. In order to provide the most accurate control data, the control flasks were trypsinized in an order dispersed within the trypsinization times of the experimental groups. The trypsinization and collection of all cells were performed in the minimum time allowable in order to prevent DNA oxidation within this time period.

2.3. DNA isolation

DNA was isolated as described by a previously reported procedure with a minor modification (Gupta, 1984). Collected cells were resuspended in 1 ml of 1% (w/v) sodium dodecyl sulfate, 1 mM EDTA, and incubated at 37°C for 60 min in the presence of proteinase K (500 µg/ml). At the end of incubation, 50 µl of 1 M Tris-HCl (pH 7.5) were added to the cell lysates and the samples were then extracted successively with 1 vol. of phenol, 1 vol. of phenol-chloroform-isoamyl alcohol (25:24:1) and 1 vol. of chloroform-isoamyl alcohol (24: 1). After addition of 0.1 vol. of 5 M NaCl, DNA was precipitated with 2 vols of absolute ethanol and precooled to -20°C. The samples were stored at -20°C for 24 h and then centrifuged at 2000 × *g* for 10 min. DNA samples were then washed three times with 70% (v/v) ethanol. Traces of ethanol were removed and the DNA was dissolved in water.

2.4. Preparation of DNA samples for HPLC-EC

DNA samples in 50 μ l of water ($A_{260} = 2-3$ units) were heated in boiling water for 2 min and then quenched immediately in ice water (Abelson and Simon, 1994). One unit of A_{260} quantitates 50 μ g of DNA. After the addition of 100 μ l of 30 mM sodium acetate (pH 5.3), DNA samples were digested to nucleosides with 10 μ g of nuclease P1 (1 mg/ml) at 37°C for 30 min. Then, 10 μ l of 0.5 M Tris were added to the samples and they were treated with 1.5 units of *Escherichia coli* alkaline phosphatase at 37°C for 60 min (Abelson and Simon, 1994). The resulting mixture was filtered through a 0.20- μ m pore filter and analyzed by high-performance liquid chromatography with electrochemical detection (HPLC-EC).

2.5. HPLC-EC system

The HPLC system (Shimadzu) comprised a Model LC-6A pump, a Rheodyne injection valve with a 20- μ l injection filling loop, and a Model SPD-6A UV spectrophotometric detector operating at an absorption wave length of 290 nm. Electrochemical detection was accomplished by a Bioanalytical Systems (West Lafayette, IN) LC-4C amperometric detector with a glassy-carbon working electrode and an Ag/AgCl reference electrode. The electrochemical detector was operated at an oxidation potential of +0.7 V. The analytical column used for the separation of oh^8dG from other components present in the hydrolyzed DNA samples was a 3- μ m C_{18} column (10 cm $\times$ 4.6 mm). The mobile phase consisted of filtered and degassed 50 mM KH_2PO_4 buffer (pH 5.5)–methanol (90:10, v/v). The separations were performed at a flow rate of 1 ml/min. Quantitation of the peaks from the HPLC-EC system was performed with two Chromatopacs, Model CR601 (Shimadzu).

2.6. Statistical analysis

InStat by GraphPad software (San Diego, CA) was used to analyze data statistically. One-way analysis of variance (ANOVA) and Student–Newman–Keuls multiple comparison tests were applied.

3. Results

Fig. 1 shows the results of the time–course study in the presence of different ALA concentrations. The data are presented in Table 1. Exposure of CHO cells to ALA resulted in an increased generation of oh^8dG . Statistically significant increases in oh^8dG levels compared to control oh^8dG levels were observed following 1 and 5 mM ALA exposure for 4, 12 and 24 h. Results indicate that increasing the ALA concentration over 1 mM does not further increase oh^8dG formation. The differences between the 1 and 5 mM ALA-induced formations of oh^8dG were statistically insignificant at all time points tested. The time–course study also indicated that ALA treatment of cells causes a linear increase in oh^8dG levels. As shown in Fig. 2, 0.5 mM ALA increases oh^8dG levels approximately twice as much as this. This increase is statistically significant when compared to the control oh^8dG levels.

Reactive oxygen intermediates (ROIs) have been known to interfere with cell proliferation by disturbing the cell cycle kinetics (Halliwell and Aruoma, 1993). To determine if ALA interferes with cell proliferation, we constructed a growth curve in the presence of 0.1, 1 and 5 mM ALA. Results showed similar rates of cell proliferation in ALA-exposed cells and control cells. This seems to indicate that cells continue to proliferate in the presence of elevated oh^8dG , thereby increasing the probability of mutations.

In order to test whether a lead-induced increase in ALA levels in vitro produces measurable DNA damage or if lead potentiates the ALA-induced generation of oh^8dG , we incubated one set of cells with lead acetate only and another set with a combination of lead acetate and ALA. As shown in Fig. 2, exposure of cells to lead caused no significant change in levels of oh^8dG . Moreover, lead combined with ALA did not show any additive effect in the formation of oh^8dG . Fig. 3 shows the result of treatment with NAC and antioxidant enzymes along with ALA. Both NAC and antioxidant enzyme addition returned the oh^8dG levels in ALA-exposed cells to the control levels.

4. Discussion

Our results indicate that exposure of CHO cells to ALA induces oxidative DNA damage. A linear increase in oh^8dG levels was observed following exposure of cells to 1 and 5 mM ALA. Both 1 and 5 mM ALA induced oxidative DNA damage to a similar extent, suggesting that ALA-induced damage to DNA reaches a plateau at a certain concentration, and further increasing the ALA concentration does not generate any additional oxidative damage to DNA. Previous *in vitro* studies showed that the addition of transition metal ions into solutions containing ALA and DNA promotes the formation of oh^8dG several fold (Hiraku and Kawanishi, 1996). In this respect, it could be suggested that the rate of intracellular free radical generation by ALA oxidation is limited by the simultaneous availability of the transition elements. In the presence of limited metal ions, ALA-induced oxidative DNA damage reaches a plateau and further increases in ALA levels do not generate additional free radicals. Another possibility is that exposure of cells to ALA may inhibit cell proliferation due to genera-

tion of free radicals that result in oxidative damage to cellular macromolecules. A reduced rate of cell proliferation by higher ALA concentrations thus prevents further DNA damage by decreasing the frequency of DNA exposure to a cellular environment with a relatively higher free radical content during replication. To test this possibility, the rate of cell proliferation in the presence of different ALA concentrations was investigated. However, we did not see an inhibition of cell proliferation after a 24-h ALA exposure (data not shown). An important outcome was that the cells continued to proliferate at a similar rate to control cells in the presence of elevated DNA damage. This may increase the frequency of DNA point mutations and support the previous hypothesis by other researchers that ALA may play a role in carcinogenesis (Fraga et al., 1994; Hiraku and Kawanishi, 1996).

Our results (Fig. 2) indicated that exposure of cells to lead acetate or lead acetate and ALA together does not induce oxidative DNA damage above control or ALA levels, respectively. There are many explanations for this result. To the best of our knowledge, lead has never been shown to

Fig. 1. Cells from exponentially growing cultures were established in F-12 media supplemented with 10% FCS. After 4 h of incubation, cells were treated with 0.1, 1 or 5 mM ALA. Cells were then further incubated for 4, 12 or 24 h. At the end of the incubation, cells were collected by trypsinization and DNA was isolated for oh^8dG measurement. Results are the mean $\pm$ S.D. of three separate experiments. Results from 1 mM and 5 mM ALA have values of $P < 0.05$ compared to control values.

Table 1
oh⁸dG formation in ALA-exposed cells^a

Time (h)	oh ⁸ dG/dG ± S.D. (× 10 ⁵)			
	Control	0.1 mM ALA	1 mM ALA	5 mM ALA
4	1.35 ± 0.09	1.67 ± 1.39	5.46 ± 1.69	4.15 ± 0.68
12	4.70 ± 0.64	4.38 ± 1.55	9.86 ± 0.06	7.67 ± 1.97
24	6.52 ± 1.55	7.38 ± 0.99	14.26 ± 1.81	13.17 ± 1.77

^a Results are the mean ± S.D. of three separate experiments. 1 mM and 5 mM ALA have values of $P < 0.05$ compared to control values. The experimental conditions for these data are the same as those outlined in Fig. 1.

directly increase free radical formation. However, lead is known to inhibit ALA dehydratase activity, thereby leading to an accumulation of ALA (Haeger-Aronsen et al., 1971; Ribarov and Bochev, 1982; Gibbs et al., 1991). It is ALA that is proposed to auto-oxidize and then increase hydroxyl free radicals, and therefore ALA that is usually studied as the direct-acting agent. Furthermore, it has been shown that, in the presence of hydroxyl free radicals, oh⁸dG formation is lowered by the formation of ring ruptured species, 2,6-diaminohydroxy-5-formamidopyrimidine (FapyGua) (Aruoma and Halliwell, 1995). Therefore, it is possible that FapyGua formation is favored over oh⁸dG formation in the presence of lead. Moreover, lead may accelerate the repair processes involved, showing less oh⁸dG formation. Although our results show no increase in oh⁸dG formation after lead acetate and ALA treatment, this does not mean there is no increase in DNA damage. Although oh⁸dG formation is a widely studied parameter of DNA damage, it is obviously not the only one (Aruoma and Halliwell, 1995). Once DNA damage by ALA was established, possible protective agents were analyzed. Due to the mediation of the intracellular response to oxidative stress by the enzymes SOD and CAT (Cerruti et al., 1988), administration of these enzymes was performed to evaluate these possible antioxidant capabilities. SOD and CAT cannot cross the cell membrane, and their protective effects are expected due to their scavenging abilities of superoxide and hydrogen peroxide. Superoxide is not proposed to induce DNA damage through DNA strand breaks or heme oxidation; therefore, any damage is most likely due to

the hydrogen peroxide (Aruoma and Halliwell, 1995). Hydrogen peroxide can cross the cell membrane and can be converted to hydroxyl free radicals, which can then generate oh⁸dG. The marked decrease in formation of oh⁸dG after

The effect of lead on oh⁸dG formation in the presence and absence of ALA

Fig. 2. Cells from exponentially growing cultures were established in F-12 media supplemented with 10% FCS. After 4 h of incubation, cells were treated with 0.1 and 0.5 mM ALA in the presence and absence of lead acetate (100 µg/ml). Cells were then further incubated at 37°C for an additional 24 h. At the end of the incubation, cells were collected by trypsinization and DNA was isolated for oh⁸dG measurement. Results are the mean ± S.D. of three separate experiments. Results from 0.5 mM ALA have values of $P < 0.05$ compared to corresponding control values.

The effect of NAC and antioxidant enzymes on ALA-induced oxidative DNA damage

Fig. 3. Cells from exponentially growing cultures were established in F-12 media supplemented with 10% FCS. After 4 h of incubation, cells were treated with 1 mM ALA in the presence and absence of 1 mM NAC and 10 units/ml of each of the antioxidant enzymes (SOD and CAT). Cells were then further incubated at 37°C for an additional 12 h. At the end of the incubation, cells were collected by trypsinization and DNA was isolated for oh⁸dG measurement. Results are the mean ± S.D. of three separate experiments. *Results from 1 mM ALA have values of $P < 0.05$ compared to control values.

administration of SOD and CAT indicates that ALA induces oxidative stress in the CHO cells, as well as DNA damage. Another possible remedy for this oxidative stress due to ROIs would be replenishment of the glutathione (GSH) pool. However, GSH does not readily cross the outer cell membrane, and direct GSH supplementation is not possible (Moldeus, 1994). To avert this limitation, intracellular cysteine pools are usually elevated by NAC administration. NAC is involved in the rate-limiting step of GSH synthesis, and is a well-known antioxidant with an established high toxicity threshold in vivo (Janes and Routledge, 1992). NAC can cross the cell membrane; therefore, it has a protective effect inside the cell. The protective effect of NAC on the

formation of oh⁸dG seen in this study, in conjunction with our previous studies (Ercal et al., 1996; Neal et al., 1997), suggests that the production of ROIs, leading to oh⁸dG formation, is caused by ALA. The amount of oh⁸dG in cells treated with only NAC or SOD and CAT were similar to control levels, as expected. Under the assumption that lead-induced free radical formation is mainly due to the accumulation of ALA, we suggest that NAC may be helpful in cases of lead poisoning and diseases such as acute intermittent porphyria to prevent DNA damage during oxidative stress.

Acknowledgements

This study was supported by NIH grant 1R15ES09497-01.

References

- Abelson, J.N., Simon, M.I., 1994. Oxygen radicals in biological systems. *Methods Enzymol.* 234.
- Aruoma, O.I., Halliwell, B., 1995. Immunopharmacology of free radical species. In: Editor, A. (Ed.), *DNA Damage by Free Radicals: Carcinogenic Implications*. Academic Press, San Diego.
- Cerruti, P.A., Knrputza, G., Larsson, R.L., Muehlemater, D., Crawford, D., Amstad, P., 1988. Physiological and pathological effects of oxidants in mouse epidermal cells. *Ann. New York Acad. Sci.* 551, 75–82.
- Demasi, M., Penatti, C., DeLucia, R., Bechara, J.H., 1996. The prooxidant effect of 5-aminolevulinic acid in the brain tissue of rats: implications in neuropsychiatric manifestations in porphyrias. *Free Radic. Biol. Med.* 20 (3), 291–299.
- Ercal, N., Treeratphan, P., Lutz, P., Hammond, T.C., Matthews, R.H., 1996. *N*-Acetylcysteine protects Chinese Hamster Ovary (CHO) cells from lead-induced oxidative stress. *Toxicology* 108, 57–64.
- Fraga, C.G., Onuki, J., Lucasoli, F., Bechara, E.J.H., Di Mascio, P., 1994. 5-Aminolevulinic acid mediates the in vivo and in vitro formation of 8-hydroxy-2'-deoxyguanosine in DNA. *Carcinogenesis* 15, 2241–2244.
- Gibbs, P.N.B., Gore, M.G., Jordan, P.M., 1991. Investigation of the effect of metal ions on the reactivity of thiol groups in human 5-aminolevulinic dehydratase. *Biochem. J.* 225, 573–580.
- Gupta, R.C., 1984. Nonrandom binding of the carcinogen *N*-hydroxy-2-acetylaminofluorene to repetitive sequences of rat liver DNA in vivo. *Proc. Natl. Acad. Sci. USA* 81, 6943–6947.

- Haeger-Aronsen, B., Abdulla, M., Fristedt, B.I., 1971. Effect of lead on delta aminolevulinic acid dehydratase activity in red blood cells. *Arch. Environ. Health* 23, 440–445.
- Halliwell, B., Aruoma, O.I., 1993. *DNA and Free Radicals*. Ellis Horwood, New York, pp. 211–222.
- Hermes-Lima, M., Valle, G.R.V., Vercesi, A.E., Bechara, E.J.H., 1991. Damage to rat liver mitochondria promoted by delta-aminolevulinic acid-generated reactive oxygen species: connections with acute intermittent porphyria and lead poisoning. *Biochim. Biophys. Acta* 1056, 57–63.
- Hindmarsh, J.T., 1986. The porphyrias: recent advances. *Clin. Chem.* 32, 1255–1263.
- Hiraku, Y., Kawanishi, S., 1996. Mechanism of oxidative DNA damage induced by delta-aminolevulinic acid in the presence of copper ion. *Cancer Res.* 56, 1786–1793.
- Janes, J., Routledge, P., 1992. Recent developments in the management of paracetamol (acetaminophen) poisoning. *Drug Safety* 7, 170–177.
- Moldeus, P., 1994. *N-Acetylcysteine*. *Methods Enzymol.* 234, 482–492.
- Monteiro, H.P., Abdalla, D.S.P., Augusto, O., And Bechara, E.J.H., 1989. Generation of active oxygen species during coupled autoxidation of oxyhemoglobin and delta-aminolevulinic acid. *Biochim. Biophys. Acta* 881, 100–106.
- Neal, R., Yang, P., Fiechtel, J., Yildiz, D., Gurer, H., Ercal, N., 1997. Pro-oxidant effects of delta-aminolevulinic acid on Chinese hamster ovary cells. *Toxicol. Lett.* 91, 169–178.
- Ribarov, S.R., Bochev, P.G., 1982. Lead–hemoglobin interaction as a possible source of reactive oxygen species—a chemiluminescent study. *Arch. Biochem. Biophys.* 213, 288–292.