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Carcinogen-Induced, Free Radical-Mediated Reduction in Microsomal Membrane Fluidity: Reversal by Indole-3-propionic Acid

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Chromium (Cr) is a well established carcinogen, with Cr(III) accounting for much of the intracellular oxidative damage that this transition metal induces. Indole-3-propionic acid (IPA), a melatonin-related molecule, is a reported antioxidant and free radical scavenger. Concentration (1, 10, 100, 500, or 1000 μM) and time (15, 30, 45, 60, or 90 min)-dependent effects of Cr(III) in the presence of H_2O_2 (0.5 mM), as well as the protective effect of IPA on Cr(III)-induced alterations in membrane fluidity (the inverse of membrane rigidity), as an index of membrane damage, were estimated by fluorescence spectroscopy. Cr(III), in a concentration- and a time-dependent manner, decreased membrane fluidity, with marked effects at a concentration of 500 μM and 60 min of incubation. IPA (5, 3, or 1 mM) prevented the Cr(III)-induced decrease in membrane fluidity. It is concluded that the carcinogen Cr(III), in the presence of H_2O_2, generates free radicals, which decrease membrane fluidity in rat microsomal membranes. Membrane alterations are pharmacologically prevented by the antioxidant IPA.

KEY WORDS: Indole-3-propionic acid; chromium; carcinogen; membrane fluidity; microsomes; oxidative damage.

INTRODUCTION

Although a trace element essential for optimal cellular physiology, chromium (Cr) is also considered an environmental toxin. Hexavalent chromium [Cr(VI)], the primary toxic form, is a known environmental and occupational carcinogen in humans and has been used to experimentally induce cancer in animals (IARC, 1990; Cohen *et al.*, 1993). Cr(VI) is used in a number of occupational settings, *e.g.*, in the production of chromates, chromium plating, chromate pigment manufacture, and the production of cement and stainless steel (IARC, 1990). After entering cells via active anion-transport channels, Cr(VI) is readily reduced to its trivalent form Cr(III). Because of its limited ability to diffuse through cellular membranes, Cr(III) accumulates in cells (Snow, 1992). The carcinogenic activity of chromium is thought to be due to macromolecular damage caused by reactive intermediates arising in the course of its intracellular reduction to Cr(III) and/or by Cr(III) itself (Snow, 1992). Since antioxidants alter the carcinogenicity of Cr(III), it is widely held that the toxicity of this metal involves the generation of free radicals (Shi *et al.*, 1993; Tsou *et al.*, 1996; Zhitkovich *et al.*, 1996).

Tryptophan-derived indole compounds have been widely investigated as antioxidants and as free radical svavengers (Reiter, 1997, 1998; Karbownik *et al.*, 2000a; Reiter *et al.*, 2000a,b) and, due to their ability to protect DNA from oxidative damage, they are potential anticarcinogens (Blask *et al.*, 1999; Petranka *et al.*, 1999; Reiter, 1999; Romero *et al.*, 1999). Indole-3-propionic acid (IPA) is a deamination product of tryptophan and

[1] Key to abbreviations: Cr(III), trivalent chromium; Cr(VI), hexavalent chromium; H_2O_2, hydrogen peroxide; IPA, indole-3-propionic acid; ·OH, hydroxyl radical; TMA-DPH, 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene *p*-toluene sulfonate.

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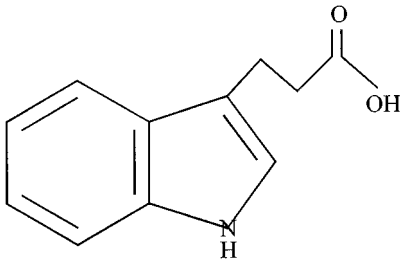

Fig. 1. The chemical structure of indole-3-propionic acid (IPA).

possesses, like another indole melatonin, a heterocyclic aromatic ring structure (Fig. 1). IPA is measurable in plasma and cerebrospinal fluid (Young *et al.*, 1980; Morita *et al.*, 1992). Although not as thoroughly investigated as an antioxidant as melatonin (Reiter *et al.*, 2000a,b; Tan *et al.*, 2000a), in two recent studies IPA has been reported to be equivalent to melatonin in scavenging free radicals and protecting against oxidative damage (Chyan *et al.*, 1999; Poeggeler *et al.*, 1999). These findings suggest that IPA may potentially protect against oxidative damage due to free radical generators, such as the carcinogen Cr(III).

Because of their high content of lipids, which are easily oxidized, cellular membranes constitute important targets for free radicals as well as for antioxidants. Compounds, which experimentally prevent membranes from the damaging effects of free radicals, may be considered as therapeutic agents. One major index of free radical damage to cellular membranes is a change in their fluidity (Yu *et al.*, 1992; Garcia *et al.*, 1997, 1998, 1999; Karbownik *et al.*, 2000b,c). Thus, we designed the current study to evaluate the membrane altering effects of Cr(III) and the possible protective actions of IPA on Cr(III)-induced changes in hepatic microsomal membranes.

MATERIALS AND METHODS

Chemicals

IPA, Cr(III) chloride hexahydrate ($CrCl_3 \cdot 6\ H_2O$), hydrogen peroxide (H_2O_2) and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma (St. Louis, Missouri). 1-[4-(Trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene *p*-toluene sulfonate (TMA–DPH) was obtained from Molecular Probes (Eugene, Oregon). Other chemicals used were of analytical grade and came from commercial sources. IPA was diluted in ethanol and TMA-DPH was diluted in tetrahydrofuran (THF) and water. The final concentrations of ethanol and THF in the incubation volume were 2.67 and 0.53%, respectively, and the concentration of TMA-DPH was 88.9 nM.

Animals

The procedures used in the study were approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing at that time ~250 g were purchased from Harlan (Houston, Texas) and housed in plexiglas cages (three animals per cage) in a windowless room with automatically regulated temperature ($22 \pm 2^\circ$C) and lighting (14 h light/10 h dark, with light on from 06.00 to 20.00 h). The animals received standard chow (Ralston Purina Co., Inc., St. Louis, Missouri) and water *ad libitum*. After 1 week of acclimatization, the rats were killed by decapitation and the liver was collected, frozen on solid CO_2, and stored at -80°C until assay.

Microsomal Membrane Isolation

Liver microsomal membranes were isolated as previously described (Yu *et al.*, 1992). Liver was homogenized in 140 mM KC1/20 mM HEPES buffer (pH 7.4) (1:10 w/v) and, thereafter, centrifuged at 1,000 $\times g$ for 10 min at 4°C. The pellets containing nuclei were removed and the supernatant was centrifuged at 105,000 $\times g$ for 60 min at 4°C. The pellets containing both microsomes and mitochondria were resuspended in the buffer and centrifuged at 10,000 $\times g$ for 15 min at 4°C. The supernatant, which contained exclusively the microsomal fraction, was centrifugated at 105,000 $\times g$ for 60 min at 4°C. Following the last centrifugation, the final microsomal pellets were suspended in 140 mM KCl/20 mM HEPES buffer (1:1, v/v) and kept at -80°C until used in the experiments.

Measurement of Protein

Protein was measured using the method of Bradford (1976), with bovine albumin as the standard.

Incubation of Microsomal Membranes

Microsomes (0.5 mg/ml microsomal protein) were resuspended in 50 mM Tris-HCl buffer (pH 7.4) and were incubated in the water bath at 37°C for 1 h in the presence of Cr(III) and H_2O_2 (0.5 mM); Cr(III) concentrations were either 1, 10, 100, 500, or 1000 μM. On the basis of the

concentration-dependent effect of Cr(III), a concentration of 500 μM was selected for the subsequent study. In the second study, which was designed to test for the time-dependent effects of Cr(III), microsomal membranes were incubated in the presence of Cr(III) (500 μM) + H_2O_2 (0.5 mM) at 37°C for either 15, 30, 45, 60, or 90 min. On the basis on this study, 60 min was chosen as the optimal incubation time with Cr(III) + H_2O_2. The main experiment consisted of two phases; first, microsomal membranes were incubated in the presence of IPA in concentrations of either 0.01, 0.1, 0.3, 1, 2, or 5 mM for 30 min at 37°C. This reaction was stopped by placing the samples on ice. Next, microsomes were incubated in the presence of Cr(III) (500 μM) + H_2O_2 (0.5 mM) for 60 min at 37°C. This reaction was stopped by addition of EDTA (2 mM).

Measurement of Membrane Fluidity (the Inverse of Membrane Rigidity)

Membrane fluidity was measured in duplicates (Yu *et al.*, 1992). Immediately after incubation, two aliquots (1-ml each) of microsomal membranes containing 0.5 mg protein were suspended in 50 mM Tris-HCl buffer (pH 7.4) (final volume 3 ml), vortexed for 1 min in the presence of TMA-DPH, and then incubated while shaking at 37°C for 30 min to ensure the uniform incorporation of the probe into the membranes. Fluorescence measurements were performed using a Perkin-Elmer LS-50 Luminescence Spectrometer equipped with a circulating water bath to maintain the temperature of cuvette at 22 ± 0.1°C during the assay. Excitation and emission wavelengths of 360 and 430 nm were used, respectively. The emission intensity of vertically polarized light was detected by an analyzer oriented parallel (I_{V_V}) or perpendicular (I_{V_H}) to the excitation plane. A correction factor for the optical system (G) was used. Polarization (P) was calculated as follows:

$$P = \left(I_{V_V} - GI_{V_H}\right) / \left(I_{V_V} + GI_{V_H}\right)$$

Because an inverse relationship exists between membrane fluidity and polarization, membrane fluidity is expressed as the inverse of P (1/P) (Yu *et al.*, 1992).

Statistical Analyses

Results are expressed as means ± SE. The data were statistically analyzed using a one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls test. Statistical significance was determined at a level of < 0.05.

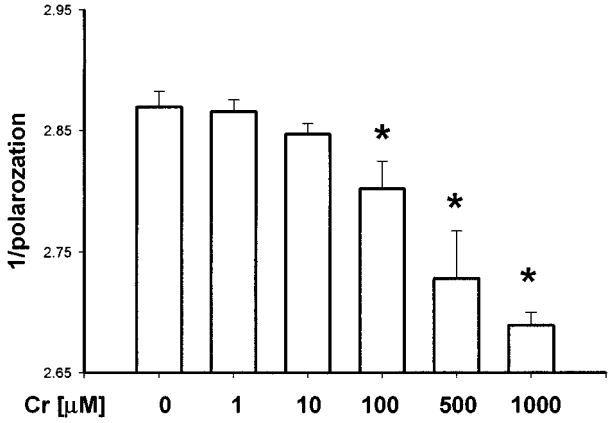

Fig. 2. Membrane fluidity (the inverse of membrane rigidity), expressed as an inverse of polarization (1/polarization) in hepatic microsomal membranes incubated in the presence of Cr(III) at concentrations of 1, 10, 100, 500, or 1000 μM for 60 min. Bars represent the mean ± SE of three independent experiments; $^*p < 0.05$ vs. control (in the absence of Cr).

RESULTS

Effects of Cr(III) Applied in Different Concentrations Plus H_2O_2 (0.5 mM) on Fluidity in Microsomal Membranes

The incubation of microsomal membranes in the presence of Cr(III) resulted in the concentration-dependent reduction in membrane fluidity, with statistically significant differences being achieved for concentrations of 1000, 500, and 100 μM (Fig. 2). Since a 500 μM concentration of Cr(III) caused a pronounced decrease in membrane fluidity, which was not significantly different than that caused by 1000 μM Cr(III), the 500 μM concentration was selected for the subsequent studies.

Effect of 500 μM Cr(III) Plus 0.5 mM H_2O_2 on Fluidity in Microsomal Membranes Incubated for Different Time Intervals

Figure 3 summarizes the time-dependent effect of 500 μM Cr(III) on membrane fluidity. Incubation of 30 min or longer significantly increased membrane fluidity. Based on these findings, a 60-min incubation time was selected for the subsequent studies.

Effect of IPA on Cr(III)-Induced Membrane Rigidity

Figure 4 shows a significant decrease in membrane fluidity when microsomes were incubated in the presence of 500 μM Cr(III) plus 0.5 mM H_2O_2 for 60 min. When microsomes were incubated in the presence of IPA

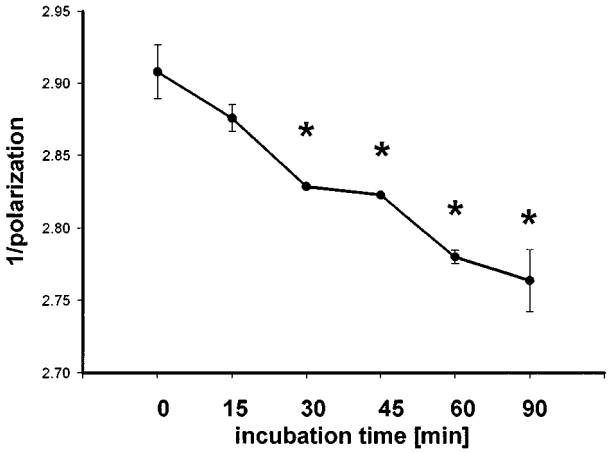

Fig. 3. Membrane fluidity (the inverse of membrane rigidity), expressed as an inverse of polarization (1/polarization) in hepatic microsomal membranes incubated in the presence of Cr(III) (500 μM) for 15, 30, 45, 60, or 90 min. Points represent the mean ± SE of three independent experiments; $^{*}p < 0.05$ vs. control (not incubated).

for 30 min, the indole, in the concentration-dependent manner, prevented Cr(III)-induced changes in membrane fluidity, with statistical difference obtained for IPA at concentrations of 5, 3, and 1 mM.

DISCUSSION

At physiological pH, in the presence of H_2O_2, Cr(III) has been shown to generate the hydroxyl radical

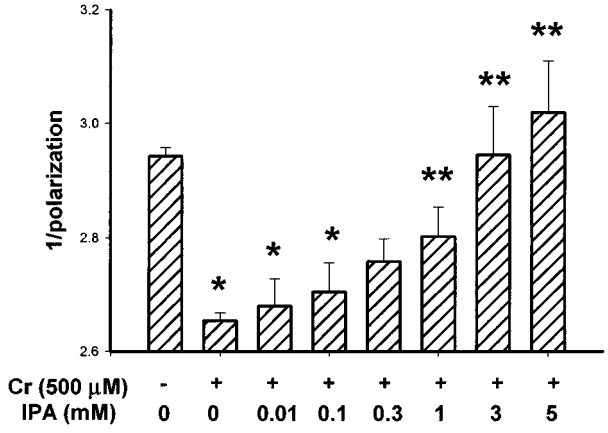

Fig. 4. Membrane fluidity (the inverse of membrane rigidity), expressed as an inverse of polarization (1/polarization) in hepatic microsomal membranes preincubated in the presence of indole-3-propionic acid (IPA) (0.01, 0.1, 0.3, 1, 2, or 5 mM) and, then, incubated with Cr(III) (500 μM) for 60 min. Bars represent the mean ± SE of three independent experiments; $^{*}p < 0.05$ vs. control (in the absence of any treatment); $^{**}p < 0.05$ vs. microsomes exposed to Cr (500 μM).

(·OH) as well as lipid hydroperoxide-derived free radicals (Shi *et al.*, 1993). This is significant in terms of cancer initiation, since ·OH is highly toxic and easily damages macromolecules (Halliwell, 1992).

With regard to cellular macromolecules, the evidence is clear that Cr(III) induces DNA damage, likely due to its ability to generate free radicals (Lloyd *et al.*, 1998; Qi *et al.*, 2000a,b); one result of this damage may be cancer. Cellular membranes are also important targets for carcinogens and for anticancer drugs (Arancia and Donelli, 1991). Because biological membranes are primarily composed of polyunsaturated fatty acids, they constitute a major site of lipid peroxidation. Lipid peroxidation is a chain reaction involving various free radicals and reactive oxygen species and the resulting metabolites (products of lipid peroxidation) can contribute to DNA damage and, consequently, to carcinogenesis (Cheeseman, 1993; Burcham, 1998). Structural changes in cellular membranes due to lipid peroxidation and, in addition, to peroxidation of membrane proteins, cause cross linking between adjacent lipid and protein molecules, thereby disrupting molecular motion in the membrane, and changing membrane fluidity (Yu *et al.*, 1992; Chen and Yu, 1994). Furthermore, damaged lipid products within the nuclear membrane, in particular, can lead to alterations in nuclear DNA, which can lead to cancer (Cheeseman, 1993). Thus, Cr(III)-related oxidative changes in membranes are relevant to the carcinogenic potential of this transition metal.

To date, only limited information is available concerning the influence of Cr(III) on cellular membranes. Electron paramagnetic resonance spectroscopy revealed changes in the fluidity of yeast plasma membranes due to Cr(III) exposure. The authors of these reports (Belagyi *et al.*, 1999; Pesti *et al.*, 2000) provide several potential explanations for their finding, including that the Cr(III) cation bound to the surface components of the membranes and thereby disturbed their physiology, changed the electric charge of the cell surface, induced lipid peroxidation, and disturbed membrane function and homeostasis. In any case, cell death was the result. It is not clear to what extent these primarily physical properties of Cr(III) modify membrane fluidity of mammalian cells. The changes observed in the present study, wherein Cr(III)-decreased fluidity in rat hepatic microsomal membranes, were likely due to the induced oxidative stress, since Cr(III), in the presence of H_2O_2, causes generation of the highly toxic ·OH.

The Fenton reaction, typically initiated by ferrous ions ($Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + {\cdot}OH + H_2O$), is induced by other transition metal ions as well. In the presence of H_2O_2, Cr(III) is highly effective, when compared to several other transition metal ions and to Cr(VI), in inducing free radical generation and DNA damage

(Lloyd *et al.*, 1998). The Fenton reaction initiated by Cr(III) is proposed to be the mechanism whereby this transition metal induced changes in membrane fluidity in the current study. ·OH are known to be sufficiently reactive to initiate lipid peroxidation and to alter membrane fluidity.

Herein, we show for the first time that Cr(III), in the presence of H_2O_2 decreases fluidity of mammalian hepatic microsomal membranes. As previously noted, Cr(III) in the presence of H_2O_2, at the same concentrations and incubation times as used here, also readily damages purified calf thymus DNA (Qi *et al.*, 2000a,b).

Cr(III)-induced damage of calf thymus DNA is prevented by melatonin, a molecule structurally similar to IPA (Qi *et al.*, 2000a). No tryptophan-related indoles, with the exception of IPA, have been examined in terms of their ability to prevent chromium-induced damage to microsomal membranes.

To what extent our findings can be extrapolated to conditions *in vivo* remains to be determined. Considering that Cr(III) does not cross cellular membranes, but causes intracellular damage (Snow, 1992), suggests that the Cr(III)-induced alterations in the plasma membrane secondarily lead to intracellular damage.

The mechanisms by which IPA preserves microsomal membranes from oxidative abuse are likely complex. IPA has been shown to readily protect primary neurons, neuroblastoma cells, and rat brain against oxidative damage due to β-amyloid protein, to H_2O_2 or to an inhibitor of superoxide dismutase (a key antioxidative enzyme), and to effectively scavenge ·OH with the rate constant of 7.8 to 8.0×10^{10} M^{-1}/s^{-1} (Chyan *et al.*, 1999; Poeggeler *et al.*, 1999). While reacting with ·OH, IPA also acts synergistically with another well known antioxidant, glutathione (Poeggeler *et al.*, 1999), to reduce oxidative damage. Moreover, glutathione is known to form stable complexes with Cr(III) (Denniston and Uyeki, 1987), thereby likely reducing Cr(III)-related toxicity. Besides scavenging the ·OH, a chemiluminescence study revealed that IPA also quenches superoxide anion radical ($O_2^{-\cdot}$) (Hardeland *et al.*, 1999). On the other hand, IPA seems to be a poor chain-breaking antioxidant and peroxyl radical scavenger (Poeggeler *et al.*, 1999; Karbownik *et al.*, unpublished data 2000). Thus, IPA's protective actions in the current study are likely due to its ability to scavenge radicals, such as the ·OH, which initiate lipid peroxidation.

Both IPA and melatonin act as endogenous electron donors, primarily detoxifying reactive radicals, but, at the same, time they do not undergo autooxidation in the process of redox-recycling or in the presence of transition metals. This is believed to be due to the fact that they lack a free hydroxyl group (Candeias *et al.*, 1995). Indeed, IPA is not converted to reactive intermediates with prooxidative activity (Chyan *et al*., 1999).

IPA, because of its structural similarity to melatonin, might theoretically stimulate antioxidant enzymes, since melatonin does so (Reiter *et al.*, 2000a). This could be of importance for protecting against Cr(III)-induced oxidative damage *in vivo*, although this was not examined in current *in vitro* studies. It also known that melatonin directly scavenges H_2O_2 (Tan *et al.*, 2000a,b), an action that could be shared by IPA. Indeed, the ability of IPA to prevent membrane fluidity changes (as reported here), as well as to reduce DNA damage (Tsou *et al.*, 1996; Qi *et al*., 2000a,b) due to Cr(III) (which occurs when H_2O_2 is present) could be a result of IPA neutralizing H_2O_2.

The structural similarities and antioxidative properties of IPA and melatonin suggest that the former compound, like melatonin (Blask *et al.*, 1999; Reiter, 1999; Reiter *et al.*, 2000), may be a promising agent to reduce damage to membranes. It is concluded that the carcinogen Cr(III), in the presence of H_2O_2, decreases membrane fluidity in rat microsomal membranes and that this change is pharmacologically reduced by IPA.

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