Pressure promotes DNA synthesis in rat cultured vascular smooth muscle cells.

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Research Article

High blood pressure is one of the major risk factors for atherosclerosis. In this study, we examined the effects of pressure on cell proliferation and DNA synthesis in cultured rat vascular smooth muscle cells. Pressure without shear stress and stretch promotes cell proliferation and DNA synthesis in a pressure-dependent manner. Pressure-induced DNA synthesis was inhibited significantly by the phospholipase C (PLC) inhibitor 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate, the protein kinase C inhibitor H-7, 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine, staurosporine, and the tyrosine kinase inhibitor ([3,4,5-trihydroxyphenyl]methylene)propanedinitrile. To clarify whether activation of PLC and calcium mobilization are involved in pressure-induced DNA synthesis, production of 1,4,5-inositol trisphosphate (IP3) and intracellular Ca2+ was measured. Pure pressure increased IP3 and intracellular Ca2+ in a pressure-dependent manner. The increases in both IP3 and intracellular Ca2+ were inhibited significantly by 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate. This study demonstrates a novel cellular mechanism whereby pressure regulates DNA synthesis in vascular smooth muscle cells, possibly via activation of PLC and protein kinase C.

Pressure Promotes DNA Synthesis in Rat Cultured Vascular Smooth Muscle Cells

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Abstract

High blood pressure is one of the major risk factors for atherosclerosis. In this study, we examined the effects of pressure on cell proliferation and DNA synthesis in cultured rat vascular smooth muscle cells. Pressure without shear stress and stretch promotes cell proliferation and DNA synthesis in a pressure-dependent manner. Pressure-induced DNA synthesis was inhibited significantly by the phospholipase C (PLC) inhibitor 2-nitro-4-carboxyphenyl-*N,N*-diphenylcarbamate, the protein kinase C inhibitor H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine, staurosporine, and the tyrosine kinase inhibitor ([3,4,5-trihydroxyphenyl]methylene)propanedinitrile. To clarify whether activation of PLC and calcium mobilization are involved in pressure-induced DNA synthesis, production of 1,4,5-inositol trisphosphate (IP_3) and intracellular Ca^{2+} was measured. Pure pressure increased IP_3 and intracellular Ca^{2+} in a pressure-dependent manner. The increases in both IP_3 and intracellular Ca^{2+} were inhibited significantly by 2-nitro-4-carboxyphenyl-*N,N*-diphenylcarbamate. This study demonstrates a novel cellular mechanism whereby pressure regulates DNA synthesis in vascular smooth muscle cells, possibly via activation of PLC and protein kinase C. (*J. Clin. Invest.* 1994. 93:1975–1980.) Key words: atherosclerosis • mechanoreception • 1,4,5-inositol trisphosphate • phospholipase C • Ca^{2+}

Introduction

Abnormal proliferation of vascular smooth muscle cells plays an important role in the development of hypertension and atherosclerosis. A large number of growth factors, cytokines, and vasoregulatory molecules have been reported to participate in the pathogenesis of atherosclerosis and vascular remodeling (1). In addition to humoral factors, recent studies have demonstrated that physical or mechanical factors such as shear stress and stretch (or tension) contribute to vascular remodeling (2–5). The physical forces exerted on the blood vessel wall by the passage of intravascular blood are flow and pressure. Pressure is exerted radially at right angles to the axis of flow and leads to tangenital strain on the wall, causing the wall to stretch (tension). However, in studies using vessels or vascular rings, it is impossible to separate effects due to pure pressure from those due to stretch (or tension) induced by pressure. Recently, we devised a method in which pure pressure could be applied to cultured cells without shear stress and stretch (6). The purpose of this study was to examine the hypothesis that pressure alone can cause vascular remodeling. We have demonstrated that pressure alone promotes cell proliferation and DNA synthesis in cultured rat vascular smooth muscle cells. We have also shown that pressure causes hydrolysis of the phosphoinositides and calcium mobilization.

Methods

Materials. 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (H-7),[1] *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA1004), *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride (W-7), and *N*-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride (W-5) were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). 2-Nitro-4-carboxyphenyl-*N,N*-diphenylcarbamate (NCDC) and staurosporine were obtained from Sigma Chemical Co. (St. Louis, MO). ([3,4,5-Trihydroxyphenyl]methylene)propanedinitrile (tyrphostin 1) was from Calbiochem-Novabiochem Corp. (La Jolla, CA). Fura-2/AM (the acetoxymethyl ester of Fura-2) was from Dojindo Laboratories (Kumamoto, Japan). FCS was from Mitsubishi Kasei Corp. (Tokyo, Japan). Plastic 25-cm^2 flasks were obtained from Corning Inc. (Corning, NY). Glass chamber slides (flaskette chamber) and 175-cm^2 film panel flasks (EZIN flasks) were purchased from Nunc, Inc. (Naperville, IL).

Growth of vascular smooth muscle cells. In our preparation, the cell population was contaminated with small numbers of endothelial cells that were identified by cobblestone-like morphology and the presence of anticoagulant Factor VIII–related antigen. Endothelial cells produce various vasoactive substances in response to mechanical stress (2, 6–10). These contaminating cells could have affected the response of vascular smooth cells to pressure. Therefore, we cloned the cells to obtain pure smooth muscle cells. We used rat aortic smooth muscle cells (RACS-1) originally obtained from Wistar rats (11, 12). The cells were negative for anticoagulant Factor VIII–related antigen. Cells were passaged eight times to complete the cloning and then stored in liquid nitrogen. Therefore, passage eight was the point at which cells were frozen in liquid nitrogen. RACS-1 were grown to confluence in media composed of Earle's M199 (Gibco Laboratories, Grand Island, NY) and 10% FCS. RACS-1 from passages 11–13 were used.

Pressure loading apparatus. A pressure loading apparatus was set up as reported previously (6), with some modifications. In the cell proliferation experiments and DNA synthesis experiments, 12- and 96-well plates were placed in a film panel flask by peeling off the upper plastic film and then tightly resealing the flask. The flasks were clamped tightly between two iron plates, and the top of the 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,

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1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; HA1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride; IP_3, 1,4,5-inositol trisphosphate; NCDC, 2-nitro-4-carboxyphenyl-*N,N*-diphenylcarbamate; PKC, protein kinase C; PLC, phospholipase C; RACS-1, rat aortic cultured smooth muscle cells; tyrphostin 1, ([3,4,5-trihydroxyphenyl]methylene)propanedinitrile; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride.

and a pressure valve. Compressed He gas was pumped in to raise the internal pressure. In the 1,4,5-inositol trisphosphate (IP_3) production experiments, RACS-1 were seeded in 25-cm^2 flasks. The top of the flask was sealed with a rubber cap, and He gas was pumped in as mentioned above. In the intracellular calcium measurement experiments, RACS-1 were seeded on a glass chamber flask and pressurized as mentioned above. 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, such as oxygen, nitrogen, and carbon dioxide, were kept constant. The partial pressure of oxygen and pH of the incubation medium ranged from 154±4 to 158±6 mmHg and from 7.3±0.1 to 7.4±0.2, respectively. There were no significant changes in these values throughout the experiments, consistent with Boyle-Charles' law.

Proliferation. Cells were cultured in 12-well multiwell plates in M199 medium containing 10% FCS. After cells had attached to their substrata, the culture medium was changed to serum-free media, which were again changed 12 h later. Cells were exposed to M199 containing 0.1% FCS for growth experiments 24 h after plating. Cell number determinations were performed 24 and 48 h in quadruplicate after each treatment. The partial pressure of oxygen and pH of the medium showed no significant changes for 48 h after the application of pressure. Medium replenishment was required every other day, inevitably causing the elevated pressures of the plates to fall to atmospheric pressure. Cell proliferation assays were examined at 24 and 48 h to circumvent the effects of this pressure–depressurization cycle on cell function.

DNA synthesis. DNA synthesis was estimated with an immunocytochemical assay kit using monoclonal antibromodeoxyuridine (BrdU) antibody to detect BrdU incorporation in cellular DNA (RPN 210; Amersham Corp., Tokyo, Japan) (13). In brief, cells were seeded in a 96-well plate (1×10^3 cells/well, 0.32 cm^2/well) and cultured in the presence of 10% FCS. 3 d later, the growth medium was replaced with a chemically defined medium consisting of Earle's M199, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite. The medium was changed daily for 3 d. After the last medium change, test agents and pressure were added.

Measurement of IP_3 production. Production of IP_3 in RACS-1 was measured with a protein-binding assay kit (TRK 1000; Amersham Corp.). After pressure had been applied for a designated time in each study, a 20% (vol/vol) volume of ice-cold 20% perchloric acid was injected into the flasks with a syringe and needle by piercing the caps. The flasks were then kept on ice for 20 min. The cells were scraped and sonicated. Next, the cell lysates were centrifuged for 30 min at 4°C; the supernatant was carefully titrated to pH 7.5 with 10 N KOH. After removal of $KClO_4$ by centrifugation, 100 μl supernatant was assayed.

Measurement of intracellular Ca^{2+}. RACS-1 cultured on a glass slide chamber flask were loaded with Fura-2/AM. The glass chamber flask was mounted on the stage of an inverted microscope equipped with quartz optics. Fura-2 was excited alternately with light at 340 and 380 nm, and the intensity of the emitted light was measured with a photomultiplier tube through a 500-nm bandpass filter. Fluorescence measurement was performed with an Olympus OSP system, as described previously (Olympus Optical, Tokyo, Japan) (14). The signal ratio at two excitation wavelengths was determined after subtracting autofluorescence. Measurement of fluorescence intensity was performed at a rate of 20 points/s.

Statistical analysis. The results are expressed as means±SEM, and statistical significance was assessed by t test. Values of $P < 0.05$ were considered significant.

Results

Effect of pressure on cell proliferation in RACS-1. To promote a slow sustained rate of growth, RACS-1 were grown in M199 with 0.1% FCS and then were pressurized for 24 and 48 h. There were no significant changes in cell number at 24 h, but pressurization for 48 h significantly increased numbers at both 40 and 80 mmHg (Fig. 1 *a*). The increase in cell number at 80 mmHg was ~ 45% of that observed with 10% FCS. Cells were examined by light microscopy and the trypan blue exclusion test at the end of experiments. The cells were morphologically intact, and numbers of detached cells were negligible throughout the experiments. The cell viability of RACS-1, as assessed with the trypan blue exclusion test, was > 90% throughout the experiments.

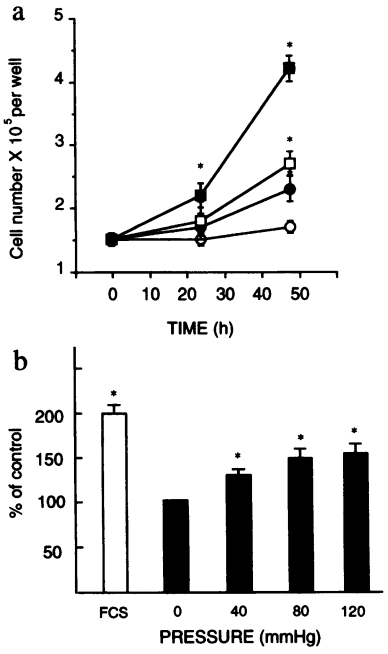

Figure 1. Effect of pure pressure on cell proliferation (*a*) and DNA synthesis (*b*) in RACS-1. In cell proliferation studies (*a*), RACS-1 were pressurized in a medium with 0.1% FCS, and cell numbers were counted at 24 and 48 h after the application of pressure. Open circles indicate atmospheric pressure, closed circles indicate 40 mmHg. Open squares indicate 80 mmHg, closed squares indicate 10% FCS at atmospheric pressure. In DNA synthesis experiments (*b*), RACS-1 were pressurized for 24 h under the indicated pressures in a serum-free medium, and DNA synthesis was determined immunocytochemically with anti-BrdU antibody. The effects of 10% FCS on DNA synthesis in RACS-1 were determined under atmospheric pressure (pressure = 0). On the y-axis, 100% represents the OD at 410 nm in cultures that were at atmospheric pressure without FCS. Values are means±SEM ($n = 8$). *$P < 0.05$ vs 0 time.

Effect of pure pressure on DNA synthesis in RACS-1. It is known that cultured vascular smooth muscle cells produce and secrete PDGF, which stimulates mitogenesis in vascular smooth muscle cells (15–17). Therefore, even in the absence of exogenously added growth factors, vascular smooth muscle cells synthesize DNA through autocrine mechanisms. This may account for DNA synthesis occurring at 0 mmHg (=atmospheric condition) after serum-free culture for 3 d (Fig. 1 *b*). FCS (10%) increased DNA synthesis twofold as compared with the basal level (0 mmHg). Pure pressure from 40 to 120 mmHg also promoted DNA synthesis in a pressure-dependent manner. The promoting effect of 120 mmHg on DNA synthesis was ~ 50% that of FCS. To examine the effect of a pressure pulse on DNA synthesis, cells were pressurized for 5 to 10 min and then depressurized to atmospheric pressure. The pressure pulses at 40 and 120 mmHg for 5 and 10 min produced no promoting effect on DNA synthesis (for 5 min: 0 mmHg 100±5% of control, 40 mmHg 104±8% of control, 120 mmHg 103±10% of control; for 10 min: 0 mmHg 100±5% of control, 40 mmHg 105±7% of control, 120 mmHg 105±10% of control, $n = 8$). To investigate the contribution of humoral factors which can be secondarily secreted in response to pressure, RACS-1 were incubated at atmospheric pressure for 24 h with a conditioned medium obtained from the cells that had been exposed to both 0 and 120 mmHg for 24 h. The conditioned

medium exposed to 120 mmHg showed no promoting effect on DNA synthesis as compared with the medium exposed to 0 mmHg (120 mmHg 104±8% of control vs 0 mmHg 100±5% of control, n = 8). At the end of each experiment, cells were examined by light microscopy and the trypan blue exclusion test. The cells were morphologically intact, and numbers of detached cells were negligible. The cell viability of RACS-1, as assessed with the trypan blue exclusion test, was > 90% throughout the experiments.

Involvement of phospholipase C (PLC), protein kinase C (PKC), and calmodulin in pressure-induced DNA synthesis. To investigate the mechanism of pressure-induced DNA synthesis, RACS-1 were pretreated with the PLC inhibitor NCDC (18, 19) and then pressurized. Pretreatment with 200 μM of NCDC significantly inhibited pressure-induced DNA synthesis at both 40 and 120 mmHg (Fig. 2). Although there was no significant difference between the effects of 3 μM H-7 (20) and HA1004 (Fig. 3 *a*), 30 μM H-7 significantly inhibited pressure-induced DNA synthesis at both 40 and 120 mmHg, as compared with equimolar amounts of HA1004 (Fig. 3 *b*). Additionally, pretreatment with staurosporine (5 nM) significantly inhibited pressure-induced DNA synthesis at 120 mmHg (vehicle 147±5% of control vs staurosporine 105±10% of control, n = 8). Next, RACS-1 were pretreated with the selective calmodulin inhibitor W-7 (21) and with W-5 as the negative control and then were pressurized. Although there was no significant inhibition of pressure-induced DNA synthesis at 3 μM of either W-5 or W-7 (Fig. 4 *a*), 30 μM of W-7 significantly inhibited pressure-induced DNA synthesis at 40 and 120 mmHg, as compared with equimolar amounts of W-5 (Fig. 4 *b*). Although cells treated with W-5 were morphologically intact and viability was > 90%, according to trypan blue test results, at both 3 and 30 μM, a portion of the cells treated with the higher concentration (30 μM) of W-7 detached from the plate and was stained with trypan blue. To investigate the pathway of activation of PLC in pressure-induced DNA synthesis, RACS-1 were pretreated with a tyrosine kinase inhibitor, tyrphostin 1 (10 μM), which significantly inhibited pressure-induced DNA synthesis at 120 mmHg (vehicle 152±5% of control vs tyrphostin 1 107±9% of control, n = 8).

Effect of pure pressure on IP_3 production. Norepinephrine (20 μM) stimulated rapid formation of IP_3 at 30 s (3.3-fold rise from basal level), but the concentration returned to the basal level within 60 s (Fig. 5). Pure pressure (80 mmHg) signifi-

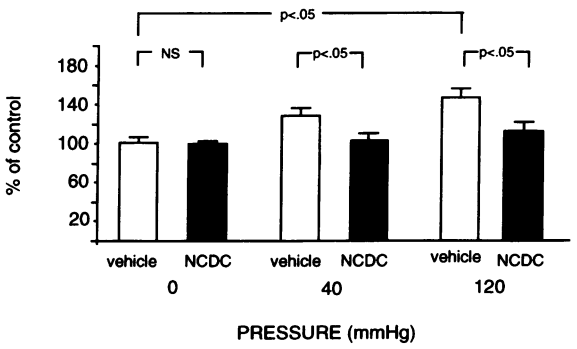

Figure 2. Effect of the PLC inhibitor NCDC on pressure-induced DNA synthesis. On the y-axis, 100% represents the OD at 410 nm in cultures that were at atmospheric pressure without FCS. Values are means±SEM (n = 8). $P < 0.05$ vs control.

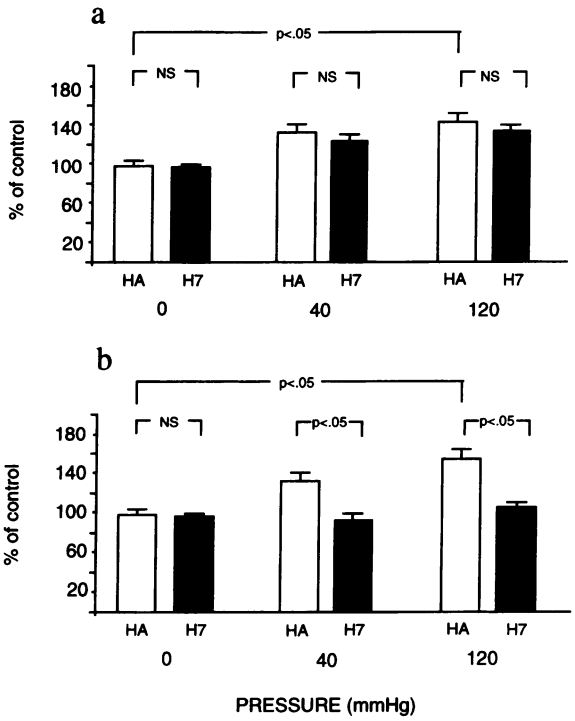

Figure 3. Effect of the nonselective protein kinase inhibitor HA1004 (*HA*) and the selective PKC inhibitor H-7 on pressure-induced DNA synthesis at 3 (*a*) and 30 μM (*b*) of each drug. On the y-axis, 100% represents the OD at 410 nm in cultures that were at atmospheric pressure without FCS. Values are means±SEM (n = 8). $P < 0.05$ vs control.

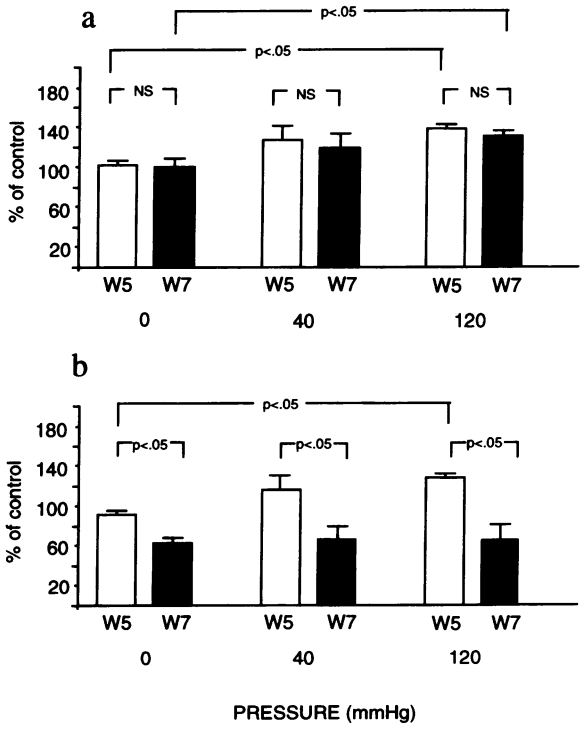

Figure 4. Effect of selective calmodulin inhibitor W-7 and its negative control W-5 on pressure-induced DNA synthesis at 3 (*a*) and 30 μM (*b*). On the y-axis, 100% represents the OD at 410 nm in cultures that were at atmospheric pressure without FCS. Values are means±SEM (n = 8). $P < 0.05$ vs control.

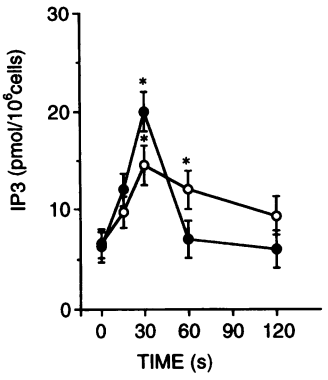

Figure 5. Effect of norepinephrine and pressure (80 mmHg) on IP_3 production as a function of time in RACS-1. After the addition of norepinephrine or pressure, RACS-1 were incubated for the indicated times, and IP_3 generation was determined with an IP_3-binding protein assay. Open circles indicate 80 mmHg, closed circles indicate 20 μM norepinephrine. Each point with a bar indicates a mean±SEM ($n = 4$). *$P < 0.05$ vs each 0 time value.

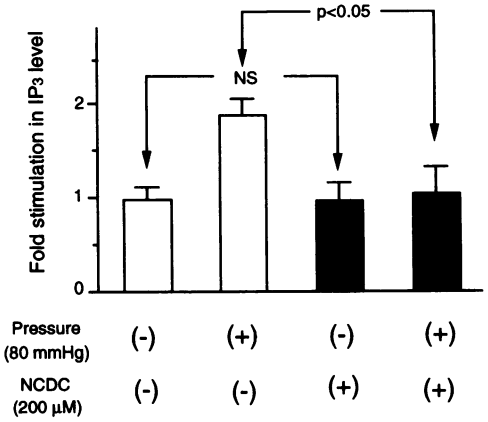

Figure 7. Effect of PLC inhibitor NCDC on pressure-induced IP_3 production. After pretreatment with 200 μM NCDC, RACS-1 were pressurized to 80 mmHg for 30 s, and IP_3 generation was determined. Values are means±SEM of five samples. $P < 0.05$ vs control.

cantly increased IP_3 at 30 s after initiating pressure loading (2.4-fold rise from basal level). After this initial peak, the level of IP_3 decreased but was still significantly elevated at 60 s (Fig. 5). Fig. 6 shows the effect on the production of IP_3 of increasing pressure from 40 to 160 mmHg. The IP_3 level was measured at 30 s after initiation of the pressure increase. IP_3 production was increased significantly in a pressure-dependent manner. Although NCDC, a PLC inhibitor, did not affect the basal IP_3 content, pressure-induced IP_3 production was inhibited significantly by NCDC (Fig. 7).

Pressure-induced changes in intracellular Ca^{2+}. To determine whether IP_3 production causes changes in intracellular Ca^{2+}, RACS-1 were loaded with Fura-2/AM, and the resulting fluorescence was measured. Pressure increased the calcium signal from 40 to 160 mmHg in a pressure-dependent manner (Fig. 8 *a*). After pressure loading, RACS-1 were depressurized and challenged with 20 μM norepinephrine. Norepinephrine increased the calcium signal, confirming cell viability despite pressurization. Successive pressurizations at 120 mmHg caused calcium signals of similar magnitude (Fig. 8 *b*). The sustained phase was maintained during pressurization for at least 20 min (data not shown). The pressure-induced calcium signal increase was abolished completely by pretreatment with NCDC, as in the case of IP_3 production (Fig. 8 *c*). On the other hand, with incubation in Ca^{2+}-free medium in the presence of 2 mM EGTA, the pressure-induced calcium signal was decreased slightly but not abolished completely (Fig. 8 *d*). These results suggest that the main source of Ca^{2+} may be intracellular.

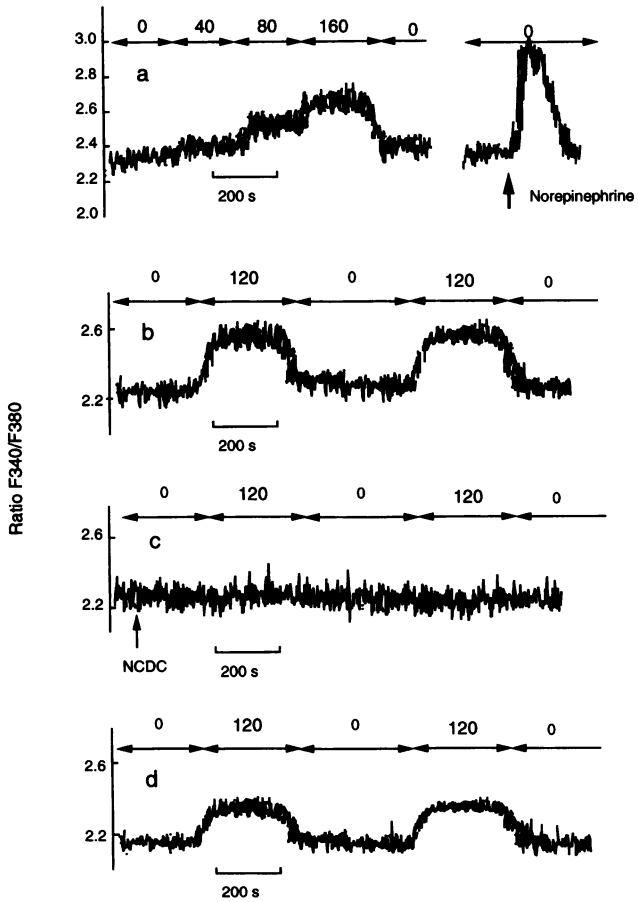

Figure 8. Representative traces of Ca^{2+} Fura-2 fluorescence induced by pressure in RACS-1. (*a*) RACS-1 loaded with Fura-2 were pressurized from 40 to 160 mmHg and depressurized to 0 mmHg. After a 180-s interval, the RACS-1 were challenged with 20 μM of norepinephrine. (*b*) RACS-1 were pressurized from 0 to 120 mmHg, then depressurized to 0, again increased to 120 mmHg, and finally depressurized. (*c*) RACS-1 were pretreated with NCDC (200 μM) and pressurized as in *b*. (*d*) RACS-1 were incubated in Ca^{2+}-free medium in the presence of 2 mM EGTA and pressurized as in *b*. These results are representative of four similar experiments.

Discussion

In this study, we have demonstrated that pressure promotes cell proliferation and DNA synthesis with minimal participa-

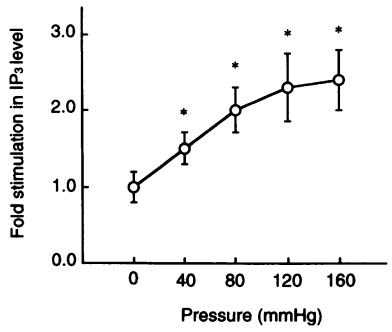

Figure 6. Pressure dose–response curve for IP_3 production in RACS-1. Various levels of pressure were applied to RACS-1 for 30 s, and IP_3 generation was determined. Values are means±SEM of five samples. *$P < 0.05$ vs control.

tion of shear stress, stretch, or humoral factors. Pressure-induced DNA synthesis was inhibited significantly by NCDC. Pressure-induced DNA synthesis was not inhibited by 3 μM of H-7 but was inhibited significantly by staurosporine (5 nM) and 30 μM of H-7, as compared with equimolar amounts of HA1004. The K_i of H-7 for cGMP-dependent protein kinase (PKG), cAMP-dependent protein kinase (PKA), and PKC were 5.8, 3.0, and 6.0 μM, respectively. The K_i of HA1004 for PKG, PKA, and PKC were 1.3, 2.3, and 40 μM, respectively. Moreover, pressure-induced DNA synthesis was inhibited significantly by a tyrosine kinase inhibitor, tyrphostin 1 (22). These results suggest that activation of PLC and PKC plays an important role in pressure-induced DNA synthesis and that pressure-induced activation of PLC can be mediated by activation of tyrosine kinase. Pressure-induced DNA synthesis at 40 and 120 mmHg, as compared with 0 mmHg, was inhibited significantly by W-7 as compared with W-5, and these results are compatible with a previous report (23) that calmodulin is involved in DNA synthesis in vascular smooth muscle cells. Tomita et al. (23) reported that 10–100 μM W-7 significantly inhibited DNA synthesis but they did not evaluate cell viability. Our results show that 30 μM W-7, but not W-5, caused cells to detach from the plate and be stained with trypan blue even at atmospheric pressure. These results suggest that the calcium-calmodulin system plays an important role not only in pressure-induced DNA synthesis but also in cell attachment and the integrity of plasma membranes in vascular smooth muscle cells.

PKC is physiologically activated by diacylglycerol, which is released by hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLC, and IP_3 is the coproduct. The primary function of IP_3 is to mobilize calcium from internal stores. To investigate the signaling pathways of pressure-induced DNA synthesis, IP_3 production and intracellular calcium were measured directly. Pressure increased IP_3 production and intracellular calcium in a pressure-dependent manner. Moreover, these responses were abolished completely by the PLC inhibitor NCDC. NCDC, a serine esterase inhibitor, has a well characterized specificity for the active site of PLC and inhibits phosphoinositide-specific PLC (18). These results suggest that the first step of pressure-sensitive mechanoreception may be activation of PLC.

Enhanced PLC activity and elevated intracellular calcium have been found in aortic vascular smooth muscle cells obtained from spontaneously hypertensive rats (24, 25). However, increased PLC activity and increased intracellular calcium are already observed in prehypertensive spontaneously hypertensive rats (24, 26). Whether increased PLC activity in hypertensive animals is a primary genetic defect or is caused secondarily by hypertension remains controversial. Our results suggest the possibility that alterations in PLC activity and intracellular calcium can be caused secondarily by high blood pressure in genetically normotensive animals. At the molecular level, however, the pathogenesis in hypertensive animals remains to be elucidated.

In contrast to the norepinephrine-induced rise in IP_3, the pressure-induced IP_3 increases are of longer duration. Nollert et al. (27) reported that the shear stress–induced increase in IP_3 in human umbilical vein endothelial cells persists for several minutes after an increase in shear stress, although other agonist-induced IP_3 levels, such as those induced by histamine, thrombin, and bradykinin, return to near basal levels within 1 to 2 min after exposure to the agonist. Pressure-induced calcium responses were sustained and also differed from those of norepinephrine. When cultured renal distal tubule cells (A6 cells) were exposed to a flow stimulus, the cytosolic calcium concentration increased after a lag period of $\sim$ 30 s and was sustained (28). Ando et al. (29) also reported that flow-induced changes in intracellular calcium levels of endothelial cells are sustained. These slow and sustained responses can be characteristic aspects of mechanical stimuli, such as flow and pressure, which have a longer duration than brief stimuli, such as stretch.

Although confluent RACS-1 attached to a solid flask can be subjected to pure pressure with minimal participation of pressure-induced stretch, we cannot exclude completely the possibility that pressure-induced stretch occurred. Kulik et al. (30) reported that stretch-induced IP_3 production in vascular smooth muscle cells was increased after 25 s of a 20% stretch and returned to the control level within 45 s, and that stretch-induced IP_3 production was not inhibited by 20 ng/ml pertussis toxin. We have also found that 20 ng/ml of pertussis toxin did not inhibit pressure-induced IP_3 production at 80 mmHg (data not shown), but the difference in response time to stimuli between pressure and stretch-induced IP_3 production may distinguish pressure from stretch stimuli.

Research interest is focusing increasingly on the roles of various hemodynamic stress modalities in the etiology and pathogenesis of atherosclerosis. Many studies of endothelial cells as mechanotransducers have been reported (31, 32), whereas mechanotransduction other than stretch in vascular smooth muscle cells has not been investigated thoroughly (33). Vascular smooth muscle cells are covered with endothelial cells, are therefore not in direct contact with blood flow, and do not receive shear stress. The mechanical stresses that are transmitted to vascular smooth muscle cells are pressure and pressure-induced stretch. Our study is the first to demonstrate that pressure promotes cell proliferation and DNA synthesis in vascular smooth muscle cells, a process closely related to vascular remodeling. This novel mechanism may contribute to the pathophysiology of the hypertension-associated vascular remodeling underlying atherosclerosis.

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