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Evidence for Free Radical Generation Due to NADH Oxidation by Aldehyde Oxidase during Ethanol Metabolism

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Several studies associate ethanol hepatic toxicity to the generation of reactive oxygen species. Ethanol metabolism by alcohol dehydrogenase (ADH) originates acetaldehyde and NADH, with the subsequent increase of the NADH/NAD^+ ratio. Some authors have suggested that the oxidation of acetaldehyde by aldehyde oxidase (AO) may be responsible for oxyradical generation during ethanol metabolism. In this study we demonstrated that AO acts not only upon acetaldehyde but also upon NADH, with superoxide anion radical ($O_2^{\cdot-}$) formation. The apparent K_m of NADH for AO was approximately 28 μM, a much smaller value than the one reported for acetaldehyde (1 mM). The NADH oxidation by AO promoted the $O_2^{\cdot-}$ generation and the ADP-Fe^{3+}-dependent microsomal lipid peroxidation in a NADH and AO concentration-dependent manner. If in these experiments NADH is substituted by ethanol, NAD^+, and ADH, a higher level of lipid peroxidation will be obtained. To explain this observation a vicious cycle which increases the oxyradical production is suggested: ADH reduces NAD^+ to NADH, which is oxidized by AO, generating reactive oxidative species plus NAD^+ available again for reduction by ADH. From the studies which were done in the presence of some antioxidants it was observed that the addition of SOD and/or catalase did not inhibit lipid peroxidation, but these results do not exclude the participation of reactive oxygen species. Our studies indicate that the NADH oxidation by AO may play a role in ethanol-induced generation of reactive oxygen species, contributing to its hepatotoxicity.

Key Words: **aldehyde oxidase; ethanol metabolism; free radicals; reduced nicotinamide adenine dinucleotide.**

Lipid peroxidation is an oxygen free radical-mediated process which has been implicated in alcohol-induced liver injury (1–6). However, the mechanisms by which ethanol metabolism leads to free radical production remains unclear.

The first stable product of ethanol metabolism is acetaldehyde and several studies showed that the metabolism of acetaldehyde via aldehyde oxidase (AO)[2] (EC 1.2.1.3) may play a role in ethanol-induced free radical injury (7, 8). The enzyme can use several electron acceptors but molecular oxygen is the physiological oxidant (9). When molecular oxygen is the electron acceptor, it is largely divalently reduced to produce H_2O_2. However, a part of the oxygen is univalently reduced, generating superoxide anion radical ($O_2^{\cdot-}$) (10).

During ethanol metabolism there is an increase in the NADH/NAD^+ ratio, which is responsible for several metabolic disturbances (11, 12). The accumulation of NADH results from ethanol oxidation by alcohol dehydrogenase (ADH) into acetaldehyde and from acetaldehyde oxidation into acetate by aldehyde dehydrogenase (2, 11, 12).

It is known that AO can also catalyze the oxidation of a wide variety of nitrogen-containing heterocycles, such as the nicotinamide adenine dinucleotide (10, 13, 14). Thus, the metabolism of NADH by AO might also contribute to ethanol-induced free radical injury.

In the present paper we studied the $O_2^{\cdot-}$ generation during NADH oxidation by AO and compared it with the $O_2^{\cdot-}$ generation, which results from acetaldehyde oxidation by AO. We evaluated the ability of the NADH/AO system to promote ADP-Fe^{3+}-dependent lipid peroxidation and compared the extent of lipid peroxidation,

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[2] Abbreviations used: Abs, Absorbance; ADH, alcohol dehydrogenase; AO, aldehyde oxidase; DETAPAC, diethylenetriaminepentaacetic acid; GSH, L-glutathione; NBT, nitroblue tetrazolium; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid; TBARS, TBA-reactive substances; TCA, trichloroacetic acid; XO, xanthine oxidase.

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obtained in the presence of NADH, with those obtained in the presence of either ethanol/NAD^+/ADH or acetaldehyde.

MATERIALS AND METHODS

Chemicals. β NADH (sodium salt, grade III from yeast), β NAD^+ (sodium salt, grade VII from yeast), ADP [di(monocyclohexylammonium) salt, grade V from equine muscle], equine liver alcohol dehydrogenase, bovine erythrocytes, superoxide dismutase, bovine liver catalase, NBT and GSH were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest purity available from Merck (Darmstadt, Germany).

Aldehyde oxidase activity. AO was partially purified from rat liver mainly as described by Shaw and Jayatilleke (7, 8) and according to the procedure of Branzoli and Massey (15). In summary, the liver was homogenized in 0.05 M potassium phosphate buffer, pH 6.8, with 1 mM EDTA. The homogenate was rapidly heated to 55°C in a water bath set at 75°C and was maintained at that temperature for 10 min. It was then chilled in ice to 0°C and centrifuged at 14,000*g* for 40 min. at 4°C. The supernatant was saturated 50% (w/v) with ammonium sulfate and the precipitate was collected by centrifugation at 14,000*g*. The precipitated protein was resuspended in 0.05 M phosphate buffer (pH 7.8) and the solution was clarified by centrifugation.

AO activity from the crude extract was measured by the reduction of ferricyanide using acetaldehyde as electron donor, according to the method of Hall and Krenitsky (10). Activities were determined at 30°C in 0.14 M potassium phosphate buffer, pH 6.8, containing 0.4 mM EDTA, 1 mM potassium ferricyanide, 10 mM acetaldehyde, and 20 μM allopurinol to inhibit any contaminating xanthine oxidase (XO). When both allopurinol and menadione, an AO inhibitor (16), were added, the reduction of ferrycianide was completely inhibited. Thus the assay with menadione represents the contaminating XO activity and the assay with allopurinol represents the AO activity. One unit of enzyme activity is defined as the amount of enzyme which will catalyze the transformation of 1 mmol of acetaldehyde per minute under our assay conditions.

The apparent K_m of AO for NADH, as determined by NADH disappearance, was calculated using the direct linear plot of Eisenthal and Cornish-Bowden (17).

Assay of $O_2^{\cdot-}$ generation during acetaldehyde and NADH oxidation catalyzed by AO. $O_2^{\cdot-}$ generation was estimated following the reduction of ferricytochrome c, at 550 nm, in comparison with a sample containing also superoxide dismutase (SOD) directly in a double-beam spectrophotometer. The reactive mixtures were constituted by 2 mM acetaldehyde or NADH, 0.15 mM ferricytochrome c, 20 μM allopurinol, SOD (535 units/cm^3), and AO in 0.14 M potassium phosphate buffer (pH 6.8) containing EDTA (0.4 mM). NADH and AO concentrations are indicated in the legends of figures. $O_2^{\cdot-}$ generation during 2 mM acetaldehyde oxidation by 0.033 units/cm^3 of AO was also estimated using 1.75 mM nitroblue tetrazolium (NBT) instead of cytochrome c as indicator compound. Measurements of ferrocytochrome c concentrations were made spectrophotometrically at 550 nm using: $\Delta\epsilon_{550}$ = 21,000 M^{-1} cm^{-1}.

Preparation of microsomes. Male Sprague–Dawley rats weighing 250–300 g were fasted for 18–20 h before being sacrificed. Liver microsomes were prepared by differential centrifugation as described by Searl and Willson (18). Each microsomal preparation was obtained from three rat livers. The protein content was determined by the method of Lowry *et al.* (19).

Lipid peroxidation assays. ADP-Fe^{3+}-dependent microsomal lipid peroxidation was promoted by the following systems: NADH plus AO, acetaldehyde plus AO, and a NADH generating system (consisting of ethanol, NAD^+, and ADH) plus AO.

The extent of lipid peroxidation was determined through the thiobarbituric acid (TBA) method and was also followed by measuring the oxygen consumption using a Clark-type oxygen electrode.

The NADH/AO and acetaldehyde/AO reactive systems were constituted by NADH (variable concentrations) or 2 mM acetaldehyde, 20 μM allopurinol, 2 mM ADP–0.1 mM $FeCl_3$, and microsomes (1 mg protein/cm^3) in 0.1 M potassium phosphate buffer (pH 7.4). In the ethanol/NAD^+-ADH/AO reactive system 2 mM ethanol, NAD^+ (0.2 or 0.3 mM), and ADH (0.3 units/cm^3) were used instead of acetaldehyde or NADH. Reactions were initiated by the addition of 0.033 units/cm^3 AO and the assays were incubated at 37°C for 30 min. Controls were done in the absence of AO. After incubation, reactions were stopped by the addition of TCA to a final concentration of 2%.

The TBA-reactive substances (TBARS) were estimated after another addition of TCA (to a final concentration of 6%) and of 15 mM TBA. The mixtures were centrifugated and the supernatants were heated in a boiling water bath for 10 min. The absorbance was measured at 532 nm.

To evaluate the oxygen consumption during the lipid peroxidation the same systems were prepared as described above and it was measured immediately after AO addition. Controls were done in absence of AO.

Antioxidant study. The effects of antioxidants on microsomal lipid peroxidation promoted during 0.3 mM NADH oxidation by AO, were studied through the TBA method already described. The basic reaction mixture was as described to the NADH/AO system. SOD (200 units/cm^3), catalase (400 units/cm^3), and 2.5 mM GSH were added just prior to the addition of microsomes and without any previous incubation.

Analysis of the data. All measurements were performed in duplicate or triplicate. Except where indicated, experiments presented under Results are representative of at least four independent experiments.

RESULTS

Aldehyde Oxidase Activity

The enzyme solutions obtained had activities between 1 and 2 units/cm^3 and were stable for several days when maintained at 4°C. All the extracts obtained presented a negligible XO activity.

Superoxide Anion Generation

The difference between the rate of ferricytochrome c reduction in the absence and in the presence of SOD was used as a measure of $O_2^{\cdot-}$ generation during NADH or acetaldehyde oxidation catalyzed by AO. The initial rate of $O_2^{\cdot-}$ generation during 2 mM acetaldehyde oxidation by AO is higher than that observed during NADH oxidation. However, after the first minutes, there is a progressive decay in the rate of cytochrome c reduction by the acetaldehyde/AO system, and the reduction curve reaches a plateau (Fig. 1). This effect may be due to the reoxidation of cytochrome c by H_2O_2 generated during the reaction but a similar effect was verified with NBT as the indicator compound. Using either cytochrome c (results not shown) or NBT (Fig. 1, inset) an enhancement in the rate of $O_2^{\cdot-}$ production by the acetaldehyde/AO system was observed only if more AO is added. The yield of $O_2^{\cdot-}$ production (estimated through the accumulation of ferrocytochrome c) after 30 min of reaction

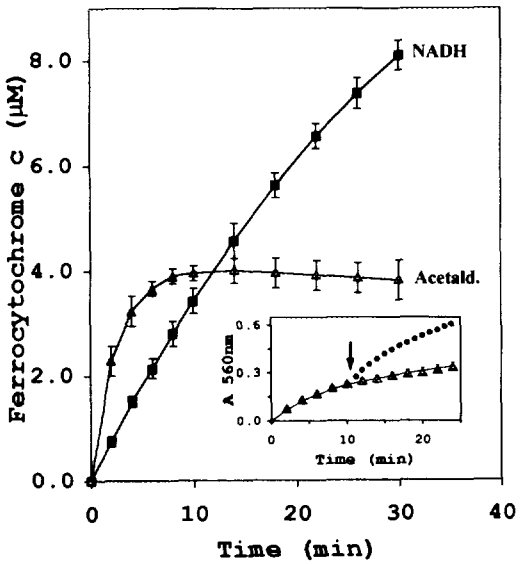


FIG. 1. Superoxide anion generation during acetaldehyde or NADH oxidation by aldehyde oxidase. The reactive mixture contained 0.14 M potassium phosphate buffer, pH 6.8, 0.4 mM EDTA, 0.15 mM ferricytochrome c, 20 μM allopurinol, 0.033 units/cm^3 aldehyde oxidase, and 0.3 mM NADH or 2 mM acetaldehyde. The assays were done against a reference assay containing 535 units/cm^3 SOD in a double-beam spectrophotometer. (Inset) NBT reduction by the acetaldehyde/AO system in the same reactive conditions but using 1.75 mM NBT instead of ferricytochrome c. The arrow indicates a second addition of AO to the acetaldehyde/AO system. Data presented are means ± SD from six independent experiments.

time for various concentrations of NADH (0.05 to 0.3 mM) and of AO (0.022 to 0.100 units/cm^3) are shown in Fig. 2 (inset) and Fig. 3 (inset), respectively. Above those NADH and AO concentrations the reoxidation of cytochrome c by H_2O_2 generated in the course of reactions is not negligible. In addition, it is known that the reduction efficiency of cytochrome c is less than unity due to rapid, spontaneous dismutation of $O_2^{\cdot-}$ at pH 7.4 (20). Consequently, both rates and extents of reduction are lower limits for rates and extents of $O_2^{\cdot-}$ production.

NADH Oxidation

To know if the product of the NADH oxidation by AO is NAD^+, the NADH consumption was followed measuring the decrease in absorbance at 340 nm. After all the NADH has been oxidized by AO, NAD^+-dependent alcohol dehydrogenase plus ethanol were added and an increase in the absorbance at 340 nm was observed (data not shown). Such increase can be due only to the reduction of NAD^+ which is associated to the ethanol oxidation. These results enable us to conclude that the product resulting from NADH oxidation catalyzed by AO is NAD^+.

The apparent K_m of NADH for AO was approximately 28 μM.

Microsomal Lipid Peroxidation

The NADH oxidation by AO, in the presence of ADP–Fe^{3+} complex, promotes microsomal lipid peroxidation, evaluated by the formation of TBARS. This is dependent on the concentration of NADH (Fig. 2) and of AO (Fig. 3).

The lipid peroxidation increases with increasing concentrations of NADH whether aldehyde oxidase is present or not, but in its presence lipid peroxidation is higher. As shown in Fig. 3, increasing the amount of aldehyde oxidase beyond 0.1 units/cm^3 has an inhibitory effect on TBARS formation. The oxygen consumption resulting from lipid peroxidation induced by 0.2 mM or 0.3 mM NADH (Fig. 4) confirms the results which were obtained through the TBA method.

The reconstituted system ethanol/NAD^+-ADH, which generates NADH and acetaldehyde, also catalyses, in the presence of ADP–Fe^{3+} and AO, the microsomal lipid peroxidation (Figs. 5 and 6). The lipid peroxidation obtained with the reconstituted system is higher than those induced through the NADH or acetaldehyde oxidation by AO. The lipid peroxidation induced through 2 mM acetaldehyde oxidation by AO is much lower than that induced by 0.2 or 0.3 mM NADH (Fig. 6).

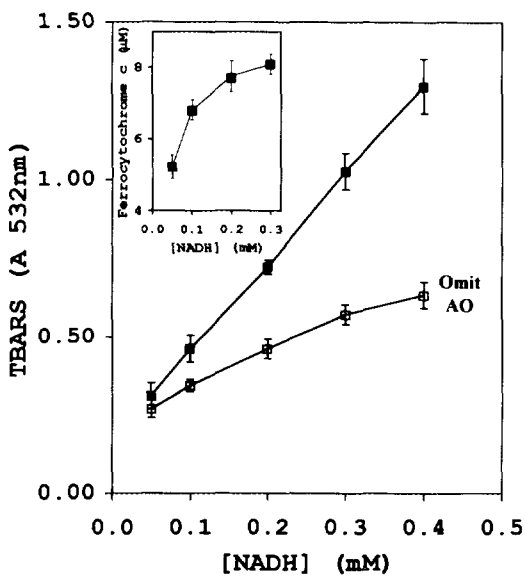


FIG. 2. Effect of NADH concentration on NADH/AO-promoted microsomal lipid peroxidation measured as TBARS. Microsomes (1 mg protein/cm^3) in 0.1 M potassium phosphate buffer, pH 7.4, were incubated at 37°C with NADH, 0.033 units/cm^3 aldehyde oxidase, 20 μM allopurinol, and 2 mM ADP–0.1 mM Fe^{3+} for 30 min. After incubation the TBARS were determined as described under Materials and Methods. (Inset) Effect of NADH concentration on the yield of $O_2^{\cdot-}$ generation during NADH oxidation by 0.033 units/cm^3 AO after 30 min of reaction time, estimated through the reduction of cytochrome c as described in the legend of Fig. 1. Data presented are means ± SD from six independent experiments.

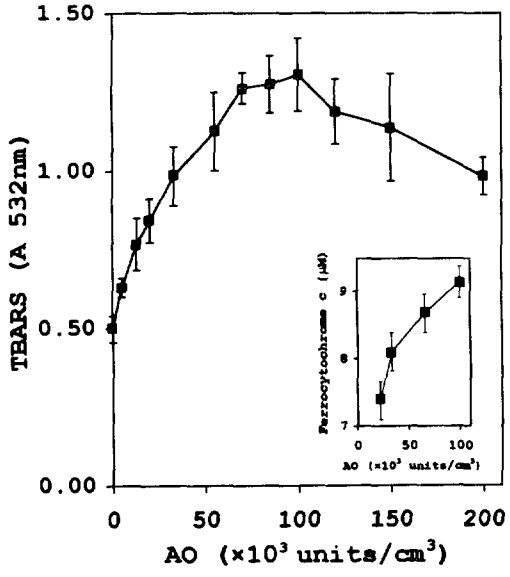


FIG. 3. Effect of aldehyde oxidase concentration on NADH/AO-promoted microsomal lipid peroxidation measured as TBARS. Incubation conditions were as described in Fig. 2 with 0.3 mM NADH. (Inset) Effect of AO concentration on the yield of O_2^- generation during 0.3 mM NADH oxidation after 30 min of reaction time, estimated through the reduction of cytochrome c as described in the legend of Fig. 1. Data presented are means ± SD from six independent experiments.

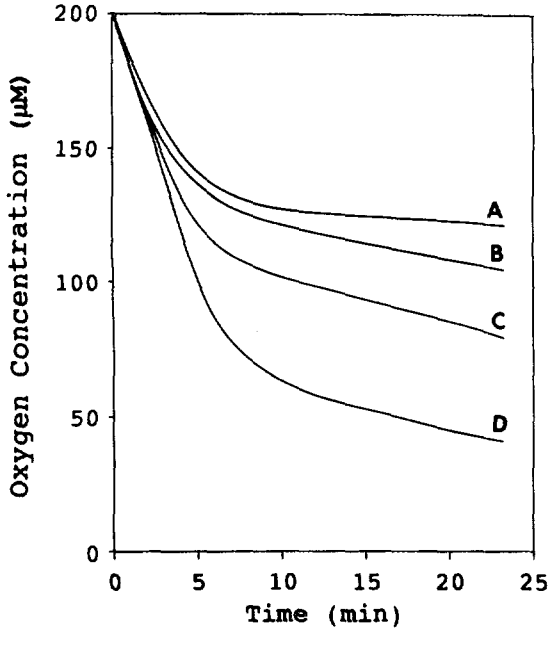


FIG. 4. Oxygen consumption during NADH/AO-induced lipid peroxidation. The reactive mixtures contained 0.1 M potassium phosphate buffer, pH 7.4, 20 μM allopurinol, 2 mM ADP–0.1 mM Fe^{3+}, 1 mg/cm³ microsomal protein, 0.2 or 0.3 mM NADH, and 0.033 units/cm³ aldehyde oxidase. (A) 0.2 mM NADH in the absence of AO, (B) 0.3 mM NADH in the absence of AO, (C) 0.2 mM NADH, (D) 0.3 mM NADH.

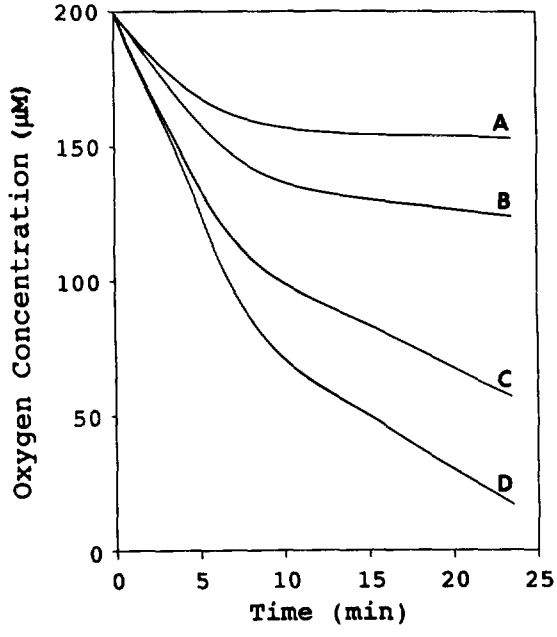


FIG. 5. Oxygen consumption during microsomal lipid peroxidation promoted by the ethanol/ADH-NAD^+/AO system. The reactive mixtures contained 0.1 M potassium phosphate buffer, pH 7.4, 20 μM allopurinol, 2 mM ADP–0.1 mM Fe^{3+}, 0.2 or 0.3 mM NAD^+, 0.3 units/cm³ ADH, 2 mM ethanol, 1 mg/cm³ microsomal protein, and 0.033 units/cm³ AO. (A) 0.2 mM NAD^+ in the absence of AO, (B) 0.3 mM NAD^+ in the absence of AO, (C) 0.2 mM NAD^+, (D) 0.3 mM NAD^+.

Effects of Antioxidants on Microsomal Lipid Peroxidation Induced during NADH Oxidation

The ADP–Fe^{3+}-dependent lipid peroxidation induced during NADH oxidation by AO was also studied in the

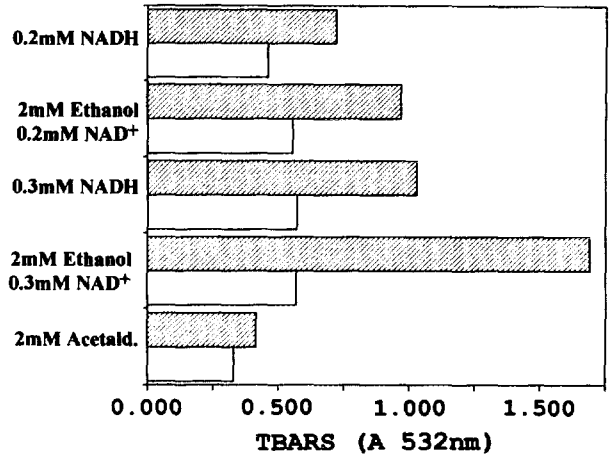


FIG. 6. Comparative study of microsomal lipid peroxidation promoted by the ethanol/ADH-NAD^+/AO, NADH/AO, and acetaldehyde/AO systems, measured as TBARS. The reactive mixtures of the NADH/AO and ethanol/ADH-NAD^+/AO systems were as described previously in Figs. 2 and 5 and they were incubated at 37°C for 30 min. The acetaldehyde/AO system contained 2 mM acetaldehyde instead of NADH. ▨, complete systems; □, systems without AO.

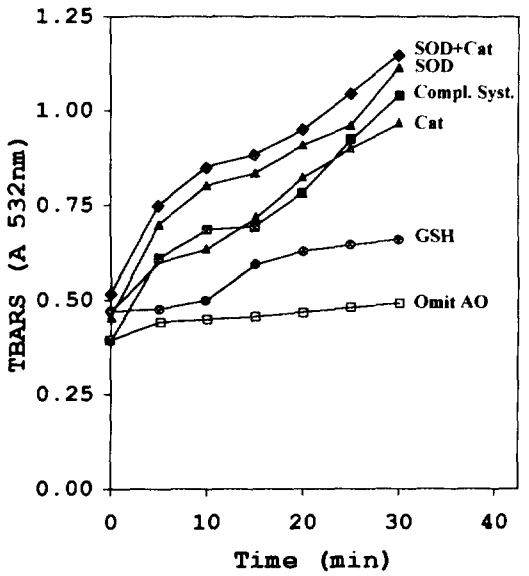


FIG. 7. Effects of antioxidants on microsomal lipid peroxidation promoted during NADH oxidation by AO. The standard reaction mixture was as described in Fig. 2 with 0.3 mM NADH. Additional components were 200 units/cm^3 SOD, 400 units/cm^3 catalase (Cat), and 2.5 mM GSH.

presence of several antioxidants (Fig. 7). Under our experimental conditions SOD, catalase, and SOD plus catalase did not show a protective effect on the production of TBARS. The addition of GSH, a nonspecific antioxidant, inhibited significant lipid peroxidation.

DISCUSSION

Several reports have shown that NADPH and NADH can promote the interaction of iron chelates with microsomes to yield oxidizing species capable of initiating lipid peroxidation (21–25). The mechanisms responsible for those processes must involve NADH cytochrome b_5 reductase and/or cytochrome P450 reductase (24, 25). Our studies confirmed that NADH promotes the iron-dependent microsomal lipid peroxidation but the presence of AO enhanced significantly the extent of lipid peroxidation. The NADH oxidation by AO generated $O_2^{\cdot-}$ and promoted the ADP–Fe^{3+}-dependent lipid peroxidation in a NADH and AO concentration dependent manner. As it is well known, iron chelates are required for the promotion of lipid peroxidation and its reduction precedes the initiation of peroxidation (26, 27). In our system the reduction of ADP–Fe^{3+} via $O_2^{\cdot-}$ generated by AO is a possible pathway and a relationship between the $O_2^{\cdot-}$ production and lipid peroxidation is supported by our results.

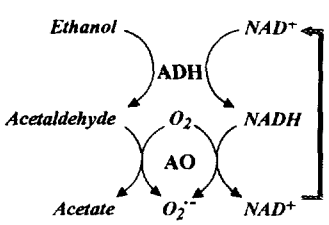
The NADH/AO system is considerably more effective than the acetaldehyde/AO system in order to promote the ADP–Fe^{3+}-dependent microsomal lipid peroxidation (Fig. 6), which is in agreement with the relative ability of both systems to generate $O_2^{\cdot-}$ in our experimental conditions.

While the NADH/AO system generates $O_2^{\cdot-}$ at approximately a steady-state level during 30 min, the acetaldehyde/AO system generates a burst of $O_2^{\cdot-}$ which immediately declines after 4 min. The decay observed in the rate of cytochrome c reduction may be due to the reoxidation of reduced cytochrome c by H_2O_2 generated during the course of the reaction. However the assay with NBT indicates that another factor may be involved once the same type of curve was observed. The increase of $O_2^{\cdot-}$ generation, which was observed when more AO was added to the acetaldehyde/AO system, suggests that the enzyme is inactivated during acetaldehyde oxidation. In fact, AO has been shown to be inactivated by H_2O_2 (16), which is generated during acetaldehyde oxidation by AO. According to the high K_m (1 mM) of acetaldehyde for AO, a great production of H_2O_2 will be expected for 2 mM concentration of acetaldehyde. Once AO has a much lower K_m for NADH (28 μM), it will appear that NADH oxidation by AO would be a more likely pathway of oxyradical generation during ethanol oxidation.

A decrease on lipid peroxidation was observed for high aldehyde oxidase concentrations. A similar decrease was also verified on lipid peroxidation systems with xanthine oxidase (28, 29). This inhibitory effect of high concentrations of superoxide-generating enzymes on lipid peroxidation may be due to the overproduction of $O_2^{\cdot-}$ and of H_2O_2 which will be able to inactivate the enzymes.

When microsomes were incubated with ethanol, NAD^+, and ADH instead of NADH, in the presence of ADP–Fe^{3+} and AO, an even higher level of lipid peroxidation was obtained. This fact may be due to the oxidation, by AO, not only of NADH but also of acetaldehyde, both generated during ethanol oxidation by ADH. However, only when acetaldehyde reaches a concentration greater than 1 mM will it be oxidized by AO (K_m = 1 mM) and, in our experimental conditions, the acetaldehyde/AO system is not effective in promoting lipid peroxidation, as was discussed before.

An alternative hypothesis for explaining the high lipid peroxidation level observed during ethanol oxidation through the NAD^+-dependent ADH in the presence of AO, will be the existence of a continuous source of



SCHEME I

NADH and acetaldehyde. Our results showed that NADH oxidation by AO produces NAD^+. Further, xanthine oxidase, a flavoenzyme structurally similar to AO and with overlapping substrate specificities, catalyzes the oxidation of NADH to NAD^+ (10, 13). This dehydrogenation reaction is clearly different in character from the hydroxylation reactions which are characteristic of both enzymes. Thus, if NADH is oxidized to NAD^+, a vicious cycle can be activated and will perpetuate the continuous generation of reactive oxygen species (Scheme I).

From the studies which were done with antioxidants it was observed that the addition of SOD and/or catalase did not inhibit lipid peroxidation. This was also observed by Dicker and Cederbaum (25) when they studied the effect of SOD and catalase on the NADH-dependent production of TBARS by microsomes. The lack of inhibition by SOD or catalase does not exclude the participation of reactive oxygen species. This was also verified with several systems by other authors and by ourselves when we studied the oxidative inhibition of red blood cell ATPases by glyceraldehyde (30). Probably the lack of effect of SOD and catalase may reflect a limited acessibility of those enzymes to the sites of $O_2^{\cdot-}$ generation in the hydrophobic microsomal membrane. The membrane peroxidation may be site localized and will occur where iron is associated with the membrane, involving a short-lived species reacting at this site, without diffusion into the solution (27). This site will not be acessible to SOD and to catalase that would otherwise inhibit the reaction. The inhibition of lipid peroxidation by GSH, measured as TBARS, may be due to the formation of conjugates with lipid peroxidation-derived aldehydes or to a radical scavenging action (31, 32).

Evidence of a role for AO in ethanol-induced lipid peroxidation in the rat was already shown by Shaw and Jayatilleke (7, 8). Inhibition of AO and XO by feeding tungstate decreased to a much larger extent the generation of lipid peroxidation products than the inhibition of XO by allopurinol.

In conclusion, our results show that NADH oxidation catalyzed by AO generates $O_2^{\cdot-}$ and, in the presence of catalytic iron, promotes microsomal lipid peroxidation. Thus we suggest that the increased availability of NADH resulting from ethanol metabolism may turn the NADH oxidation by AO into an important pathway to ethanol-dependent free radical generation and contribute to the hepatotoxicity of ethanol.

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