Impaired flow-induced dilation of coronary arterioles of dogs fed a low-salt diet: roles of ANG II, PKC, and NAD(P)H oxidase

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Huang A, Yan C, Suematsu N, Cuevas A, Yang YM, Kertowidjojo E, Hintze TH, Kaley G, Sun D. Impaired flow-induced dilation of coronary arterioles of dogs fed a low-salt diet: roles of ANG II, PKC, and NAD(P)H oxidase. Am J Physiol Heart Circ Physiol 299: H1476–H1483, 2010. First published September 10, 2010; doi:10.1152/ajpheart.01250.2009.—Low-salt (LS) diet has been considered to be beneficial in the prevention and treatment of hypertension; however, it also increases plasma angiotensin (ANG) II and may cause adverse cardiovascular effects, such as endothelial dysfunction. We assessed endothelial function of coronary arterioles and vascular superoxide production, as a function of LS diet. Dogs were fed with LS (0.05% NaCl) or a normal-salt (NS, 0.65% NaCl) diet for 2 wk. There were threefold increases in plasma ANG II, associated with a 60% reduction in flow-induced dilation (FID) in coronary arterioles of LS compared with NS dogs. In vessels of NS dogs, FID was primarily mediated by nitric oxide (NO), as indicated by an eliminated FID by Nω-nitro-l-arginine methyl ester (l-NAME). In vessels of LS dogs, however, FID was eliminated. Administration of apocynin, a NAD(P)H oxidase inhibitor, partially restored FID and additional l-NAME eliminated FID. Generation of superoxide, measured with dihydroethidium, was significantly greater in vessels of LS than in NS dogs, which was further increased in response to ANG II or phorbol 12,13-dibutyrate, an agonist of protein kinase C (PKC). The enhanced superoxide was normalized by apocynin, losartan (a blocker of angiotensin type 1 receptor), and chelerythrine chloride (an antagonist of PKC). Western blotting indicated an upregulation of gp91phox and p47phox, associated with increased expression of phosphorylated PKC in vessels of LS dogs. In separate experiments, dogs were fed simultaneously with LS and losartan (LS + Losa) for 2 wk. There was a significant increase in plasma ANG II in LS + Losa dogs, which, however, was associated with normal FID and gp91phox expression in coronary arterioles. In conclusion, LS led to endothelial dysfunction, as indicated by an impaired flow-induced dilation caused by decreasing NO bioavailability, a response that involves angiotensin-induced activation of PKC that, in turn, activates vascular NAD(P)H oxidase to produce superoxide.

low-salt diet; angiotensin; nicotinamide adenine dinucleotide phosphate oxidase; superoxide; flow-induced dilation

EXCESSIVE ACTIVATION OF THE renin-angiotensin-aldosterone system (RAAS), as a major player in the pathogenesis of cardiovascular diseases, has been extensively investigated. The question of whether restriction of sodium intake reduces the incidence of cardiovascular events is still a major issue of controversy (2). Experimental studies and clinical trials have yielded considerable heterogeneity concerning the effects of low-salt diet on blood pressure, as well as cardiovascular morbidity and mortality (3, 10, 14, 16). Sodium restriction, along with a reduction in blood pressure, is associated with increases of other, potentially adverse, cardiovascular effects, such as increases in sympathetic and RAAS activities (14). Given the direct and indirect adverse effects of excess angiotensin (ANG) II on vascular, myocardial, and renal tissues (27), the activation of RAAS may compromise the beneficial effects of sodium restriction on blood pressure and consequently increase cardiovascular risk (2).

An imbalance between the generation of vasoactive mediators, such as increased superoxide formation and decreased nitric oxide (NO) production promotes vascular damage, including endothelial dysfunction, inflammation, and atherosclerosis, in all of which stimulation of vascular NAD(P)H oxidase-derived superoxide by ANG II may be a pathogenetic factor (15, 38, 44). Increased superoxide production contributes to endothelial dysfunction via inactivating NO and transforming it into the prooxidant peroxynitrite, which may further cause eNOS uncoupling and reduced NO bioavailability (41, 42). In this context, it was reported that, as a consequence of sodium restriction, there was an accelerated development of atherosclerosis in aorta of apolipoprotein E-deficient mice (19).

Also, the loss of the counterregulatory effects of ANG-(1–7) toward ANG II were proposed to be involved in the detrimental effects of sodium restriction (33). Additionally, vascular dysfunction, as evidenced by reduced acetylcholine-induced vasodilation (6) and enhanced phenylephrine-induced vasoconstriction (13), in aorta of rats fed a LS diet was also reported. These studies, however, provided no mechanistic insight and, moreover, were conducted on conduit vessels that are not primarily responsible for the regulation of peripheral vascular resistance and tissue perfusion. Thus, in the present study, we aimed to evaluate endothelial function of coronary arterioles by determination of flow/shear stress-induced responses to elucidate the mechanism underlying endothelial dysfunction in dogs fed a low-salt diet.

MATERIALS AND METHODS

Animals

Male mongrel dogs (25–28 kg) were fed with a normal-sodium diet (NS, 0.65% of NaCl; n = 7), a low-sodium diet (LS, 0.05% of NaCl; n = 8), or LS plus losartan (LS + Losa; 2 mg·kg⁻¹·day⁻¹ orally, n = 2) for 2 wk after a thoracotomy for implantation of instruments, for measurements of pressure and flow, and for blood sampling (37). Dogs were anesthetized with pentobarbital sodium (25 mg/kg iv). The heart was removed, and the left ventricular free walls were obtained and placed in cold MOPS-buffered (pH 7.4) physiological salt solution (PSS). Hemodynamics reported and the tissues used here were also used in another study, previously published (35). Experimental
Superoxide and Endothelial Dysfunction

Protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conform to the current guidelines of the National Institutes of Health and the American Physiological Society for the care and use of laboratory animals.

Measurement of Plasma ANG II

Plasma concentration of ANG II was measured with peptide enzyme immunoassay (Peninsula Laboratories).

Isolation of Arterioles

Isolation of arteriolar branches of the left anterior descending coronary artery was performed with the use of microscissors and an operating microscope (Olympus, Lake Success, NY) (37). Segments of subepicardial arterioles, ~1 mm in length, were separated from the adhering cardiac muscle by careful dissection and were transferred to a vessel chamber containing Krebs bicarbonate-buffered PSS at room temperature. The vessel chamber contained two glass microcannulas, which were connected to two pressure-servo syringe systems (Living Systems, Burlington, VT). The vessel chamber was connected to a reservoir through a suffusion pump. The isolated vessels were incubated in PSS (37°C and pH 7.4) for at least 30 min before experiments.

Experimental Protocols

Flow-induced dilation. The intravascular pressure of cannulated arterioles was maintained at 60 mmHg. After vessels developed spontaneous tone, flow-diameter relationships were obtained in control conditions and in the presence of apocynin (10⁻⁵ M), an inhibitor of NAD(P)H oxidase, or N⁶-nitro-L-arginine methyl ester (Υ-NAM, 3 x 10⁻⁴ M), an inhibitor of nitric oxide synthase. Perfsufat flow was increased from 0 to 20 μl/min (maximal ~20 dyn/cm² shear stress) in steps of 5 μl/min. Each flow step was maintained for 3–5 min to allow the vessels to reach steady-state conditions before their diameter was measured. Inhibitors were added to the vessel chamber and incubated with vessels for at least 30 min before flow-induced dilation was reassessed.

Adenosine- or sodium nitroprusside-induced dilation. Vasodilation induced by the endothelium-independent agent adenosine (ADO, 10⁻⁹ to 10⁻⁴ M) was recorded at 60 mmHg of perfusion pressure in no-flow conditions. The agent was added to the vessel chamber, and final concentrations are reported. In separate experiments, sodium nitroprusside (SNP, 10⁻¹⁰ to 10⁻⁵ M)-induced dilation was also assessed. At the conclusion of the experiments, passive diameter (PD) of the arterioles, at 60 mmHg, was obtained in calcium-free PSS containing EGTA (1 mM).

Superoxide Detection

Superoxide production in coronary arterioles of NS and LS dogs was determined by two assays.

Assay 1. Superoxide formation in the endothelium and smooth muscle cells of isolated coronary arterioles was assessed by using dihydroethidium (DHE) staining with confocal fluorescent imaging (Bio-Rad MRC 1024ES/Olympus 1 x 70). Three images of the endothelial and smooth muscle layers were consistently obtained per vessel segment. All images were taken with an UPlanFL x40 objective and identical program settings. A histogram of full-sized fluorescent intensity of the image measured, which corresponds to the level of superoxide.

Assay 2. Quantitative superoxide formation in coronary arterioles was assessed by using DHE and an HPLC/fluorescence detector-based assay to determine 2-hydroxyethidium (2-EOH), a superoxide-induced oxidative product of DHE (11, 43). Briefly, coronary arterioles were isolated and perfused with MOPS-buffered PSS. Intravascular pressure was maintained constant at 60 mmHg. DHE (10⁻⁵ M) was then administered intraluminally to control vessels and to those that had been pretreated with apocynin (10⁻⁵ M) for 30 min. After a 1-h incubation, excess DHE was washed out from the vessels. Vessels were then removed, pulverized in liquid nitrogen, and homogenized in a 1:1 mixture of acetonitrile and water. After centrifugation, the supernatant was collected for HPLC analysis; the remaining tissues were dissolved in 1 N NaOH for the protein measurement with the Bio-Red Protein Assay. In separate experiments, superoxide formation in vessels was determined in control and after incubation of vessels with ANG II (10⁻⁷ M) for 30 min, with or without apocynin, losartan [blocker of angiotensin type 1 receptor (AT₁R), 2 x 10⁻⁶ M], or chelerythrine chloride [CLT, a protein kinase C (PKC) antagonist, at 2 x 10⁻⁶ M]. Superoxide formation in the vessels was also determined in control and after incubation of vessels with phosphol 12,13-dibutyrate (PDBu) for 45 min (PKC agonist, 10⁻⁵ M), or PDBu plus CLT. In the last protocol, superoxide formation in coronary arteries isolated from LS + Losa dogs was determined in control and after incubation of vessels with PDBu (45 min), or PDBu plus losartan or CLT.

Twenty-microliter samples or 2-EOH standards were separated by a HPLC system (PU-2080 Plus; Jasco) with a C-18 reverse-phase column (5 μm, 4.6 x 250 mm, Ultrasphere ODS; Beckman). The mobile phase was composed of 37% acetonitrile and 0.1% trifluoroacetic acid and run at a flow rate of 1 ml/min. The fluorescent signal of 2-EOH was detected at 480 nm (excitation) and 580 nm (emission) with a fluorescence detector (FP2020 Plus; Jasco). 2-EOH standards were synthesized from potassium nitrosodisulfonate as previously described (45). Standard curves of 2-EOH (0.3–10 pmol) were generated and used to calculate vascular superoxide production as picomoles per milligram protein (pmol/mg) in response to 1 h of incubation with 10 μM DHE.

Western Blot Analysis

Four to six single coronary arterioles were pooled as one sample. Equal amounts of total protein from samples were loaded on a 10% SDS-PAGE and transferred to a PVDF membrane. Membranes were probed with primary antibodies of gp91phox, p47phox, AT₁R, and PKC A-P (A-P detection of all PKC family members) (all from Santa Cruz), and phospho-PKC (p-PKC-βII/Ser⁶⁶⁰) (Cell Signaling), as well as β-actin (Sigma-Aldrich, St. Louis, MO) or β-tubulin (Santa Cruz). Immunoreactive bands were detected with an appropriate second antibody and visualized with a chemiluminescence kit (Pierce, Rockford, IL). Specific bands were normalized to glyceraldehyde-3-phosphate dehydrogenase, β-actin, or β-tubulin.

Statistical Analysis

Data are expressed as means ± SE. Statistical significance was calculated by Student’s t-test and by repeated measures of two-way ANOVA, followed by Tukey-Kramer multiple-comparison test. Significance level was taken at P < 0.05.

RESULTS

The characteristics of coronary arterioles of NS, LS, and LS + Losa dogs are shown in Table 1. Active diameter and PD of arterioles in the three groups of dogs were comparable. As a consequence, the basal tone of vessels, expressed as a percentage of their PD, was not different in these three groups.
Superoxide, NAD(P)H, AT1R, and PKC smooth muscle function.

Flow-induced dilation in vessels of LS dogs. L-NAME or NO by superoxide most likely accounts for the impaired groups of dogs (Fig. 1, SNP or ADO was unchanged between the vessels of the two groups of dogs. In addition, arteriolar response to arteriolar dilations in response to LS diet

Endothelium-dependent (flow/shear stress-induced) and -independent (ADO- and SNP-induced) responses were assessed (Fig. 1) in coronary arterioles of LS and NS dogs. Flow-induced dilation was significantly attenuated in vessels of LS (10% of PD, Fig. 1B) compared with those of NS (30% of PD, Fig. 1A) dogs. The endothelial mediator responsible for the flow-induced dilation in vessels of NS dogs was NO, as indicated by the elimination of the responses by  \textit{l-NAME}. To clarify the possible role of superoxide, flow-induced dilation was performed in the presence of apocynin. We found that apocynin had no significant effect on flow-induced dilation of NS vessels but significantly increased the response in LS vessels (19% of PD); the increased portion of the responses was then abolished by \textit{l-NAME}, indicating that inactivation of NO by superoxide most likely accounts for the impaired flow-induced dilation in vessels of LS dogs. \textit{l-NAME} or apocynin did not significantly affect the basal tone of arterioles in the two groups of dogs. In addition, arteriolar response to SNP or ADO was unchanged between the vessels of the two groups of dogs (Fig. 1, C and D), suggesting a comparable smooth muscle function.

In separate experiments, dogs were fed simultaneously with LS diet and losartan for 2 wk to clarify whether in vivo

Superoxide, NAD(P)H, AT1R, and PKC

Based on the results showing an apocynin-dependent restoration of flow-induced dilation of LS vessels, we measured superoxide production in coronary arterioles of both groups of dogs. By using an HPLC/fluorescence detector of 2-EOH (Fig. 2, A and B), we demonstrated that, in line with the functional results shown in Fig. 1, the increased superoxide in LS vessels was prevented by apocynin, whereas apocynin did not affect superoxide levels of NS vessels. These results were further confirmed by confocal fluorescent images (Fig. 2, C and D), showing a significantly greater staining for superoxide in both endothelial and smooth muscle layers of LS compared with those of NS vessels.

As a major source of vascular superoxide, expression of key components of the NAD(P)H oxidase, gp91\textsuperscript{phox} and p47\textsuperscript{phox}, was determined. Western blot analysis (Fig. 3) shows a significant upregulation of gp91\textsuperscript{phox} and p47\textsuperscript{phox} in coronary arterioles of LS dogs.

To clarify the specific mechanism underlying LS-induced ANG II to increase vascular superoxide, we determined superoxide formation in coronary vessels of NS and LS dogs treated with ANG II in control conditions and after inhibition of NAD(P)H oxidase, AT1R, or PKC with apocynin, losartan, and CLT, respectively (Fig. 4A). We found that, compared with untreated vessels, superoxide was further increased in ANG II-treated vessels. The increase was significantly greater in vessels of LS than that of NS. This ANG II-induced increase in superoxide was prevented by apocynin, losartan, or CLT, indicating that ANG II, through its interaction with the AT1R, activates NAD(P)H oxidase to produce superoxide via a PKC-dependent mechanism. To further evaluate the specific role of PKC, superoxide was measured in NS and LS vessels that were treated with PDBu, an agonist of PKC. Similar to ANG II, PDBu significantly increased superoxide production, with a significantly greater increase in LS than in NS, which was then prevented by CLT (Fig. 4B).

Western blot analysis revealed that the expression of AT1R (Fig. 5, A and B) and PKC (Fig. 5, C and D) was similar between the vessels of two groups of dogs, but the expression of phosphorylated PKC (Fig. 5, C and D) was significantly greater in LS than in NS vessels. These data suggest that ANG II-stimulated activation of vascular PKC is responsible for the upregulation and activation of NAD(P)H oxidase and increase of superoxide in coronary arterioles, as a result of the LS.

Role of In Vivo Treatment with Losartan in LS-Induced Endothelial Dysfunction

In separate experiments, dogs were fed simultaneously with LS diet and losartan for 2 wk to clarify whether in vivo

<table>
<thead>
<tr>
<th>values are the means ± SEM; n, no. of dogs/no. of isolated arterioles. AD, active diameter; PD, passive diameter; MAP, mean aortic pressure. *Significant difference from dogs fed a normal-salt diet.</th>
<th>Low Salt</th>
<th>Low Salt + Losartan</th>
<th>Normal Salt</th>
</tr>
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<tbody>
<tr>
<td>AD, ( \mu m )</td>
<td>83.7 ± 5.9</td>
<td>102.1 ± 8.7</td>
<td>80.5 ± 4.7</td>
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<tr>
<td>PD, ( \mu m )</td>
<td>133.7 ± 5.2</td>
<td>158.1 ± 14.4</td>
<td>130.3 ± 4.7</td>
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<tr>
<td>Basal tone, %PD</td>
<td>62.9 ± 4.4</td>
<td>64.5 ± 3.6</td>
<td>61.8 ± 2.7</td>
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<tr>
<td>MAP, mmHg</td>
<td>97.6 ± 4.3*</td>
<td>110.8 ± 3.1</td>
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Plasma ANG II and Mean Aortic Pressure

Plasma ANG II was 3.7 ± 0.9 pg/ml in NS and 14.7 ± 2.8 pg/ml in LS dogs. The increase in circulating ANG II started after the 1st wk and was maintained during the entire period of LS diet. Mean aortic pressure (MAP) was significantly reduced after the 1st wk and was maintained during the entire period of LS treatment. Mean aortic pressure (MAP) was significantly reduced after the 1st wk and was maintained during the entire period of LS treatment.

**Table 1. Characteristics of coronary arterioles of dogs**
treatment with losartan is able to prevent LS-activated signaling in ANG II-induced oxidative stress and endothelial dysfunction. Similar to dogs fed with LS alone, plasma concentration of ANG II in losartan-treated LS dogs increased from 5.08 ± 0.2 to 16.54 ± 0.08 pg/ml. However, flow-induced dilation (25% of PD) was similar to that in NS dogs (Fig. 6A). Superoxide formation in coronary arteries (1.5 ± 0.3 nmol/mg protein) was not different in that of NS dogs (1.8 ± 0.2 nmol/mg protein) but increased significantly in response to PDBu (Fig. 6B). Additional losartan did not, but CLT did, inhibit PDBu-induced superoxide in the vessels. The expression of gp91phox in losartan-treated dogs was similar to that in NS dogs (Fig. 6, B and C). These results confirm the role of ANG II in the mediation of LS-induced endothelial dysfunction.

**DISCUSSION**

The major finding of the present study is that endothelial response to shear stress, a primary physiological stimulus for the release of endothelial NO to control arteriolar tone, is impaired in coronary arterioles of dogs fed LS diet. The signaling cascade responsible for the endothelial dysfunction involves the ANG II/AT1R-dependent activation of PKC, which, in turn, upregulates NAD(P)H oxidase to produce superoxide, leading to a reduced NO bioavailability, as manifested by the attenuated flow-induced dilation.

Activity of the RAAS is regulated by sodium intake. Salt depletion is a potent stimulus for the secretion of renin and the generation of angiotensin, as well as aldosterone (8). However, the mechanism contributing to the failure of hyperreninemia to elevate blood pressure has not been convincingly explained. Indeed, in the present study, we observed a significant reduction of MAP in LS compared with NS dogs, which was paradoxically associated with a threefold increase in plasma...
ANG II. Therefore, our results provide strong evidence in favor of the hypothesis that LS intake initiates blood pressure-independent effects on the cardiovascular system. We demonstrated previously that arterial/arteriolar endothelium contributes to circulatory homeostasis and tissue perfusion by the flow/shear-stress-dependent regulation of vascular resistance (21) through release of endothelial NO. Flow-induced dilation of coronary arterioles has not yet been investigated in dogs with sodium restriction, preventing the assessment of the effects of ANG II on shear stress-dependent mechanisms in the coronary circulation. Motivated by this, flow-induced dilation of coronary arterioles was assessed. As demonstrated previously, NO is the primary mediator in coronary arteries of dogs (37). Thus, the significantly attenuated flow-induced dilation in coronary arterioles of LS dogs (Fig. 1) is indicative of an impaired NO-mediated portion of the response. Consistent with our findings, an impaired ACh-initiated NO-mediated vasodilation was also reported in aorta of rats fed LS (6). Because the endothelium-independent dilator responses in vessels of both groups of dogs were similar, we hypothesized that an impaired NO availability was responsible for the attenuated flow-induced dilation of LS vessels. To further characterize this response, the role of superoxide in this endothelial dysfunction was evaluated by inhibition of vascular NAD(P)H oxidase, since, among the many enzymatic sources of superoxide, NAD(P)H oxidase appears to be most prevalent in the vasculature (23, 41). The results indicated that apocynin significantly restored the attenuated dilator responses to shear stress in LS vessels and that the restored portion of the responses was inhibited by L-NAME, indicating that NAD(P)H-derived superoxide impairs flow-induced dilation through scavenging of NO. However, shear stress-induced release of NO (36) and superoxide-induced eNOS nitration (42) were not determined in the present study. Thus, effects of LS on eNOS function (NO production) need to be further investigated. In our recent study, we found that in vivo administration of veratrine initiated NO-mediated increases in coronary blood flow in NS dogs. In LS dogs, however, the veratrine-induced increases in coronary blood flow were reduced by 44% and were completely reversed by ascorbic acid or apocynin (35). In line with these results, Fig. 2 demonstrates that vascular (including the endothelium and smooth muscle layers) generation of superoxide was indeed significantly greater in LS than NS dogs. This was normalized by apocynin, confirming that NAD(P)H oxidase is the major source of superoxide, although we could not, in the present study, exclude the possibility that apocynin has additional antioxidant effects besides the inhibition of NAD(P)H oxidase.

![Figure 4](http://ajpheart.physiology.org/)

**Fig. 4.** Superoxide production assessed by HPLC/fluorescence detector of 2-hydroxyethidium in coronary arterioles of NS and LS dogs in control (C) and in response to ANG II (10^-7 M) and ANG II plus apocynin (10^-5 M), losartan (Losa, 2 x 10^-6 M), or chelerythrine chloride (CLT, 2 x 10^-6 M) (A); and in control and in response to phorbol 12,13-dibutyrate (PDBu, 10^-3 M) and PDBu plus CLT (B) (n = 5–7 dogs/group). *Significant difference from NS dogs. #Significant difference from corresponding controls. †Significant difference from those treated with ANG II alone or PDBu alone.

![Figure 5](http://ajpheart.physiology.org/)

**Fig. 5.** Protein expression of angiotensin type 1 (AT1) receptor (A and B) and protein kinase C (PKC) and phosphorylated PKC (p-PKC-ßII/Ser660) (C and D) in coronary arterioles of dogs. Densitometry data were summarized from three blots. *Significant difference from NS dogs.
oxidase is a multicomponent enzyme consisting of membrane-bound gp91phox and p22phox, three cytoplasmic subunits including p47phox, p67phox, and p40phox, and the small GTPase Rac 1/2 (4). Vascular endothelial cells express all of these components, as well as gp91phox homologs nox1, nox4, and nox5 (1, 23, 25). Of the numerous vasoactive agents regulating vascular NAD(P)H oxidase, ANG II appears to be one of the most important. ANG II activates NAD(P)H oxidase via translocation of cytosolic p47phox, p67phox, and p40phox to membrane-associated gp91phox and p22phox, leading to assembly and activation of the oxidase to generate superoxide (31, 39). Additionally, we also observed in the present study that ANG II is able to control the expression of NAD(P)H oxidase subunits (23) by increased protein expression of gp91phox and p47phox in LS coronary arterioles (Fig. 3). A specific role of p47phox in functionally active NAD(P)H oxidase was shown by studies using p47phox knockout (KO) mice. These studies indicated that vascular smooth muscle cells isolated from p47phox-KO mice failed to produce superoxide in response to ANG II (24). Moreover, ANG II-stimulated superoxide production was completely absent in coronary microvascular endothelial cells isolated from p47phox-KO mice, which could be restored by transfection of the cells with p47phox cDNA (26). These studies strongly support our conclusions that LS-initiated increases in plasma ANG II stimulate the production of superoxide, and that increased production of reactive oxygen species (ROS) leads to eNOS uncoupling, which, in turn, further reduces NO synthesis and enhances superoxide production (22), although we indicated recently that, in physiological conditions, aldosterone evokes an endothelium-dependent NO-mediated vasodilation (17). In the present study, we were particularly interested in the role of AT1R, which participates in ANG II-dependent regulation of superoxide generation (9, 29). AT1R serves as a control point for regulating the ulterior effects of ANG II on its target tissue. This issue was well clarified by a previous study showing that superoxide-induced endothelial dysfunction in aged cerebral arteries, represented by an impaired NO-mediated vasodilation, was absent in AT1R-KO mice of the same age (30). Our results also provide evidence showing that the increased production of superoxide in ANG II-treated vessels was reversed by blockade of AT1R (Fig. 4). The expression/activity of AT1R links, mechanistically, a variety of cardiovascular diseases, such as hypertension and hypercholesterolemia. For instance, low-density lipoprotein upregulates AT1R via posttranscriptional mRNA stabilization (32) that could serve as an explanation for the association between hyperlipidemia and hypertension. In the present study, vascular protein expression of AT1R was unchanged after LS diet for 2 wk, but its essential role in the mediation of ANG II-induced superoxide production was evidenced by the normalization of the responses by in vitro treatment of LS vessels (Fig. 4A) and in vivo treatment of LS dogs with losartan (Fig. 6). To further clarify whether the ANG II/AT1R accounts directly for the upregulation of vascular NAD(P)H oxidase and superoxide production, and, if so, what mechanism(s) is involved, we tested generation of superoxide in LS vessels that were additionally treated in vitro with ANG II. As expected, the vessels, having been subjected to ANG II, produced significantly more than the originally elevated basal level of superoxide. The additional increase was normalized by CLT (Fig. 4A), suggesting that activation of PKC plays a critical role...
in the signaling cascade. The specific role of PKC in the responses was then further evaluated by exposure of the vessels to the PKC agonist PDBu. Indeed, PDBu increased superoxide production (Fig. 4B) to the level induced by ANG II (Fig. 4A). PKC is a family of serine-threonine kinases, among which class/conventional PKC isoforms (cPKC-α, -βI, -βII, and -γ) are the main subgroups present in endothelial cells (28). Inhibition of PKC activity is associated with suppression of ROS production in a variety of vascular cell types (18, 40), suggesting that the activation of PKC and generation of intracellular ROS are interdependent. Activated PKC stimulates NAD(P)H oxidase activity through phosphorylation of p47phox (5, 7). In this context, PKC-dependent phosphorylation of p47phox has been reported to be essential for platelet-derived growth factor-stimulated ROS generation in human umbilical venous endothelial cells (34). Consistent with these findings, we demonstrated significantly increased phosphorylation at the βII/Ser660 position, but not unphosphorylated PKC (Fig. 5, C and D), confirming further the essential role of PKC activation in ANG II-dependent upregulation of superoxide synthesis in LS vessels (Fig. 4). Although phosphorylation of p47phox, downstream to PKC activation during the signaling cascade for ANG II-dependent regulation of NAD(P)H oxidase, was not measured in the present study, its role is implied by upregulation of p47phox (Fig. 3), accompanied by an increased PKC activity (Fig. 5). Thus, the ANG II-AT1R-PKC-NAD(P)H-superoxide signal transduction cascade is likely to account for the generation of superoxide in LS vessels. It is noteworthy that PKC-dependent generation of superoxide was also observed in ANG II-treated NS vessels, implying the universal nature of the response. Moreover, the increased activation of PKC (phosphorylated PKC, Fig. 5) contributes to a greater increase in the production of superoxide in LS vessels in response to ANG II. Interestingly, a recently published study revealed a novel mechanism in phagocytic cells by which the metabolite of losartan blocks NADPH oxidase-mediated superoxide production by inhibiting PKC (12), although the significance of these findings in vascular endothelial cells needs to be clarified.

In conclusion, LS for 2 wk increases coronary vascular superoxide production that decreases NO bioactivity to impair the mechanism of endothelium-mediated responses to shear stress. The specific signal transduction pathway involves an ANG II-dependent upregulation of NAD(P)H oxidase, via AT1R-mediated activation of PKC. It is noted that normal dogs were used in the present study, whereas in patients with hypertension or heart failure hormone levels affecting salt retention may already be altered. Nevertheless, in regard to the net effect of LS intake, results of our study suggest that a dietary recommendation of salt restriction should take into account its multiple consequences for cardiovascular function in devising a clinical strategy for the prevention/treatment of cardiovascular diseases.

REFERENCES


