High Pressure Induces Superoxide Production in Isolated Arteries Via Protein Kinase C–Dependent Activation of NAD(P)H Oxidase

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Background—Oxidative stress seems to be present in all forms of hypertension. Thus, we tested the hypothesis that high intraluminal pressure (P_i) itself, by activating vascular oxidases, elicits increased superoxide ($O_2^{\cdot-}$) production interfering with flow-induced dilation.

Methods and Results—Isolated, cannulated rat femoral arterial branches were exposed in vitro (for 30 minutes) to normal P_i (80 mm Hg) or high P_i (160 mm Hg). High P_i significantly increased vascular $O_2^{\cdot-}$ production (as measured by lucigenin chemiluminescence and ethidium bromide fluorescence) and impaired endothelium-dependent dilations to flow; these effects could be reversed by superoxide dismutase. Administration of the NAD(P)H oxidase inhibitor diphenyleneiodonium, apocynin, the protein kinase C (PKC) inhibitor chelerythrine or staurosporin or the removal of extracellular Ca^{2+} during high P_i treatment prevented the increases in $O_2^{\cdot-}$ production, whereas administration of losartan or captopril had no effect. High P_i resulted in significant increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in the vascular wall (fura 2 fluorescence) and phosphorylation of PKCα (Western blotting). The PKC activator phorbol myristate acetate significantly increased vascular $O_2^{\cdot-}$ production, which was inhibited by superoxide dismutase, diphenyleneiodonium, chelerythrine, or removal of extracellular Ca^{2+}. Both high P_i and phorbol myristate acetate increased the phosphorylation of the NAD(P)H oxidase subunit $p47^{phox}$.

Conclusion—High P_i itself elicits arterial $O_2^{\cdot-}$ production, most likely by PKC-dependent activation of NAD(P)H oxidase, thus providing a potential explanation for the presence of oxidative stress and endothelial dysfunction in various forms of hypertension and the vasculoprotective effect of antihypertensive agents of different mechanisms of action. (***Circulation*. 2003;108:1253-1258.**)

Key Words: signal transduction ■ hypertension ■ angiotensin ■ endothelium ■ peripheral vascular disease

Numerous studies in humans[1] and animals[2–6] suggest that increased superoxide ($O_2^{\cdot-}$) production contributes significantly to the functional alterations of arteries present in hypertension. In peripheral arteries and arterioles, increased levels of $O_2^{\cdot-}$ have been shown to decrease the bioavailability of the endothelium-derived vasodilator nitric oxide to flow,[6,7] thereby contributing to the maintenance of elevated peripheral resistance. 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 the humoral factors, angiotensin has been suggested to increase $O_2^{\cdot-}$ generation in vascular cells.[3,4,8,9] However, oxidative stress seems to be present in virtually all forms of hypertension,[4,5] including low-renin hypertension,[10] despite the differences in plasma levels of circulating factors. Importantly, in angiotensin-infused rats, reduction of blood pressure with hydralazine or spironolactone (which is unlikely to affect angiotensin levels) normalized aortic $O_2^{\cdot-}$ production.[8,11] Reduction of blood pressure in rats infused with both angiotensin and norepinephrine by hydralazine also normalized increased plasma levels of 8-epi-prostaglandin $F_{2\alpha}$, a marker of oxidative stress in vivo.[12] Thus, it is logical to hypothesize that high intraluminal pressure (P_i) itself can also promote vascular $O_2^{\cdot-}$ generation in hypertension. This idea is congruent with previous observations that even short-term increases in P_i, both in vivo and in vitro, impair endothelial function,[13–16] an effect that can be prevented by superoxide dismutase (SOD).[17] However, the possible role of high P_i alone in the upregulation of arterial $O_2^{\cdot-}$ production and the underlying signaling mechanisms has not yet been elucidated.

To test the hypothesis that the presence of high P_i per se, by activating vascular oxidases, elicits oxidative stress, we investigated pressure-induced $O_2^{\cdot-}$ production in isolated arteries in vitro, a condition in which neurohumoral factors are absent. Also, we aimed to elucidate the contribution of possible vascular sources of $O_2^{\cdot-}$, including NAD(P)H oxi-

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dases,[4,5] and the role of mechanosensitive pathways, such as Ca^{2+} and protein kinase C (PKC)–dependent signaling mechanisms.[2,3,18]

Methods

Vessel Isolation and Functional Studies

Functional studies were conducted on pressurized branches of the femoral artery (diameter, ≈200 μm) that were isolated from male Wistar rats (n=20; 14 weeks old; Charles River Laboratories, Wilmington, Mass), as previously described.[17,19,20] At normal P_i (80 mm Hg), flow-induced dilations were obtained; then, P_i was increased to 160 mm Hg for 30 minutes. In other experiments, vessels were exposed to high P_i in the presence of SOD (200 U/mL), apocynin (3×10^{-4} mol/L, a NAD(P)H oxidase inhibitor[5,21]), losartan (10^{-6} mol/L, an angiotensin II type 1 receptor inhibitor), or captopril (10^{-6} mol/L, an angiotensin-converting enzyme inhibitor). After the high P_i exposure, P_i was returned to 80 mm Hg, and flow-induced responses were reassessed. In separate experiments, arteries were incubated with Ca^{2+}-free solution during high P_i treatment, then flow-induced responses were reassessed after readministering Ca^{2+} (at 80 mm Hg).

Measurement of Vascular $O_2^{\cdot-}$ Levels

Proximal arterial segments were isolated and cannulated, and the production of $O_2^{\cdot-}$ in response to step increases in P_i (from 0 mm Hg to 80 mm Hg or from 0 mm Hg to 160 mm Hg) was continuously measured by the real-time lucigenin (10 μmol/L) chemiluminescence method using a vessel-perfusion system coupled to a photomultiplier-based detector.[21] Pressure-induced changes in lucigenin chemiluminescence were also measured in the presence of 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 PKC and PKC-induced NAD(P)H activity[3]) or in the absence of extracellular Ca^{2+}.

In separate experiments, arteries were cannulated and pressurized to 80 mm Hg or 160 mm Hg (for 30 minutes) in the absence or presence of SOD (200 U/mL), chelerythrine (10^{-6} mol/L), apocynin (3×10^{-4} mol/L), staurosporine (10^{-6} mol/L, an inhibitor of PKC), Tiron (10 mmol/L, a $O_2^{\cdot-}$ scavenger), or losartan (10^{-6} mol/L) or in the absence of extracellular Ca^{2+}. Then, post-pressure treatment lucigenin chemiluminescence was measured with a scintillation counter, as previously described.[22] Also, in some experiments, lucigenin chemiluminescence was compared in arteries exposed to 80 mm Hg and 120 mm Hg. Further, arterial $O_2^{\cdot-}$ production was also assessed after incubation with the PKC activator phorbol myristate acetate (PMA; 10^{-6} mol/L, for 30 minutes, at 37°C) in the absence and presence of chelerythrine, SOD, or DPI or after removal of extracellular Ca^{2+}. The effect of ionomycin (10^{-6} mol/L, a Ca^{2+} ionophore) on vascular $O_2^{\cdot-}$ levels was also determined.

Ethidium Bromide Fluorescence

Hydroethidine was used to localize pressure-induced $O_2^{\cdot-}$ production, as described previously.[22] Identical branches of the left and right femoral arteries were cannulated, pressurized (to 80 or 160 mm Hg), and exposed to hydroethidine (2×10^{-6} mol/L, at 37°C, for 15 minutes). They were then embedded, snap-frozen in liquid nitrogen, and cryosectioned. The sections were immunostained for smooth muscle α-actin. We used 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) for nuclear staining, and fluorescent images were collected. Normal and high P_i-exposed vessels were processed and imaged in parallel.

Measurement of Endothelial and Smooth Muscle Intracellular Ca^{2+}

The smooth muscle or the endothelium of isolated, cannulated arteries were loaded with the Ca^{2+}-sensitive fluorescent indicator fura 2 acetoxy-methyl, as described previously.[20] Changes in intracellular Ca^{2+} $\{[Ca^{2+}]_i\}$ in the arterial endothelium and smooth muscle in response to increases in P_i (from 0 to 80 mm Hg or to 160 mm Hg) were measured by the ratiometric fluorescence method using the Ionoptix Microfluorimeter System (Ionoptix Co).[20] Responses were also reassessed in the absence of extracellular Ca^{2+}.

Detection of Pressure-Induced PKCα Phosphorylation by Western Blotting

Phosphorylation of PKCα was detected by Western blotting, as described previously.[23] Arteries were cannulated in a dual-vessel chamber (model CH/1/QT, Living System Instrumentation), pressurized to 80 mm Hg or 160 mm Hg (for 30 minutes at 37°C), and then snap-frozen in liquid nitrogen. Nonpressurized vessels served as controls. Protein samples were prepared[22,23] in the presence of Na_3VO_4 (1 mmol/L) and phosphatase inhibitors (Sigma). Equal amounts of protein (8 μg) were electrophoresed on 10% SDS-PAGE gels, and Western blotting was performed using a phosphorylation-specific primary antibody for PKCα (Cell Signalling Technology, Beverly, Mass; 1:1000) and anti-rabbit IgG-horseradish peroxidase secondary antibody (Amersham Biosciences, Piscataway, NJ; 1:4000). Anti-β-actin was used for normalization.

Immunofluorescent Labeling of Phospho-PKCα

Isolated arteries pressurized to 160 mm Hg were treated with phosphatase inhibitors, embedded, snap-frozen in liquid nitrogen, and cryosectioned (thickness, 4 μm). Immunofluorescent labeling[22] was performed with phospho-specific anti-PKCα primary antibody. DAPI was used for nuclear staining.

Immunoprecipitation and Detection of Pressure-Induced p47^{phox} Phosphorylation by Western Blotting

Isolated arteries were pressurized (for 30 minutes at 37°C) to 0, 80, or 160 mm Hg or 160 mm Hg plus 10^{-6} mol/L chelerythrine or 0 mm Hg plus 10^{-6} mol/L PMA, snap-frozen in liquid nitrogen, and lysed in a radio immunoprecipitation (RIPA) lysis buffer (Upstate USIA Inc) containing phosphatase inhibitors. Proteins were incubated with protein A beads (Amersham; 1 hour at 4°C) and with anti-p47^{phox} primary antibody (5 μg, overnight at 4°C). Then, the samples were centrifuged (12 000*g*), the pellet was washed (3× RIPA and 1× 50 mmol/L Tris, pH 8.0), and the proteins were

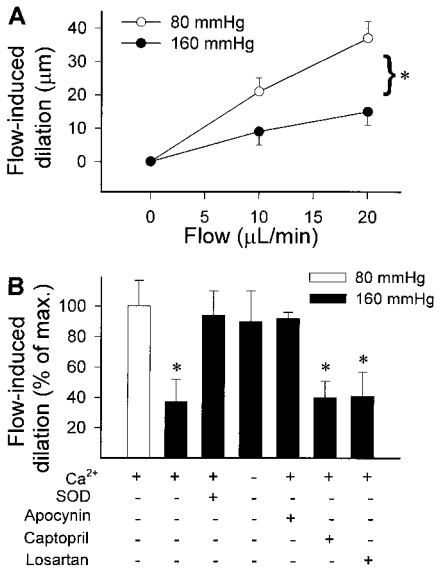

Figure 1. A, Flow-induced dilations in rat femoral arterial branches under control conditions (80 mm Hg) and after high P_i treatment (160 mm Hg). B, Effect of high P_i treatment on flow (20 μL/min)-induced dilation (expressed as percentage of maximum response) in the absence or presence of superoxide dismutase (SOD; 200 U/mL), apocynin (3×10^{-4} mol/L), losartan (10^{-6} mol/L), or captopril (10^{-6} mol/L) or in the absence of extracellular Ca^{2+} (n=4 to 12). Data are normalized to the control mean values. *$P<0.05$.

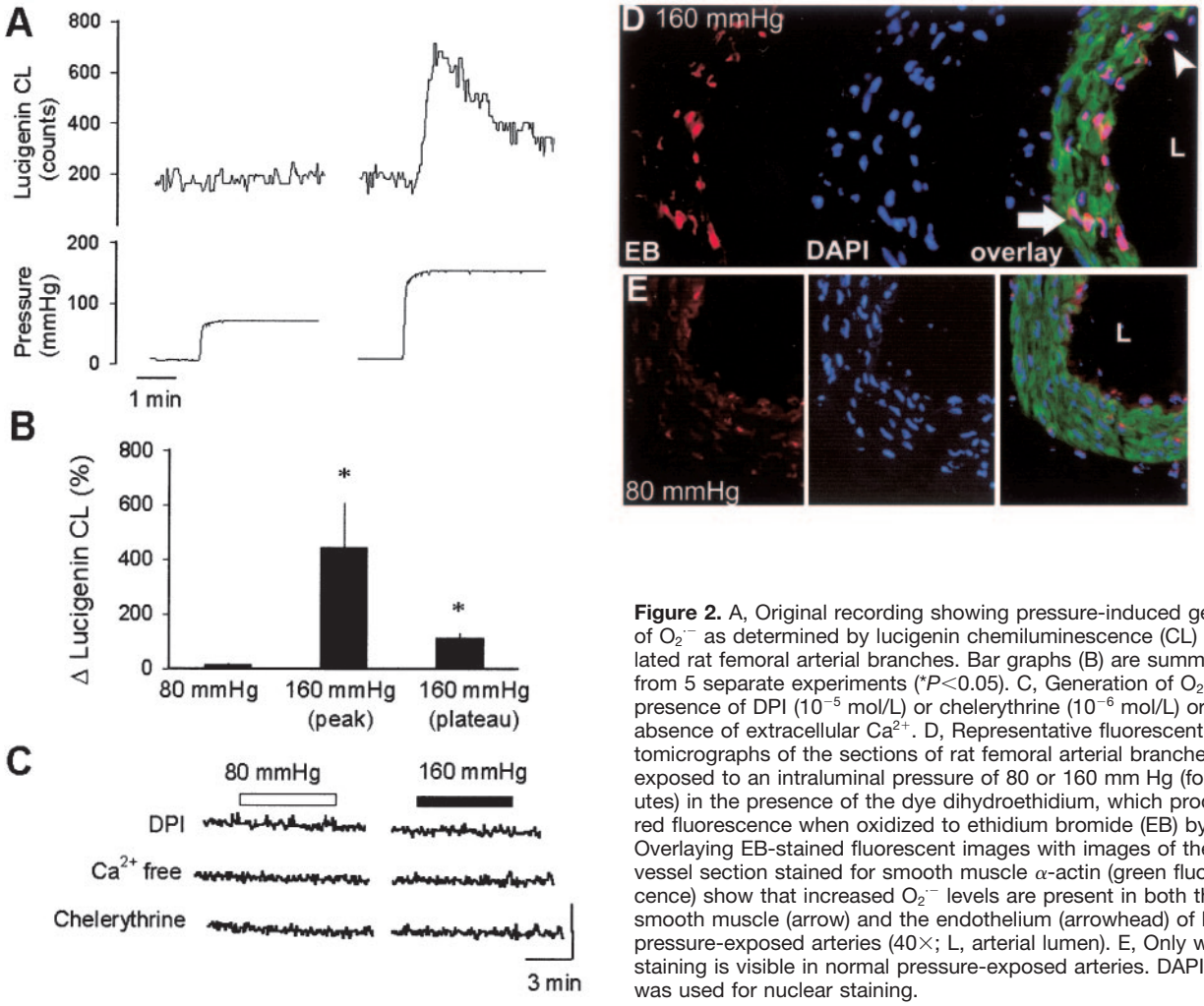

Figure 2. A, Original recording showing pressure-induced generation of $O_2^{\cdot-}$ as determined by lucigenin chemiluminescence (CL) in isolated rat femoral arterial branches. Bar graphs (B) are summary data from 5 separate experiments (*$P<0.05$). C, Generation of $O_2^{\cdot-}$ in the presence of DPI (10^{-5} mol/L) or chelerythrine (10^{-6} mol/L) or in the absence of extracellular Ca^{2+}. D, Representative fluorescent photomicrographs of the sections of rat femoral arterial branches exposed to an intraluminal pressure of 80 or 160 mm Hg (for 30 minutes) in the presence of the dye dihydroethidium, which produces a red fluorescence when oxidized to ethidium bromide (EB) by $O_2^{\cdot-}$. Overlaying EB-stained fluorescent images with images of the same vessel section stained for smooth muscle α-actin (green fluorescence) show that increased $O_2^{\cdot-}$ levels are present in both the smooth muscle (arrow) and the endothelium (arrowhead) of high pressure-exposed arteries (40×; L, arterial lumen). E, Only weak EB staining is visible in normal pressure-exposed arteries. DAPI (blue) was used for nuclear staining.

separated by SDS-PAGE, transferred to membranes, and probed with a primary antibody against phosphorylated serine residues (1:1000, 1 hour, Cell Signaling) and normalized for $p47^{phox}$ content.[22,23]

Materials

All chemicals, if otherwise not specified, were obtained from Sigma-Aldrich Co, and solutions were prepared on the day of the experiment. Final concentrations are reported in the text.

Data Analysis

Lucigenin chemiluminescence data and densitometric ratios were normalized to the respective control mean values. Selective portions of representative Western blots are shown for clarity. Data are expressed as the mean±SEM. Statistical analyses of data were performed by Student's *t* test or by 2-way ANOVA followed by the Tukey post hoc test, as appropriate. $P<0.05$ was considered statistically significant.

Results

Flow-Induced Dilation

In control conditions, increases in intraluminal flow elicited significant dilations. These dilations were significantly reduced after the vessels were exposed to high P_i (Figure 1). Previous studies demonstrated that agonist-induced nitric oxide–mediated dilations are also impaired in vessels that were previously exposed to high P_i in a similar manner.[17] Administration of SOD or apocynin (but not losartan or captopril) or removal of extracellular Ca^{2+} prevented the high P_i–induced impairment of flow-induced dilation (Figure 1).

Vascular $O_2^{\cdot-}$ Production

Real-time lucigenin chemiluminescence measurements showed that an increase in P_i from 0 mm Hg to 80 mm Hg resulted in minimal changes in vascular $O_2^{\cdot-}$ production. In contrast, exposure to high P_i (160 mm Hg) elicited biphasic increases in vascular $O_2^{\cdot-}$ generation, with a rapid peak increase followed by a plateau phase (Figures 2A and 2B). Incubation of the vessels with DPI or chelerythrine or removal of extracellular Ca^{2+} prevented high P_i–induced increases in $O_2^{\cdot-}$ levels (Figure 2C). Scintillation counter measurements showed that levels of $O_2^{\cdot-}$ remained significantly increased in vessels exposed to 160 mm Hg compared with arteries exposed to 80 mm Hg (Figure 3A). Pressurization of arteries to 120 mm Hg also resulted in moderate but significant increases (14±4%) in $O_2^{\cdot-}$ generation. Increased $O_2^{\cdot-}$ generation in high (160 mm Hg) P_i–pretreated vessels was inhibited by cotreatment with SOD, DPI, chelerythrine, apocynin, staurosporin, or Tiron or by removal of extracellular Ca^{2+} (Figures 3A and 3B), thus eliminating the difference between the groups. Increased $O_2^{\cdot-}$ generation in high P_i–treated

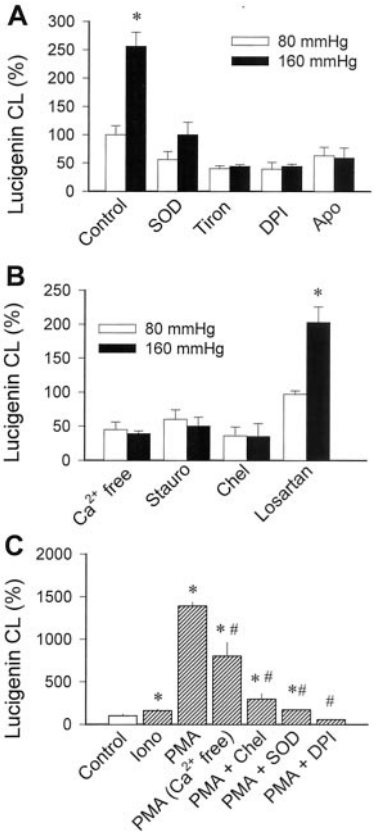

Figure 3. A and B, Generation of $O_2^{\cdot-}$, as determined by lucigenin chemiluminescence (CL) in isolated rat femoral arterial branches exposed to 80 or 160 mm Hg intraluminal pressure under control conditions or in the presence of SOD (200 U/mL), DPI (10^{-5} mol/L), chelerythrine (Chel; 10^{-6} mol/L), apocynin (Apo; 3×10^{-4} mol/L), losartan (10^{-6} mol/L), staurosporine (Stauro; 10^{-6} μmol/L), or Tiron (10 mmol/L) or in the absence of extracellular Ca^{2+}. C, $O_2^{\cdot-}$ production in rat femoral arterial branches under control conditions and in the presence of PMA (10^{-6} mol/L), PMA in the absence of extracellular Ca^{2+}, PMA plus chelerythrine (Chel), PMA plus DPI, PMA plus SOD, or ionomycin (Iono; 10^{-6} mol/L). Data are normalized to the control mean value. *$P<0.05$ vs control, #$P<0.05$ vs PMA alone (n=4 to 5).

vessels was unaffected by losartan (Figure 3B). Incubation of control arteries with PMA (for 30 minutes) significantly increased $O_2^{\cdot-}$ production, which could be inhibited by administration of chelerythrine, SOD, or DPI or by removal of extracellular Ca^{2+} (Figure 3C). The Ca^{2+} ionophore ionomycin also significantly increased vascular $O_2^{\cdot-}$ generation, although its effect was smaller than that of PMA (Figure 3C).

Hydroethidine Fluorescence

In sections of arteries exposed to 160 mm Hg, ethidium bromide staining of nuclei was stronger than in sections of arteries exposed to 80 mm Hg (representative ethidium bromide fluorescent images are shown in Figures 2D and 2E). Overlaying of ethidium bromide–stained fluorescent images (red) with images of the same vessel sections stained for smooth muscle α-actin (green) showed that high P_i increased $O_2^{\cdot-}$ levels in both the media and intima of arteries (Figure 2D).

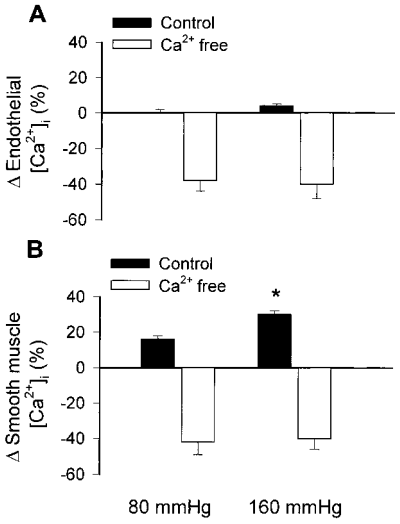

Figure 4. Pressure-induced changes in $[Ca^{2+}]_i$ in the arterial endothelium (A) and smooth muscle (B) in the presence and absence of extracellular Ca^{2+}. Data are mean±SEM. *$P<0.05$ (n=5 to 6).

Measurement of Endothelial and Smooth Muscle $[Ca^{2+}]_i$

Smooth muscle $[Ca^{2+}]_i$ was significantly greater at 160 mm Hg P_i than at 80 mm Hg P_i (Figure 4B), which corresponded to our previous findings,[20] whereas increases in P_i had only a slight effect on $[Ca^{2+}]_i$ in the endothelium (Figure 4A). In the absence of extracellular Ca^{2+}, both endothelial and smooth muscle $[Ca^{2+}]_i$ were substantially decreased and were unaffected by changes in P_i (Figures 4A and 4B).

Pressure-Induced Phosphorylation of PKCα

Increases in P_i resulted in significantly increased phosphorylation of PKCα in arteries (Figure 5A). In arteries pressurized to 160 mm Hg, PKCα phosphorylation was significantly greater than in vessels exposed to 80 mm Hg (Figure 5A). Immunofluorescent labeling of sections of arteries pressurized to 160 mm Hg showed that phospho-PKCα was predominantly located in the media and the intima, whereas immunostaining of the adventitia was less evident (Figure 5B).

Pressure-Induced Phosphorylation of p47^{phox}

The phosphorylation of p47^{phox} in isolated arteries was analyzed by detecting phosphorylation of serine residues after p47^{phox} immunoprecipitation and SDS-PAGE. Pressurization of arteries resulted in significantly increased phosphorylation of p47^{phox} compared with nonpressurized vessels (Figure 5C), as determined by densitometry analysis. In arteries pressurized to 160 mm Hg, p47^{phox} phosphorylation was substantially greater than in vessels exposed to 80 mm Hg. Chelerythrine prevented high P_i–induced p47^{phox} phosphorylation, whereas PMA stimulation of nonpressurized vessels triggered the phosphorylation of p47^{phox} (Figure 5C).

Discussion

The new findings in the present study are that in high P_i–exposed isolated arteries, there is (1) an increased chelerythrine- and DPI-sensitive, Ca^{2+}-dependent $O_2^{\cdot-}$ production that impairs flow-induced nitric oxide–mediated dilation; (2) elevated smooth muscle $[Ca^{2+}]_i$ and enhanced phosphorylation of PKCα;

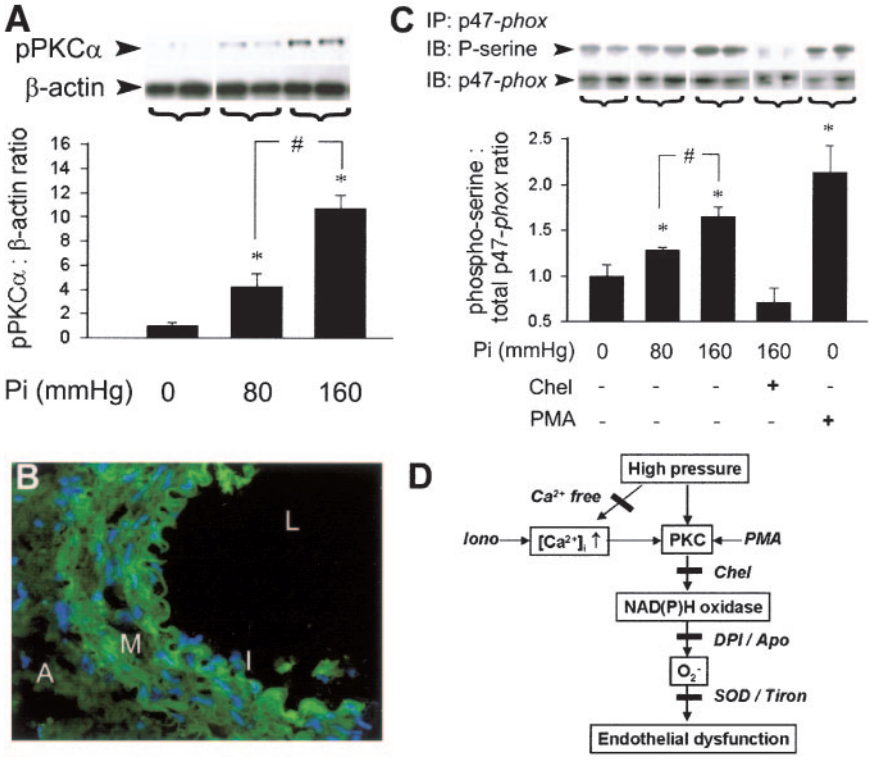

Figure 5. A, Western blot analysis of pressure-induced PKCα phosphorylation in isolated arteries. Bar graphs are summary data of normalized densitometric ratios. (n=3 to 4 vessels for each group). Data are mean±SEM. *$P<0.05$. B, In rat femoral arterial branches pressurized to 160 mm Hg, immunofluorescent staining for phospho-PKCα (green) was predominantly localized in the media (M) and the intima (I), whereas immunostaining of the adventitia (A) was less evident. L indicates arterial lumen (40×). DAPI (blue) was used for nuclear staining. C, Immunoprecipitation (IP) of the p47^{phox} NAD(P)H oxidase subunit from sam ples of rat femoral arterial branches pressurized to 0, 80, or 160 mm Hg under control conditions or exposed to 160 mm Hg in the presence of chelerythrine (Chel; 10^{-6} mol/L). Arteries treated with PMA (10^{-6} mol/L) served as positive controls. Phosphorylated serine residues and total p47^{phox} were detected by Western blotting (IB indicates immunobinding). Bar graphs are summary data of normalized densitometric ratios. (n=4 vessels for each group). $P<0.05$ vs control, #$P<0.05$, 80 vs 160 mm Hg. D, Proposed scheme for high pressure-induced arterial $O_2^{\cdot-}$ production by upregulation of Ca^{2+}- and PKC-dependent of phosphorylation and activation of the NAD(P)H oxidase. Apo indicates apocynin.

and (3) enhanced chelerythrine-sensitive phosphorylation of the p47^{phox} subunit of NAD(P)H oxidase.

Pressure-Induced Increase in Arterial $O_2^{\cdot-}$ Generation

In isolated arteries, SOD-reversible endothelial dysfunction could be elicited in vitro in the absence of circulating factors by increasing P_i to a hypertensive level (Figure 1), thus confirming our previous findings.[17] Other studies also showed that in the coronary and cerebral circulation, short-term increases in P_i in vivo also impair endothelial function.[13–16] Mechanosensitive production of reactive oxygen species has also been linked to pressure-induced constriction of arterioles.[24,25] To provide further evidence for high P_i–induced oxidative stress, we demonstrated with real-time lucigenin chemiluminescence measurements that high P_i itself induced significant increases in arterial $O_2^{\cdot-}$ generation (Figures 2A and 2B), whereas normal levels of P_i had minimal effect. High P_i induced a rapid peak increase in $O_2^{\cdot-}$ production that was followed by a plateau phase (Figure 2A). Importantly, arterial $O_2^{\cdot-}$ levels measured after exposure to high P_i also remained elevated (Figure 3A). We have found a similar, sustained $O_2^{\cdot-}$ generation recently in vascular ring preparations after short-term stretching,[21] thus suggesting that mechanical activation-induced $O_2^{\cdot-}$ production is not readily reversible. High P_i increased vascular $O_2^{\cdot-}$ production in the endothelium and the smooth muscle, as shown by ethidium bromide staining (Figure 2D), thus suggesting that similar mechanosensitive pro-oxidant pathways are present in both cell types.

Role of NAD(P)H Oxidase

Because high P_i–induced endothelial dysfunction (Figure 1) and increased $O_2^{\cdot-}$ generation (Figures 2C and 3A) could be prevented by apocynin and DPI, it is likely that high P_i itself activates NAD(P)H oxidases. The in vivo importance of high P_i–induced activation of oxidases is supported by recent findings showing increased NAD(P)H oxidase-dependent vascular $O_2^{\cdot-}$ production in virtually all forms of hypertension,[2,5,7,8,26] which can be reduced or prevented by lowering P_i.[8,11] Studies on cultured endothelial and smooth muscle cells[27] and on aortic rings[21] showing stretch-induced $O_2^{\cdot-}$ generation also suggest that mechanosensitive pathways are coupled to NAD(P)H oxidase activity in both the arterial endothelium and smooth muscle. Previous studies suggested a role for locally produced angiotensin in vascular oxidative stress and modulation of flow-mediated responses.[28] Thus, we incubated the vessels with losartan and captopril to exclude the contribution of a local renin-angiotensin system, yet high P_i still activated $O_2^{\cdot-}$ generation (Figure 3B) and induced endothelial dysfunction (Figure 1), providing further evidence for the direct role of high P_i.

Role of Ca^{2+} and PKC-Dependent Signal Transduction

We found that removal of Ca^{2+} and inhibition of PKC prevented high P_i-induced $O_2^{\cdot-}$ production (Figures 3A and 3B), indicating a role for mechanosensitive Ca^{2+} signaling and PKC-dependent pathways in high P_i–induced upregulation of NAD(P)H oxidase activity. This idea is consistent with the findings that high P_i elicited significantly greater increases in smooth muscle $[Ca^{2+}]_i$ than normal levels of P_i (Figure 4). The important role of Ca^{2+} signaling is also supported by the finding that administration of a Ca^{2+} ionophore resulted in significantly increased arterial $O_2^{\cdot-}$ production (Figure 3C). Further, pharmacological activation of PKC^2 elicited substantial increases in arterial NAD(P)H oxidase-derived $O_2^{\cdot-}$ generation (Figure 3C). Because removal of Ca^{2+} during high-pressure treatment prevented endothelial dysfunc-

tion (Figure 1) and increases in $O_2^{\cdot-}$ production in high P_i–exposed arteries (Figure 3A) and phorbol ester–stimulated $O_2^{\cdot-}$ generation in normotensive arteries could also be reduced by removal of Ca^{2+} (Figure 3C), it is likely that Ca^{2+}-dependent PKC isoforms are involved in the high P_i–induced upregulation of arterial NAD(P)H oxidase activity. This idea is further supported by the findings that high P_i elicited phosphorylation of PKCα[23] (Figure 5A), the most abundant Ca^{2+}–dependent PKC isoform in arteries. It seems that high P_i induces phosphorylation and activation of PKCα in both the endothelium and smooth muscle, as indicated by immunostaining (Figure 5B) that leads to increased $O_2^{\cdot-}$ production in both cell types (Figure 2D).

The activity of NAD(P)H oxidases is thought to be regulated by phosphorylation of the cytosolic regulatory $p47^{phox}$ subunit[29] that facilitates association of the membrane-bound and cytoplasmic subunits of the enzyme.[21] We found that high P_i substantially increased phosphorylation of serine residues of $p47^{phox}$, an effect that could be prevented by inhibition of the serine/threonine kinase PKC[2,3] (Figure 5C). Correspondingly, pharmacological activation of PKC resulted in phosphorylation of the $p47^{phox}$ subunit (Figure 5C) and increased NAD(P)H oxidase-dependent $O_2^{\cdot-}$ production (Figure 3C).

Thus, we conclude that hypertensive levels of P_i itself are a sufficient stimulus for increased arterial $O_2^{\cdot-}$ production, likely by inducing Ca^{2+}- and PKC-dependent of phosphorylation and activation of the NAD(P)H oxidase (Figure 5D). This mechanism may contribute to the increased $O_2^{\cdot-}$ levels and endothelial dysfunction in hypertension, regardless of its cause. In addition, high P_i–induced NAD(P)H oxidase function in vivo is likely also stimulated by increased pulsatility[27,30] and/or by increased levels of humoral factors, such as angiotensin II,[3,29] that increase PKC activity via G protein-coupled receptors. One can speculate that chronic high P_i–induced $O_2^{\cdot-}$ generation by stimulating redox-sensitive gene expression also contributes to structural remodeling, because a reduction of systemic blood pressure could reverse morphological changes.[31] Finally, our findings also provide an explanation for the similar vasculoprotective effects of blood pressure lowering by pharmacological treatments with different mechanisms of action (eg, calcium antagonists, diuretics, and ACE inhibitors) in various forms of human hypertension.

Acknowledgments

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