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SHORT COMMUNICATION

Effect of 5-aminolevulinic acid on the expression of carcinogenesis-related proteins in cultured primary hepatocytes

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Abstract

Acute intermittent porphyria (AIP) is a heme pathway disorder caused by a decrease in the activity and synthesis of porphobilinogen deaminase. Thus, the first heme precursor 5-aminolevulinic acid (ALA) accumulates in the liver. Reactive oxygen species (ROS) resulting from ALA oxidation may be correlated to a higher incidence of hepatocellular carcinoma (HCC) in AIP patients. However, the molecular mechanisms of this relationship have not been thoroughly elucidated to date. In this study, we investigated the effect of increasing levels of ALA on the expression of proteins related to DNA repair, oxidative stress, apoptosis, proliferation and lipid metabolism. Primary rat hepatocytes were isolated by the collagenase perfusion method, lipoperoxidation was evaluated by a TBA fluorimetric assay and Western blotting was used to assess protein abundance. The data showed that ALA treatment promoted a dose-dependent increase of p53 expression, downregulation of Bcl-2, HMG-CoA reductase and OGG1 and an increase in lipoperoxidation. There was no alteration in the expression of the transcription factor NF-κB, catalase and superoxide dismutase. ALA oxidation products induced protein regulation patterns, suggesting the interconnection of cellular processes, such as the intrinsic pathway of apoptosis, redox homeostasis, cell proliferation, lipid metabolism and DNA repair. This study helps to elucidate the molecular mechanisms of hepatotoxicity mediated by ALA pro-oxidant effects and supports the hypothesis that ALA accumulation correlates with a higher incidence of hepatic carcinogenic events.

Keywords 5-Aminolevulinic acid · Acute intermittent porphyria · Hepatocellular carcinoma · Protein expression · Reactive oxygen species · Primary rat hepatocytes

Abbreviations

Bcl-2	β-Cell lymphoma 2
ALA	5-Aminolevulinic acid
8-oxodGuo	8-Oxo-7,8-dihydro-2´-deoxyguanosine
OGG1	8-Oxoguanine DNA glycosylase I
AIP	Acute intermittent porphyria
CAT	Catalase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCC	Hepatocellular carcinoma
HMG-CoAr	Hydroxymethylglutaryl-CoA reductase
NF-κB	Nuclear factor κB
ROS	Reactive oxygen species
SOD	Superoxide dismutase
p53	Tumor protein p53

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Introduction

Porphyrias are acquired or inherited metabolic diseases identified by alterations in the activity or the reduced synthesis of heme biosynthetic pathway enzymes, such as porphobilinogen deaminase [1]. Among these diseases, lead poisoning, hereditary tyrosinemia and acute intermittent porphyria (AIP) are characterized by an increase of 5-aminolevulinic acid (ALA) in the urine and plasma. ALA, the first precursor of the heme complex, accumulates heavily in the brain and liver of AIP patients [2]. This accumulation can be the cause of the typical clinical manifestations in AIP patients, such as neuromuscular weakness, neuropsychiatric alterations, attacks of abdominal pain [3] and an increased hepatocellular carcinoma (HCC) incidence [4].

In vitro, ALA oxidation catalyzed by iron/ferritin generates reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH), and the aldehyde 4,5-dioxovaleric acid (DOVA), as final products. In fact, ALA induces several DNA lesions, such as strand breaks in plasmid DNA [5], increases the level of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) and radical-induced base degradation products in vitro and in the rat spleen and liver DNA [6, 7]. Moreover, it is reported that ALA induces chromosomal aberrations [8] and mutagenicity by the SOS Chromotest and Ames test [9]. Moreover, ALA impairs the mitochondrial transmembrane potential and damages the inner membrane of isolated rat liver mitochondria through a Ca^{2+}-dependent mechanism [10], which plays a significant role in signaling pathways of apoptosis, proliferation and carcinogenesis [11, 12]. In addition, exposing PC12 and SVNF cells to ALA leads to lesions in nuclear and mitochondrial DNA. ALA produces DNA lesions and alters the morphology of CHO and HepG2 cells, due to apoptosis induction [13]. This evidence suggests that ALA is an endogenous source of ROS which, in turn, overwhelms antioxidant defense mechanisms, resulting in damage to biomolecules and the regulation of signaling pathways involved in the cell survival checkpoints [14] and the carcinogenic process [5, 15]. Although the mechanism for ALA-induced cell transformation and carcinogenesis seems to be the mutations that originate from unrepaired or misrepaired DNA lesions [16], damage to other biomolecules, such as signaling proteins, DNA repair enzymes and lipids, may also play crucial roles in all steps of the tumorigenesis process [17], possibly through ALA derivatives.

The objective of this work was to investigate the effect of ALA on lipoperoxidation and on the expression of selected mammalian proteins related to apoptosis (tumor protein p53 and β-cell lymphoma 2), oxidative stress (nuclear factor κB, catalase, and superoxide dismutase), cell proliferation (β-cell lymphoma 2 and tumor protein p53), DNA repair (tumor protein p53 and 8-oxoguanine DNA glycosylase I) and lipid metabolism (hydroxymethylglutaryl-CoA reductase). To assess ALA's capacity to alter hepatic protein abundance, we chose primary rat hepatocytes (PRH) as our in vitro model. These cells maintain most normal hepatic functions, including drug-metabolizing enzymes, which makes them a suitable model for chemical toxicity assessments. We chose a short period of treatment (2 h) to monitor the early onset of the alterations, considering the high chemical reactivity of ROS and previous DNA damage studies, which established the prooxidant hypothesis of ALA's mechanism. Our data help to elucidate ALA-induced liver injury and substantiate its hypothesized relation to HCC development in symptomatic AIP patients.

Materials and methods

Animals

Male Wistar rats (6–8 weeks old, 200–250 g) were fed and given water ad libitum and were obtained from the Butantan Institute animal house. The experimental procedure was approved by the animal ethics committee of Butantan Institute (CEUAIB 755/10) and was performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and the associated guidelines, the EU Directive 2010/63/EU for animal experiments and complied with the ARRIVE guidelines.

Rat primary hepatocytes: isolation and culture

Rat primary hepatocytes were isolated and cultured following a collagenase perfusion method as detailed elsewhere [18].

Hepatocyte treatments

Immediately after isolation, the primary hepatocytes (2.0×10^6 cells) were seeded in a 25 cm^2 flask with 5 mL media (DMEM, 10% fetal bovine serum and penicillin:streptomycin 100 UI/mL:0.1 mg/mL). The PRH were incubated for 16 h at 37 °C, with 5% CO_2 in a humidified atmosphere prior to treatment. The cells were exposed to ALA (5–100 mM) in serum-free DMEM to prevent the enhancement or suppression of the chemical stimuli. The hepatocytes were treated for 2 h and were washed with PBS and lysed with ice-cold RIPA buffer containing a proteases and phosphatases inhibitors cocktail (Thermo Scientific, MA, USA). The cell lysates were immediately analyzed or

frozen at − 20 °C for further analyses. We chose a short period of treatment (2 h) in order to monitor the early onset of the alterations, considering the high chemical reactivity of ROS and for comparisons with previous studies, which established the pro-oxidant mechanism hypothesis [6, 19]. We used a high concentration of ALA, considering that it is an endogenous molecule, which is not cytotoxic per se, and it is hypothesized that it acts as a pro-oxidant only when it is accumulated in the liver of AIP patients.

Protein concentration measurement

The protein concentration of the lysates was determined by the Coomassie protein assay kit (Pierce Chemical Co., IL, USA) as described elsewhere [20].

Lipoperoxidation

Lipid peroxidation was evaluated by a microscale TBA fluorimetric assay, which was modified from Mihara & Uchiyama [21]. Briefly, 50 µL of the sample was mixed with 1 mL of phosphoric acid and 50 µL of BHT. One milliliter of a 0.67% TBA solution was added to the reaction mixture, which was stirred and incubated in boiling water for 30 min. After being cooled at room temperature, the pink chromogen was extracted by adding 1 mL of butanol and stirring for 30 s. The samples were centrifuged at 680x g for 5 min. The fluorescence of the butanol phase was measured at 515 nm (excitation wavelength) and 553 nm (emission wavelength). The lipid peroxidation was expressed as a percentage of the micromolar of tetraepoxypropane (TEP) equivalents per millimolar of protein.

Western blotting

The extracted proteins (40 µg) were mixed with loading buffer and were heat-denatured at 95 °C water for 2 min, separated on a 12.5% SDS-PAGE gel for 2 h at 100 V and were transferred to a polyvinylidene fluoride (PVDF) membrane using a semidry system at 20 V for 30–45 min in transfer buffer (12 mM Tris, 96 mM glycine, 20% methanol, pH 8.3). Bovine serum albumin (1%), containing 0.1% Tween-20, was used to block the membranes overnight at 4 °C, and they were later incubated with primary antibodies targeting NF-κB (1:1000) and p53, (5 µg/µL) from Abcam, Bcl-2 (1:1000), catalase (1:1000) and OGG1 (1:1000) from Thermo Scientific, and superoxide dismutase (1:2000) and HMG-CoA reductase (1 µg/mL) from Millipore overnight at 4 °C. After three washes with TBS-T for 5 min, the membranes were incubated with an HRP-conjugated second antibody (Goat anti-rabbit IgG (H + L) 1:5000 or Goat anti-mouse IgG1 1:4000) for 1 h at room temperature. Using enhanced chemiluminescence, the bands were revealed using the SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific, MA, USA) and were visualized at ChemiDoc MP (Bio-Rad Lab, CA, USA). GAPDH or β-actin were used as the loading controls. The image acquisition, band detection and analyses were performed using Image Lab 5.0 Software (BioRad Lab, CA, USA).

Statistical analysis

The data are represented as the mean ± SEM. A one-way ANOVA and Dunnett's multiple comparison tests were used to analyze the significant differences between the groups.

Results and discussion

PRH were stimulated with increasing concentrations of ALA, which promoted an increase in lipoperoxidation and caused the regulation of oxidative stress-related proteins associated with the onset of carcinogenesis. ALA promoted a dose-dependent increase in p53 protein expression and decreased the protein expressions of Bcl-2, HMG-CoAr and OGG1. No significant alteration in the expression of NF-ƙB (p105/p50), CAT or SOD was found. It is essential to note that ROS levels are supposed to remain high in all of the tested concentrations.

The oxidative cellular environment induced by ALA on the PRH resulted in an upregulation pattern for p53 and a downregulation pattern for Bcl-2 (Figs. 1, 2). Reactive oxygen species causes activation and direct mutations in p53, which acts as a tumor suppressor [22]. More than 50% of human cancers, especially at the advanced stages, present mutations in the p53 gene or a loss of protein function [23]. The protein p53 plays a pivotal role in sensing and removing nuclear and mitochondrial DNA (mitoDNA) oxidative damage, preventing mutations and genetic instability by mediating DNA repair, cell cycle arrest, senescence and cell death [24]. In the event of failed DNA repair, p53 represses Bcl-2, a known survival signal, and initiates extrinsic and/or intrinsic apoptosis by the transactivation of pro-apoptotic proteins [25]. In fact, the expression of Bcl-2 was significantly decreased in the primary hepatocytes treated with ALA (Fig. 2).

Proteins in the Bcl-2 family are the central regulators of the mitochondrial pathway of apoptosis, acting by sequestering the pro-apoptotic proteins (Bak and Bax) responsible for the formation of the oligomer channels on the outer membrane of the mitochondria, through which the release of cytochrome c to the cytosol occurs [26]. Alterations in this protein indicate that the intrinsic pathway could be the mechanism of apoptosis induced by ALA in hepatocytes [13]. Under different redox states, Bcl-2 promotes, modulates and optimizes mitochondrial respiration [27] and it

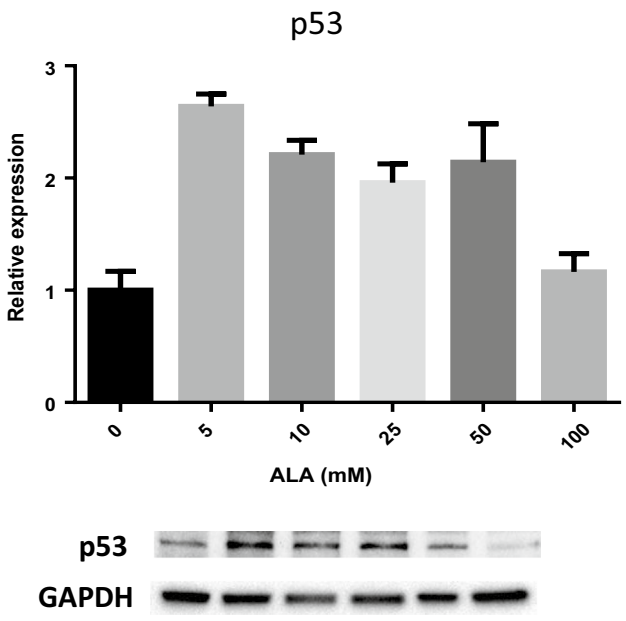

Fig. 1 Effect of ALA on p53 expression in primary hepatocytes treated with the indicated concentrations (5–100 mM) for 2 h. The protein extracts were subjected to Western blotting using p53 and GAPDH primary antibodies and an HRP-conjugated goat anti-mouse IgG1 secondary antibody. The emitted chemiluminescence signals were quantified. The experiment was performed in four independent cell preparations. The results are expressed as the ratio of p53/GAPDH (mean ± SEM) relative to the control, which was set at 1. *p < 0.05, **p < 0.01 versus the control

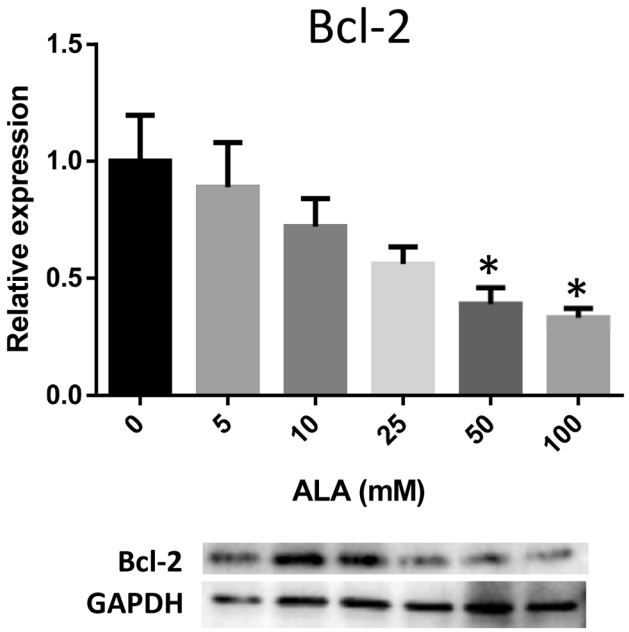

Fig. 2 Effect of ALA on Bcl-2 expression in primary hepatocytes treated with the indicated concentrations (5–100 mM) for 2 h. The protein extracts were subjected to Western blotting using Bcl-2 and GAPDH primary antibodies and an HRP-conjugated goat anti-rabbit Ig (H + L) secondary antibody. The emitted chemiluminescence signals were quantified. The experiment was performed in four independent cell preparations. The results are expressed as the ratio of Bcl-2/GAPDH (mean ± SEM) relative to the control, which was set at 1. *p < 0.05, **p < 0.01 versus the control

demonstrates an anti-proliferative activity in mouse hepatocarcinogenesis [28]. These properties contribute to the ALA oxidative effects and emphasize the important roles of mitochondrial bioenergetics and ROS in the onset and/or maintenance of carcinogenesis [29].

Usually, apoptosis prevents cancer development by eliminating damaged cells or malignant-transformed cells. Nevertheless, in certain liver diseases, such as hepatitis B and C, alcoholic liver disease, and non-alcoholic steatohepatitis, hepatocyte apoptosis is frequently observed [30], and it seems to be mechanistically linked to HCC through the activation of the mitochondrial apoptotic pathway, which enhances ROS production.

Members of the Bcl-2 family are also described to exert an inhibitory influence on cell cycle entry, a function genetically separated from its anti-apoptotic role [31]. It seems that there is a correlation between the overexpression of the Bcl-2 protein and a lower proliferative rate in tumors [32]. Studies in mice show a tumor suppressor effect in diethylnitrosamine- or c-Myc-induced hepatocarcinogenesis due to the anti-proliferative activity of Bcl-2 [28]. Proteins of Bcl-2 also regulate migration, invasion and cancer metastasis [33]. The co-expression of Bcl-2 and Twist promotes cell migration and invasion in hepatocarcinoma cells [34]. Considering that Bcl-2 also suppresses lipid peroxidation [35] and increases antioxidant enzyme expression [36], the absence of an alteration in CAT and SOD expressions (Fig. 3) and the increased lipid peroxidation (Fig. 4) were in accordance with the decreased Bcl-2 protein and with the unaltered NF-κB protein expression (Fig. 5). After a two-hour treatment with ALA, the cells might already had overcome the initial phase of the ROS-triggered antioxidant signaling pathways. Alterations in the protein expression of p53 and Bcl-2 may explain several of the pro-oxidative cellular effects of ALA, such as lipoperoxidation [37], mitochondrial dysfunction [10, 38] and apoptosis [9]. Moreover, it is important to emphasize that the total lipid content, the oxidized lipid products and the secondary products of lipoperoxidation are extremely important in the carcinogenesis process [39].

The induction of DNA lesions by ALA and their molecular characterization have already been extensively studied [6, 13, 40]. Nevertheless, little is known regarding the effect of ALA in other biomolecules, such as proteins, enzymes and lipids. Our results indicate that DNA repair and lipid metabolism might also be involved in ALA's toxic mechanism along with the downregulation of HMG-CoAr (Fig. 6) and OGG1 (Fig. 7). The rate-limiting step in the synthesis of cholesterol is the reduction of HMG-CoA to mevalonate,

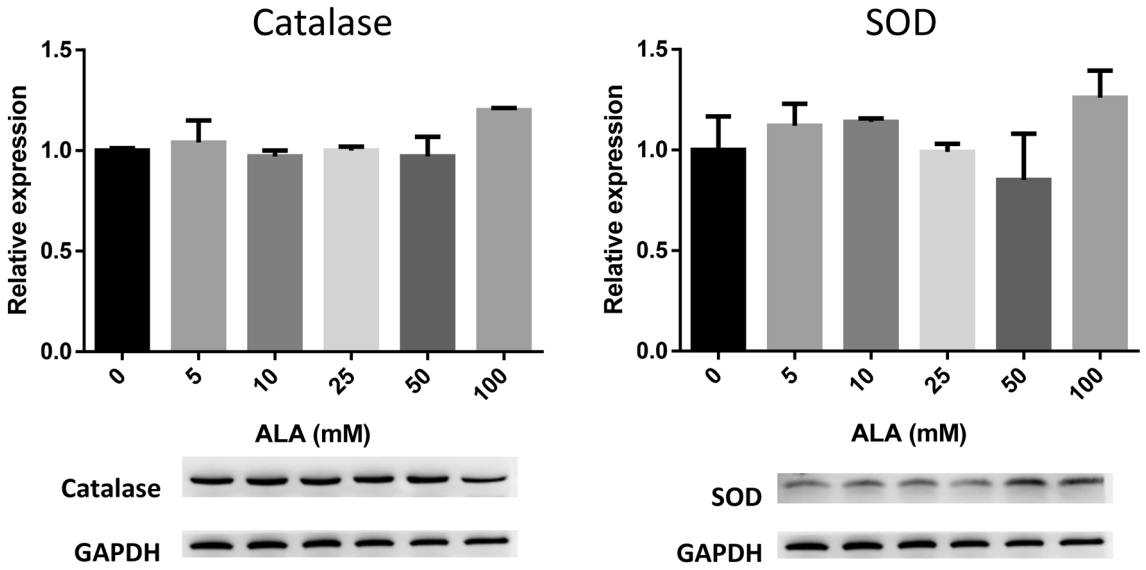

Fig. 3 Effect of ALA on catalase (CAT) and superoxide dismutase (SOD) expressions in primary hepatocytes treated with the indicated concentrations (5-100 mM) for 2 h. CAT, SOD and GAPDH primary antibodies, and a chemiluminescent HRP-conjugated goat anti-rabbit Ig (H + L) secondary antibody was used in the Western blotting. The results are expressed as ratio of CAT or SOD/GAPDH (mean ± SEM) relative to the control, which was set at 1; the data are derived from four independent experiments. *p < 0.05, **p < 0.01 versus the control

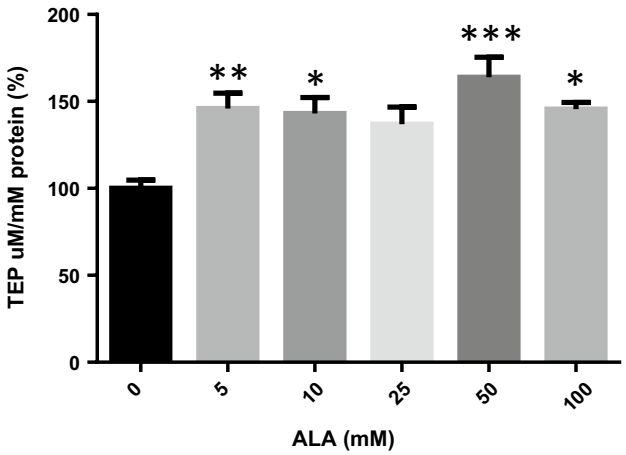

Fig. 4 Microscale TBA fluorimetric assay in primary hepatocytes induced by ALA (0–100 mM). The lipid peroxidation is expressed as a percentage of micromolar of tetraepoxypropane (TEP) equivalents per millimolar of protein. The experiment was performed in four independent cell preparations. The results are expressed as the mean ± SEM relative to the control, set at 100%. *p < 0.05, **p < 0.01, ***p < 0.001 versus the control

which is catalyzed by the enzyme HMG-CoAr [41]. The cholesterol level in tumor cells is higher than in surrounding cells, and the deregulated growth demand leads to an alteration in its synthesis and regulation [42]. Actually, an enhanced cholesterol requirement is closely related to a high cell proliferation [43] and tumor growth [44]. Cancer tissues display a decrease in the expression of the cholesterol exporter ATP binding cassette transporter A1, an increase in cholesterol uptake via LDL receptors, an upregulation of HMG-CoAr and a loss of its feedback inhibition [45]. Moreover, the trafficking of cholesterol to the mitochondria is increased [46], contributing to mitochondrial dysfunction, chemotherapy resistance and the metabolic reprogramming of tumor cells [47, 48]. Accordingly, HCC presents a cholesterol dependency [49] and metabolic models show that "among 101 metabolites relevant to HCC development, 30% are related to cholesterol biosynthesis" [50]. Indeed, the use of statins has a protective association with the risk of developing liver cancer [51]. In addition, it is hypothesized that the accumulation of cholesterol in the mitochondria alters membrane fluidity, permeability and organization, affecting the function of resident proteins, pore formation, cytochrome c release and the interactions between Bcl-2 proteins, which leads to mitochondria impairment [48].

An elevated level of 8-oxodGuo in DNA was already measured in peritumoral tissue in HCC [52]. Moreover, increased oxidative DNA damage is associated with hepatocarcinogenesis in patients with nonalcoholic steatohepatitis (NASH); the level of 8-oxodGuo in the liver of NASH patients who concomitantly present HCC is higher than the NASH patients without HCC [53]. Although no direct and specific molecular mechanisms can be established regarding the exact role of oxidatively induced DNA lesions, the conversion of the misrepaired or unrepaired lesions to mutations seems to be the leading force conducting cells to transformation and cancer development [52, 54]. Usually,

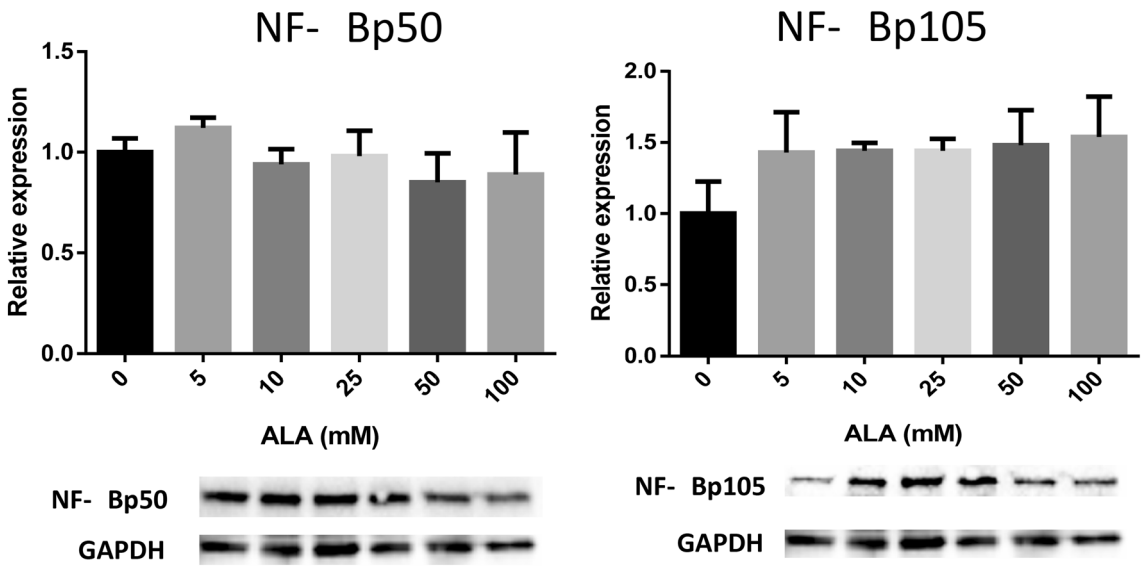

Fig. 5 Effect of ALA on NF-κB 105/50 expression in primary hepatocytes treated with the indicated concentrations (5–100 mM) for 2 h. The experiment was performed in four independent cell preparations. NF-κB and GAPDH primary antibodies and a chemiluminescent HRP-conjugated goat anti-rabbit secondary antibody were used in the Western blotting. The results are expressed as the ratio of NF-κB/GAPDH (mean ± SEM) relative to the control, which was set at 1; that data are derived from four independent experiments. *p < 0.05, **p < 0.01 versus the control

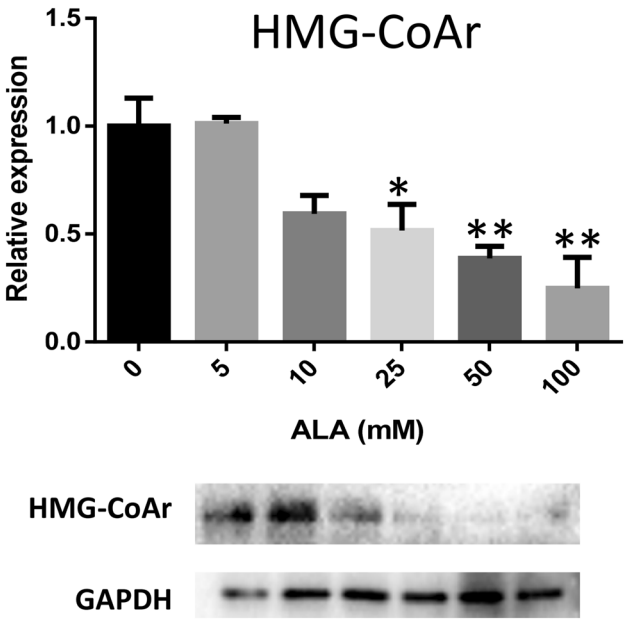

Fig. 6 Effect of ALA on HMG-CoAr expression in primary hepatocytes treated with the indicated concentrations (5–100 mM) for 2 h. HMG-CoAr and GAPDH primary antibodies and a chemiluminescent HRP-conjugated goat anti-rabbit Ig (H + L) secondary antibody were used in the Western blotting. The results are expressed as the ratio of HMG-CoAr/GAPDH (mean ± SEM) relative to the control, which was set at 1; the data are derived from 4 independent experiments. *p < 0.05, **p < 0.01 versus the control

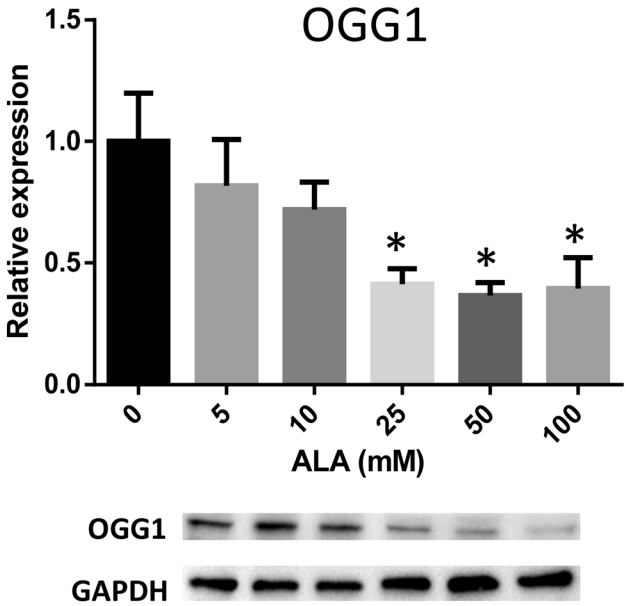

Fig. 7 Effect of ALA on OGG1 expression in primary hepatocytes treated with the indicated concentrations (5-100 mM) for 2 h. OGG1 and GAPDH primary antibodies and a chemiluminescent HRP-conjugated goat anti-rabbit Ig (H + L) secondary antibody were used in the Western blotting. The results are expressed as the ratio of OGG1/GAPDH (mean ± SEM) relative to the control, which was set at 1; that data are derived from four independent experiments. *p < 0.05, **p < 0.01 versus the control

8-oxodGuo causes a transversion from guanine (G) to thymidine (T), modulating gene expression by either changing the sequence context of the transcription factors binding sites or by directly altering DNA–protein interactions [55]. In fact, NF-κB classically binds to DNA sequences that contains runs of guanines [55, 56]. The access of NF-κB to its binding motifs can also be affected by architectural changes in DNA induced by OGG1 binding to 8-oxodGuo. Thus, the transcriptional machinery encounters a new epigenetic regulatory mechanism to modulate a timely cellular response to oxidative exposure [55], possibly including the one caused by ALA oxidation.

It is important to emphasize that the cellular processes promoted by ROS do not increase in a linear manner. Following the hierarchical oxidative stress model [57], a low oxidative stress induces the expression of antioxidant enzymes (CAT and SOD), an intermediate level of oxidative stress triggers a microenvironment inflammatory response by NF- ƙB and AP-1 activation, and a high level of ROS affects the mitochondrial PT pore and disrupts the electron transfer, thereby resulting in apoptosis or necrosis [57].

Hikita et al. [54] proposed that an enhanced ROS production aggravates oxidative stress events and induces oxidative DNA lesions, especially 8-oxodGuo, which leads to mutations, such as the C > A/G > T transversion, and ultimately leads to HCC development. This hypothesis accounts for the underlying mechanisms for ALA-induced carcinogenesis. A harmful build-up of ALA in the hepatic tissue of AIP patients boosts ROS production, especially hydrogen peroxide and hydroxyl radical. This oxidative cellular environment leads to a redox homeostasis imbalance and regulates the expression of stress-related carcinogenic proteins, such as p53, Bcl-2, HMG-COAr and OGG1. This scenario is congruent with the hypothesis that ROS-induced tumor promoting events may occur in AIP patients, thereby inducing the onset of HCC.

Conclusions

Using primary rat hepatocytes, we demonstrated that increasing the levels of ALA during a short-term treatment (2 h) altered the expression pattern of oxidative stress-related proteins. ALA induced lipoperoxidation and promoted an increase in p53 expression and a downregulation of Bcl-2, HMG-CoAr and OGG1 proteins. Taken together, our results indicate that ALA's pro-oxidative properties induce a redox homeostasis imbalance, proapoptotic events, and cell proliferation and interfere with lipid metabolism and DNA repair processes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The experimental procedure was approved by the animal ethics committee of Butantan Institute (CEUAIB 755/10) and was performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and the associated guidelines, the EU Directive 2010/63/EU for animal experiments and complied with the ARRIVE guidelines.

Research involving human participants All authors declare that this article does not contain any studies with human participants performed by any of the authors.

References

1. Karim Z, Lyoumi S, Nicolas G et al (2015) Porphyrias: a 2015 update. Clin Res Hepatol Gastroenterol 39:412–425. https://doi.org/10.1016/j.clinre.2015.05.009
2. Besur S, Schmeltzer P, Bonkovsky HL (2015) Acute porphyrias. J Emerg Med 49:305–312. https://doi.org/10.1016/j.jemermed.2015.04.034
3. Bissell DM, Wang B (2015) Acute hepatic porphyria. J Clin Transl Hepatol 3:17–26. https://doi.org/10.14218/JCTH.2014.00039
4. Sardh E, Wahlin S, Björnstedt M et al (2013) High risk of primary liver cancer in a cohort of 179 patients with acute hepatic porphyria. J Inherit Metab Dis 36:1063–1071. https://doi.org/10.1007/s10545-012-9576-9
5. Onuki J, Medeiros MH, Bechara EJ, Di Mascio P (1994) 5-Aminolevulinic acid induces single-strand breaks in plasmid pBR322 DNA in the presence of Fe^{2+} ions. Biochim Biophys Acta 1225:259–263
6. Fraga CG, Onuki J, Lucesoli F et al (1994) 5-Aminolevulinic acid mediates the in vivo and in vitro formation of 8-hydroxy-2′-deoxyguanosine in DNA. Carcinogenesis 15:2241–2244
7. Douki T, Onuki J, Medeiros MHG et al (1998) Hydroxyl radicals are involved in the oxidation of isolated and cellular DNA bases by 5-aminolevulinic acid. FEBS Lett 428:93–96. https://doi.org/10.1016/S0014-5793(98)00504-3
8. Fiedler DM, Eckl PM, Krammer B (1996) Does delta-aminolaevulinic acid induce genotoxic effects? J Photochem Photobiol B 33:39–44
9. Onuki J, Rech CM, Medeiros MHG et al (2002) Genotoxicity of 5-aminolevulinic and 4,5-dioxovaleric acids in the Salmonella/microsuspension mutagenicity assay and SOS chromotest. Environ Mol Mutagen 40:63–70. https://doi.org/10.1002/em.10083
10. Homedan C, Schmitt C, Laafi J et al (2015) Mitochondrial energetic defects in muscle and brain of a Hmbs−/− mouse model of acute intermittent porphyria. Hum Mol Genet 24:5015–5023. https://doi.org/10.1093/hmg/ddv222
11. Ciccarese F, Ciminale V (2017) Escaping death: mitochondrial redox homeostasis in cancer cells. Front Oncol 7:117. https://doi.org/10.3389/fonc.2017.00117

12. Singh KK (2006) Mitochondria damage checkpoint, aging, and cancer. Ann N Y Acad Sci 1067:182–190. https://doi.org/10.1196/annals.1354.022
13. Onuki J, Chen Y, Teixeira PC et al (2004) Mitochondrial and nuclear DNA damage induced by 5-aminolevulinic acid. Arch Biochem Biophys 432:178–187. https://doi.org/10.1016/j.abb.2004.09.030
14. Sies H (2017) Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: oxidative eustress. Redox Biol 11:613–619. https://doi.org/10.1016/j.redox.2016.12.035
15. Onuki J, Teixeira PC, Medeiros MHG, Di Mascio P (2002) Danos ao DNA promovidos por ácido 5-aminolevulínico: Possível associação com o desenvolvimento de carcinoma hepatocelular em portadores de porfiria aguda intermitente. Quim Nova 25:594–608. https://doi.org/10.1590/S0100-40422002000400015
16. Olinski R, Gackowski D, Rozalski R et al (2003) Oxidative DNA damage in cancer patients: a cause or a consequence of the disease development? Mutat Res 531:177–190
17. Rani V, Deep G, Singh RK et al (2016) Oxidative stress and metabolic disorders: pathogenesis and therapeutic strategies. Life Sci 148:183–193. https://doi.org/10.1016/j.lfs.2016.02.002
18. Guguen-Guillouzo C, Guillouzo A (1996) No Title. In: Guillouzo A, Guguen-Guillouzo C (eds) Isolated and cultured hepatocytes. Les Editions INSERM and John Libbey Eurotext, Paris, pp 1–12
19. Onuki J, Teixeira PC, Medeiros MHG et al (2002) Is 5-aminolevulinic acid involved in the hepatocellular carcinogenesis of acute intermittent porphyria? Cell Mol Biol 48:17–26
20. Noble JE (2014) Quantification of protein concentration using UV absorbance and coomassie dyes. Methods Enzymol 536:17–26. https://doi.org/10.1016/B978-0-12-420070-8.00002-7
21. Mihara M, Uchiyama M (1978) Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 86:271–278
22. Hofseth LJ, Hussain SP, Harris CC (2004) p53: 25 years after its discovery. Trends Pharmacol Sci 25:177–181. https://doi.org/10.1016/j.tips.2004.02.009
23. Hollstein M, Sidransky D, Vogelstein B, Harris CC (1991) p53 mutations in human cancers. Science 253:49–53
24. Levine AJ (1997) p53, the cellular gatekeeper for growth and division. Cell 88:323–331
25. Miyashita T, Krajewski S, Krajewska M et al (1994) Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene 9:1799–1805
26. Kirkin V, Joos S, Zörnig M (2004) The role of Bcl-2 family members in tumorigenesis. Biochim Biophys Acta 1644:229–249. https://doi.org/10.1016/j.bbamcr.2003.08.009
27. Chong SJF, Low ICC, Pervaiz S (2014) Mitochondrial ROS and involvement of Bcl-2 as a mitochondrial ROS regulator. Mitochondrion 19:39–48. https://doi.org/10.1016/j.mito.2014.06.002
28. Pierce RH, Vail ME, Ralph L et al (2002) Bcl-2 expression inhibits liver carcinogenesis and delays the development of proliferating foci. Am J Pathol 160:1555–1560. https://doi.org/10.1016/S0002-9440(10)61101-7
29. Takaki A, Yamamoto K (2015) Control of oxidative stress in hepatocellular carcinoma: helpful or harmful? World J Hepatol. https://doi.org/10.4254/wjh.v7.i7.968
30. Cabrera R, Limaye A, Cabrera R (2012) Hepatocellular carcinoma: current trends in worldwide epidemiology, risk factors, diagnosis, and therapeutics. Hepatic Med Evid Res 4:19. https://doi.org/10.2147/HMER.S16316
31. Vairo G, Innes KM, Adams JM (1996) Bcl-2 has a cell cycle inhibitory function separable from its enhancement of cell survival. Oncogene 13:1511–1519
32. Murphy KL, Kittrell FS, Gay JP et al (1999) Bcl-2 expression delays mammary tumor development in dimethylbenz(a) anthracene-treated transgenic mice. Oncogene 18:6597–6604. https://doi.org/10.1038/sj.onc.1203099
33. Um H-D (2016) Bcl-2 family proteins as regulators of cancer cell invasion and metastasis: a review focusing on mitochondrial respiration and reactive oxygen species. Oncotarget 7:5193–5203. https://doi.org/10.18632/oncotarget.6405
34. Sun T, Sun B, Zhao X et al (2011) Promotion of tumor cell metastasis and vasculogenic mimicry by way of transcription coactivation by Bcl-2 and Twist1: a study of hepatocellular carcinoma. Hepatology 54:1690–1706. https://doi.org/10.1002/hep.24543
35. Hockenbery DM, Oltvai ZN, Yin XM et al (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 75:241–251
36. Ellerby LM, Ellerby HM, Park SM et al (1996) Shift of the cellular oxidation-reduction potential in neural cells expressing Bcl-2. J Neurochem 67:1259–1267
37. Emanuelli T, Pagel FW, Porciúncula LO, Souza DO (2003) Effects of 5-aminolevulinic acid on the glutamatergic neurotransmission. Neurochem Int 42:115–121. https://doi.org/10.1016/S0197-0186(02)00074-8
38. Laafi J, Homedan C, Jacques C et al (2014) Pro-oxidant effect of ALA is implicated in mitochondrial dysfunction of HepG2 cells. Biochimie 106:157–166. https://doi.org/10.1016/j.biochi.2014.08.014
39. Rossin D, Calfapietra S, Sottero B et al (2017) HNE and cholesterol oxidation products in colorectal inflammation and carcinogenesis. Free Radic Biol Med. https://doi.org/10.1016/j.freeradbiomed.2017.01.017
40. Douki T, Onuki J, Medeiros MHG et al (1998) DNA alkylation by 4,5-dioxovaleric acid, the final oxidation product of 5-aminolevulinic acid. Chem Res Toxicol 11:150–157. https://doi.org/10.1021/tx970157d
41. Goldstein JL, Brown MS (1990) Regulation of the mevalonate pathway. Nature 343:425–430. https://doi.org/10.1038/343425a0
42. Garcia-Ruiz C, Mari M, Colell A et al (2009) Mitochondrial cholesterol in health and disease. Histol Histopathol 24:117–132. https://doi.org/10.14670/HH-24.117
43. Lo Sasso G, Celli N, Caboni M et al (2010) Down-regulation of the LXR transcriptome provides the requisite cholesterol levels to proliferating hepatocytes. Hepatology 51:1334–1344. https://doi.org/10.1002/hep.23436
44. Dang CV (2012) Links between metabolism and cancer. Genes Dev 26:877–890. https://doi.org/10.1101/gad.189365.112
45. Casey SC, Amedei A, Aquilano K et al (2015) Cancer prevention and therapy through the modulation of the tumor microenvironment. Semin Cancer Biol 35:S199–S223. https://doi.org/10.1016/j.semcancer.2015.02.007
46. Montero J, Morales A, Llacuna L et al (2008) Mitochondrial cholesterol contributes to chemotherapy resistance in hepatocellular carcinoma. Cancer Res 68:5246–5256. https://doi.org/10.1158/0008-5472.CAN-07-6161
47. Ribas V, García-Ruiz C, Fernández-Checa JC (2016) Mitochondria, cholesterol and cancer cell metabolism. Clin Transl Med 5:22. https://doi.org/10.1186/s40169-016-0106-5
48. Maxfield FR, Tabas I (2005) Role of cholesterol and lipid organization in disease. Nature 438:612–621. https://doi.org/10.1038/nature04399
49. Borena W, Strohmaier S, Lukanova A et al (2012) Metabolic risk factors and primary liver cancer in a prospective study of 578,700 adults. Int J Cancer 131:193–200. https://doi.org/10.1002/ijc.26338
50. Agren R, Mardinoglu A, Asplund A et al (2014) Identification of anticancer drugs for hepatocellular carcinoma through personalized genome-scale metabolic modeling. Mol Syst Biol 10:721
51. Singh S, Singh PP (2014) Statins for prevention of hepatocellular cancer: one step closer? Hepatology 59:724–726. https://doi.org/10.1002/hep.26614

52. Kryston TB, Georgiev AB, Pissis P, Georgakilas AG (2011) Role of oxidative stress and DNA damage in human carcinogenesis. Mutat Res 711:193–201. https://doi.org/10.1016/j.mrfmmm.2010.12.016
53. Tanaka S, Miyanishi K, Kobune M et al (2013) Increased hepatic oxidative DNA damage in patients with nonalcoholic steatohepatitis who develop hepatocellular carcinoma. J Gastroenterol 48:1249–1258. https://doi.org/10.1007/s00535-012-0739-0
54. Hikita H, Kodama T, Tanaka S et al (2015) Activation of the mitochondrial apoptotic pathway produces reactive oxygen species and oxidative damage in hepatocytes that contribute to liver tumorigenesis. Cancer Prev Res 8:693–701. https://doi.org/10.1158/1940-6207.CAPR-15-0022-T
55. Pan L, Zhu B, Hao W et al (2016) Oxidized guanine base lesions function in 8-oxoguanine DNA glycosylase-1-mediated epigenetic regulation of nuclear factor κB-driven gene expression. J Biol Chem 291:25553–25566. https://doi.org/10.1074/jbc.M116.751453
56. Ghosh S, Karin M (2002) Missing pieces in the NF-kappaB puzzle. Cell 109(Suppl):S81–S96
57. Tonks NK (2005) Redox redux: revisiting PTPs and the control of cell signaling. Cell 121:667–670. https://doi.org/10.1016/j.cell.2005.05.016