High-calcium diet enhances vasorelaxation in nitric oxide-deficient hypertension

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Jolma, Pasi, Jarkko Kalliovalkama, Jari-Petteri Tolvanen, Peeter Kőöbi, Mika Kähönen, Nina Hutri-Kähönen, Xiumin Wu, and Ilkka Pörsti. High-calcium diet enhances vasorelaxation in nitric oxide-deficient hypertension. Am J Physiol Heart Circ Physiol 279: H1036–H1043, 2000.—Because the effects of calcium supplementation on arterial tone in nitric oxide-deficient hypertension are unknown, we investigated the influence of elevating dietary calcium from 1.1 to 3.0% in Wistar rats treated with L-N^G-nitro-arginine methyl ester (L-NAME; 20 mg · kg^-1 · day^-1) for 8 wk. A high-calcium diet attenuated the development of hypertension induced by L-NAME and abrogated the associated impairments of endothelium-independent mesenteric arterial relaxations to nitroprusside, isoproterenol, and cromakalim. Endothelium-dependent relaxations to acetylcholine during nitric oxide synthase inhibition in vitro were decreased in L-NAME rats and improved by calcium supplementation. The inhibition of cyclooxygenase by diclofenac augmented the responses to acetylcholine in L-NAME rats but not in calcium + L-NAME rats. When hyperpolarization of smooth muscle was prevented by KCl precontraction, the responses to acetylcholine during combined nitric oxide synthase and cyclooxygenase inhibition were similar in all groups. Furthermore, superoxide dismutase enhanced the acetylcholine-induced relaxations in L-NAME rats but not in calcium + L-NAME rats. In conclusion, calcium supplementation reduced blood pressure during chronic nitric oxide synthase inhibition and abrogated the associated impairments in endothelium-dependent and -independent arterial relaxation. The augmented vasorelaxation after increased calcium intake in L-NAME hypertension may be explained by enhanced hyperpolarization and increased sensitivity to nitric oxide in arterial smooth muscle and decreased vascular production of superoxide and vasoconstrictor prostanoids.

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pendent and sensitive to changes in dietary sodium intake (28). Because increased dietary Ca\(^{2+}\) intake is known to effectively reduce blood pressure in volume-dependent hypertension (5, 30), the present investigation was designed to test the hypothesis whereby Ca\(^{2+}\) supplementation would counteract the elevation of blood pressure and the associated vascular changes elicited by long-term 1-NAME treatment in Wistar rats. Special attention was paid to evaluate the roles of different endothelium-derived mediators in the vasodilator responses and to elucidate the possible functional changes in arterial smooth muscle.

**METHODS**

**Animals and Experimental Design**

Male Wistar rats were housed four to a cage (40 × 70 × 25 cm) in an experimental animal laboratory (illuminated 0600–1800, temperature +22°C), with free access to drinking fluid and food pellets (Ewos, Södertälje, Sweden). The systolic blood pressures of the conscious animals held in plastic restrainers were measured at +28°C by the tail-cuff method (model 129, blood pressure meter; IITC, Woodland Hills, CA), with an acclimatization period of 30 min preceding the measurements. At 11 wk of age, the rats were divided into four groups (n = 10) of equal mean systolic blood pressures. The 1-NAME and calcium + 1-NAME groups were treated with 1-NAME (20 mg·kg\(^{-1}\)·day\(^{-1}\)) added to the drinking fluid (tap water). 1-NAME solutions were prepared daily and given in light-proof bottles. The control and calcium groups received normal tap water. The standard chow in the control and 1-NAME groups contained 1.1% Ca\(^{2+}\), and the modified chow in the calcium and calcium + 1-NAME groups contained 3% Ca\(^{2+}\). The extra Ca\(^{2+}\) was supplied as the carbonate salt. The two chows were identical except for the Ca\(^{2+}\) contents. 1-NAME administration, dietary Ca\(^{2+}\) supplementation, and blood pressure measurements were continued for 8 wk; thereafter the rats were anesthetized by intraperitoneal administration of urethane (1.3 g/kg) and exsanguinated. The hearts were removed and weighed, and the superior mesenteric arteries were excised. The experimental design of the study was approved by the Animal Experimentation Committee of the University of Tampere (Tampere, Finland). Moreover, the investigation conforms to the “Guiding Principles for Research Involving Animals.”

**Mesenteric Arterial Responses In Vitro**

The inhibitory effect of orally administered 1-NAME on acetylcholine (ACh)-induced relaxation is known to decline during successive responses in isolated arterial preparations from 1-NAME-treated rats (14). In preliminary experiments, we observed that vasorelaxation to ACh from 1-NAME rats clearly improved during repeated challenges. Therefore, we tested the in vitro concentrations of 0.1–100 μM 1-NAME and found that 100 μM 1-NAME was needed to prevent the enhancement of ACh-induced relaxation during four repetitions. Thus the in vitro experiments were performed in the presence of 100 μM 1-NAME as given below.

Five successive sections (3 mm in length) of the main superior mesenteric artery from each animal were cut; in the three distal rings the endothelium was left intact, and from the two proximal pieces it was removed (4). The rings were placed between small hooks and suspended in an organ bath chamber in physiological salt solution (PSS; pH 7.4) containing (in mM) 119.0 NaCl, 25.0 NaHCO\(_3\), 11.1 glucose, 1.6 CaCl\(_2\), 4.7 KCl, 1.2 KH\(_2\)PO\(_4\), and 1.2 MgSO\(_4\) and aerated with 95% O\(_2\)-5% CO\(_2\). The rings were initially equilibrated for 1.5 h at +37°C with a resting preload of 1.5 g, which, in preliminary experiments, was found to render maximal contractions in all groups. The force of contraction was measured with isometric transducers (FT 03 transducer and model 7 E polygraph; Grass Instrument, Quincy, MA). We usually confirm the presence of intact endothelium by a clear relaxation to 1 μM ACh in 1 μM norepinephrine (NE)-precontracted vascular rings and the absence of endothelium by the lack of this response (4). However, in the present study, the responses to ACh were almost completely absent in the 1-NAME group, and therefore no vascular preparations were excluded from the study. In the course of the study, each vascular preparation was allowed a 30-min equilibration period in PSS between the responses. When a response was elicited in the presence of an inhibitor or an enzyme, this was also present in the PSS during the preceding equilibration period.

**Sensitivity of arterial contractions to KCl and Ca\(^{2+}\).** The concentration-response curves of endothelium-denuded rings to KCl were cumulatively determined (24). In solutions containing high concentrations of potassium (20–125 mM), NaCl was equimolarly replaced with KCl. The rings were then repeatedly contracted with 10 μM NE in Ca\(^{2+}\)-free PSS to deplete Ca\(^{2+}\) stores and challenged with 125 μM KCl in Ca\(^{2+}\)-free PSS; thereafter Ca\(^{2+}\) was cumulatively added, and the contraction was registered (25). The responses were performed in the presence of 0.1 mM 1-NAME.

**Endothelium-independent relaxations to exogenous NO, activation of β-adrenoceptors, and opening of ATP-sensitive K\(^{+}\) channels.** Relaxations to nitroprusside (NP), isoproterenol, and cromakalim were examined in endothelium-denuded rings precontracted with 1 μM NE. Thereafter, the relaxations to NP were examined in rings precontracted with 50 mM KCl. The responses in the 1-NAME and calcium + 1-NAME groups were performed in the presence of 0.1 mM 1-NAME.

**Contractile responses induced by NE during NOS and cyclooxygenase inhibition.** Concentration-response curves for NE were determined in endothelium-intact rings in the presence of 0.1 mM 1-NAME and in the presence of 1-NAME and 3 μM diclofenac (24).

**Endothelium-mediated relaxations during NOS inhibition: influence of cyclooxygenase inhibition and eliminated hyperpolarization.** Relaxations to ACh in the presence of 0.1 mM 1-NAME and in the presence of 1-NAME plus 3 μM diclofenac were examined in endothelium-intact rings precontracted with 1 μM NE. The responses to ACh in the presence of 1-NAME plus 3 μM diclofenac were also elicited in rings precontracted with 50 mM KCl.

**Effect of scavenging of oxygen-derived free radicals (superoxide and hydrogen peroxide) on endothelium-mediated relaxation.** Relaxations to ACh in the presence of 0.1 mM 1-NAME were examined in endothelium-intact mesenteric arterial rings precontracted with 1 μM NE. The responses to ACh were also elicited in the presence of 1-NAME plus 50 U/ml superoxide dismutase (SOD) and in the presence of 1-NAME and SOD plus 100 U/ml catalase.

**Data Presentation and Analysis of Results**

The EC\(_{50}\) for NE, KCl, and Ca\(^{2+}\) in each ring was calculated as a percentage of maximal response and presented as the negative logarithm of the concentration of the agonist producing 50% of maximal contractile force (pD\(_{50}\)), which was also used in the statistical analysis. The relaxations in re-
Cromakalim, isoproterenol hydrochloride, L-NAME hydrochloride, and diclofenac were considered significant when *P < 0.05 (ANOVA for repeated measurements).

**Drugs**

The following drugs were used: ACh chloride, catalase, cromakalim, isoproterenol hydrochloride, L-NAME hydrochloride, and SOD (Sigma Chemical, St. Louis, MO); L-NE L-hydrogentartrate, NP (Fluka Chemie, Buchs, Switzerland); and diclofenac (Voltaren injection solution; Ciba-Geigy, Basle, Switzerland). For the preparation of stock solutions, the compounds used in the in vitro studies were dissolved in distilled water, with the exception of cromakalim (in 50% ethanol). All solutions were freshly prepared before use and protected from light.

**RESULTS**

**Blood Pressure, Heart Weight, and Body Weight**

Long-term administration of L-NAME resulted in an elevation of blood pressure, which reached its maximum within 4 wk, and Ca$^{2+}$ supplementation clearly attenuated the development of hypertension. The mean systolic blood pressure in the control and calcium groups remained stable and comparable throughout the investigation. After 8 wk of study, the blood pressures in the experimental groups were as follows (means ± SE): control 139 ± 6, calcium 146 ± 5, L-NAME 198 ± 6, and calcium + L-NAME 175 ± 7 mmHg (Fig. 1). The heart weight-to-body weight ratios in the other groups did not differ from those of the control group. The final body weights in the Ca$^{2+}$-supplemented groups were not significantly different from control but were lower than in the L-NAME group (Table 1).

**Endothelium-Independent Relaxations**

To make proper interpretations from the endothelium-dependent relaxations, the vasodilatory properties of arterial smooth muscle were examined. The relaxations of endothelium-denuded NE-precontracted rings to NP, isoproterenol, and cromakalim, the vasodilators acting via the formation of NO; activation of β-adrenoceptors; and opening of ATP-sensitive K+ channels (K$_{ATP}$), respectively, were impaired in L-NAME rats when compared with all other groups (Fig. 2, A–D). All of these impairments in vasorelaxation were abrogated by Ca$^{2+}$ supplementation. Furthermore, the relaxation to isoproterenol was even more pronounced in the calcium group when compared with the control group (Fig. 2C). In addition, when hyperpolarization of smooth muscle was prevented by precontractions with 50 mM KCl (24), the relaxations to NP were impaired in the L-NAME group, whereas in the two Ca$^{2+}$-supplemented groups, the relaxations were more pronounced than in the control group (Fig. 2B).

**Endothelium-Dependent Relaxations**

The relaxations induced by ACh in NE-precontracted arterial rings in the presence of NOS inhibition in vitro were markedly impaired in the L-NAME group when compared with the control group, whereas these responses in the Ca$^{2+}$-supplemented groups were enhanced when compared with the control group (Fig. 3A). The addition of the cyclooxygenase (COX) inhibitor diclofenac to the organ bath enhanced the relaxations to ACh in the L-NAME and control groups (*P < 0.05) but not in the Ca$^{2+}$-supplemented groups. Diclofenac also abolished the difference in the ACh response between the control and the Ca$^{2+}$-supplemented groups, whereas the relaxations still remained.

**Table 1. Experimental group data at close of study**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Calcium</th>
<th>L-NAME</th>
<th>Calcium + L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>432 ± 16</td>
<td>402 ± 10†</td>
<td>440 ± 11</td>
<td>401 ± 15†</td>
</tr>
<tr>
<td>Heart wt, mg</td>
<td>1,148 ± 27</td>
<td>1,013 ± 22‡</td>
<td>1,234 ± 24‡</td>
<td>1,058 ± 27‡</td>
</tr>
<tr>
<td>Heart wt-to-body wt ratio, mg/g</td>
<td>2.7 ± 0.1</td>
<td>2.5 ± 0.1†</td>
<td>2.8 ± 0.1</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>400 ± 7</td>
<td>401 ± 9†</td>
<td>377 ± 5*</td>
<td>356 ± 4†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8–10 for all groups. L-NAME, N$^{	ext{G}}$-nitro-l-arginine methyl ester. *P < 0.05 compared with the control group and †P < 0.05 compared with the L-NAME group (Bonferroni test).
impaired in the L-NAME group when compared with the others (Fig. 3B). The responses to ACh were almost abolished in all groups when induced in KCl-precontracted rings in the presence of L-NAME and diclofenac (Fig. 3C). When L-NAME and SOD were added to the organ bath, the relaxations to ACh were enhanced in the L-NAME group (Fig. 3, compare A and D; P < 0.05), but the responses remained impaired when compared with the other groups. In addition, SOD augmented the relaxation to ACh in the control group (P < 0.05), whereby the difference in response to ACh between the control group and the Ca2+ -supplemented groups was abrogated (Fig. 3D). The further addition of catalase had no effect on the relaxation to ACh in any of the study groups (not shown). It is noteworthy that the effects of diclofenac and SOD addition on vasoconstriction to ACh in vitro did not statistically differ from each other (Fig. 3, B and D).

Vasoconstrictor Responses

The contractile experiments were performed to elucidate the possible differences in vasoconstrictor sensitivity, which may curtail the results on arterial relaxation. Both in the presence of L-NAME and in the presence of L-NAME and diclofenac, the vascular rings of the study groups showed comparable sensitivity to NE (i.e., pD2 values). In the presence of L-NAME, the endothelium-denuded rings of the control and L-NAME groups showed similar contractile sensitivity to KCl, whereas the sensitivity was somewhat higher in the Ca2+ -supplemented groups. Arterial sensitivity to the addition of Ca2+ during depolarization with 125 mM KCl was similar in the calcium and control groups but was higher in the calcium group when compared with the L-NAME and calcium + L-NAME groups (Table 2).

DISCUSSION

Chronic inhibition of NOS is a novel experimental model of hypertension (7). In the present study, oral L-NAME administration resulted in a marked hypertension, which reached its maximum within 4 wk, whereas Ca2+ supplementation attenuated the elevation of blood pressure. This agrees with previous experiments on dietary Ca2+ in other models of experimental hypertension (17, 30). However, the
The antihypertensive effect of a high-Ca\(^{2+}\) diet in L-NAME-induced hypertension has not been previously investigated, and this study for the first time showed that the impairments of endothelium-dependent and -independent arterial relaxation associated with NO-deficient hypertension can be abrogated by increased Ca\(^{2+}\) intake.

The heart weight-to-body weight ratios did not differ between the L-NAME and control rats (Table 1), which agrees with some of the previous reports (2, 3, 6), although contradictory findings have been published (34, 35). The lack of marked increase in relative cardiac weight in this model of hypertension is proposed to result from the L-NAME treatment, possibly blocking cardiovascular growth processes (6, 29, 41). Despite the absence of cardiac hypertrophy in the L-NAME group, the high-Ca\(^{2+}\) diet reduced absolute heart weights in this study, although the heart weight-to-body weight ratios were not affected (Table 1). Previously, increased Ca\(^{2+}\) intake has attenuated weight gain in hypertensive animals (30, 44) and in patients with essential hypertension (40), which could result from reduced body fat content after the high-Ca\(^{2+}\) diet (32). Correspondingly, we found that the Ca\(^{2+}\)-supplemented rats gained less weight than the L-NAME group.

The arterial relaxations induced by the NO donor NP have been found to be enhanced (15, 20) or to remain unaffected in L-NAME-hypertensive rats (9, 27). However, in our study, the L-NAME rats showed attenuated relaxations to NP in both NE- and KCl-precontracted endothelium-denuded rings, suggesting that the sensitivity of arterial smooth muscle to NO was decreased. In addition, the relaxations induced by the \(\beta\)-adrenoceptor agonist isoproterenol and the K\(_{ATP}\) opener cromakalim were impaired in the L-NAME rats. Therefore, NO-deficient hypertension was associated with attenuated vasorelaxation via cGMP, cAMP, and the opening of K\(_{ATP}\), which suggests a general impairment of relaxation in arterial smooth muscle. These mechanisms of vasodilatation are all associated with changes in cellular Ca\(^{2+}\) metabolism and modulation of K\(^{+}\)-channel activity in arterial smooth muscle (23, 43). Because these impairments have also been described in spontaneously hypertensive rats (44), they are likely to result from the elevation of blood pressure. This view is supported by the fact that the present antihypertensive effect of increased dietary Ca\(^{2+}\) was accompanied by a clear improvement of these changes. It is noteworthy that in the normotensive rats, the high-Ca\(^{2+}\) diet also improved relaxations to isoproterenol and to NP in KCl-precontracted arterial rings (Fig. 2).
The chemical antagonism between superoxide anions and NO is an important modulator of vascular tone. In addition, superoxide can inhibit the vascular production of superoxide as well as the generation of NO by the NOS enzyme, which is critical for the production of vasodilator factors. Increased production of vasoconstrictor prostanooids may contribute to the impaired vasodilation in L-NAME-hypertensive rats (27). In the present study, the inhibition of COX by diclofenac enhanced the relaxations to ACh in the L-NAME and control groups, suggesting that COX-derived contractile factors were involved in these responses. Ca^{2+} supplementation appeared to reduce the production of these factors in L-NAME rats, because the relaxation to ACh after diclofenac was augmented in the L-NAME group, whereas no significant change was detected in the calcium + L-NAME group. The release of vasoconstrictor prostanooids was probably also reduced in the control rats by the high-Ca^{2+} diet, because the addition of diclofenac augmented the relaxations to ACh in the control group, whereas the relaxations in the calcium group were not affected (Fig. 3). In addition, decreased arterial superoxide production may also have contributed to the enhanced endothelium-mediated vasodilation after diclofenac administration, because COX is a significant source of superoxide (26).

The endothelium-dependent relaxations, which remain resistant to NOS and COX inhibition, are mediated by another vasoactive autacoid, EDHF (12). The chemical characteristics of EDHF remain unknown, but functionally this factor is a K^+ channel opener (12), the action of which can be inhibited by K^+ channel blockers or by depolarization of the cell membrane with high concentrations of K^+ (1). Although all of the present groups showed distinct NOS- and COX inhibitor-resistant relaxations to ACh, the remaining responses in the L-NAME group were attenuated when compared with all other groups, whereas the responses in the calcium + L-NAME group did not differ from control (Fig. 3). Thus Ca^{2+} supplementation prevented the impairment of endothelium-dependent hyperpolarization in L-NAME-treated rats. The precontraction of arterial rings with KCl almost abolished the remaining NOS- and COX inhibitor-resistant relaxations to ACh, suggesting that these responses were indeed mediated by EDHF. Decreased endothelium-dependent hyperpolarization has previously been observed in various

### Table 2. Parameters of contractile responses of isolated mesenteric arterial rings in experimental groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Calcium</th>
<th>L-NAME</th>
<th>Calcium + L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>pD2 (-log M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+E with L-NAME</td>
<td>6.80 ± 0.09</td>
<td>6.54 ± 0.15</td>
<td>6.73 ± 0.11</td>
<td>7.19 ± 0.27</td>
</tr>
<tr>
<td>+E with L-NAME and diclofenac</td>
<td>6.29 ± 0.12</td>
<td>6.04 ± 0.22</td>
<td>5.98 ± 0.08</td>
<td>6.24 ± 0.14</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pD2 (-log M)</td>
<td>1.47 ± 0.01†</td>
<td>1.40 ± 0.02</td>
<td>1.48 ± 0.02*‡</td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pD2 (-log M)</td>
<td>3.63 ± 0.06</td>
<td>3.73 ± 0.08†‡</td>
<td>3.52 ± 0.06</td>
<td>3.51 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8–10 for all groups. +E and -E, endothelium-intact and endothelium-denuded arterial rings, respectively. pD2 is the negative logarithm of the concentration of agonist producing 50% of maximal contractile force. *P < 0.05 compared with the control group, †P < 0.05 compared with the L-NAME group, and ‡P < 0.05 compared with the calcium + L-NAME group (Bonferroni test).
forms of experimental hypertension (genetic, renal, mineralocorticoid-NaCl) (16, 30, 45), and the present results suggest that the same holds true for L-NAME-induced hypertension.

Impaired endothelium-dependent hyperpolarization could result from decreased endothelial release of EDHF or from reduced sensitivity of smooth muscle to EDHF. The present results, whereby the relaxations induced by the K\textsubscript{ATP} opener cromakalim were attenuated in L-NAME rats, suggest that the sensitivity of smooth muscle to hyperpolarizing factors was decreased. Furthermore, isoproterenol has been reported to hyperpolarize arterial smooth muscle via K\textsubscript{ATP} and Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (36, 42). Thus the present finding, whereby relaxation to isoproterenol was impaired in L-NAME rats, is in agreement with the view of reduced hyperpolarization of arterial smooth muscle in these rats.

The present study confirmed the earlier finding, whereby chronic L-NAME hypertension does not affect NE-induced vasoconstrictor responses (27). Previously, 3-wk-long administration of L-NAME has decreased arterial sensitivity to KCl in rats (15, 20), but we observed no differences between the L-NAME and control rats in the contractions induced by KCl in this 8-wk study. Ca\textsuperscript{2+} supplementation was without effect on the constrictor sensitivity of arterial rings to NE, whereas the arterial preparations from the Ca\textsuperscript{2+}-supplemented groups showed somewhat higher sensitivity to KCl-induced contractions when compared with the control and L-NAME groups (Table 2). However, the deviations in the contractile responses between the study groups were small. Therefore, changes in vasoconstrictor sensitivity could not explain the clear differences in arterial relaxation between the study groups.

In conclusion, chronic L-NAME hypertension was associated with a clear impairment of endothelium-dependent and -independent vasorelaxation in the mesenteric artery of the Wistar rat. Increased dietary Ca\textsuperscript{2+} intake attenuated the development of hypertension and abrogated the impairments of endothelium-dependent and -independent vasodilatation. The reduced blood pressure and the improved vasorelaxation after Ca\textsuperscript{2+} supplementation in NO-deficient hypertension may be explained by enhanced arterial hyperpolarization, increased sensitivity to NO in smooth muscle, and decreased vascular production of superoxide and vasoconstrictor prostanoids.

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