Salt-induced ANG II suppression impairs the response of cerebral artery smooth muscle cells to prostacyclin

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Zhu, Jiaxuan, Ming Yu, Jill Friesema, Tianjian Huang, Richard J. Roman, and Julian H. Lombard. Salt-induced ANG II suppression impairs the response of cerebral artery smooth muscle cells to prostacyclin. Am J Physiol Heart Circ Physiol 288: H908–H913, 2005. First published October 14, 2004; doi:10.1152/ajpheart.00795.2004.—Recent studies have demonstrated that cerebral arteries of male Sprague-Dawley rats maintained on an HS (4% NaCl) or a low-salt diet (0.4% NaCl) for 3 days. The stable prostacyclin analog iloprost (10 ng/ml) inhibited serotonin (0.1–10 μM)-induced contractions and the increase in intracellular Ca2+ concentration ([Ca2+]i) in VSMC isolated from arteries of animals fed the low-salt diet. In contrast, iloprost had no effect on serotonin-induced contractions and increases in [Ca2+]i in VSMC isolated from arteries of rats fed the HS diet. Preventing the fall in ANG II levels in rats fed a HS diet by infusion of a low dose of ANG II (5 ng·kg−1·min−1) restored the inhibitory effect of iloprost on serotonin-induced contractions and increases in [Ca2+]i, and VSMC from rats fed the HS diet. These results indicate that ANG II suppression secondary to elevated dietary salt intake impairs vascular relaxation and Ca2+ regulation by prostacyclin.

Previous studies demonstrated that serotonin increases the release of Ca2+ from intracellular stores and increases the influx of extracellular Ca2+ into cerebral VSMC (VSMC) (12, 18, 22). Purdy and Arendshorst (19) demonstrated that the stable prostacyclin analog iloprost attenuates the initial inositol 1,4,5-trisphosphate-mediated release of intracellular Ca2+ in response to ANG II in cultured VSMC isolated from preglomerular resistance vessels. However, activation of L-type Ca2+ channels by BAY K 8644 was unaffected by iloprost treatment in the study of Purdy and Arendshorst.

In light of previous studies showing impaired responses of MCAs and other vessels to iloprost and other vasodilator stimuli in rats fed a HS diet (6, 13, 15, 20), we hypothesized that the impaired relaxation in response to iloprost in rats fed a HS diet results from an intrinsic defect in the ability of the VSMC themselves to respond to the agonist. The purposes of the present study were to determine 1) whether the inhibitory effect of iloprost on contractile responses to serotonin and serotonin-induced increases in intracellular Ca2+ concentration ([Ca2+]i) are impaired in individual VSMC isolated from cerebral arteries of rats fed a HS diet, 2) whether the impaired response to iloprost could be restored by maintaining normal ANG II levels in rats fed a HS diet by intravenous infusion of ANG II, and 3) whether the AT1 receptor antagonist losartan abolishes the ability of ANG II infusion to restore iloprost-induced inhibition of VSM responses to serotonin in VSMC from cerebral arteries of rats fed a HS diet.

METHODS
Experiments were performed on 10- to 12-wk-old male Sprague-Dawley rats that were maintained in the American Association for Accreditation of Laboratory Animal Care-approved Animal Resource Center at the Medical College of Wisconsin. The Medical College of Wisconsin animal care committee approved all procedures used in the study. Four experimental groups were utilized. The first two groups consisted of rats fed a HS (4% NaCl) or a low-salt (LS, 0.4% NaCl) diet for 3 days. The other two groups of rats were maintained on a HS diet for 3 days and then infused with ANG II (5 ng·kg−1·min−1) or ANG II + losartan (20 μg·kg−1·min−1), an ANG II AT1 receptor blocker, for another 3 days to prevent the fall in circulating ANG II levels normally seen in rats fed a HS diet.

Surgical preparation. Rats were anesthetized with an intramuscular injection containing ketamine HCl (78.0 mg/kg) and acepromazine maleate (2.2 mg/kg). Under sterile conditions, chronic indwelling catheters were placed in the femoral artery and vein and advanced into the abdominal aorta (for blood pressure measurement) or into the inferior vena cava (to allow infusion of ANG II or ANG II + losartan). The catheters were secured in the vessel with a 3-0 suture (Ethicon, Somerville, NJ). The catheters were filled with heparin

A NUMBER OF STUDIES HAVE SHOWN that short-term and chronic exposure to a high-salt (HS) diet impair the response of arterioles and resistance arteries to vasodilator stimuli such as reduced Po2, acetylcholine, and prostacyclin (1, 5, 7, 8, 13, 15). For example, pial arterioles of normotensive animals fed a HS diet fail to dilate in response to acetylcholine or the stable prostacyclin analog iloprost (10 ng/ml) inhibited serotonin (0.1–10 μM)-induced contractions and the increase in intracellular Ca2+ concentration ([Ca2+]i) in VSMC isolated from arteries of animals fed the low-salt diet. In contrast, iloprost had no effect on serotonin-induced contractions and increases in [Ca2+]i in VSMC isolated from arteries of rats fed the HS diet. Preventing the fall in ANG II levels in rats fed a HS diet by infusion of a low dose of ANG II (5 ng·kg−1·min−1) restored the inhibitory effect of iloprost on serotonin-induced contractions and increases in [Ca2+]i, and VSMC from animals fed the HS diet. These results indicate that ANG II suppression secondary to elevated dietary salt intake impairs vascular relaxation and Ca2+ regulation by prostacyclin.

salt intake; hypertension; angiotensin; vascular smooth muscle; calcium; serotonin

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sodium (500 U/ml), tunneled subcutaneously and exteriorized at the back of the neck. The catheters were protected by passage through a flexible spring and were connected to a swivel outside the cage, allowing the rat to move freely within the cage. After surgery, the rat received an injection of penicillin G (300,000 U/kg im) to prevent infections and was allowed a 3-day recovery period before the beginning of the experiment.

Blood pressure was monitored daily by arterial catheters attached via a hydraulic swivel to pressure transducers (Argon, Athens, TX) for direct measurement of blood pressure. The transducer output was fed through a signal-conditioning amplifier (Stemtech, Houston, TX), digitized at 100 samples/s, and analyzed with software to compute systolic, diastolic, and mean arterial pressure and heart rate (Apollo Computer, Chelmsford, MA). Each daily pressure measurement represents the average of data obtained for 60 s during a period of continuous recording (±1 h), for which a pulse pressure >10 mmHg was maintained.

Isolation of cerebral artery smooth muscle cells. On the day of the acute experiment, rats were anesthetized with pentobarbital sodium (50 mg/kg). The brain was removed, and cerebral arteries were dissected and placed in a Ca<sup>2+</sup>-free solution containing (in mM) 119 NaCl, 4.7 KCl, 1.17 MgSO<sub>4</sub>, 20 MgCl<sub>2</sub>, 1.18 NaH<sub>2</sub>PO<sub>4</sub>, 0.03 EDTA, 2 EGTA, and 5.5 dextrose. VSMC were isolated as described by Jackson et al. (11). Briefly, the vessels were incubated for 15 min in a dissociation solution containing (in mM) 145 NaCl, 4 KCl, 1 MgCl<sub>2</sub>, 10 HEPES, and 0.05 CaCl<sub>2</sub> with 0.1% albumin and transferred to the same solution containing 1.5 mg/ml papain (14 U/ml) and 1 mg/ml dithiothreitol for an additional 10 min. The vessels were then placed in another dissociation solution containing elastase (0.5 mg/ml), soybean trypsin inhibitor (10,000 U/ml), and collagenase (196 U/ml) for 10 min at 37°C. After these incubations, the vessels were triturated with a transfer pipette to the free smooth muscle cells. The vessel suspension was centrifuged at 1,000 g for 2 min, and the pellet was resuspended in physiological salt solution (PSS; in mM: 119 NaCl, 4.7 KCl, 1.17 MgSO<sub>4</sub>, 1.6 CaCl<sub>2</sub>, 1.18 NaH<sub>2</sub>PO<sub>4</sub>, 24 NaHCO<sub>3</sub>, 0.026 EDTA, and 5.5 glucose). For Ca<sup>2+</sup> measurements, cells were stored at 4°C.

Measurement of VSMC length. Freshly dispersed VSMC were prepared as described above and placed in a chamber containing PSS on the stage of a Nikon inverted microscope. Elongated, spindle-shaped VSMC were identified by video microscopy. Serotonin (0.1–10 μmol/l) was added to the chamber in the presence or absence of iloprost (10 ng/ml), and the cell images were captured with a video copy processor (Mitsubishi) before and after stimulation with different concentrations of serotonin. Cell lengths were determined from the captured video images with use of a map wheel calibrated against a stage micrometer.

Measurement of [Ca<sup>2+</sup>]i in VSMC. Experiments were performed to determine the effect of iloprost in VSMC isolated from cerebral arteries of rats fed the LS diet, rats fed the HS diet alone, and rats fed the HS diet and infused with ANG II with and without losartan. In these experiments, changes in [Ca<sup>2+</sup>]i, in response to 10 μmol/l serotonin were measured in the presence and absence of iloprost (10 ng/ml). Cells were loaded for 45 min at room temperature with 5 μmol/l fura 2-AM (Molecular Probes, Eugene, OR) prepared in PSS containing 0.1% albumin. The cells were then transferred to a 1-ml perfusion chamber filled with PSS equilibrated with 95% O<sub>2</sub>-5% CO<sub>2</sub> and maintained at 37°C. After 30 min of equilibration at 37°C, the cells were observed with an inverted Nikon TS 100 microscope using a ×40 infinity-corrected ultraviolet fluorescence objective (S Fluor) with a numerical aperture of 0.90. [Ca<sup>2+</sup>]i was measured using an imaging system (InCyt Im2, Intracellular Imaging, Cincinnati, OH) mounted on the microscope. [Ca<sup>2+</sup>]i values were based on fluorescence intensity ratios obtained using excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. [Ca<sup>2+</sup>]i values were calculated from a standard curve using solutions with known Ca<sup>2+</sup> concentration. The standard curve was adjusted every month using standard Ca<sup>2+</sup> solutions (Molecular Probes). The resulting standard curve showed very little variation from day to day and provided an excellent proportional relation between the ratio of 340- to 380-nm wavelength and Ca<sup>2+</sup> concentration. This method, which is easy and reproducible, is based on the method reported by Wahl et al. (21) and was subsequently utilized in a number of studies by other investigators (16, 26, 27). It is used as an alternative to the minimum-to-maximum wavelength ratiometric (R<sub>min</sub>/R<sub>max</sub>) method of Grynkiewicz et al. (10), because it circumvents the difficulties in obtaining the R<sub>max</sub> value, which often requires the use of metabolic poisons to reduce cellular ATP level and the prevention of pH changes due to Na<sup>+</sup>/H<sup>+</sup> exchange.

Statistics. Values are means ± SE. The significance of any differences in serotonin-induced changes in cell length or [Ca<sup>2+</sup>]i in VSMC.

RESULTS

Blood pressure in rats infused with ANG II with or without losartan. As previously reported (20, 23) arterial blood pressures were unaffected by the HS diet. Mean arterial pressure was ~116 ± 2 mmHg in animals fed the LS diet and 117 ± 5 mmHg in animals fed the HS diet. Figure 1 shows arterial blood pressure in rats fed the HS diet before and during infusion of ANG II in the presence and absence of losartan. In the present study, ANG II infusion in animals fed the HS diet caused an elevation in arterial pressure that was prevented by coinfusion of losartan. The latter observation contrasts with those of previous studies (23, 24), in which the same dose of ANG II failed to increase mean arterial pressure in animals fed the 4% NaCl diet. This observation suggests that this dose of ANG II is on the borderline for being a pressor dose in the presence of this level of elevated dietary salt intake.

Effect of iloprost on serotonin-induced contractions of VSMC. Addition of serotonin to the chamber caused a concentration-dependent contraction of cerebral artery smooth muscle cells isolated from rats fed the LS or the HS diet (Figs. 2–4). Iloprost (10 ng/ml) inhibited serotonin-induced contractions in VSMC from rats fed the LS diet but had no effect on serotonin-induced contractions of cells isolated from rats fed the HS diet (Fig. 3). Chronic intravenous infusion of a low dose of ANG II restored the inhibitory effect of iloprost on serotonin-induced
contractions in VSMC isolated from rats fed the HS diet (Fig. 4). Blockade of the ANG II AT\(_1\) receptor with losartan eliminated the ability of ANG II to restore the inhibitory effect of iloprost on serotonin-induced contractions of VSMC from rats fed the HS diet (Fig. 4).

**Effect of iloprost on serotonin-induced changes in [$Ca^{2+}$]\(_i\), in cerebral artery smooth muscle cells.** A representative trace of the effects of serotonin on [$Ca^{2+}$]\(_i\) in VSMC isolated from cerebral arteries of rats fed the LS and HS diets is presented in Fig. 5. Serotonin produced a transient increase in [$Ca^{2+}$]\(_i\), followed by a sustained plateau phase, in VSMC from rats fed the HS or LS diet. This response is similar to those previously reported (12, 18, 22).

Figure 6 summarizes the effects of iloprost on [$Ca^{2+}$]\(_i\) in the various groups. There were no differences in resting [$Ca^{2+}$]\(_i\) in any of the groups, and resting [$Ca^{2+}$]\(_i\) was unaffected by iloprost in any of the groups (Fig. 6A). Iloprost significantly attenuated the transient peak increase of [$Ca^{2+}$]\(_i\) in response to serotonin in smooth muscle cells isolated from cerebral arteries of rats fed the LS diet but not in smooth muscle cells from rats fed the HS diet (Fig. 6B). ANG II infusion restored the inhibitory effect of iloprost on the transient [$Ca^{2+}$]\(_i\) peak in response to serotonin in cells from rats fed the HS diet, and losartan blocked the ability of ANG II to restore iloprost-induced inhibition of the rapid [$Ca^{2+}$] increase in smooth muscle cells from animals fed the LS diet ($P < 0.05$) but had no effect on plateau [$Ca^{2+}$] values in serotonin-treated smooth muscle cells from cerebral arteries of any of the groups of rats fed the HS diet (Fig. 6C). ANG II infusion (with or without losartan) had no discernible effect on plateau [$Ca^{2+}$] values in smooth muscle cells from rats fed the HS diet.

**DISCUSSION**

A number of recent studies have indicated that elevations in dietary salt intake lead to impaired vascular relaxation in response to different vasodilator agonists and to reduced PO\(_2\) in arterioles, resistance arteries, and conduit vessels of normotensive rats (2, 4, 6–8, 13–15, 20, 28). Previous studies have indicated that prostacyclin release is a crucial mediator of the VSM hyperpolarization and relaxation in cerebral, skeletal muscle, and coronary resistance arteries during exposure to
reduced \( \text{PO}_2 \) (8, 15, 17). In MCAs of rats fed a normal salt diet, VSM hyperpolarization during hypoxia appears to be mediated via the opening of ATP-sensitive K\(^+\) channels in response to endothelium-derived prostacyclin acting on the VSMC (14).

In contrast, MCAs isolated from rats fed a HS diet exhibit a paradoxical vasoconstriction and depolarization of the VSMC after a reduction in \( \text{PO}_2 \) (15). These findings suggest that vascular responses to prostacyclin may be altered during exposure to a HS diet (14). The latter observation is consistent with the observation that vascular relaxation and VSM hyperpolarization in response to the prostacyclin analog iloprost are attenuated in isolated resistance arteries of rats fed a HS diet (7, 8, 15).

The present study examined whether an altered response to prostacyclin exists at the level of individual VSMC from cerebral arteries of rats fed a HS diet and sought to determine whether any intrinsic alterations in VSM function in vessels from rats fed a HS diet involve changes in intracellular \( \text{Ca}^{2+} \) from intracellular stores and/or in \( \text{Ca}^{2+} \) entry from the extracellular fluid. In these experiments, the failure of iloprost to block cell contraction in response to serotonin and to inhibit serotonin-induced \([\text{Ca}^{2+}]_i\) transients that occur in response to serotonin. The latter observation is consistent with the ability of iloprost to inhibit the contractile response to serotonin in VSMC from rats fed the LS diet. This inhibition of serotonin-induced contraction by iloprost in smooth muscle cells isolated from rats fed the LS diet could be due to inhibition of \( \text{Ca}^{2+} \) release from the sarcoplasmic reticulum, a reduction in the filling of membrane \( \text{Ca}^{2+} \) stores, or an enhanced transport of \( \text{Ca}^{2+} \) from the cytoplasm, leading to an attenuation of the rapid \( \text{Ca}^{2+} \) peak. Iloprost also had a significant inhibitory effect on the \([\text{Ca}^{2+}]_i\) transient from animals fed the HS diet. Taken together, these observations provide new evidence suggesting that iloprost has a reduced ability to inhibit \( \text{Ca}^{2+} \) release from membrane stores as well as an impaired ability to inhibit the influx of \( \text{Ca}^{2+} \) into the VSMC.
suggest that a HS diet may impair the function of the prostacyclin receptor itself, the heterotrimeric G protein, linking receptor activation to downstream signal transduction mechanisms, or the coupling of the G protein to the prostacyclin receptor, resulting in an impaired ability to increase cAMP levels in the VSMC. The latter alterations are similar to changes previously reported in experimental models of hypertension, such as the spontaneously hypertensive rat (3, 9) and the reduced renal mass-hypertensive rat (6).

Previous studies (25) demonstrated that cAMP inhibits phenylephrine-induced increases in inositol phosphates in aortic smooth muscle cells (including inositol 1,4,5-trisphosphate, which mediates Ca\(^{2+}\) release from intracellular stores during agonist stimulation). Because iloprost-induced increases in cAMP are impaired in resistance arteries from rats fed a HS diet (8, 15), we believe that the reduced ability of iloprost to attenuate the initial transient increase in Ca\(^{2+}\) in response to serotonin is most likely due to the impaired ability of the compound to increase cAMP levels in the arterial smooth muscle cells (3, 9, 25). In a similar fashion, an impaired ability to increase cAMP levels in the VSMC could also contribute to the loss of iloprost-induced hyperpolarization of the VSM membrane via opening of ATP-sensitive K\(^+\) channels that has been previously reported in MCAs of rats fed a HS diet (15). This impaired hyperpolarization would, in turn, reduce the ability of iloprost to inhibit Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels.

In the present study, we observed that preventing the fall in plasma ANG II levels in rats fed the HS diet restored the inhibitory effect of iloprost on serotonin-induced contractions and the reduction by iloprost of the initial [Ca\(^{2+}\)] transient in response to serotonin. This protective action of ANG II to restore the inhibitory effects of iloprost on serotonin-induced contractions and increases in [Ca\(^{2+}\)] in VSMC from rats fed the HS diet was blocked by losartan. Taken together, these findings indicate that a salt-induced suppression of plasma ANG II levels, with a subsequent loss of AT\(_1\) receptor activation in the smooth muscle cells, contributes to reduced vascular relaxation in response to prostacyclin due to impaired regulation of [Ca\(^{2+}\)]. These alterations could make a major contribution to the impaired vascular relaxations in response to prostacyclin and hypoxia that have been reported previously in resistance arteries of animals fed a HS diet (23, 24).

Previous studies of altered vascular responses during increased dietary salt intake have established that endothelial function is altered in response to a HS diet (13, 28) and have indirectly suggested that vascular relaxation mechanisms are impaired in the smooth muscle cells. The novel aspects of the present study are the demonstration that short-term (3 days) ingestion of the HS diet reduces the ability of iloprost to inhibit serotonin-induced contractions and increases in [Ca\(^{2+}\)] in VSMC from cerebral arteries of rats fed the HS diet (13, 28).

Although the precise mechanisms contributing to the reduced ability of iloprost to inhibit serotonin-induced contraction and increases in [Ca\(^{2+}\)] in VSMC isolated from rats fed a HS diet are not clear, the results of earlier studies (8, 14)

\[ \text{Baseline [Ca}^{2+}] \]

**A**

**B**

**C**

Fig. 6. Effect of iloprost on resting [Ca\(^{2+}\)], (A), transient peak in [Ca\(^{2+}\)], in response to serotonin (B), and sustained plateau phase of elevated [Ca\(^{2+}\)], in response to serotonin (C) in VSMC isolated from rats fed LS or HS diet and in rats fed HS diet and infused with a low dose (5 ng·kg\(^{-1}\)·min\(^{-1}\) iv) of ANG II in the presence and absence of losartan (20 μg·kg\(^{-1}\)·min\(^{-1}\)) to block ANG II AT\(_1\) receptors. Values are means ± SE of 18–26 cells from 5 to 6 animals.

*Significantly different from corresponding value in the absence of iloprost.
infusion restores the ability of iloprost to inhibit the rapid transient increase in \([\text{Ca}^{2+}]_i\), in response to serotonin in arterial smooth muscle cells from rats fed the HS diet, and this restorative effect of ANG II infusion on iloprost-induced inhibition of the transient increase in \([\text{Ca}^{2+}]_i\) in response to serotonin is blocked by losartan (Fig. 6B). Taken together, the findings of the present study provide additional evidence that elevated dietary salt intake can lead to an impaired coupling between membrane receptors and vascular relaxation mechanisms that prevent increases in \([\text{Ca}^{2+}]_i\) and VSM contraction in response to vasoconstrictor agonists such as serotonin. The demonstration of salt-induced decreases in the reactivity of resistance arteries to vasodilator stimuli in normotensive animals may reveal previously unknown defects in the ability of normotensive individuals on a HS diet to regulate blood flow and respond to circulatory stresses such as hypoxia, hemorrhage, and exercise.

**REFERENCES**