Changes in (Na + K)-Adenosine Triphosphatase Activity and Ultrastructure of Lung and Kidney Associated With Oxidative Stress Induced by Acute Ethanol Intoxication*

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Study and objectives: (Na + K)-adenosine triphosphatase (ATPase) activity, oxidative stress parameters, and morphologic characteristics of the lung and kidney of rats under acute ethanol intoxication were assessed to investigate the pathogenic mechanism of tissue damage. Design and interventions: Adult rats were given ethanol (5.5 g/kg) 3 h before performing the biochemical and morphologic studies. Oxidative stress was assessed by measuring the levels of reduced glutathione (GSH) and glutathione disulfide (GSSG), the activities of key antioxidant enzymes (ie, catalase [CAT], superoxide dismutase [SOD], and glutathione peroxidase [GSH-Px]) and malondialdehyde production. (Na + K)-ATPase, a membrane-bound enzyme, also was assayed. Results: In the lung, ethanol increased MDA production by 60%, decreased GSH levels by 33%, decreased SOD and GSH-Px activity by 10%, and decreased (Na + K)-ATPase activity by 55%, whereas CAT activity was unaltered. Impaired surfactant secretion and cell adhesion of lung epithelial cells were found. In the kidney, ethanol did not influence the activity of (Na + K)-ATPase or lipid peroxidation, despite the reduction of both GSH and the GSH/GSSG ratio. Focally thickened glomerular basement membrane, apoptosis of foot processes, and tubulointerstitial fibrosis were found. Conclusions: These data suggest that oxidative stress plays a role in mediating the ethanol-induced down-regulation of lung (Na + K)-ATPase. GSH depletion seems to be a major determinant of this effect. Independent mechanisms seem to account for the morphologic alterations of these organs. (CHEST 2002; 121:589–596)

Key words: antioxidant enzymes; ethanol; glutathione; kidney; lung; (Na + K)-adenosine triphosphatase; malondialdehyde

Abbreviations: ATPase = adenosine triphosphatase; CAT = catalase; GSH = glutathione; GSSG = glutathione disulfide; GSH-Px = glutathione peroxidase; MDA = malondialdehyde; ROS = reactive oxygen species; SOD = superoxide dismutase

The generation of reactive oxygen species (ROS) is a crucial step in the pathogenesis of tissue damage.1 The antioxidant defense system operates through enzymatic and nonenzymatic components,2 but it can be overwhelmed by metabolic derangements causing oxidative stress. Thus, consequences of the attack of biomolecules by ROS, such as lipid peroxidation, could result, thereby altering the structure of biological membranes and plasma lipoproteins.

Despite the fact that ethanol is known to induce oxidative stress,3 the mechanisms impairing organs such as the lung and kidney have not been determined. Previously, glutathione (GSH) depletion was found in the kidneys of rats subjected to acute ethanol intoxication,4 and lipid peroxidation was related to this effect. Furthermore, an epidemiologic study5 has revealed an association between alcohol abuse and ARDS. Recently, alterations of GSH homeostasis by chronic alcohol abuse, in human...
lung, were suggested to be involved in the mechanism of ARDS. In fact, ethanol impairs both the surfactant secretion and the GSH homeostasis of type-II pneumocytes in lung alveoli, thereby increasing the susceptibility of the lung to acute edematous injury in the rat. In addition, ethanol ingestion enhances the endotoxin-induced lung injury due to the activation of matrix-degrading enzymes, thus causing increased permeability of the alveolar epithelial barrier. In addition, ethanol may alter the regulation of the activity of (Na + K)-adenosine triphosphatase (ATPase), an enzyme that participates in lung fluid clearance by exerting the active transport of sodium. In turn, ethanol in vitro inhibits (Na + K)-ATPase, but this activity increases after chronic ethanol treatment in several organs, including the kidney. There are no data reported in the lung. Alternatively, Dobrota et al. suggested an oxidative modification of (Na + K)-ATPase by oxidative stress, a molecular mechanism that could operate in vivo during acute ethanol intoxication. In view of these considerations, the aim of the present investigation was to characterize the response of the antioxidant systems of the lung and the kidney to the oxidative challenge of acute ethanol intoxication. Also, the occurrence of oxidative stress was related to the effects on (Na + K)-ATPase activity and the ultrastructural characteristics of the organs.

Materials and Methods

Animals

Adult male Wistar albino rats (Rattus norvegicus) weighing 200 to 250 g (ICBM; Programa de Farmacología Molecular y Clínica, Facultad de Medicina, Universidad de Chile; Santiago, Chile) were given free access to pellet chow (Champion; Santiago, Chile) and water before the experiments. Acute ethanol intoxication was induced by an intraperitoneal injection of ethanol in normal saline solution, at a dose of 5.5 g ethanol per kilogram of body weight, 3 h prior to performing the morphologic and biochemical studies (ethanol group). The control animals were injected with an equal volume of normal saline solution. Blood samples were obtained, under anesthesia, by puncturing the carotid artery. The management of rats was carried out according to internationally accepted rules of ethics.

Blood Ethanol Levels and Plasma Antioxidant Capacity

Blood ethanol levels were measured by an enzymatic method based on the spectrophotometric determination of NADH, which is formed by ethanol oxidation. Plasma antioxidant capacity was assessed by the method of Benzie and Strain, based on the ferric reducing ability of plasma.

Biochemical Assays

The animals were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally) and were perfused with Earle’s balanced salt solution (Sigma Chemical Co; St. Louis, MO; pH, 7.40). Homogenates of the lung, renal cortex, and papilla were prepared in either 0.25 M sucrose, for the determination of superoxide dismutase (SOD) [EC 1.15.1.1] activity, or 1.15% KCl-0.010 M Tris (pH, 7.40), for the determination of the activities of both catalase (CAT) [EC 1.11.1.6] and glutathione peroxidase (GSH-Px) [EC 1.11.1.9]. GSH and glutathione disulfide (GSSG) were assayed by fluorometry, according to the method of Hissin and Hilf. An assay for lipid peroxides was performed spectrophotometrically at 532 nm by the thiobarbituric acid reaction, at pH 3.5, followed by the separation of malondialdehyde (MDA) through solvent extraction with a mixture of butanol/pyridine (15:1, v/v). The activity of (Na + K)-ATPase (EC 3.6.1.3) was assayed by the method of Katz and Epstein, using whole homogenates in buffer (pH, 6.80) containing the following: 0.25 M sucrose; 30 mM histidine; and 2.4 mM sodium deoxycholate (freshly prepared). The homogenates were filtered in a double layer of gauze prior to incubation at 37°C, for 15 min, in a medium containing the following: 100 mM NaCl; 20 mM KCl; 6 mM MgCl2; 6 mM ATP disodium salt (vanadium-free); and 10 mM imidazole (pH, 7.50). The activity of (Na + K)-ATPase was calculated from the difference between the amount of inorganic phosphate released in the presence and in the absence of KCl and was expressed as micromoles of inorganic phosphate per milligram of protein per hour. Total protein content was measured by the method of Lowry et al.

Ultrastructural Studies

Electron microscopy studies of the kidneys and lungs were carried out on 2-mm vertical slices. The samples were fixed for transmission electron microscopy in 3.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH, 7.3) at 4°C for 3 h and were postfixed for 1 h in 2% osmium tetroxide prepared in the same buffer. The fixed samples were dehydrated in ascending grades of ethanol, were cleared in propylene oxide, and were embedded in Araldite 502. Sections were stained with uranyl acetate and lead citrate prior to examination under an electron microscope (EM 100; Zeiss; Göttingen, Germany). For orientation purposes, semi-thin sections were stained with 1% toluidine blue in 1% sodium tetraborate and were examined by light microscopy. Semi-thin sections were prepared by using an ultramicrotome (OM-U2; Riechert; Germany).

Statistical Analysis

The results were expressed as the mean ± SEM. The sources of variation were analyzed by unpaired Student’s t test. The differences were considered statistically significant at p < 0.05.

Results

Plasma Antioxidant Capacity and Blood Ethanol Levels

After 3 h of acute ethanol exposure, a decreased antioxidant capacity of plasma, as assessed by the levels of ferric reducing ability (control group [n = 8], 280.5 ± 7.8 μM; ethanol group [n = 9], 160.3 ± 3.5 μM) was found (p < 0.05). At the same time, the blood ethanol levels of the ethanol group reached values of 108.3 ± 6.8 mM (n = 12).

Lipid Peroxidation and (Na + K)-ATPase Activity

The effects of acute ethanol intoxication on lipid peroxidation, as assessed by MDA production, and...
(Na + K)-ATPase activity of rat lungs and kidneys are shown in Figure 1. In the lung, ethanol increased MDA production by 60% and decreased (Na + K)-ATPase activity by 55% (p < 0.05), whereas no significant changes were observed in the renal cortex and papilla.

**Activities of Antioxidant Enzymes**

Table 1 shows the effects of acute ethanol intoxication on the activities of antioxidant enzymes of the lung, renal cortex, and papilla of adult rats. In the lung, after 3 h of ethanol treatment, the activities of both SOD and GSH-Px were diminished by 11% (p < 0.05), whereas that of CAT showed no changes. In the kidney, these parameters were not modified.

**Levels of GSH and GSSG**

The effect of acute ethanol exposure on the content of GSH and GSSG, and on the GSH/GSSG ratio in the lung, renal cortex, and renal papilla is shown in Table 2. In the lung, after 3 h of acute ethanol administration, the level of GSH and the GSH/GSSG ratio decreased by 20% and 26%, respectively, compared with control values (p < 0.05). In the kidneys of ethanol-treated rats, both renal regions showed decreased levels of GSH and lower GSH/GSSG ratios. GSH was diminished in the renal cortex by 33% and in the renal papilla by 56%, and the GSH/GSSG ratio was diminished in renal cortex by 38% and in the renal papilla by 71% (p < 0.05).

**Ultrastructural Characteristics**

Figure 2 shows a comparison of the ultrastructural characteristics of rat kidneys from the control group and the ethanol group. The glomerular capillary wall of the kidney exposed to ethanol showed endothelial cell swelling, apoptotic podocytes, increased mesangial secretion of extracellular matrix, and basement membrane thickening. Besides, the distal convoluted tubule wall showed abundant mitochondria, an absence of transport vacuoles, and active fibroblast secretion of extracellular matrix, as shown by the polymerization of tropocollagen molecules into microfibrils, which were observed transversally.

After ethanol treatment, type-II pneumocytes in the lung air-blood barrier showed numerous pale intracellular lamellar bodies without the dark central core observed in controls. In addition, the intercellular junctions of type-I pneumocytes were altered, showing spaces between these epithelial cells.

**DISCUSSION**

The data presented indicate that acute ethanol intoxication causes differential effects in lung and kidney parameters that are related to oxidative stress and tissue morphologic characteristics. While both organs showed morphologic alterations, the lung exhibited decreased (Na + K)-ATPase activity, which was associated with decreased GSH content and GSH/GSSG ratio, and decreased lipid peroxidation response, whereas only a derangement of the GSH homeostasis was observed in the renal cortex and papilla. This lack of association in the kidney may be explained on the basis of the activity of antioxidant enzymes, which, together with the scavenger effect of GSH would be enough to compensate the effects of ROS and/or acetaldehyde derived from ethanol oxidation.

Oxidative stress induced in the lung by ethanol ingestion can be analyzed on the basis of both enzymatic and nonenzymatic pathways for ROS clearance. Considering that a primacy of GSH in the oxidant/antioxidant status of the lung has been reported under normal conditions, ethanol-induced GSH depletion may render lipids more susceptible to ROS attack. This view is supported by the significant increase in lung lipid peroxidation found in the
ethanol group, an effect that may be facilitated by the secondary reduction in the activity of SOD and glutathione peroxidase achieved by the ethanol challenge.

Diminution in lung (Na+ K)-ATPase activity associated with increased lipid peroxidation could be related to an impairment in the optimal interaction of (Na+ K)-ATPase with membrane phospholipids, considering that its activity is modulated by the microenvironment given by the physicochemical properties of the membranes into which it is inserted.24 In addition, the contribution of oxidative modifications of thiol groups of the enzyme itself may be of importance,25 as well as the direct inhibitory effect of ethanol reported in vitro.26 Therefore, acute ethanol exposure in vivo also could result in an impairment of sodium transport across the alveolar epithelium. It is important to consider that the present report shows the data of (Na + K)-ATPase activity measured in whole-lung homogenates having various cell types. However, since this enzyme inhibition is a general effect of ethanol,27 in order to assess the contribution of alveolar pneumocytes to the impairment of lung liquid clearance, further studies of (Na + K)-ATPase activity following the isolation of these epithelial cells are required. Although no edematous enlargement of the ground substance space of interstitium was found in the air-blood barrier after 3 h of ethanol exposure, its later appearance cannot be ruled out, since the occurrence of lung edema has been reported in oxidative stress using other models.9,28 Furthermore, an ethanol-induced activation of two lung matrix metalloproteinases was reported in rats during endotoxemia,8 providing the basis for another contributory factor in the pathophysiology of ethanol-induced pulmonary edema. This finding is consistent with the spaces found by us between the epithelial lung cells of rats in the ethanol group (Fig 3, bottom right, d).

The retention of surfactant by the alveolar type-II pneumocytes of rats in the ethanol group deserves special mention. The presence of numerous pale intracytoplasmatic bodies lacking the central core, within the type-II pneumocytes of the ethanol group (Fig 3, top right, b) accounts for an intrinsic lamellar body defect. Also, it is suggested that the cellular effects of increased lipid peroxidation may lead to a functional damage of the apical microvilli membrane of type-II pneumocytes, thereby eliciting an impairment of the extrusion of lamellar bodies by these pneumocytes of rats in the ethanol group (Fig 3, bottom right, d).

Table 1—Effects of Acute Ethanol Intoxication on the Activities of Antioxidant Enzymes of Rat Lung and Kidney*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CAT, k/mg protein</th>
<th>SOD, U/mg protein</th>
<th>GSH-Px, U/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>0.085 ± 0.003 (8)</td>
<td>5.42 ± 0.20 (12)</td>
<td>0.253 ± 0.010 (12)</td>
</tr>
<tr>
<td>Ethanol group</td>
<td>0.093 ± 0.007 (8)</td>
<td>4.82 ± 0.121 (10)</td>
<td>0.228 ± 0.0081 (12)</td>
</tr>
<tr>
<td>Renal cortex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>0.33 ± 0.02 (8)</td>
<td>10.86 ± 0.62 (8)</td>
<td>0.305 ± 0.017 (8)</td>
</tr>
<tr>
<td>Ethanol group</td>
<td>0.33 ± 0.04 (8)</td>
<td>10.46 ± 0.47 (8)</td>
<td>0.297 ± 0.026 (8)</td>
</tr>
<tr>
<td>Renal papilla</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>0.022 ± 0.004 (8)</td>
<td>8.29 ± 0.64 (8)</td>
<td>0.124 ± 0.008 (8)</td>
</tr>
<tr>
<td>Ethanol group</td>
<td>0.021 ± 0.005 (8)</td>
<td>7.88 ± 0.35 (8)</td>
<td>0.138 ± 0.016 (8)</td>
</tr>
</tbody>
</table>

*Values given as mean ± SEM (No. of rats). k = first-order kinetic constant for the breakdown of hydrogen peroxide.
†p < 0.05 between control and ethanol groups (unpaired Student’s t test).

Table 2—Effects of Acute Ethanol Intoxication on the Levels of GSH, GSSG, and the GSH/GSSG Ratio of Rat Lung and Kidney*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>GSH, μmol/g</th>
<th>GSSG, μmol/g</th>
<th>GSH/GSSG Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>0.915 ± 0.042 (9)</td>
<td>0.285 ± 0.018 (9)</td>
<td>3.21 ± 0.16 (9)</td>
</tr>
<tr>
<td>Ethanol group</td>
<td>0.732 ± 0.0231 (8)</td>
<td>0.308 ± 0.040 (8)</td>
<td>2.38 ± 0.121 (8)</td>
</tr>
<tr>
<td>Renal cortex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>1.464 ± 0.072 (9)</td>
<td>0.240 ± 0.028 (9)</td>
<td>6.10 ± 0.65 (9)</td>
</tr>
<tr>
<td>Ethanol group</td>
<td>0.975 ± 0.0311 (8)</td>
<td>0.255 ± 0.028 (8)</td>
<td>3.82 ± 0.021 (8)</td>
</tr>
<tr>
<td>Renal papilla</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>0.867 ± 0.048 (8)</td>
<td>0.133 ± 0.031 (8)</td>
<td>6.52 ± 0.23 (8)</td>
</tr>
<tr>
<td>Ethanol group</td>
<td>0.381 ± 0.0361 (8)</td>
<td>0.200 ± 0.071 (8)</td>
<td>1.90 ± 0.181 (8)</td>
</tr>
</tbody>
</table>

*Values given as means ± SEM (No. of rats).
†p < 0.05 between control and ethanol groups (unpaired Student’s t test).
Figure 2. Electron micrographs of the kidneys of rats from the control group and the ethanol group. The glomerular capillary wall of a control rat (top, a) shows the primary processes (*) and the foot processes (arrowheads) of a normal podocyte, the glomerular basement membrane, and the capillary endothelium (arrow) [original ×66,000]. Instead, the kidney of an ethanol rat (middle left, b) shows a podocyte having an apoptotic nucleus with dark cytoplasm at the foot processes (arrow heads), swelling endothelial cytoplasm (E), and focally thickened basement membrane (arrows) [original ×42,000]. Moreover, in the ethanol group it is possible to observe that the lamina rara externa (arrowheads) is focally thickened and that there are deposits of dense material (arrow) along their outer margins and a fusion of the foot processes (*) over the lamina rara externa (middle right, c). The mesangium (M) supports the capillary network of the tuft (original ×37,000). The distal convoluted tubule wall near the collecting tubules of a control rat (bottom left, d) shows the microvilli at its apical surface (arrow), the vacuole (v), the mitochondria (m), and a capillary (C) vessel near the basal surface (original ×10,500). A kidney from a rat in the ethanol group (bottom right, e) shows a distal convoluted tubular wall with abundant mitochondria (m). Note the capillary vessel near the basal surface and an active fibroblast (F) secreting protocollagen molecules into the intercellular matrix and their polymerization as tropocollagen into microfibrils observed transversally (arrows) [original ×10,500]. The sections were stained with uranyl acetate and lead citrate.
cells. Consequently, lamellar bodies, otherwise forming tubular myelin, would be retained in intracytoplasmatic vesicles, a feature that may blunt the formation of monolayer surfactant in the alveolar space. However, further studies, such as those determining the phospholipid composition of BAL effluents, are necessary to support this hypothesis.

It is of interest to note that the lack of surfactant secretion in alveoli could have other effects. It is known that surfactant suppresses the NF-κB activation in human monocytic cells, a response that also is expected to occur in alveolar type-II pneumocytes. Therefore, a lack of suppression of NF-κB activation, caused by acute ethanol exposure, could trigger an inflammatory response (Fig 3, bottom left, c), thereby further contributing to the pathologic processes involved in the pathogenesis of pulmonary edema occurring during acute ethanol intoxication.

It is known that ultrastructural alterations could be triggered by changes in the intracellular redox state, which is reflected in either the depletion of thiols or in an increased number of ROS. In fact, a ROS may behave as a second messenger, favoring the activation of transcription factors under redox control, such as NF-κB and AP-1. The unfavorable consequences of these cellular events include in-

Figure 3. Electron micrographs of the lungs of rats from the control group and the ethanol group. Top left, a: the air-blood barrier of a control rat is shown. It is possible to observe two capillaries, each containing an erythrocyte (*). The wall between the left capillary lumen and the alveolus is extremely thin, and it is composed of three layers. The central part is represented by a basement membrane (arrow heads), which remains in direct connection with the epithelial type-I pneumocyte (N) and the endothelial cell (E). The arrow shows a type-II pneumocyte protruding into the alveolar space and the extrusion of a lamellar electron dense inclusion, or multilamellar body. Positioned at the core of the latter, dense areas that represent the early formation of tubular myelin (surfactant) can be noted (original ×44,000). Instead, in the lung of an ethanol rat (top right, b), it is possible to observe two type-II pneumocytes showing numerous pale intracellular lamellar bodies that lack the central core corresponding to initial tubular myelin (arrows). Also, an alteration of the junctional complex formed by type-I pneumocytes can be observed, allowing for spaces between these epithelial cells (arrowheads) (original ×46,000). Bottom left, c: the image shows the interalveolar wall of a control rat, the endothelium (E), and the occluding junction, which is an important component of the junctional complexes between the epithelial cells (arrows) (original ×39,600). Instead, the lung of a rat from the ethanol group (bottom right, d) shows intercellular spaces (arrows) and an erythrocyte (er) in the alveoilocapillary membrane (original ×46,000). The sections were stained with uranyl acetate and lead citrate.
flammation, fibrosis, or apoptosis and could contribute to the explanation of lung ultrastructural alterations found in the present report (Fig 3). Alternatively, a free radical derived from ethanol itself has been suggested as a factor of cytotoxicity. In summary, after 3 h of ethanol exposure the lung shows an association of increased MDA levels with decreased GSH levels, but the kidney does not, although in both organs a drop in GSH levels was found. The lung seems to have more vulnerability than the kidney to ethanol-induced oxidative stress, which likely is due to its lower GSH/GSSG ratio together with a lower profile of antioxidant enzymes. In the lung, an increased lipid peroxidation was associated with a markedly decreased activity of (Na + K)-ATPase, an enzymatic effect accounting for the reduced fluid clearance by lung epithelia involved in the pathogenesis of lung edema during acute ethanol intoxication. Also, the ultrastructural modifications found in the lung are consistent with an impairment of surfactant secretion, which likely is mediated by ROS.

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