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EARLY INHIBITION OF THE Na^+/K^+-ATPase ION PUMP DURING ACETAMINOPHEN-INDUCED HEPATOTOXICITY IN RAT

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The status of Na^+ regulation was examined during early stages of alkylation insult to rat liver. Na^+/K^+-ATPase activity in plasma membranes declined by 52% within 3 hr of treatment with 850 mg/kg acetaminophen. This loss preceded the release of alanine aminotransferase (2880 ± 1550 U/ml) and necrosis (2+) seen at 24 hr. Activities of 5'-nucleotidase and Mg^{2+}-ATPase and recovery of plasma membranes were comparatively unchanged at 3 hr. Because damage to Na^+/K^+-ATPase appeared early in the pathogenesis of acetaminophen hepatotoxicity, loss of hepatocellular Na^+ regulation could represent one of the critical molecular consequences of lethal alkylation by acetaminophen.

Hepatotoxic doses of acetaminophen produce marked disruption of Ca^{2+} regulation in the liver (1-4). Intracellular Ca^{2+} accumulation has been proposed as the specific lethal consequence of macromolecular damage caused by alkylation and anoxic injury (5,6). In support of this hypothesis, data of Tsokos-Kuhn et al. (7) show diminished plasma membrane Ca^{2+}-ATPase activity as an early disturbance in rats overdosed with acetaminophen. However, protein alkylation by acetaminophen occurs in all subcellular fractions and appears to be a comparatively indiscriminate event (8). It is logical to expect that the regulatory loss described for Ca^{2+} extends to other essential ions. This speculation is supported by observations that an influx of Na^+ precedes the accumulation of Ca^{2+} in models of ischemic cell death (5). Considerations such as these gave rise to the present study examining the activity Na^+/K^+-ATPase, a plasma membrane protein that regulates Na^+ extrusion from hepatocytes. We particularly wished to establish whether early damage occurs to the Na^+ pump and whether a loss in activity precedes biochemical and morphologic signs of cell injury, as required of an event that may relate causally to necrosis.

MATERIALS AND METHODS

Materials and Animals

Acetaminophen, ouabain, L-lactic dehydrogenase (type XI), pyruvate kinase (type III) were purchased from Sigma Chemical Co. (St. Louis, MO). All other

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chemicals were analytical grade or higher. Male Sprague-Dawley rats weighing 275-325 g (Blue Spruce Farms, Altamont, NY) were maintained on hardwood shavings and given free access to food and water. Animals received 850 mg/kg acetaminophen i.p. (in 0.9% NaCl at 37° C) or the same volume of vehicle and were sacrificed at 3 or 24 hr.

Isolation of Liver Plasma Membranes

Liver plasma membranes were isolated by the discontinuous sucrose gradient method essentially as described by Neville (9) through step 11. Preparations were washed twice and suspended in 25 mM histidine HCl containing 0.25 M sucrose and 1.0 mM EDTA (pH 7.5). The final concentration of membranes was 1-2 mg protein per ml, as determined by the method of Lowry et al. (10) using bovine serum albumin as a standard.

Na^+-K^+-ATPase and 5'-Nucleotidase Assays

Assays were performed on the day of sampling. Na^+/K^+-ATPase activity in membranes (5-20 μg of protein/ml) was determined by an NADH-coupled enzyme assay (11,12). NADH oxidation and declining absorbance at 340 nm reflected linear rates of ATP hydrolysis. Na^+/K^+-ATPase activity was measured as the ouabain-inhibitable component of total Mg^{2+}-ATPase activity. 5'-Nucleotidase activity was determined in liver homogenate and plasma membranes (5-20 μg protein/ml) incubated at 37° C for 30 min in buffer containing 50 mM Tris HCl (pH 7.5), 10 mM magnesium chloride and 5 mM AMP (11). Inorganic phosphate release was monitored at 820 nm after terminating enzyme activity with cold 10 % trichloroacetic acid and adding Chen's reagent (13).

Assessment of Hepatocellular Injury

Alanine aminotransferase activity (ALT) in serum was determined by kit (Sigma Chemical Co., St. Louis, MO) and served as a biochemical index of liver damage. Liver necrosis was quantitated by light microscopic evaluation of formalin-fixed tissue sections stained with hematoxylin and eosin (14). Samples were coded and the examiner blinded to treatment status. Necrosis was graded according to the following scale : 0 = absent, 1^+ = less than 6 %, 2^+ = 6 to 25 %, 3^+ = 26 to 50 %, and 4^+ = greater than 50 % of hepatic parenchymal cells exhibiting necrosis.

Data Analysis and Statistics

All results are expressed as mean ± standard deviation unless indicated otherwise. Comparison of groups was made by Student's t-test or the Mann-Whitney test (15). Differences were considered more likely to be a result of acetaminophen treatment rather than chance variation when $p < 0.05$.

RESULTS

Within 3 hr after acetaminophen overdose, rats exhibited no change in body weight or liver weight. Importantly, these animals also showed no biochemical evidence of generalized plasma membrane damage, as reflected in normal ALT activity in serum (Table 1). By 24 hr, however, serum ALT activity was markedly increased and most rats had suffered substantial hepatic necrosis (2+). Total protein concentration in liver homogenate was slightly enriched at 3 hr but activity of 5'-nucleotidase, a plasma membrane marker, did not differ between the treated and control groups (Table 2). Similarly, the activities of 5'-nucleotidase and Mg^+-ATPase and the recovery of plasma membrane were relatively unchanged at 3 hr (Table 2). In contrast, plasma membrane Na^+/K^+-ATPase activity was inhibited by 52% at this time.

Table 1. Early Effects of Acetaminophen Overdose on the Liver of Sprague-Dawley Rats. All values are mean ± SD (n = 5-7) except necrosis scores (median with range)

PARAMETER [a]	CONTROL	ACETAMINOPHEN	% CHANGE
BODY WEIGHT	315 ± 18.2	312 ± 12.0	- 1
LIVER WEIGHT	9.73 ± 0.83	9.80 ± 0.60	+ 1
PLASMA ALT			
3 hr	55 ± 21	72 ± 9	+ 31
24 hr	52 ± 7	2880 ± 1550 [b]	+ 5470
HEPATIC NECROSIS			
24 hr	0 (0-1+)	2+ (0-2+) [b]	

[a] Weight given in g, ALT in Wroblewski-LaDue U/ml, and necrosis in graded scores from 0 (no necrosis) to 4+ (> 50% cell death).
[b] $p < 0.01$ vs. control (Mann-Whitney test)

Inhibition of the Na^+ pump may not represent selective damaging of Na^+ regulation because the ratio of Na^+/K^+-ATPase and 5'-nucleotidase activities in acetaminophen-treated animals declined only 20 % from that in control animals (Table 2). Nonetheless, these results indicate that substantial

Table 2. Early Effect of Acetaminophen Overdose on Na^+/K^+-ATPase Activity of Rat Liver Plasma Membrane. Details as in Table 1

PARAMETER [a]	CONTROL	ACETAMINOPHEN	% CHANGE
Homogenate			
5'-Nucleotidase	2.86 ± 0.49	2.74 ± 0.53	- 4
Total Protein	137 ± 16	152 ± 11 [b]	+ 12
Plasma Membrane			
Membrane Recovery	126 ± 8	127 ± 27	+ 1
5'-Nucleotidase	20.1 ± 9.91	14.8 ± 9.54	- 26
Mg^{2+}-ATPase	411 ± 119	308 ± 156	- 25
Na^+/K^+-ATPase	45.9 ± 10.4	21.9 ± 3.01 [b]	- 52
$\frac{Na+/K+\text{-ATPase}}{5'\text{-Nucleotidase}}$	2.65 ± 1.10	2.12 ± 1.27	- 20

[a] Units: enzyme activity (μmol P_i/mg protein/hr), membrane recovery (μg protein/g liver), total protein (mg/g liver)
[b] $p < 0.001$ vs. control (Student's t-test).

inhibition of plasma membrane Na^+/K^+-ATPase activity appeared as an early event in acetaminophen-induced hepatotoxicity.

DISCUSSION

Recent work from this laboratory shows that acetaminophen overdose in mice is accompanied by the expected increase in total hepatic calcium, and perhaps more importantly that cytosolic Ca^{2+} activity rises in unison with the alkylation of protein macromolecules (3,4). Depletion of glutathione could contribute to this inhibition in extrusion and rise in activity of Ca^{2+}, but this explanation is unlikely considering the failure of buthionine sulfoximine to produce changes in the biological Ca^{2+} indicator, glycogen phosphorylase a (4). Altered calcium homeostasis in vivo is more apt to be associated with alkylation of critical regulatory molecules. Tsokos-Kuhn et al. (7) find that acetaminophen and several other alkylating hepatotoxins impair the plasma membrane Ca^{2+} pump and show that this may be a non-viable regulatory loss. However, macromolecular alkylation by acetaminophen is widespread (8) and other regulatory sites may also become severely damaged. Several studies suggest that loss of calcium regulation at the endoplasmic reticulum (16) and the mitochondria (1,17) can account for toxic accumulation of cytosolic calcium ion. Trump and Berezesky (5) have speculated that an early increase in sodium may trigger or potentiate the Ca^{2+} accumulation needed for irreversible cell injury. In addition, Schanne and Moore (18) confirm the presence of a sodium-calcium exchanger in plasma membrane and show that it can transport Ca^{2+} into the hepatocyte when intracellular sodium ion concentration rises.

These studies provide the rationale for the present work showing the decline in plasma membrane Na^+/K^+-ATPase activity long before there was biochemical or morphologic evidence of cell necrosis. Whether this decrement in activity results from specific alkylation of the pump itself cannot be determined from these results. It is noteworthy that although plasma membrane 5′-nucleotidase and Mg^{2+}-ATPase were not significantly inhibited by acetaminophen, they decreased in activity by 25 %. Together with the reported decrease in plasma membrane Ca^{2+}-ATPase activity (7), it would appear that acetaminophen overdose causes nonspecific arylation of plasma membrane proteins and that this is accompanied by widespread plasma membrane disfunction. The hypothesis that a specific ion pump located on the plasma membrane or elsewhere is individually responsible for lethal cellular damage has not been adequately tested.

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