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Chronic High Pressure-Induced Arterial Oxidative Stress

Involvement of Protein Kinase C-Dependent NAD(P)H Oxidase and Local Renin-Angiotensin System

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Regardless of the underlying pathological mechanisms oxidative stress seems to be present in all forms of hypertension. Thus, we tested the hypothesis that chronic presence of high pressure itself elicits increased arterial $O_2^{\cdot-}$ production. Hypertension was induced in rats by abdominal aortic banding (Ab). Rats with Ab had elevated pressure in vessels proximal and normal pressure in vessels distal to the coarctation, yet both vascular beds were exposed to the same circulating factors. Compared to normotensive hind limb arteries (HLAs) hypertensive forelimb arteries (FLAs) exhibited 1) impaired dilations to acetylcholine and the nitric oxide donor *S*-nitroso-*N*-acetyl-D,L-penicillamine that were restored by administration of superoxide dismutase; 2) an increased production of $O_2^{\cdot-}$ (measured by lucigenin chemiluminescence and ethidium bromide fluorescence) that was inhibited or reduced by superoxide dismutase, the NAD(P)H oxidase inhibitors diphenyleneiodonium and apocynin, or the protein kinase C (PKC) inhibitors chelerythrine and staurosporine or by the angiotensin-converting enzyme (ACE) inhibitor captopril; and 3) increased ACE activity. In organ culture, exposure of isolated arteries of normotensive rats to high pressure (160 mmHg, for 24 hours) significantly increased $O_2^{\cdot-}$ production compared to that in arteries exposed to 80 mmHg. High pressure-induced $O_2^{\cdot-}$ generation was reduced by inhibitors of ACE and PKC. Incubation of cultured arteries with angiotensin II elicited significantly increased $O_2^{\cdot-}$ generation that was inhibited by chelerythrine. Thus, we propose that chronic presence of high pressure itself can elicit arterial oxidative stress, primarily by activating directly a PKC-dependent NAD(P)H oxidase pathway, but also, in part, via activation of the local renin-angiotensin system. ***(Am J Pathol 2004, 165:219–226)***

In several forms of hypertension increased arterial superoxide ($O_2^{\cdot-}$) production has been shown to decrease the bioavailability of endothelium-derived vasodilator nitric oxide (NO), thereby contributing to the maintenance of elevated peripheral resistance.[1–6] However, the stimuli and mechanisms underlying increased $O_2^{\cdot-}$ production in hypertension are not completely understood.

The vascular effects of hypertension are complex and are likely to be induced, at least in part, by increased levels of neurohumoral factors. Among them, angiotensin II has been suggested to increase $O_2^{\cdot-}$ generation in vascular cells.[3,4,7] However, oxidative stress seems to be present in virtually all forms of hypertension[4,5] (including low-renin hypertension,[8,9] genetic hypertension, angiotensin II-induced hypertension,[3,4] renovascular hypertension,[2,10] pheochromocytoma-related hypertension[11]) despite the differences in plasma levels of circulating factors. Importantly, it has been reported that in angiotensin II-infused rats reduction of blood pressure with hydralazine or spironolactone (that are unlikely to affect angiotensin II levels) normalized aortic $O_2^{\cdot-}$ production.[7,12]

Thus, it is logical to hypothesize that high intraluminal pressure itself promotes vascular $O_2^{\cdot-}$ generation in hypertension. This idea is congruent with our recent findings that *in vitro* acute increases in pressure up-regulate arterial $O_2^{\cdot-}$ production by activating NAD(P)H oxidase.[13] Previous studies also showed that short-term increases in pressure both *in vivo* and *in vitro* impair endothelial function,[14–16] an effect that can be prevented

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by superoxide dismutase (SOD).[17] To test the role of chronic exposure to high pressure *in vitro* in the up-regulation of arterial $O_2^{\cdot -}$ production, isolated arteries maintained in vessel culture were pressurized to normotensive or hypertensive levels. To test the differential role of chronic presence of high pressure *in vivo*, hypertension was induced in rats by abdominal aortic banding (Ab). The Ab model of hypertension provides the advantage that blood vessels proximal to the coarctation are exposed to high pressure, whereas in distal vascular beds pressure is close to normotensive levels. Because both vascular beds are exposed to the same circulating factors, the *in vivo* effects of chronic presence of high blood pressure on vascular $O_2^{\cdot -}$ production could be independently assessed.

Materials and Methods

Suprarenal Aortic Constriction Hypertension

In male Wistar-Hannover rats ($n = 11$) Ab hypertension was induced according to the protocol of Taconic Biotechnology Co. In brief, animals were anesthetized with intraperitoneal sodium pentobarbital and a dorsal midline incision was made. The abdominal aorta was exposed and a constriction (~0.7 mm in diameter) was applied cranial to both renal arteries. Then, the abdomen and skin were closed and animals were allowed to recover. Successful Ab was indicated by an undetectable pulse pressure measured by the tail cuff method after the operation. Weight-matched sham-operated control animals (C, $n = 11$) were subjected to the same procedure, without Ab. Animals were monitored for 6 weeks after the operation. Systolic blood pressure was measured by the tail cuff method once a week. All subsequent experiments were performed 6 weeks after operation,[18,19] when plasma angiotensin II levels are close to normal values in rats with suprarenal Ab.[19] Animals were heparinized and anesthetized (intraperitoneal sodium pentobarbital), the left carotid and femoral artery were cannulated, and arterial pressures were simultaneously recorded. Then the animals were sacrificed and the hearts were excised and weighed.

Vessel Isolation and Functional Studies

Arteries of the forelimbs (FLAs) and the hind limbs (HLAs) of control and Ab rats were exposed (brachial and femoral arteries, respectively) and branches supplying the skeletal muscle were isolated for further studies. In isolated, pressurized FLA and HLA branches (diameter, ~200 μm) dilations to acetylcholine and to the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine in the absence and presence of SOD (200 U/ml) were measured, as described.[17] Responses to the phosphodiesterase inhibitor papaverine (10^{-4} mol/L) were also obtained.

Measurement of Vascular Superoxide Levels

Vascular $O_2^{\cdot -}$ production was assessed by the lucigenin chemiluminescence method as previously described.[20] In brief, the vessels were placed in scintillation vials containing HEPES-buffered (10 mmol/L, pH 7.4) physiological salt solution (PSS) and lucigenin (10 μmol/L) chemiluminescence was measured in a liquid scintillation counter (LS-6000IC; Beckman, Fullerton, CA) in the absence or presence of SOD (200 U/ml) or diphenyleneiodonium (DPI) [10^{-5} mol/L, an inhibitor of flavoprotein-containing oxidases, including NAD(P)H oxidases] or chelerythrine [10^{-6} mol/L, an inhibitor of protein kinase C (PKC) and PKC-induced NAD(P)H activity[3]] or in the absence of extracellular Ca^{2+}. In separate experiments, $O_2^{\cdot -}$ production in FLAs of Ab rats was determined in the absence and presence (30 minutes incubation) of apocynin [3×10^{-4} mol/L, an inhibitor of superoxide production by NAD(P)H oxidases[5,21]] or *N*ω-nitro-L-arginine-methyl-ester (L-NAME) (3×10^{-4} mol/L, an inhibitor of NO synthesis[20]) or captopril [10^{-4} mol/L, an angiotensin-converting enzyme (ACE) inhibitor, 1 hour incubation] or staurosporine (10^{-6} mol/L, an inhibitor of PKC), or Tiron (10 mmol/L, a superoxide scavenger). Scintillation counts were obtained 15 to 20 minutes after addition of vessels (averaged) and background-corrected values were normalized to tissue weight.

Ethidium Bromide Fluorescence

Hydroethidine was used to localize superoxide production, as described.[20] Samples exposed to hydroethidine in the presence of SOD, Tiron, or DPI served as control. In other experiments arterial segments from the same control rat were incubated in the absence or presence of phorbol myristate acetate (PMA) (10^{-6} mol/L), PMA plus SOD, or PMA plus DPI and then were incubated with hydroethidine.

Measurement of Vascular ACE Activity

ACE activity was measured in vascular homogenates in the absence or presence of the ACE inhibitors captopril or enalaprilat using the synthetic ACE-specific substrate hyppuryl histidyl leucine by a high pressure liquid chromatography (HPLC)-based assay according to the protocols of Meng and colleagues[22] and Koiter and colleagues.[23] In brief, vessels were pulverized in liquid nitrogen and homogenized (at 0°C for 1 minute) in 0.02 mol/L potassium phosphate buffer (pH 8.3). One hundred μL of homogenate containing 10 mg of vascular tissue[23] was added to 500 μl of reaction mixture[24] (300 mmol/L NaCl, 10^{-4} mol/L $CoCl_2$, in 0.1 mol/L phosphate buffer, pH 8.3) in three test tubes, stored on ice. Samples were incubated with or without captopril (10^{-4} mol/L) or enalaprilat (10^{-4} mol/L) for 15 minutes. The reaction was started by adding hyppuryl histidyl leucine (final concentration, 5 mmol/L) to the reaction mixture and transferring the tubes to a 37°C water bath. After 15 minutes of incubation generation of the product (hippuric acid, HA) was stopped by adding 250 μL of HCl (1 mol/L) then the internal standard *N*-benzoyl-L-alanine (BA, 0.1 mg/sample) was added. To extract HA and BA 1 ml of ethyl acetate was added to the sample according to the

method of Cushman and Cheung.[24] After vortexing for 1 minute and 10 minutes of centrifugation (3000 × *g*), 0.8 ml of the ethyl acetate layer was transferred to a glass tube and evaporated at 60°C. The residue was dissolved in 150 μL mobile phase. Twenty-five μL of the sample was analyzed with the System Gold HPLC system (Beckman Coulter, *www.beckman.com*) equipped with C_{18} guard and 150 × 2.0-mm MiniBore Ultrasphere 5μC_{18} column (Beckman Coulter) and a UV detector set at 228 nm. The mobile phase consisted of sodium acetate buffer (10 mmol/L, pH 4.0) with 10% methanol and 7.5% acetonitrile (flow-rate, 1.0 ml/min). Peaks for HA and BA were identified by comparison with retention times of standard compounds. Standard solutions of HA and BA were prepared daily by dissolving HA and BA in the HPLC mobile phase and diluted serially to provide calibration standards. Under the conditions described above, a clear separation of HA and BA was achieved. Quantification was performed by the 32Karat software (Beckman Coulter) measuring peak areas of HA (elution time, 2.7 minutes) in relation to the internal standard BA (elution time, 3.5 minutes) at 228 nm. Measurements were performed in duplicate. The intra-assay coefficient of variation was 4%. Doubling of the incubation time doubled the generation of HA showing the linearity of the assay. ACE activity was expressed in units (U), defined as the formation of nmol HA per minute at 37°C per g vascular tissue wet weight. The site-specific ACE inhibitors captopril and enalaprilat eliminated the HA peak in the samples showing the specificity of the assay to ACE activity.

Detection of 3-Nitrotyrosine

To characterize $ONOO^-$ formation in vascular samples we used the modified method of Csiszar and colleagues[20] and Ungvari and colleagues[25] using a dot-blot system that allows for the determination of the total 3-nitrotyrosine content in all proteins without molecular size limitations. Immunolabeling of membranes was performed using a primary antibody against nitrated tyrosine residues of proteins (1:100; Cell Signaling Technology Co, Beverly, MA) and a goat anti-rabbit secondary antibody conjugated to biotin (Vector Laboratories, Burlingame, CA). 3-Nitrotyrosine was localized in arterial sections by immunohistochemistry as described.[20,25] Sections were stained using an avidin-biotinylated enzyme complex (ABC Vectastain, Vector Laboratories, www.vectorlabs.com) and Vector-DAB (diaminobenzidine tetrahydrochloride) substrate, counterstained with hematoxylin.

Vessel Culture Studies

To investigate vascular $O_2^{\cdot -}$ generation under controlled conditions isolated FLAs of control rats were maintained in vessel culture. In brief, arteries were cannulated on both sides in a stainless steel vessel culture chamber (Danish Myo Technology, www.dmt.dk) under sterile conditions and continuously superfused with F12 medium (Life Technologies, Inc., Grand Island, NY) containing antibiotics (100 UI/L penicillin, 100 mg/L streptomycin, and 10 μg/L fungizone) and supplemented with 5% fetal calf serum (Boehringer-Mannheim, Indianapolis, IN) according to the modified technique of Bakker and colleagues[26] and Bolz and colleagues,[27,28] as previously described.[29] Arteries were exposed to 80 mmHg or 160 mmHg (to mimic hypertensive conditions) for 24 hours in the absence or presence of enalaprilat (10^{-4} mol/L), captopril (10^{-4} mol/L), or chelerythrine (10^{-6} mol/L). In separate experiments, vessels were incubated with the PKC activator PMA (10^{-6} mol/L, for 30 minutes in oxygenated PSS, at 37°C) or with angiotensin II (10^{-8} to 10^{-6} mol/L, for 24 hours) in the absence or presence of chelerythrine, SOD, or DPI. Minimal intraluminal flow was maintained only to renew the culture medium within the intraluminal space and maintaining minimal shear stress (<0.5 dyn/cm^2). At the end of the culture period vascular $O_2^{\cdot -}$ generation was determined.

Table 1. Hemodynamic Data and Heart Weight

Parameter	Sham-operated	Ab rats
MAP, carotid artery (mmHg)	92 ± 2	139 ± 9*†
MAP, femoral artery (mmHg)	87 ± 3	69 ± 9
SBP (tail cuff, mmHg)	127 ± 1	n.d.
HR (tail cuff, BPM)	416 ± 10	n.d.
BW (g)	322 ± 9	333 ± 9
HW/BW (mg/g)	2.6 ± 0.1	3.8 ± 0.4*

Hemodynamic data and heart weight in sham-operated control (n = 4 to 11) and aortic-banded (n = 4 to 11) rats. MAP, mean arterial pressure measured in anesthetized animals; SBP, systolic blood pressure measured in awake animals by the tail cuff method; HR, heart rate; BPM, beats per minute, n.d., nondeterminable; BW, body weight; HW, heart weight. Values are mean ± SEM.

*$P < .05$ *versus* control.

†$P < .05$ *versus* femoral artery.

Data Analysis

Lucigenin chemiluminescence data and densitometric ratios were normalized to the respective control mean values. Data are expressed as means ± SEM. Statistical analyses of data were performed by Student's *t*-test or by two-way analysis of variance followed by the Tukey post hoc test, as appropriate. $P < 0.05$ was considered statistically significant.

Results

Ab rats had elevated mean arterial blood pressure proximal and normal blood pressure distal to the coarctation (Table 1), as previously reported.[30]

Arterial Dilations

Dilations to ACh and *S*-nitroso-*N*-acetyl-D,L-penicillamine in Ab FLAs were significantly decreased as compared to responses of HLAs of the same rats (Figure 1). Previous studies also showed impaired ACh-induced relaxation in high pressure-exposed thoracic segments, but not normal pressure exposed abdominal segments, of the aorta of Ab rats.[30] Administration of SOD did not affect significantly ACh- (not shown) or *S*-nitroso-*N*-acetyl-D,L-penicillamine-

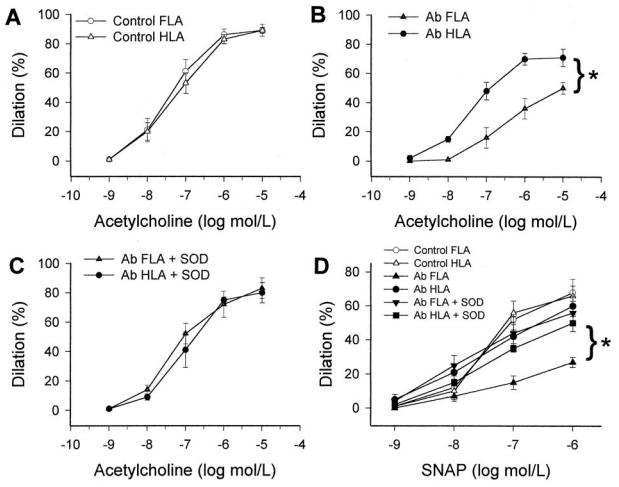

Figure 1. A–D: Dilations to acetylcholine and to *S*-nitroso-*N*-acetyl-D,L-penicillamine in isolated FLAs and HLAs of sham-operated control and Ab rats in the absence and presence of SOD (200 U/ml) (n = 4 to 7 rats). Data are mean ± SEM. *, $P < 0.05$.

induced responses in FLAs and HLAs of control rats and in HLAs of Ab rats, whereas it restored responses of Ab FLAs (Figure 1, C and D). Arterial dilations to papaverine did not differ among the four groups of vessels (not shown).

Vascular Superoxide Production

Under basal conditions, lucigenin chemiluminescence in FLAs was significantly higher than in HLAs of the same Ab rats or in FLAs and HLAs from control rats (C FLA, 23.4 × 10^3 counts/mg/minute; Figure 2A). Increased $O_2^{\cdot -}$ generation in Ab FLAs was inhibited by SOD or DPI eliminating the difference between the groups (Figure 2A). Increased $O_2^{\cdot -}$ generation by Ab FLAs was also blocked by apocynin, staurosporine, and Tiron, whereas it was not significantly affected by L-NAME (Figure 3A). Captopril also elicited a partial, but significant, reduction of $O_2^{\cdot -}$ generation in Ab FLAs (Figure 3A), whereas chelerythrine and removal of extracellular Ca^{2+} completely

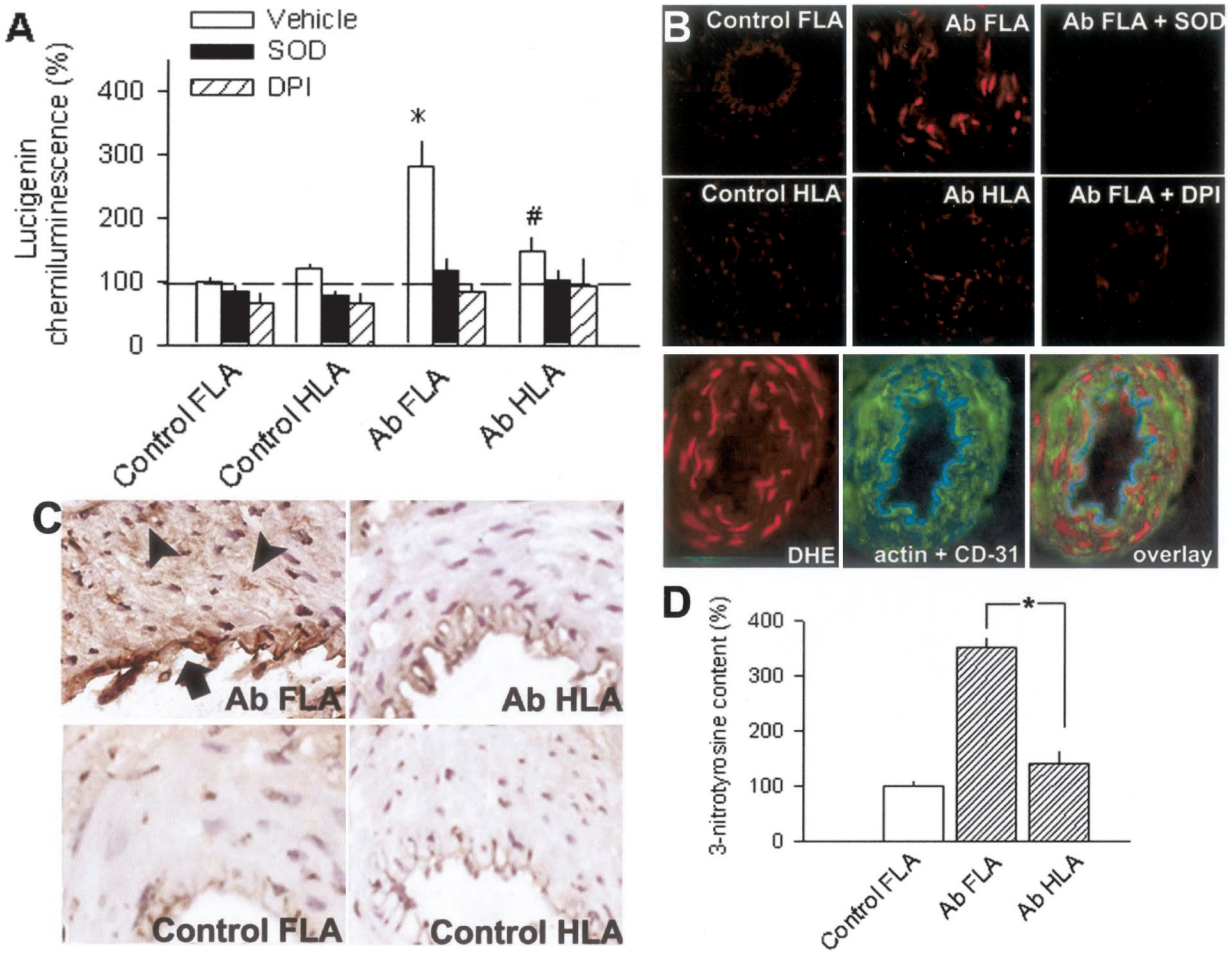

Figure 2. A: Generation of $O_2^{\cdot -}$ as determined by lucigenin chemiluminescence in isolated FLAs and HLAs of sham-operated control or Ab rats under control conditions and in the presence of SOD (200 U/ml) or DPI (10^{-5} mol/L). Data are normalized to the mean value of the control FLA group. *, $P < 0.05$ *vs.* control and #, $P < 0.05$ *vs.* FLA (n = 4 to 15 rats). **B: Top:** Representative fluorescent photomicrographs of control and Ab FLAs and HLAs labeled with the dye dihydroethidium, which produces a red fluorescence when oxidized to ethidium bromide (EB) by $O_2^{\cdot -}$. Sections of Ab FLA incubated with DPI or SOD are also shown. **Bottom:** Overlaying EB-stained fluorescent images of an Ab FLA with images of the same vessel section stained for α-smooth muscle actin (green) and the endothelium-specific marker CD-31 (blue) show that increased $O_2^{\cdot -}$ levels are present both in the smooth muscle and the endothelium of Ab FLA. Similar findings were observed in three separate experiments. **C:** Hypertensive Ab FLA showed more prevalent immunostaining for 3-nitrotyrosine (brown reaction product, DAB staining) that was localized both to the endothelium (**arrow**) and the media (**arrowheads**), than normotensive vessels (Ab HLA, control FLA and HLA). Hematoxylin counterstaining. **D:** Summary data for Western blot analysis of protein tyrosine nitration in normotensive and hypertensive vessels (n = 3 in each group). *, $P < 0.05$. Original magnifications: ×40 (**B**); ×20 (**C**).

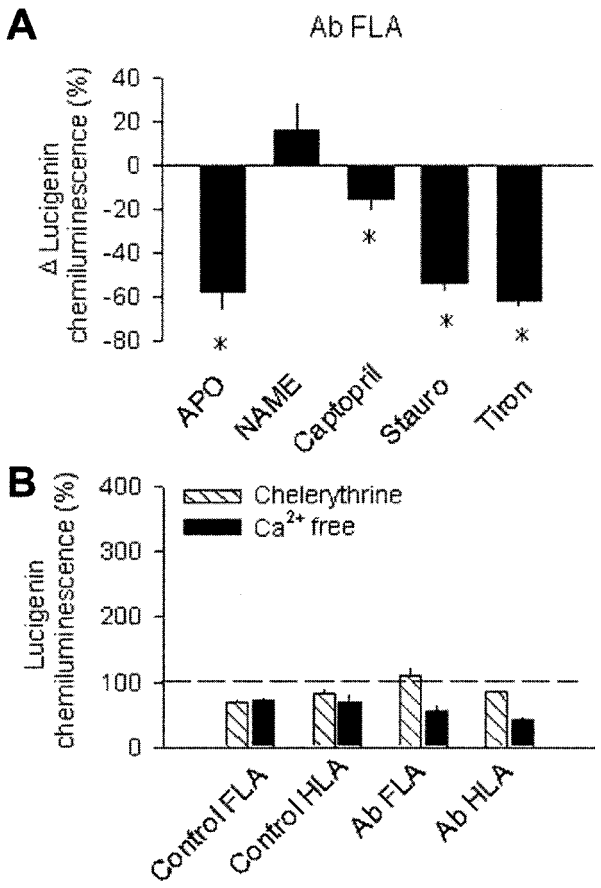

Figure 3. A: Changes in $O_2^{\cdot -}$ generation in isolated FLAs and HLAs of sham-operated control or Ab rats in response to apocynin (Apo, 3×10^{-4} mol/L), L-NAME (3×10^{-4} mol/L), captopril (10^{-4} μmol/L), staurosporine (10^{-6} μmol/L), or Tiron (10 mmol/L). *, $P < 0.05$ ($n = 4$ to 7 rats). **B:** $O_2^{\cdot -}$ generation in FLAs and HLAs of control and Ab rats in the presence of chelerythrine (Chel, 10^{-6} mol/L) or in the absence of Ca^{2+}. Data are normalized to the mean value of the untreated control FLA group. *, $P < 0.05$ ($n = 5$ to 7).

inhibited increased $O_2^{\cdot -}$ generation in Ab FLAs and eliminated the differences between the groups (Figure 3B). In Ab FLAs ($n = 3$) the relative number of ethidium bromide (EB)-positive nuclei was significantly increased both in the media and intima of Ab FLAs (Figure 2B). Incubation of vessels with DPI or SOD prevented EB staining (Figure 2B).

Vascular 3-Nitrotyrosine Content

In FLAs of Ab rats there was a significantly increased 3-nitrotyrosine content (Figure 2D), as shown by Western blotting. Enhanced immunostaining for 3-nitrotyrosine in FLAs of Ab rats was localized both to the endothelium and media (Figure 2C). The media of Ab HLAs and arteries of control rats were relatively free from 3-nitrotyrosine immunoreactivity. In control experiments there was no evidence of nonspecific immunostaining.

Vascular ACE Activity

ACE activity was significantly greater in FLAs than in HLAs of Ab rats and could be inhibited by enalaprilat (not shown) and captopril (Figure 4).

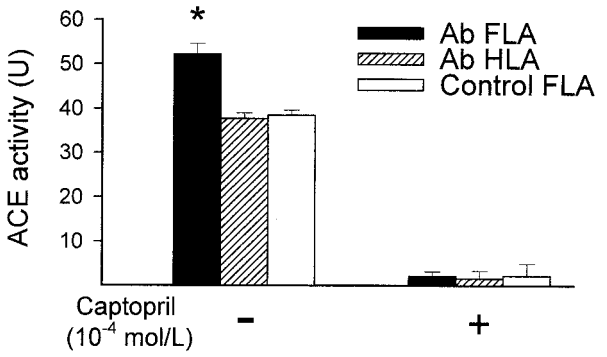

Figure 4. Vascular ACE activity, measured by a HPLC-based assay, in FLAs and HLAs of sham-operated control and Ab rats. Data are mean ± SEM. *, $P < 0.05$.

Vessel Culture Studies

Lucigenin chemiluminescence measurements showed that chronic pressurization of cultured arteries to hypertensive levels (160 mmHg) elicited significant increases in vascular $O_2^{\cdot -}$ generation, as compared to that in vessels exposed to 80 mmHg (Figure 5A). Increased $O_2^{\cdot -}$ generation in high pressure-treated vessels was partly reduced by incubation with enalaprilat (Figure 5A), which still left a large portion of $O_2^{\cdot -}$ generation intact and did not eliminate the difference between the groups. Similar results were obtained with captopril as well (not shown). Additional administration of chelerythrine completely inhibited pressure-induced increases in $O_2^{\cdot -}$ production (Figure 5A). Incubation of cultured arteries with angiotensin II (Figure 5B) or pharmacological activation of PKC with PMA (Figure 5C) also elicited concentration-dependent increases in $O_2^{\cdot -}$ generation that were inhibited by chelerythrine, DPI, or SOD.

Discussion

The main findings of the present study are that chronic presence of high intraluminal pressure itself, both *in vivo* and *in vitro* enhance $O_2^{\cdot -}$ production in peripheral arteries. Using the Ab rat model of hypertension we have demonstrated that in the presence of the same circulating factors regional increases in blood pressure resulted in selective impairment of NO-mediated dilations that could be restored by SOD (Figure 1). In contrast, dilations (Figure 1) and $O_2^{\cdot -}$ generation (Figure 2A) in normotensive arteries of the same animals were similar to those in vessels from sham-operated control rats. Previous studies also showed impairment of NO-mediated relaxations in high pressure-exposed thoracic aorta and coronary arteries, but not in normal pressure-exposed abdominal aorta of Ab rats.[19,31] We demonstrated, that hypertensive arteries selectively exhibit a SOD-sensitive enhanced $O_2^{\cdot -}$ production (Figure 2A). EB-staining showed that $O_2^{\cdot -}$ production was localized both in the endothelium and the smooth muscle of hypertensive vessels (Figure 2B). The presence of elevated $O_2^{\cdot -}$ levels in both vascular layers provides an explanation for the simultaneously impaired dilations to both endothelium-derived NO and NO released from exogenous NO donors

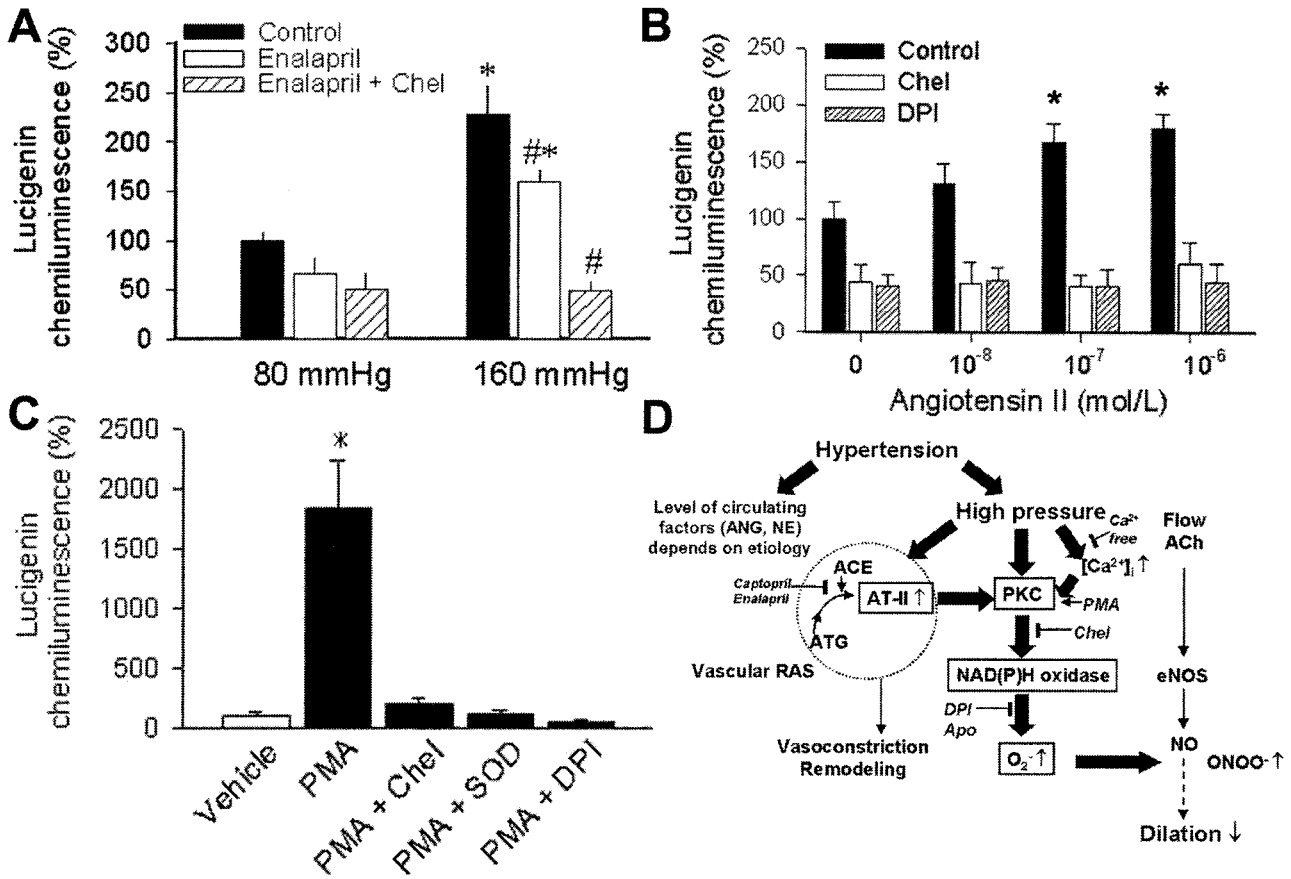

Figure 5. A: O_2^- generation in FLAs of control rats pressurized to normotensive (80 mmHg) and hypertensive levels (160 mmHg) in organ culture (24 hours) in the presence and absence of enalaprilat (10^{-4} mol/L) or enalaprilat plus chelerythrine. *, $P < 0.05$ *versus* 80 mmHg; #, $P < 0.05$ *versus* untreated ($n = 4$ to 5 rats). **B:** O_2^- generation in cultured arteries with or without incubation with angiotensin II (24 hours) in the presence and absence of chelerythrine and DPI. *, $P < 0.05$ ($n = 4$ to 5 rats). **C:** O_2^- generation in cultured arteries of control rats under control conditions and in the presence of PMA (10^{-6} mol/L), or PMA plus chelerythrine, or PMA plus DPI, or PMA plus SOD. Data are normalized to the control mean value. *, $P < 0.05$ ($n = 4$ to 5 rats). **D:** Proposed scheme for high pressure-induced activation of Ca^{2+}-PKC pathway promoting NAD(P)H oxidase-derived O_2^- production and endothelial dysfunction. High pressure-induced up-regulation of vascular RAS increases tissue concentrations of angiotensin II contributing to increased oxidative stress, vascular remodeling, and proinflammatory alterations.

(Figure 1) frequently observed in hypertensive humans and animals.[4,19,32] In hypertensive FLAs (Figure 2, C and D) and aorta[19] of Ab rats, but not in normal pressure-exposed HLAs there was an increased protein 3-nitrotyrosine content localized both to the endothelium and smooth muscle (Figure 2C), indicating that in both vascular layers there are increased O_2^- levels present *in vivo*, which scavenge endothelium-derived NO by forming $ONOO^-$. Because hypertensive and normotensive vascular beds in Ab animals are exposed to the same circulating factors, it is likely that increases in O_2^- production in hypertensive arteries are predominantly because of the prevailing high intraluminal pressure.

An important role for high pressure in up-regulation of O_2^- production is further supported by our findings that a developmental increase in blood pressure coincides with the increase in vascular O_2^- production and appearance of endothelial dysfunction[33] in spontaneously hypertensive rats (SHR). Also, in angiotensin II- and/or norepinephrine-infused rats reduction of blood pressure with hydralazine to control levels (which is unlikely to affect plasma levels of circulating factors) normalized the increased aortic O_2^- production[7] and decreased the elevated plasma levels of 8-epi-$PGF_{2\alpha}$, a marker of oxidative stress *in vivo*.[34] Although previous studies proposed that in rabbits hydralazine decreased enzymatic production of vascular O_2^- production and improved NO-induced aortic relaxations, these effects have not been correlated with hydralazine-induced changes in blood pressure.[35] Taken together, in hypertensive conditions *in vivo* high intraluminal pressure itself can increase O_2^- production, which may explain why an increased oxidative stress has been found in high pressure-exposed vessels in virtually all forms of hypertension.[2,5,7]

The primary source of O_2^- in hypertensive vessels is likely the vascular NAD(P)H oxidase, because increased lucigenin chemiluminescence of hypertensive arteries was inhibited by DPI (Figure 2A) and by apocynin (Figure 3A). Previous studies also reported an increased NAD(P)H oxidase activity in most peripheral vascular beds of animals with various forms of hypertension,[7] including genetic hypertension, angiotensin II-induced hypertension,[3,4] renovascular hypertension,[2] and low-renin hypertension.[5]

Because captopril reduced O_2^- production in hypertensive arteries of Ab rats (Figure 3A), it is likely that

increased vascular levels of angiotensin II, because of an up-regulated local renin-angiotensin system (RAS)[36] contribute to the activation of NAD(P)H oxidase. Indeed, we found that in hypertensive FLAs (Figure 4) and thoracic aorta[37] of Ab rats there is an increased ACE activity, which may be because of an increased expression of ACE,[37] although other mechanisms cannot be excluded. Also, previous studies showed that chronic inhibition of ACE in Ab hypertensive guinea pigs significantly decreased NAD(P)H oxidase activity in vascular cells.[38] In the present study vessels were exposed to high pressure only for 6 weeks, thus one can speculate that longer exposure of vessels, such as years or decades would have a more substantial effect on vascular RAS. Because inhibition of PKC (Figure 3B) substantially decreased $O_2^{\cdot-}$ production in the hypertensive vessels of Ab rats, it is likely that PKC plays an important role in chronic high pressure-induced activation of NAD(P)H oxidase *in vivo*. Correspondingly, previous studies also showed increased PKC activity[39,40] in high pressure-exposed aorta of Ab rats. It is likely that PKC phosphorylates the regulatory $p47^{phox}$ subunit of NAD(P)H oxidase,[13] which is thought to be essential to angiotensin II-induced oxidative stress.[41]

To further test the role of ACE and PKC in chronic high pressure-related $O_2^{\cdot-}$ production we pressurized isolated arteries of control rats to normal and hypertensive pressure levels in vessel culture. Compared to normotensive pressure level, chronic exposure of cultured arteries *in vitro* to high pressure resulted in an increased $O_2^{\cdot-}$ production, which was reduced, at least in part, by ACE inhibitors (Figure 5A). Thus, we propose that high intraluminal pressure-induced activation of local RAS in the vascular wall contributes to any hypertension-induced vascular oxidative stress. This view is further supported by previous studies by Bardy and colleagues[42] showing an increased angiotensin II concentration in the culture medium of high pressure-exposed cultured arteries. It is likely that angiotensin II increases vascular NAD(P)H oxidase activity via activating PKC,[3,43] because incubation of cultured arteries with exogenous angiotensin II also significantly increased arterial $O_2^{\cdot-}$ generation that could be prevented by inhibition of PKC (Figure 5B).

Importantly, however, a significant part of high pressure-induced oxidative stress appears to be independent of local and systemic RAS, because in the presence of ACE inhibitors $O_2^{\cdot-}$ production was still substantially greater in high pressure-exposed arteries than in arteries pressurized to normotensive level (Figure 5A). Importantly, this pressure-induced, ACE-independent $O_2^{\cdot-}$ generation was abolished by inhibition of PKC (Figure 5A). Previously we have demonstrated in isolated arteries that exposure to high pressure itself activates PKC[13] and in the present study we showed that pharmacological activation of PKC[2] increases vascular NAD(P)H oxidase activity (Figure 5C). These findings are in line with our recent observations that increases in pressure[13] or stretching of arteries[44] (that are unlikely to up-regulate ACE activity acutely) can elicit PKC-mediated phosphorylation and apocynin-sensitive translocation of the NAD(P)H oxidase regulatory subunit $p47^{-phox44}$ increasing NAD(P)H oxidase activity with the consequent impairment of NO-mediated dilations of vessels of normotensive rats.[17] One of the mechanisms by which high intraluminal pressure can activate PKC is an increased Ca^{2+} influx. Indeed, we have shown that high pressure *in vitro* increases $[Ca^{2+}]_i$ in the vascular wall.[13] Because in the absence of Ca^{2+}, $O_2^{\cdot-}$ production was significantly decreased both in hypertensive arteries (Figure 3B) and high pressure-exposed isolated arteries,[13] it is likely that increased Ca^{2+}-induced PKC activity is an important stimulator of vascular NAD(P)H oxidase even in the absence of activation of local RAS.

On the basis of the present and previous findings we propose (Figure 5D) that in peripheral arteries the chronic presence of high intravascular pressure increases $[Ca^{2+}]_i$ and activates PKC, increasing NAD(P)H oxidase-derived $O_2^{\cdot-}$ production. In addition, the high pressure-induced NAD(P)H oxidase activity *in vivo* is likely further modulated by the presence of pulsatility and an increased pulsatility,[45,46] such as in systolic hypertension. Chronic presence of high pressure also activates the local renin-angiotensin system, thereby increasing angiotensin II bioavailability in the vascular wall, contributing further to the activation of PKC-NAD(P)H oxidase axis. The resulting oxidative stress reduces NO-mediated regulation of arterial resistance. The high pressure-induced up-regulation of vascular RAS, together with the increased arterial $O_2^{\cdot-}$ and $ONOO^-$ production could be the initial steps leading to remodeling[47] and proinflammatory alterations of arterial wall.[48] Our findings may explain the cardiovascular protective effects attributed to ACE inhibitors in cases of human essential hypertension that are associated with low plasma angiotensin II levels. Nevertheless, the primary role of high pressure itself in the development of vascular oxidative and nitrosative stress and proinflammatory microenvironment emphasize the importance of early and effective reduction of high blood pressure by behavioral changes, dietary means, and pharmacological treatments.

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