Carvedilol Inhibits Pressure-Induced Increase in Oxidative Stress in Coronary Smooth Muscle Cells

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The cellular mechanisms by which hypertension enhances atherosclerosis are still not known in detail. Recently, evidence has been obtained that oxidative stress plays a role in the pathogenesis of pressure-induced atherosclerosis. We examined the effects of pressure on oxidative stress in cultured human coronary smooth muscle cells (SMCs). Application of increased pressure (100 mmHg) with He gas for 48 h increased oxidative stress of measured by flow cytometry by 71% and F2-isopretane by 77%. Increased pressure also increased the activities of phospholipase D (PLD), and particulate protein kinase C (PKC). The PLD inhibitor suramin 100 μmol/l, 1-butanol 40 mmol/l, and the PKC inhibitors chelerythrine 1 μmol/l and calphostin C 100 nmol/l and completely blocked the increase in oxidative stress induced by pressure. Carvedilol 1 μmol/l but not propranolol 1 μmol/l blocked pressure-induced increases in oxidative stress in cultured SMCs. These findings suggest that pressure increases oxidative stress and that carvedilol significantly inhibits pressure-induced increase in oxidative stress in cultured human coronary smooth muscle cells. (Hypertens Res 2002; 25: 419–425)

Key Words: antioxidant, pressure, leukocyte, smooth muscle, risk factors

Introduction

Pressure may result in increased oxidative stress in smooth muscle cells (SMCs), which may contribute to pressure-mediated changes in SMC function (1). The molecular pathogenesis of essential hypertension is still not known in detail, although increased oxidative stress has been reported in experimental models of hypertension (2, 3) and in patients with essential hypertension (3, 4). These findings undoubtedly have pathophysiological and therapeutic implications. If enhanced oxidative stress plays a role in the pathogenesis of atherosclerosis in hypertensive patients, an adequate antioxidant supply should be included in treatment of such patients and can be obtained with either concomitant supplementation of antioxidants, and antihypertensive compounds, or a single administration of specific antihypertensive drugs exhibiting intrinsic antioxidant properties, such as carvedilol (5).

It has been suggested that carvedilol may provide greater benefit than traditional β-blockers in the treatment of chronic heart failure because of its antioxidant effects, which synergize with its nonspecific β- and α-blocking effects (6). Carvedilol has been shown to inhibit lipid peroxidation of myocardial cell membranes and thus to protect endothelial, neuronal, and vascular smooth muscle cells from oxygen radical-mediated injury (7). Carvedilol has also been shown to scavenge peroxo and hypochlorous radicals in chemical systems in vitro (8). Carvedilol was shown to have antioxidant effects in patients treated with moderate doses of 25 mg/day as assessed by suppression of ex vivo LDL oxidation and reduction of anti-oxidized LDL antibodies in vivo (9).
Accordingly, the objectives of the present study were to determine whether elevated pressure increases oxidative stress in cultured SMCs derived from human coronary arteries and, if it does, to examine the effects of carvedilol on pressure-induced increase in oxidative stress in these cells.

**Methods**

**Materials**

$N$-acetylcysteine, suramin, 1-butanol, chelerythrine, calphostin C, PDGF (recombinant BB) and bovine serum albumin (BSA) were purchased from Sigma Chemical Corp. (St. Louis, USA). SMC basal medium (SmBM) and human coronary SMCs were purchased from Clonetics Corp. (San Diego, USA). Microchemotaxis chambers and polycarbonate filters were purchased from Neuro Probe, Inc. (Gaithersberg, USA), and Nucleopore Corp. (Pleasanton, USA), respectively. $[^3]$H$]-$ethanolamine and the protein kinase C (PKC) assay system were purchased from Amersham Japan Corp. (Tokyo, Japan). The 8-Epi-prostaglandin $F_{2α}$ (PGF$F_{2α}$) enzyme immunoassay (EIA) kit was purchased by Cayman Chemical (Ann Arbor, USA). Carboxydichlorofluorescin (CDCFH) diacetate bis-acetoxymethyl (AM) ester was purchased from Molecular Probe Corp. (Eugene, USA).

**Cell Culture**

Human coronary artery SMCs were cultured in SmBM as previously reported (10). To avoid the differences in oxidative stress due to differences in number of cell passages, the experiments in the same figure or table were performed in the same passage.

**Pressure-Loading Apparatus**

A pressure-loading apparatus was assembled as previously reported (11), with some modifications. SMCs were seeded in 25-cm$^2$ flasks. The flasks were clamped tightly between two iron plates, and the top of each flask was sealed with a rubber cap. The rubber cap was pierced with a needle connected to tubing attached to a three-way rotary valve, a sphygmomanometer, and a pressure valve. Compressed He gas was pumped in to increase the internal pressure. While the He gas was pumped in, no prepacked room air was released, so that the partial pressures of the gases originally contained in the flasks, including oxygen, nitrogen, and carbon dioxide, remained constant.

**Measurement of Lactate Dehydrogenase (LDH) and Assessment of Cytotoxicity**

The cytotoxic effects of high pressure on human coronary SMCs were assessed by LDH release using a commercially available method described by Wrobleski and LaDue (12). LDH was measured in cell culture supernatants after 24–72 h of incubation at 37°C in SmBM. Cytotoxicity was expressed at percentage of LDH activity present in supernatants of cultured cells compared to total LDH activity present in 5 $\times$ 10$^5$ cells/well incubated with 1 ml of 1% Triton X-100 for 30 min.

**Migration Assay**

Migration of SMCs was assayed using a modified version of Boyden’s chamber method with microchemotaxis chambers and polycarbonate filters, as previously reported (13). In this experiment, polycarbonate filters with pores 12 $\mu$m in diameter were used. SMCs were allowed to grow for 48 h in high (+100 mmHg)- or normal (+0 mmHg)-pressure medium with 0.5% FCS in the presence or absence of suramin or chelerythrine. Migration activity was calculated as the mean number of cells that had migrated observed in four high-power fields and is presented as the mean value of four measurements.

**Phospholipase D (PLD) Activity Measured by Ethanolamine Release**

SMCs were allowed to grow for 48 h under high or normal pressure with 0.5% FCS in the absence or presence of antioxidant, carvedilol or propranolol. SMCs in 25-cm$^2$ flasks were cultured in medium containing $[^3]$H$]-$ethanolamine (5 $\mu$Ci/ml/flask) for 24 h (the latter half of the 48 h period with pressure) to label cellular phosphatidylethanolamine. Antioxidant, carvedilol or propranolol was included during this period. After stimulation with PDGF BB 10 ng/ml for 6 h and removal of the labelling medium, fractionation of ethanolamine metabolites from the aqueous phase was performed on Dowex 50 w (H$^+$)-packed columns as previously described (14).

**Cell Fractionation and PKC Assay**

SMCs were allowed to grow for 48 h under high or normal-pressure medium with 0.5% FCS in the presence or absence of suramin or chelerythrine. After incubation with 10 ng/ml PDGF BB for 6 h, PKC activity was measured by a modified version of a method previously reported, using the Amersham PKC assay system (14).

**Assay of Intracellular Oxidative Stress**

Intracellular oxidative stress levels were measured using a fluorescent dye, CDCFH diacetate bis-AM ester. After 5 min of incubation at room temperature, the fluorescence intensity was measured by flow cytometry as previously reported (15). The excitation wavelength was 510–530 nm. Relative fluorescence intensities were calculated using untreated control cells as a standard.
Fluorescence Microscopy

SMCs were visualized through a fluorescence microscope (IX70, Olympus, Tokyo, Japan; 400 water immersion objective lens) via a camera (PM-C 35DX, Olympus,). To obtain fluorescent images, the preparation was illuminated with a 200 W mercury lamp. The light was passed through a quartz collector, heat filter and an excitation filter to epi-illuminate the preparation. Fluorescence emission from the sample was passed through a band-pass filter (515 nm) and onto the camera.

Measurement of Immunoreactive 8-epi-PGF_{2\alpha} Levels

Levels of released one F_2-isopretane, 8-epi-PGF_{2\alpha} were quantitated by a specific EIA kit. The cell supernatants (100 \mu l) were directly assayed. The assay has been proven to yield a high correlation (0.96) between known added amounts of 8-epi-PGF_{2\alpha} and the concentration measured by EIA. The antiserum used in this assay has 100% cross-reactivity with 8-epi-PGF_{2\alpha}, cross-reactivities of 0.71%, 0.66%, 0.39%, and 0.02% each with PGF_{1\alpha}, PGF_{3\alpha}, PGE_{1}, and PGE_{2}, of 0.14% and 0.16% with 6-keto-PGF_{1\alpha} and PGD_{2}, and of 0.08% and 0.09% with thromboxane B_{2} and 2,3-dinor-6-keto-PGF_{1\alpha}, respectively. The sensitivity of the assay is 8 pg/ml (16).

Statistical Methods

Statistical analysis of in vitro results was performed by analysis of variance and Student’s t-test. Values of p < 0.05 were considered to indicate statistical significance.

Results

Effect of Pressure on Migration of Human Coronary SMCs

Human coronary SMCs were pressurized for 24 h or 48 h. There were no significant changes in cell migration at 24 h. However, pressurization for 48 h at 50, 100 and 150 mmHg significantly increased cell migration. The increase in cell migration at 100 mmHg was 73% (Fig. 1A). Suramin, a PLD inhibitor, at 100 \mu mol/l, chelerythrine, a specific PKC inhibitor, at 1 \mu mol/l and N-acetylcysteine, an antioxidant (Fig. 1A), as well as carvedilol at 0.1–1 \mu mol/l but not propranolol at 0.1–1 \mu mol/l (Fig. 1B), significantly reduced the increase in SMC migration. These compounds at these concentrations had no effect on SMC migration in normal ( \leq 100 mmHg)-pressure medium (data not shown).

Involvement of PLD and PKC in Pressure- and PDGF BB-Induced SMC Response

Increases and decreases in PLD and PKC were examined by measurement of PLD and membrane-bound (particulate) PKC activities in the presence of PDGF BB. PLD (Fig. 2A) and membrane-bound PKC (Fig. 2B) activities were increased by pressure. These increases were significantly reduced by N-acetylcysteine 100 mmol/l, suramin 100 \mu mol/l, 1-butanol 40 mmol/l and carvedilol 0.1–1 \mu mol/l but not by

**Fig. 1.** Pressure-dependent effects of drugs on PDGF BB-induced human coronary smooth muscle cell (SMC) migration. Human coronary SMCs were cultured for 48 h at the indicated pressures, and migration experiments were performed as described in the Methods section. N-acetylcysteine 100 mmol/l (M), suramin 100 \mu M, 1-butanol 40 mM, chelerythrine 1 \mu M, calphostin C 0.1 \mu M (A), carvedilol 0.1–1 \mu M or propranolol 0.1–1 \mu M (B) was added to high (+100 mmHg)-pressure medium cultured for 48 h. Migration activities are expressed as means ± SD (n = 8) of the number of cells observed in four high-power fields (HPF). *p < 0.05.
propranolol 0.1–1 µmol/l, although these compounds at these concentrations had no effect on either PLD or PKC activity in normal (+ 0 mmHg)-pressure medium (data not shown). Suramin 100 µmol/l, 1-butanol 40 mM, carvedilol or propranolol at 1 µM for 48 h prior to measurement of PLD activity as described in the Methods section. Results are means ± SD (n = 8). B: The bar graph shows the distribution of the protein kinase C (PKC) activities in particulate fractions of SMCs in the presence of PDGF BB 10 ng/ml for 6 h. Results are means ± SD (n = 8). SMCs were grown to confluence and then kept in SMC basal medium containing the indicated concentrations of glucose with or without N-acetylcysteine 100 mmol/l (M), suramin 100 µM, 1-butanol 40 mM, chelerythrine 1 µM, calphostin C 0.1 µM, carvedilol or propranolol at 1 µM for 48 h prior to measurement of PKC activity as described in the Methods section. * p < 0.05. NS: not significantly different.

**Effects of Pressure on SMC Intracellular Oxidative Stress**

Chronic high-pressure treatment for 48 h increased oxidative stress by 71% (Fig. 3A). Suramin 100 µmol/l, chelerythrine 1 µmol/l, calphostin C 100 mmol/l, and N-acetylcysteine 100 mmol/l (Fig. 3A), as well as carvedilol 0.1–1 µmol/l, but not propranolol 0.1–1 µmol/l (Fig. 3B), also decreased intracellular oxidative stress. Figure 4 shows representative findings for the effects of chronic high-pressure treatment on oxidative stress in coronary SMCs as visualized by fluorescence microscopy and measured by flow cytometry.

**Effect of Pressure on 8-Epi PGF<sub>2α</sub> Levels Released by SMCs**

SMCs that underwent chronic high pressure (+ 100 mmHg) treatment for 48 h and then platelet-derived growth factor (PDGF) BB treatment for 6 h exhibited an increase in 8-epi PGF<sub>2α</sub> levels (from 5.2 ± 0.4 to 8.8 ± 0.8 ng/10<sup>6</sup> cells, n = 8, p < 0.05). This increase in 8-epi PGF<sub>2α</sub> was significantly prevented by coincubation with N-acetylcysteine 100 mmol/l (5.6 ± 0.6 ng/10<sup>6</sup> cells, n = 8, p < 0.05), suramin 100 µmol/l (5.4 ± 0.4 ng/10<sup>6</sup> cells, n = 8, p < 0.05), chelerythrine 1 µmol/l (5.6 ± 0.8 ng/10<sup>6</sup> cells, n = 8, p < 0.05), or carvedilol 1 µmol/l (5.8 ± 0.6 ng/10<sup>6</sup> cells, n = 8, p < 0.05) but not by propranolol 1 µmol/l (8.4 ± 0.8 ng/10<sup>6</sup> cells, n = 8, not significant).

**Discussion**

Mechanical forces such as shear stress (17), hydrostatic pressure (11) and stretch (18) alter the function and structure of blood vessel walls at both the cellular and molecular levels. In the present study, we found that increased static pressure potentiated PDGF BB-stimulated migration in a pressure-dependent manner. A pressure-induced increase in the migration of coronary SMCs was observed for pressures of 50 to 150 mmHg, which are equivalent to the blood pressure levels present in hypertensive patients. Our findings therefore
Fig. 3. Effects of pressure on intracellular oxidative stress measured by flow cytometry. Human coronary smooth muscle cells (SMCs) were cultured for 48 h at the indicated pressures. Intracellular oxidative stress was then measured in the presence of platelet-derived growth factor (PDGF) BB 10 ng/ml for 6 h as described in the Methods section. Mean relative fluorescence intensity was measured by flow cytometry as follows: Mean fluorescence intensity = \[\frac{\sum (\text{Fluorescence of each channel}) \times (\text{Cell number of the channel})}{\text{Total cell number}}\]. A: Means ± SD of mean fluorescence intensities (n = 8). The effects of the PLD inhibitor suramin 100 µmol/l (M), the protein kinase C (PKC) inhibitor chelerythrine 1 µM, N-acetylcysteine 100 mM (A), carvedilol 0.1 µM or propranolol 0.1–1 µM (B) on oxidative stress in cultured human coronary SMCs incubated with increased pressure for 48 h were studied. Oxidative stress was measured in the presence of PDGF BB 10 ng/ml for 6 h by flow cytometry, and expressed as fluorescence intensity. *p < 0.05.

Fig. 4. Effect of carvedilol on pressure-mediated increase in intracellular oxidative stress as measured by flow cytometry. Representative findings for the effect of carvedilol 1 µmol/l (M) on high pressure (+100 mmHg)-mediated increase in oxidative stress in smooth muscle cells (SMCs) for 48 h and in the presence of PDGF BB 10 ng/ml for 6 h are shown. Oxidative stress revealed by fluorescence microscopy was measured by flow cytometry, and expressed as fluorescence intensity.
suggest that increased pressure may promote coronary SMC migration in vivo.

We previously demonstrated the involvement of PLD and PKC in oxidative stress induced by PDGF BB (19). In the present study, suramin, 1-butanol, chelerythrine, and calphostin C each inhibited pressure-potentiated SMC migration as well as PLD or PKC activation (Figs. 1, 2). We also found that chronic high-pressure treatment for 48 h increased oxidative stress 1.7-fold, as directly measured by flow cytometry, and that suramin, 1-butanol, chelerythrine and calphostin C, which each completely inhibited pressure (+ 100 mmHg)-mediated increase in PLD and PKC activity (Fig. 2), also suppressed this increase (Fig. 3). These findings suggest that pressure increased oxidative stress through PLD and PKC activation, consistent with previous findings (14). Since it has been reported that the PKC pathway plays a role in increasing oxidative stress (15, 20), increase in oxidative stress may play a role in increasing the rate of migration (21).

In the present study, we have shown that treatment of SMCs with pressure can stimulate the release of F₂-isoprostane, 8-epi PGF₃α, a free radical-derived, non-cyclooxygenase metabolite of arachidonic acid, as an index of oxidative stress (22). This increase was suppressed by suramin and chelerythrine. These findings further confirm that oxidative stress may be increased by static pressure, possibly via PLD- and PKC-mediated process.

We showed for the first time that carvedilol directly prevents increase in oxidative stress by increased pressure, a finding not observed with propranolol. Moreover, because of its antioxidative actions, carvedilol has been reported to have potential as a novel therapeutic agent for heart failure (23). The maximum concentration of 25 mg carvedilol after oral administration was 53.4 ng/ml (about 0.2 µmol/l), which is comparable to the concentrations used in the present study (0.1–1 µmol/l) (24). Our findings therefore suggest that carvedilol directly decreases oxidative stress in vivo. And in fact, carvedilol inhibited oxidative stress of leukocytes in normotensive humans (25).

In conclusion, we found that increased static pressure increased human coronary SMC migration, possibly through PLD- and PKC-mediated increases in oxidative stress. Future therapeutic strategies for vascular protection in hypertensive patients may include direct targeting of signaling pathways to prevent oxidative stress.

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References

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