Platelet-Free Calcium and Vascular Calcium Uptake in Ethanol-Induced Hypertensive Rats

Sudesh Vasdev, Carol Ann Sampson, and V.M. Prabhakaran

This study examined the effect of moderate ethanol intake on systolic blood pressure, platelet cytosolic free calcium, aortic calcium, and rubidium-86 uptake in Wistar-Kyoto rats. Twelve Wistar-Kyoto rats, aged 6 weeks, were given 5% ethanol in drinking water the first week followed by 10% ethanol in drinking water for the next 6 weeks. Twelve control animals were given regular tap water. Systolic blood pressure in the ethanol-treated rats was significantly higher ($p<0.05$) than that in controls after 1 week and remained higher throughout the study. At 13 weeks of age, platelet cytosolic free calcium and calcium uptake by aortas were significantly higher ($p<0.001$) in ethanol-treated animals as compared with those in controls. Ethanol intake did not affect aortic ouabain-sensitive $^{86}$Rb uptake. The in vitro effect of ethanol on calcium-45 and $^{86}$Rb uptake was also investigated in aortas of untreated Wistar-Kyoto rats at 13 weeks of age. In vitro ethanol (2.5–20 mmol/l) did not significantly affect $^{45}$Ca and $^{86}$Rb uptake in rat aortas. The increases in systolic blood pressure, platelet cytosolic free calcium, and vascular calcium uptake suggest that increases in cytosolic free calcium and calcium uptake mechanisms are associated with ethanol-induced hypertension. (Hypertension 1991;18:116–122)

Epidemiological studies in humans have established that there is a positive correlation between ethanol intake and development of hypertension.\(^1\)-\(^3\) Controlled clinical studies have also shown that ethanol produces a further increase in blood pressure in patients who are already hypertensive.\(^4\)-\(^5\) Ethanol feeding of Wistar-Kyoto (WKY) and Sprague-Dawley rats for a period of 4–12 weeks causes a moderate rise in blood pressure.\(^6\)-\(^8\) However, the mechanisms by which ethanol intake elevates blood pressure is not known.

Abnormal contractile activity of the vascular smooth muscle is considered to be one cause for the development of hypertension.\(^9\) The contractile activity of vascular smooth muscle is regulated by the level of intracellular free calcium ions ($[\text{Ca}^{2+}]_i$).\(^10\)-\(^12\) It has been suggested that factors leading to an increased $[\text{Ca}^{2+}]_i$ within the vascular smooth muscle cell may be responsible for the increased contraction of the smooth muscle and the development of hypertension. Such factors may be an increased entry of calcium ions through the cell membrane via calcium channels or an increased release of calcium ions within the smooth muscle cells. Calcium influx through cell surface calcium channels is a major contributing factor to cytosolic free calcium.\(^13\)-\(^14\) Although the resting levels of $[\text{Ca}^{2+}]_i$ have not been measured in vascular smooth muscle cells from hypertensive patients or hypertensive animal models, it is reportedly increased in platelets of patients with essential hypertension\(^15\)-\(^18\) and spontaneously hypertensive and Dahl salt-sensitive hypertensive rats\(^17\)-\(^19\) and seems to relate directly to the level of systolic blood pressure.\(^15\)-\(^17\)

In the present study, we investigated the effect of an oral intake of 10% ethanol in drinking water on systolic blood pressure, platelet cytosolic free calcium, and vascular calcium uptake in Wistar-Kyoto rats.

**Methods**

**Animals, Diet, and Administration of Ethanol**

Male WKY rats from Charles River, Quebec, Canada were used in this study. All rats were given standard rat chow throughout the study and had free access to water or water/ethanol mixture. At 6 weeks of age, rats were divided into two groups: a control group (12 rats) and an ethanol group (12 rats). Animals in the control group were given regular drinking water from the tap, and the ethanol group was given 5% vol/vol ethanol in tap water in the first week and 10% ethanol in tap water from the second through the
seventh weeks. Body weight and blood pressure of all rats were recorded weekly. Food and water intakes were recorded every second day. At the end of the experiment (age 13 weeks), rats were anesthetized with intraperitoneal sodium pentobarbital (10 mg/100 g body wt). After thoracic cage resection, blood was drawn into vacutainer tubes by intracardiac puncture for serum biochemistry and platelet preparation. Thoracic aortas were excised immediately for $^{45}\text{Ca}^{2+}$ and $^{86}\text{Rb}^+$ uptake measurements.

**Laboratory Analysis**

Plasma renin and serum aldosterone were determined by standard commercially available radioimmunoassay kits from ERIA Diagnostics Pasteur, Marnes La Coquette, France and Diagnostic Products Corp., Los Angeles, Calif., respectively. Sodium, potassium, calcium, magnesium, and creatine in serum were assayed on autoanalyzers with ion-specific electrodes for sodium and potassium, compleximetric method for calcium and magnesium, and reaction rate Jaffe method for creatinine. Plasma ethanol was assayed on the TDX analyzer using an enzymatic fluorescent assay kit from Abbott Laboratories, North Chicago, Ill.

**In Vitro Effect of Ethanol on Calcium Uptake and $^{86}\text{Rb}^+$ Uptake of Wistar-Kyoto Rat Aortas**

To investigate the direct effect of ethanol on the $^{45}\text{Ca}^{2+}$ and $^{86}\text{Rb}^+$ uptake in vascular tissue, thoracic aortas from normal WKY rats aged 13 weeks were used. HEPES buffer was prepared in either distilled water or 2.5, 5, 10, and 20 mM ethanol in water and uptake was measured.

**Measurements of $[^\text{Ca}^{2+}]$, in Platelets**

Platelet cytosolic $[^\text{Ca}^{2+}]$, was measured by essentially a similar method as described previously. Blood was collected into a vacutainer tube containing acid-citrate-dextrose by cardiac puncture, and platelet rich plasma was incubated for 30-45 minutes with 5 mM bovine serum albumin at pH 7.4 and 37°C. Platelets were counted using a Coulter counter (model S-PLUS IV, Coulter Electronics Inc., Miami Lakes, Fla.) and results of $[^\text{Ca}^{2+}]$, are given as nanomoles per liter.

**Measurement of Calcium Uptake by Thoracic Aortas**

Calcium uptake by thoracic aortic tissues was measured as described previously, and thoracic aortas were excised and dissected free of connective tissue in a constantly oxygenated HEPES buffer (pH 7.4) solution containing: 150 mM NaCl, 4.5 mM KCl, 10 mM D-glucose, 5 mM HEPES, 1.5 mM CaCl$_2$, and 1 mM MgCl$_2$. Aortas were then cut into 2–3-mm long segments. After an equilibration period of 2 hours at 37°C subsequent to tissue excision, aortic rings (in tissue holders) were incubated in 5 ml of constantly oxygenated buffer containing $^{45}\text{Ca}^{2+}$ (5 μCi/ml) for 20 minutes at 37°C in a constantly shaking water bath. Subsequently, tissues were washed in cold (2°C) buffer for 2 minutes, followed by a second cold (2°C) buffer wash for 45 minutes to remove free $^{45}\text{Ca}^{2+}$. Under these conditions, efflux of intracellular calcium is prevented and only the extracellular calcium is effectively washed away. The tissues were then blotted, wet weights were measured and transferred to counting vials containing 100 μl H$_2$O and 1 ml Protosol (New England Nuclear, Boston, Massachusetts, USA), and then were placed in a water bath for 2 hours at 60°–70°C. After digestion, 100 μl glacial acetic acid was added to each vial followed by 10 ml liquid scintillation fluid (BDH Chemicals, Toronto, Canada), and the solution was counted in a Beckman liquid scintillation counter (Beckman Instruments, Fullerton, Calif.). The uptake of $[^\text{Ca}^{2+}]$ was expressed as micromoles $[^\text{Ca}^{2+}]$ per kilogram wet weight of tissue per 20 minutes. This net uptake represents total $[^\text{Ca}^{2+}]$ influx in the aortas.
Effect of Ethanol Intake on Serum Biochemistry, and Pearson correlation coefficient. Analysis of results was performed by Students t test and Pearson correlation coefficient.

Results

Effect of Ethanol Intake on Serum Biochemistry, Body Weight, Food, and Water Intake

The ethanol-treated rats consumed on average 1.1±0.2 g ethanol daily during the first week and increased their intake to 2.5±0.6 g daily by the end of the seventh week of the experimental period (Table 1). These animals consumed (mean±SD) 13±2 g rat chow per day before treatment and 14±2 g/day/rat chow by the end of the seventh week. The control group, on the other hand, increased their food consumption from 13±1 g at week 1 to 20±2 g/day/rat at week 7. Plasma ethanol levels in the treated animals at the seventh week were less than 1.25 mmol/l; the ethanol consumed by the treated animals represented extra calories in addition to those provided by their regular food intake. Ethanol was assumed to contain 7.0 kcal/g and rat chow 5.0 kcal/g in our calculations. Total caloric intake in the ethanol group (89±7 kcal/day/rat) was significantly less (p<0.05) as compared with the control group (98±11). The fluid intake for the two groups was similar: 27±2 ml/day/rat in the ethanol group and 29±3 in the control group in the beginning of the experiment but was significantly less in the ethanol group (32±3 ml/day/rat) as compared with the control group (44±2) (p<0.001) by the seventh week of the experimental period. The initial (mean±SD) body weights of control rats (127±3 g) and the ethanol group (126±5 g) were not significantly different. However, the mean value of the final body weights of the rats given ethanol (mean±SD; 305±23 g) was significantly less (p<0.01) as compared with rats given water (328±16 g) to drink. The mean values of serum sodium, potassium, calcium, magnesium, creatinine, aldosterone, and renin were not different between the two groups of rats.

Effect of Ethanol Intake on Systolic Blood Pressure of Rats

Mean±SD values of systolic blood pressure of rats in the ethanol and control groups were similar in the beginning of the experiment (age 6 weeks). The ethanol-fed rats had significantly higher blood pressure at 7, 8, 9, 10, 11, 12, and 13 weeks of age (p<0.05) as compared with control group on water (Figure 1). Mean±SD values for systolic blood pressure in the ethanol group were 106±3, 116±3, 123±3, 136±2, 144±2, 147±3, 150±3, and 153±5 mm Hg at 6, 7, 8, 9, 10, 11, 12, and 13 weeks of age, respectively. Analysis of variance of mean systolic
blood pressure values over time (weeks 6–13) was significant \((p<0.001)\) in the ethanol-treated group. The weekly increase in systolic blood pressure was significantly different by paired \(t\) test between ages 6 and 7, 7 and 8, 8 and 9, 9 and 10, 10 and 11 \((p<0.004)\), and between 11 and 12 \((p<0.027)\) weeks. There was no significant difference in systolic blood pressure between age 12 and 13 weeks.

**Effect of Ethanol on Platelet Cytosolic \([\text{Ca}^{2+}]_i\) in Wistar-Kyoto Rats**

Platelet cytosolic \([\text{Ca}^{2+}]_i\) in the ethanol-treated rats (mean±SD; 134±25 nmol/l) was significantly higher \((p<0.001)\) as compared with rats on water (75±9 nmol/l) (Table 2 and Figure 2). Figure 2 clearly shows the relation between platelet cytosolic \([\text{Ca}^{2+}]_i\), and mean systolic blood pressure between age 12 and 13 weeks.

**Effect of Ethanol Intake on Calcium Uptake in Aortas**

Calcium uptake in aortas of ethanol-treated rats at 13 weeks of age was significantly higher \((p<0.001)\) than control rats (Figure 1). The percentage increase in calcium uptake with ouabain (2 mM), calcium uptake increased significantly \((p<0.001)\) as compared with when the aortas were incubated in HEPES buffer without ouabain. This increase in calcium uptake is due to inhibition of the vascular \(\text{Na}^+\text{-K}^+\) pump with a corresponding increase in intracellular \(\text{Ca}^{2+}\) due to \(\text{Na}^+\text{-Ca}^{2+}\) exchange mechanism. The percentage increase in calcium uptake with ouabain incubation in control and ethanol-treated groups was similar (38%).

**Effect of Ethanol Intake on \(^{86}\text{Rb}\) Uptake in Aortas**

Ouabain-sensitive \(^{86}\text{Rb}\) uptake of aortas represents aortic \(\text{Na}^+\text{-K}^+\) pump activity (Table 3). Mean±SD values for total and ouabain-sensitive and ouabain-insensitive \(^{86}\text{Rb}\) uptake were not significantly different in aortas of rats given ethanol as compared with those of rats given water.

**In Vitro Effects of Ethanol on \(^{45}\text{Ca}\) and \(^{86}\text{Rb}\) Uptake in Wistar-Kyoto Rat Aortas**

Ethanol in concentration from 2 to 20 mM in HEPES buffer did not affect \(^{45}\text{Ca}\) or \(^{86}\text{Rb}\) uptake in rat aortas (Table 4). Ouabain was used in both uptake studies to inhibit ouabain-sensitive \(\text{Na}^+\text{-K}^+\) pump and acted as an indicator of the responsiveness of the tissue. In the calcium uptake studies, ouabain

### TABLE 2. Effect of Oral Intake of 10% Ethanol on Cytosolic Free Calcium in Platelets and Calcium Uptake by Aortas of Wistar-Kyoto Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Cytosolic free calcium in platelets (nmol/l)</th>
<th>Aortic calcium uptake (nmol Ca(^{2+})/kg tissue/20 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ouabain-sensitive Ouabain-insensitive</td>
</tr>
<tr>
<td>Control</td>
<td>75±9</td>
<td>419±42 580±85</td>
</tr>
<tr>
<td>Ethanol</td>
<td>134±25*</td>
<td>531±31* 735±79*</td>
</tr>
</tbody>
</table>

Values are mean±SD. \(n=12\) aortic rings from six rats in each group. Starting at 6 weeks of age, rats were given either water or 5% ethanol in first week and 10% ethanol in water from second to seventh weeks. Rats were killed at 13 weeks of age. All calcium measurements were done in normal HEPES buffer with or without 2 mM ouabain.

*Significantly different \((p<0.001)\) from control.

### TABLE 3. Effect of Oral Intake of 10% Ethanol on Rubidium-86 Uptake by Aortas of Wistar-Kyoto Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Ouabain-sensitive</th>
<th>Ouabain-insensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>315±49</td>
<td>218±40</td>
<td>97±16</td>
</tr>
<tr>
<td>Ethanol</td>
<td>289±33</td>
<td>206±39</td>
<td>83±16</td>
</tr>
</tbody>
</table>

Values are mean±SD. \(n=12\) aortic rings from six rats in each group. Starting at 6 weeks of age, rats were given either water or 5% ethanol in first week and 10% ethanol in water from second to seventh weeks. Rats were killed at 13 weeks of age. All \(^{86}\text{Rb}\) measurements were done with or without 2 mM ouabain in normal HEPES buffer.
variety of membrane transport systems that control physiology of essential hypertension is focused on a induced by alcohol.

fundamental pathogenic features of hypertension electrolytes, aldosterone, and renin. This suggests however, there was no significant difference in serum starting from 3 weeks as compared with controls.

7 week, 10% for the next 2 weeks, and 20% from week 4 to 12. In our study, rats were given ethanol at a -Ca uptake compared with calcium uptake finished with an inhibition of the Na\(^+\)-K\(^+\) pump affects Na\(^+\)-Ca\(^{2+}\) exchange producing a significant \((p<0.001)\) increase in calcium uptake compared with calcium uptake without ouabain.

Discussion

In the present study, the increase in blood pressure after ethanol feeding began after 1 week and continued over the remaining 6 weeks of the study. It has been reported by others that blood pressure elevation began after 4 weeks of ethanol feeding in Wistar and Sprague-Dawley rats: 5% ethanol in the first week, 10% for the next 2 weeks, and 20% from week 4 to 12. In our study, rats were given ethanol at a younger age; body weight 126±5 g compared with 180-300 g in other studies.\(^{6-8}\) Our results suggest that younger animals seem to be more sensitive to the effect of ethanol-induced increase in blood pressure. The plasma ethanol concentrations at the completion of our study (7 weeks of ethanol feeding) were less than 1.25 mmol/l, which is the lower limit of the sensitivity of our assay. The rats were killed and blood was collected between 9:30 and 10:30 AM. Since we cannot be sure when the animals were drinking, these ethanol concentrations may not represent their average daily blood levels. The low ethanol concentrations are also a reflection of the 10% ethanol given to the experimental group as compared with 20% ethanol given in other studies.\(^7\)

Ethanol-treated animals had slightly reduced daily caloric intake and their body weight was also lower starting from 3 weeks as compared with controls. However, there was no significant difference in serum electrolytes, aldosterone, and renin. This suggests that increased aldosterone production and enhanced renin-angiotensin pathway are unlikely to be the fundamental pathogenic features of hypertension induced by alcohol.

A large body of literature on the cellular pathophysiology of essential hypertension is focused on a variety of membrane transport systems that control the intracellular ionic milieu \((\text{Na}^+, \text{K}^+, \text{Ca}^{2+}, \text{and Mg}^{2+})\) and thus have the potential to regulate vascular smooth muscle contractile activity. Defects in one or more of these transport systems have been proposed to play an important role in the pathogenesis of hypertension.\(^{22}\) The results from the present study have shown that 10% ethanol intake leads to an increase in systolic blood pressure with an increase in platelet cytosolic \([\text{Ca}^{2+}]_c\). Platelets were chosen for this study because, besides being an easily accessible tissue, they likely represent abnormalities in calcium handling similar to those described in vascular tissue, which share many common intracellular features with platelets, including a calcium-dependent contraction-coupling process.\(^{23}\)

Ethanol intake also stimulated calcium uptake in rat aortas, thus further suggesting abnormality in calcium homeostasis. Inhibiting the vascular Na\(^+\)-K\(^+\) pump increases intracellular Na\(^+\) which, acting on plasma membrane Na\(^+\)-Ca\(^{2+}\) exchange system, could lead to increased intracellular Ca\(^{2+}\). Increased vascular smooth muscle cell Ca\(^{2+}\), by its known action on the contractile machinery, would in turn lead to contraction of vascular smooth muscle cells or increased responsiveness to vasoconstrictors. Increased vascular tone would raise blood pressure. Several groups have shown that Na\(^+\),K\(^+\)-ATPase is directly inhibited by ethanol in various tissue membranes, but only at concentrations that can be found in human serum under severe ethanol intoxication.\(^{24}\) Chronic ethanol exposure in rats has been reported to give variable results: brain Na\(^+\),K\(^+\)-ATPase activity was either increased or unaltered.\(^{24,25}\) In the present study, low concentrations of ethanol (2.5–20 mM) similar to those found in the plasma of moderate alcohol drinkers did not inhibit \(86\text{Rb}^+\) uptake in rat aortas when incubated in vitro. Ethanol feeding to WKY rats in our study also did not inhibit aortic ouabain-sensitive \(86\text{Rb}^+\) uptake, which represents the ouabain-sensitive Na\(^+\),K\(^+\) pump activity.

It has also been reported that ethanol ingestion leads to tissue magnesium depletion that in turn may inhibit the Na\(^+\)-K\(^+\) pump, which requires Mg\(^{2+}\) as a cofactor.\(^{26}\) It is possible that in our studies, Mg\(^{2+}\) ions are repleted during the 2-hour preincubation period in incubation medium that contains normal Mg\(^{2+}\) \((1 \text{ mM})\) concentration, and this may be the reason that we did not find any inhibition of the Na\(^+\)-K\(^+\) pump after ethanol ingestion.

It has also been suggested that the ethanol-induced increase in blood pressure in rats may be due to volume expansion.\(^6\) Increased circulating Na\(^+\)-K\(^+\) ATPase inhibitors due to volume expansion have been implicated in essential hypertension.\(^{27}\) In the present study, the vascular tissue responded to ouabain with an inhibition of the Na\(^+\)-K\(^+\) pump and an increase in calcium uptake. However, there was no significant difference in the increase of ouabain-sensitive calcium uptake in ethanol-treated rats as compared with control. Thus, it does not appear that an increase in intracellular \([\text{Ca}^{2+}]_c\) in ethanol-fed rats
is due to Na\(^+\)-K\(^+\) pump inhibition. The increase in intracellular cytosolic \([Ca^{2+}]\), may be due to other calcium uptake mechanisms.

There are three pathways by which \(Ca^{2+}\) can enter smooth muscle cells: through voltage-operated calcium channels, through receptor-operated calcium channels, and through calcium leak pathways.\(^{28,29}\) It is possible that an increase in aortic calcium uptake in ethanol-treated rats in the present study may be due to one or more of these mechanisms. An increase in intracellular free cytosolic \(Ca^{2+}\) can also occur through release of \(Ca^{2+}\) from internal stores by activation of polyphosphoinositol phosphate pathway.\(^{30}\) Ethanol-induced mobilization of calcium by activation of phosphoinositol-specific phospholipase C in intact hepatocytes has been reported recently.\(^{31}\) Also, it was found in another study that ingestion of ethanol in rats for 2–24 weeks led to increased total and exchangeable cellular \(Ca^{2+}\) in aortas.\(^{32}\)

The increase in calcium uptake seen in the present study could also be due to decreased calcium efflux by decreased activity of plasma membrane \(Ca^{2+}\)-ATPase, which pumps \(Ca^{2+}\) out of the cell. Others have reported inhibition of skeletal sarcoplasmic reticulum vesicles and cardiac sarcoplasmic reticulum \(Ca^{2+}\)-ATPase by ethanol, but the ethanol concentrations used were very high.\(^{24}\) It is possible that chronic ethanol feeding of rats may decrease \(Ca^{2+}\)-ATPase activity, thus causing an increase in intracellular \(Ca^{2+}\).

All the above-mentioned membrane changes leading to increase in intracellular \([Ca^{2+}]\), and hypertension may be due to altered membrane composition due to ethanol intake. Because our ethanol dose and blood ethanol levels were low, the results seen in the present study do not seem to be caused by a direct effect of ethanol on the membrane. Ethanol in concentrations from 2 to 20 mM did not affect calcium uptake in in vitro studies in aortas of control rats (Table 4). However, chronic ethanol feeding in rats has been shown to increase total and exchangeable cellular calcium in aortas.\(^{32}\) Chronic ethanol feeding may affect the membrane composition and thus membrane bound enzymes and internal ionic composition.\(^{33–39}\) Ethanol feeding has been reported to alter membrane composition by decreasing the biosynthesis of polyunsaturated fatty acids and by changes in membrane cholesterol and phospholipids.\(^{40–43}\) Ethanol has also been shown to alter membrane composition by lipid peroxidation.\(^{44,45}\) Whatever the fundamental mechanisms of hypertension may be, increased cytosolic \([Ca^{2+}]\), appears to be one of the mediating events in alcohol-induced hypertension. In view of the similarities between platelets and vascular smooth muscle cells, the observed relation between intracellular calcium in platelets, calcium uptake in aortas, and blood pressure provides further evidence for the important role of intracellular calcium in hypertension. It remains possible, however, that an increased \([Ca^{2+}]\), is a consequence rather than a cause of elevated blood pressure. Further studies using calcium channel blockers may clarify this relation.

In conclusion, the present study has demonstrated that a moderate amount of ethanol intake in Wistar-Kyoto rats causes increased systolic blood pressure, platelet cytosolic free \(Ca^{2+}\) and aortic calcium uptake. The increase in systolic blood pressure, platelet cytosolic free \(Ca^{2+}\), and vascular calcium uptake suggests that increased calcium uptake mechanisms are associated with ethanol-induced hypertension.

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