

The Effect of Anti-Hypertensive Drugs on DNA Synthesis and Proliferation of Cultured Rat Aortic Smooth Muscle Cells

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The aim of this study was to elucidate the effects of anti-hypertensive drugs, nifedipine, furosemide, hydrochlorothiazide, captopril, and atenolol on DNA synthesis and proliferation of cultured rat aortic smooth muscle cells induced by fetal calf serum. Aortic smooth muscle cells from Sprague-Dawley rats were isolated, cultured, and seeded in multi-well plates. When confluent, cells were cultured in a conditioned medium without fetal calf serum. After 72 hours, cells were cultured in the medium retaining 10% fetal calf serum with or without anti-hypertensive drugs by increasing the concentration between 10^{-8} and 10^{-4} M. DNA synthesis was assessed by [3 H]-thymidine uptake and proliferation by cell numbers using a hemocytometer. Nifedipine at a concentration of 10^{-5} M and 5×10^{-3} M inhibited serum-induced DNA synthesis significantly by 50.8% and 86.6%, respectively ($p < 0.05$). The results of cell numbers paralleled those of 3 H-thymidine incorporation. Serum-induced DNA synthesis was also reduced by 32.6% at the highest dose of furosemide (10^{-4} M), but there was no statistical significance. Hydrochlorothiazide, captopril, and atenolol did not show anti-proliferative effect throughout any of the doses. In conclusion, among the various anti-hypertensive drugs, nifedipine seems to be most beneficial in view of its direct inhibitory effect on DNA synthesis and proliferation of smooth muscle cells, as well as for its anti-hypertensive effect.

Key Words: Anti-hypertensive drugs, aortic smooth muscle cell, DNA synthesis, proliferation

Proliferation of vascular smooth muscle cells (VSMC) has been implicated in several pathologic conditions, such as atherosclerosis, hypertension and restenosis after angioplasty (Campbell and Campbell,

1985; Ross, 1986; Grünwald *et al.* 1987; Ip *et al.* 1990; Pickering *et al.* 1992). Several agents have been identified which stimulate VSMC proliferation, including platelet-derived growth factor (PDGF) (Ross *et al.* 1974; Walker *et al.* 1986; Bonin *et al.* 1989), epidermal growth factor (Orimo and Ouchi, 1990; Mokashi *et al.* 1992; Bagby *et al.* 1993; Ko *et al.* 1993), and endothelin (Hirata *et al.* 1989; Bobik *et al.* 1990). Many investigators have tried to suppress the abnormal growth of VSMC, but only a few have succeeded.

It has been demonstrated that hypertension itself stimulated arterial smooth muscle cell migration and proliferation (Owens and Schwartz, 1982; Choba-

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nian, 1990), so treatment of hypertension may be beneficial in preventing complications including atherosclerosis. In the clinic, various groups of anti-hypertensive drugs are being used to treat hypertension. They include calcium channel blockers (nifedipine, felodipine, nicardipine); diuretics (furosemide, hydrochlorothiazide); angiotensin-converting enzyme inhibitors (ACEi) (captopril, enalapril); and β -adrenergic blockers (atenolol, metoprolol). These drugs lower blood pressure by reducing circulating plasma volume or decreasing peripheral vascular resistance, but the effect on VSMC proliferation has been less-widely studied.

In this investigation, we evaluated the effect of commonly used anti-hypertensive drugs including nifedipine, furosemide, hydrochlorothiazide, captopril, and atenolol on DNA synthesis and proliferation of cultured rat aortic smooth muscle cells. DNA synthesis was assessed by [3 H]-thymidine uptake and proliferation by counting cell numbers using a hemocytometer.

MATERIALS AND METHODS

Aortic smooth muscle cell culture

Aortic smooth muscle cells were isolated from the thoracic aortas of five male Sprague-Dawley rats according to the modified method of Owens *et al.* (1986). All subsequent procedures were carried out under sterile conditions. The thoracic aortas were obtained under pentobarbital anesthesia (50 mg/kg) and were placed in Petri dish (Corning, Corning, NY, USA) that contained 4°C phosphate-buffered saline. After the blood clot and fat tissue were removed with forceps and gauze, the aortas were incubated at 37°C for 30 minutes in 7.5 ml of Eagle's minimum essential medium (EMEM) (Sigma Chemical Co., St. Louis, MO, USA) containing 1.72 mg/ml collagenase (Worthington Biochemical Co., Freehold, NJ, USA), penicillin (100 U/ml) (Sigma Chemical Co.) and streptomycin (100 mg/ml) (Sigma Chemical Co.). The adventitia and intima were removed and the remaining aortas were minced with sterile razor blades. Small pieces of aorta tissue were transferred into 15 ml tube containing 7.5 ml collagenase mixture and were incubated again at 37°C

for 1~1.5 hours with continuous shaking. The tissue was then centrifuged at $1,000 \times g$ for 5 minutes and the supernatants were removed and the cells were dispersed in EMEM. After gentle agitation and centrifuge, the supernatants were discarded again and the cells were resuspended in EMEM supplemented with 10% fetal calf serum (FCS) (Gibco, Grand Island, NY, USA) to a cell concentration of about 5×10^5 /ml, and were then plated on 35 mm² culture dishes (Corning). Cell cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂ with media changes every 2~3 days. The cells became confluent 7~10 days after inoculation and were harvested by brief exposure to trypsin/EDTA (0.25%/0.1%) and transferred into a new dish. Harvesting was repeated when the cells became confluent and we used the cells of the 3rd-to-5th passage for experiments. The cells demonstrated α 1-actin molecules by anti- α 1-actin antibody (DAKO Japan Co., Kyoto, Japan) and were not stained by anti-factor VII antigen antibody (DAKO Japan Co.).

DNA synthesis

The rate of DNA synthesis was assessed by measuring [3 H]-thymidine (Du Pont Co., Wilmington, DE, USA) incorporated into the DNA strands of smooth muscle cells. Cells were seeded into 96-well culture plates (Corning) at a density of 1×10^4 cells per well and grown to confluence. The cells were then made quiescent by substituting 10% FCS-containing medium with EMEM containing 0.1% FCS, insulin (5 μ g/ml) (Sigma Chemical Co.), transferrin (5 μ g/ml) (Sigma Chemical Co.), and sodium selenite (5 ng/ml) (Sigma Chemical Co.). After 72 hours, cells were replenished with 10% FCS-containing medium for 24 hours to stimulate mitogenesis and then were pulsed with [3 H]-thymidine (2 μ Ci/well) for 6 hours. Cells were harvested using a cell harvester (Titertek Cell Harvester 550, Flow Laboratories, Irvine, Scotland, UK), collected on glass fiber filters and placed in a 3 ml scintillation cocktail for determination of total radioactivity. The radioactivity of this solution was measured with a β -counter (TL 5000S, Beckman Instruments Inc., Fullerton, CA, USA). Experiments were performed in triplicate.

Cell proliferation

For determination of cell proliferation, cells were seeded into 24-well culture plates (Corning) at a density of 2×10^4 cells per well and grown to subconfluence. After culturing cells in serum-free medium for 72 hours, the cells were cultured for a further 5 days in EMEM containing 10% FCS. Cell counts were performed on triplicate wells by trypsinizing the cells and counting viable cells using a hemocytometer. Cell viability was checked by exclusion of 0.4% trypan blue.

The effect of anti-hypertensive drugs on DNA synthesis and proliferation of smooth muscle cells

At quiescent stage, nifedipine, furosemide, hydrochlorothiazide, captopril, and atenolol were individually added to separate wells at a concentration of 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} M respectively with 10% FCS-containing EMEM, simultaneously. The anti-proliferative effects were evaluated through [3 H]-thymidine uptake and cell numbers, as described above.

Statistical analysis

Values are expressed as mean \pm SEM. Statistical difference was assessed by one-way analysis of variance and probabilities of 0.05 or less were interpreted as being statistically significant.

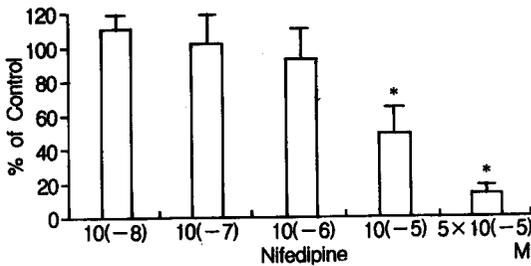


Fig. 1. The effect of nifedipine on serum-induced DNA synthesis of aortic smooth muscle cells assessed by 3 H-thymidine uptake. N: 3 independent experiments, each was in triplicate. Control: Cells incubated only with serum *: $P < 0.05$ vs. control

RESULTS

The effect of nifedipine on DNA synthesis and proliferation of aortic smooth muscle cells

[3 H]-thymidine incorporation increased from basal levels of 558.3 ± 65.1 cpm/well to 3435.7 ± 450.3 cpm/well by 10% FCS ($p < 0.001$). The effects of nifedipine on DNA synthesis of smooth muscle cells are shown in Fig. 1. FCS-induced DNA synthesis was inhibited significantly with nifedipine over a concentration of 10^{-5} M. Nifedipine at a concentration of 10^{-5} M and 5×10^{-5} M inhibited FCS-induced [3 H]-thymidine incorporation significantly by 50.8% and 86.6%, respectively ($p < 0.05$). In addition, nifedi-

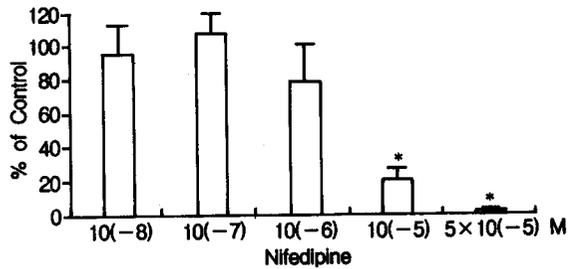


Fig. 2. The effect of nifedipine on serum-induced proliferation of aortic smooth muscle cells assessed by cell numbers. N: 3 independent experiments, each was in triplicate. Control: Cells incubated only with serum *: $p < 0.05$ vs. control

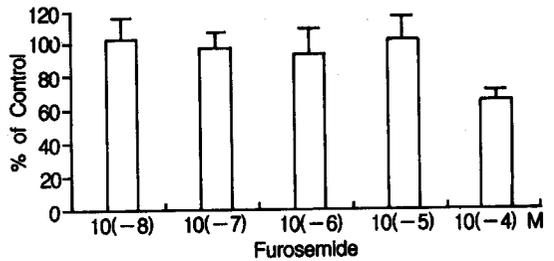


Fig. 3. The effect of furosemide on serum-induced DNA synthesis of aortic smooth muscle cells assessed by 3 H-thymidine uptake. N: 3 independent experiments, each was in triplicate. Control: Cells incubated only with serum

pine produced a significant inhibition on the increased number of cells induced by 10% FCS. After 5 days in culture, cell numbers were reduced by 77.5% with 10^{-5} M nifedipine and by 98.6% with 5×10^{-5} M nifedipine ($p < 0.05$) (Fig. 2).

The effect of furosemide on DNA synthesis and proliferation of aortic smooth muscle cells

Furosemide at a concentration less than 10^{-5} M did not inhibit FCS-induced DNA synthesis and proliferation of smooth muscle cells, but 10^{-4} M furosemide showed a small inhibitory effect on DNA synthesis by 32.6% with no statistical significance (Fig. 3).

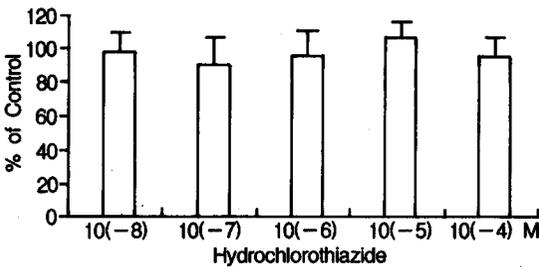


Fig. 4. The effect of hydrochlorothiazide on serum-induced DNA synthesis of aortic smooth muscle cells assessed by ^3H -thymidine uptake.

N: 3 independent experiments, each was in triplicate. Control: Cells incubated only with serum

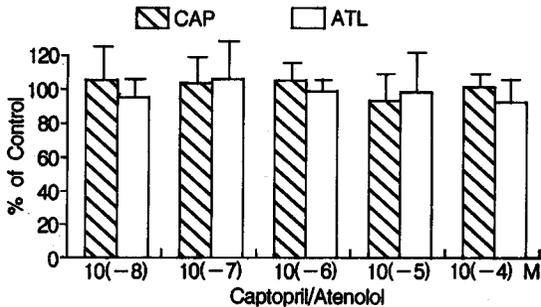


Fig. 5. The effect of captopril (CAP) and atenolol (ATL) on serum-induced DNA synthesis of aortic smooth muscle cells assessed by ^3H -thymidine uptake.

N: 3 independent experiments, each was in triplicate. Control: Cells incubated only with serum

The effect of hydrochlorothiazide, captopril, and atenolol on DNA synthesis and proliferation of aortic smooth muscle cells:

Neither of hydrochlorothiazide, nor captopril, nor atenolol had any significant effect on [^3H]-thymidine uptake induced by 10% FCS (Fig. 4, 5). The results of proliferation assessed by cell numbers paralleled the results of [^3H]-thymidine incorporation (data not shown).

DISCUSSION

Proliferation of arterial smooth muscle cells is a major event in the pathogenesis of atherosclerosis (Campbell and Campbell, 1985; Ross, 1986; Grünwald *et al.* 1987; Ip *et al.* 1990; Pickering *et al.* 1992), so an important therapeutic aim in treatment of atherosclerosis is to inhibit VSMC proliferation. Much of the work on inhibitors of VSMC proliferation has been carried out using cell culture technique. As a result, there have been many reports that heparin (Clowes and Karnovsky, 1977; Hoover *et al.* 1980; Reilly *et al.* 1986; Bärzu *et al.* 1992), transforming growth factor- β (Assoian and Sporn, 1986; Owens *et al.* 1988; Morisaki *et al.* 1991), high-dose synthetic progestogens (Spagnoli *et al.* 1990), and monoclonal antibody to PDGF (Ferns *et al.* 1991) inhibited VSMC growth successfully, but there are many limitations in using these agents for treatment of atherosclerotic patients.

Hypertension is very closely associated with atherosclerosis and many atherosclerotic patients are hypertensive and take anti-hypertensive medications. In the clinic, various anti-hypertensive drugs including calcium channel blockers (nifedipine, felodipine, nicardipine); diuretics (furosemide, hydrochlorothiazide); ACEi (captopril, enalapril); and β -adrenergic blockers (atenolol, metoprolol) are used to treat hypertension. It is well known that these drugs lower blood pressure by reducing circulating plasma volume or decreasing peripheral vascular resistance, but there have been few studies on the effect of these drugs on VSMC proliferation.

Several lines of evidence have revealed the role for Ca^{2+} in the regulation of cell proliferation (Kanbe

et al. 1983; Kaibuchi *et al.* 1986; Sibbitt, 1988), so it has been suggested that calcium channel blockers might inhibit the mitogenic signals. Nilsson *et al.* observed that nifedipine, a calcium antagonist, slowed down the rate of transformation of the SMC from a contractile to a synthetic phenotype and inhibited initiation of DNA synthesis as well as cellular proliferation induced either by 25 mg/ml PDGF or 10% newborn calf serum (Nilsson *et al.* 1985). Block *et al.* also reported that three calcium channel blockers, nifedipine, verapamil, and diltiazem, effectively inhibited PDGF-AA and -BB-induced proliferation of SMC (Block *et al.* 1989). They found that the inhibitory effect on PDGF-BB-dependent DNA synthesis was greatest with verapamil ($IC_{50}=3.3 \times 10^{-7}M$) and least with nifedipine ($IC_{50}=5.4 \times 10^{-6}M$). The anti-proliferative effect of calcium channel blockers was demonstrated *in vivo* as well as *in vitro*. Henry and Bentley tested the effects of nifedipine on atherosclerosis in cholesterol-fed rabbits and demonstrated that nifedipine reduced the percentage of intimal lesions stainable with Sudan IV from $40 \pm 5\%$ to $17 \pm 3\%$ without reducing hypercholesterolemia (Henry and Bentley, 1981). Similar data were also reported by Maggi *et al.* (1993). In this study, nifedipine inhibited DNA synthesis and proliferation of aortic SMC significantly at a concentration of more than $10^{-5}M$, and it was consistent with previous reports. Because measurement of cytosolic or phosphatidylinositol turnover was not performed in this study, the mechanism whereby nifedipine exerts its effect on FCS-induced DNA synthesis and proliferation can't be determined with certainty.

Furosemide and hydrochlorothiazide are widely-prescribed diuretics for patients with hypertension. Furosemide exerts its action by inhibiting $Na^+/K^+/Cl^-$ cotransport in the loop of Henle and hydrochlorothiazide by blocking Na^+/Cl^- cotransport mainly in the distal tubules. In addition to the diuretic action, there has been a study on the effect of furosemide on cell proliferation. Panet *et al.* reported that furosemide (0.5 mM) inhibited fibroblast growth factor (1 ng/ml)-induced vascular endothelial cell DNA synthesis by 80% and cell growth by 75% (Panet *et al.* 1994). They suggested that $Na^+/K^+/Cl^-$ cotransport might have an essential role in endothelial cell proliferation. In contrast to the results of Panet *et al.* (1994), in this study furosemide at a concentra-

tion of $10^{-4}M$ inhibited DNA synthesis of SMC by 32.6% without statistical significance. There may be a difference in signal transduction between endothelial cells and VSMC, and the maximal dose of furosemide tested in our experiment was lower than that in the study of Panet *et al.* (1994). Hadrava *et al.* observed that platelet extracts from patients receiving hydrochlorothiazide presented significantly greater growth-promoting activity on VSMC compared to those from patients treated with atenolol or captopril, but they did not explain why hydrochlorothiazide had this effect (Hadrava *et al.* 1991). Considering the results of this study, it seems that hydrochlorothiazide may modulate the growth-promoting activity released from platelets *in vivo* but may not have any effect on VSMC proliferation at the cell level. In this study, atenolol did not show any inhibitory effect on DNA synthesis and proliferation as expected.

It has been suggested by several laboratories that ACEi may have beneficial effects on decreasing the severity of atherosclerosis. Chobanian *et al.* showed that captopril (25 ~ 50 mg/kg/day) displayed a potent anti-atherogenic action by decreasing the amount of both aortic atherosclerosis and lesion cellularity (Chobanian *et al.* 1990). Similar results were obtained by Fennessy *et al.* in cholesterol-fed rabbit (Fennessy *et al.* 1994). They observed that treatment with 0.3 mg/kg/day perindopril in cholesterol-fed rabbit decreased the luminal surface area of thoracic aorta covered by lipid-filled plaques from 26.3% to 4.7%. In addition, culture studies revealed that perindopril decreased specific binding of ^{125}I -labeled β -very low density lipoprotein to passaged aortic SMC in a dose dependent manner, but had no effect on SMC proliferation. In contrast to the experimental injury model of atherosclerosis, in Goldblatt and spontaneously hypertensive rats, the increase in aortic SMC mass can be accounted for primarily by enlargement of existing cells, or cellular hypertrophy, rather than cellular proliferation (Owens and Schwartz, 1983). There have been many reports that angiotensin II was involved in this process (Geisterfer *et al.* 1988; Owens, 1989). Even though captopril did not show any anti-proliferative effect on cultured SMC in this study, which was consistent with that of Fennessy *et al.* (1994), captopril may be useful to hypertensive and atherosclerotic patients by

blocking the generation of angiotensin II.

In conclusion, among the various anti-hypertensive drugs, nifedipine seems to be most beneficial in view of its direct inhibitory effect on DNA synthesis and proliferation of aortic SMC, as well as for its anti-hypertensive effect.

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