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The involvement of reactive oxygen species and arachidonic acid in α_1-adrenoceptor-induced smooth muscle cell proliferation and migration

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1 In a previous study, we demonstrated phenylephrine-stimulated arachidonic acid (AA) release in rabbit cultured aortic smooth muscle cells. Therefore, we have investigated the functional implications of AA which are involved in the cellular response to phenylephrine, particularly proliferation and migration of rabbit cultured aortic smooth muscle cells.

2 First, to determine whether AA directly modifies proliferation and mobility of vascular smooth muscle cells (VSMCs), we exposed the cells to AA. AA induced proliferation and migration of the cells in a dose-dependent fashion. Concomitantly added catalase inhibited the proliferation and chemotaxis induced by AA of VSMCs. Conversely, aminotriazole enhanced the proliferation and migration induced by AA.

3 Secondly, we investigated whether the proliferation and migration of VSMCs by phenylephrine were related to AA and hydrogen peroxide (H_2O_2). The proliferation and chemotaxis of VSMCs by phenylephrine were inhibited by a phospholipase A_2 (PLA_2) inhibitor, or catalase.

4 Lastly, we investigated the effects of AA and phenylephrine on the content of H_2O_2 in VSMCs. AA and phenylephrine treatment led to an increase of H_2O_2 in a dose-dependent manner.

5 These results suggest that the addition of phenylephrine to the cells caused the enhancement of proliferation and migration, probably by mediating AA release and reactive oxygen species (ROS) production.

Keywords: Vascular smooth muscle cell; arachidonic acid; hydrogen peroxide; α_1-adrenoceptor; proliferation; migration

Introduction

Stimulation of adrenoceptors by endogenous catecholamines regulates a host of physiological responses in different cell types. With respect to vascular smooth muscle cells (VSMCs), we demonstrated that activation of α_1-adrenoceptors (α_1-AR) stimulated arachidonic acid (AA) release in cultured VSMCs (Nishio *et al*., 1996b). Apart from this, it has been demonstrated that activation of adrenoceptors regulates gene expression (Mark *et al*., 1990) and proliferation of the cells (Nakaki *et al*., 1990). Indeed, in this context, it has been suggested that adrenoceptor-mediated regulation of cell growth may play an important role in the development of the hyperplasia of VSMCs (Christopher & Stephen, 1992). These processes are thought to play a pivotal role in the development of atherosclerosis and in restenosis after angioplasty of human coronary arteries. However, the mechanisms responsible for these pathological changes are still incompletely understood.

An increasing body of evidence has indicated that reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) may modulate local pathological processes including atherosclerosis (Gadiparthi & Bradford, 1992). Previous studies have shown that elevated levels of ROS can trigger an intracellular signalling transduction pathway that may regulate cellular protection responses (Lo & Cruz, 1995) and ROS may function as a second messenger in the growth factor signal transduction pathway (Maitrayee *et al*., 1995).

AA and its metabolites are critical for a variety of physiological processes, such as chemotaxis (Siegel *et al*., 1982) in HL-60 cells, inflammation (Irvine, 1982), and signal transduction in FRTL-5 thyroid cells (Axelrod *et al*., 1988). In addition to these functions, AA is known to give rise to ROS through its subsequent metabolism in the brain (Coyle & Puttfarcken, 1993). Therefore, we examined whether α_1-AR stimulation results in the proliferation and migration of VSMCs and whether the proliferation and migration induced by α_1-AR stimulation are mediated by ROS production. There may be a relationship between VSMC proliferation (or migration), AA release stimulated by phenylephrine, and reactive oxygen production. To test this hypothesis, we studied the effects of AA on VSMC proliferation, migration and the content of ROS in VSMCs. Furthermore, we investigated the effect of phospholipase A_2 (PLA_2) and catalase on phenylephrine-stimulated proliferation and migration of VSMCs. We showed that the effect of phenylephrine-stimulated proliferation and migration of VSMCs may involve AA-stimulated ROS production, probably H_2O_2 production.

Methods

Cell culture

VSMCs were obtained from the thoracic aorta of the Japanese white rabbit by the method described by Bierman *et al*. (1974). The cells (1×10^5) were seeded into 35-mm diameter dishes and maintained in 2 ml of Dulbecco's modified Eagle's medium (DMEM), containing 10% foetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were used between the third and sixth passage. Cells were grown to confluence, at which time they were rendered quiescent by the DMEM medium containing 0.5% (v/v) foetal bovine serum and maintained for 72 h before experimentation.

Assay for DNA synthesis by VSMCs

The assay was performed by measuring the incorporation of [^{3}H]-thymidine into acid-insoluble materials (Dicker & Rozengurt, 1980). VSMCs were seeded at a density of 5×10^4

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cells/dish and grown to confluence. Then the medium was replaced by DMEM containing 0.5% (v/v) foetal bovine serum. Following another 3 days of cultivation, the cells were exposed to DMEM containing AA or phenylephrine in the presence of [^{3}H]-thymidine (5 μCi ml^{-1}) for 24 h. Acid-insoluble [^{3}H]-thymidine was extracted in 0.5 M NaOH and quantified by use of a liquid scintillation counter. Cell protein was quantified by the Bio-Rad protein assay (Bradford, 1976).

Cell migration assay

Migration of VSMCs was monitored in a Transwell cell culture chamber by using a polycarbonate membrane with pores of 8 μm, as described previously (Hidaka *et al.*, 1992). VSMCs were suspended in DMEM at a concentration of 2.5×10^6 cels ml^{-1}. In the standard assay, 200 μl of cell suspension in DMEM supplemented with or without various antioxidants was placed in the upper compartment of the chamber. The lower compartment contained 600 μl of DMEM supplemented with AA or phenylephrine at various concentrations as chemoattractant. Incubation was at 37°C in an atmosphere of 5% CO_2/95% air for 24 h. After incubation, nonmigrated cells on the upper surface were scraped gently, the filters were fixed in methanol and stained with 10% Giemsa stain. The number of VSMCs that had migrated to the lower surface of the filters was determined microscopically.

Detection of intracellular H_2O_2

Intracellular levels of H_2O_2 were analysed by fluorescence-activated cell sorting (FACS) by use of DCFH-DA as a probe (Lund-Johansen & Oluweus, 1992). Experiments were performed under dim light. Confluent, serum-deprived SMCs were incubated in dimethyl sulphoxide (DMSO) containing 5 mM DCFH-DA for 30 min after pretreatment with additional AA or phenylephrine for five minutes, then chilled on ice and washed with cold PBS. Washed cells were detached from culture plates by trypsin digestion. The activity of trypsin was quenched with 0.05% BSA in PBS. The fluorescent intensities of DCFH-DA for samples of 10,000 cells each were analysed by flow cytometry by use of a FACSscan flow cytometer equipped with an air-cooled argon laser.

Materials

Arachidonic acid, aminotriazole, bovine serum albumin and nitroarginine were obtained from Sigma. Aristolochic acid was from Biomol. 2′-7′-Dichlorofluorecin diacetate (DCFH-DA) from Molecular Probes and [^{3}H]-thymidine from Amersham. N-tert-butyl-2-sulphophenylnitorne (S-PBN) and N-acetyl-L-cysteine (NAC) were from Aldrich. Catalase was from Boehringer Mannheim.

Statistics

Values are expressed as the arithmetic mean ± s.e.mean. Statistical analysis of the data was performed by the one factor ANOVA-Scheffe F-test (StatView 512$^+$, version 1.0, Apple Computer, Inc). Duplicate wells were analysed for each experiment and each experiment was performed independently at least three times. $P<0.05$ was considered to be statistically significant.

Results

AA and phenylephrine stimulate DNA synthesis and chemotaxis through ROS production

Firstly, to determine the role of ROS in DNA synthesis and chemotaxis stimulated by AA and phenylephrine, VSMCs were treated with antioxidants (Figure 1). The enhancement of DNA synthesis and chemotaxis by AA and phenylephrine

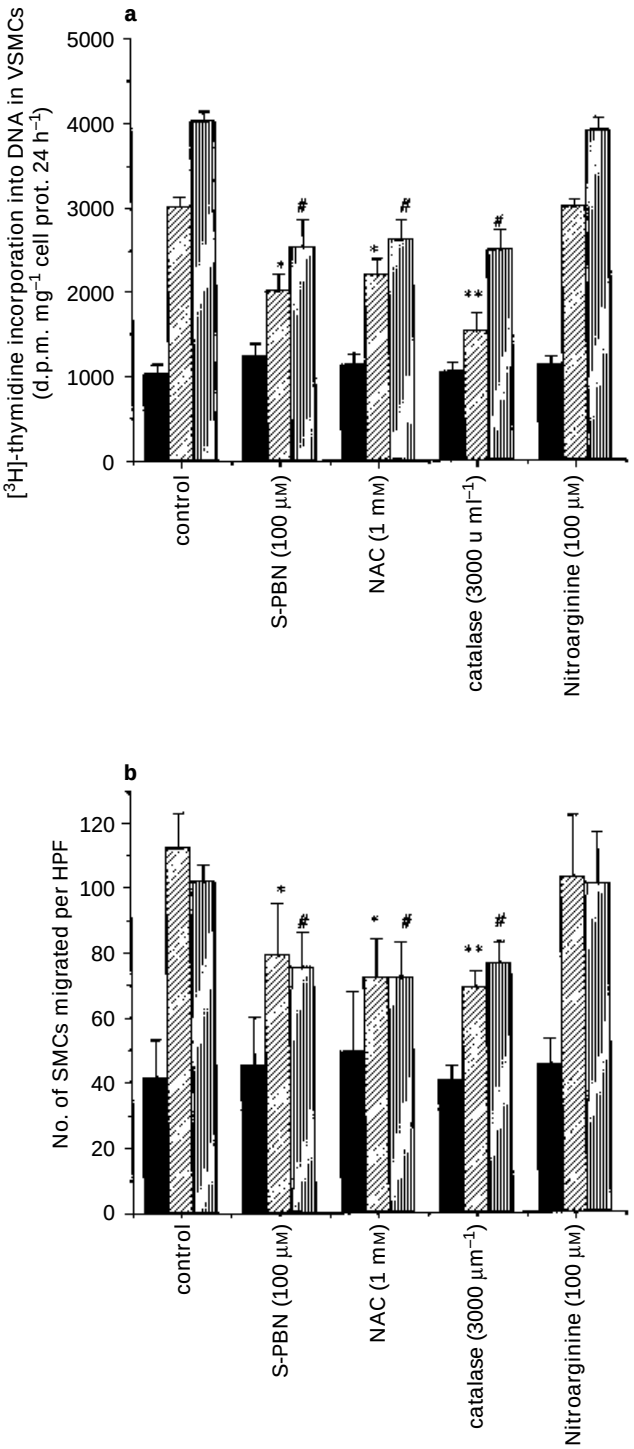

Figure 1 Antioxidants inhibit the enhancement of DNA synthesis and chemotaxis by phenylephrine and arachidonic acid (AA). Growth-arrested VSMCs were treated with AA (10 μM) or phenylephrine (10 μM) in the presence or absence of the indicated antioxidants in culture. (a) [^{3}H]-thymidine incorporation was measured over a 24 h period after the cells had been stimulated, as described in Methods. Each value represents the mean ± s.d. for three independent experiments in duplicate. *$P<0.05$ and **$P<0.01$, respectively, compared with AA alone. #$P<0.05$ compared with phenylephrine alone (a). (b) The migration of the cells was determined as described in Methods. Results were from three independent experiments in duplicate and are expressed as the number (mean ± s.d.) of migrated VSCCs per high-power field (HPF). *$P<0.05$ and **$P<0.01$ compared with AA alone. #$P<0.05$ compared with phenylephrine alone. In (a) and (b): solid columns, no addition to culture medium; diagonally hatched columns, AA (10 μM); vertically hatched columns, phenylephrine (10 μM).

were inhibited by N-tert-butyl-2-sulphophenylnitorne (S-PBN), the free-radical spin trap, and N-acetyl-L-cysteine (NAC), a glutathione precursor/radical scavenger. In addition, catalase, an enzyme that hydrolyses H_2O_2, inhibited the enhancement of DNA synthesis and migration. Nitroarginine (0.1–1 mM), a nitric oxide synthase inhibitor, did not significantly affect enhancement of DNA synthesis and chemotaxis of VSMCs by AA and phenylephrine. These results suggest that ROS derived from H_2O_2 partly mediate the enhancement of DNA synthesis and migration by AA and phenylephrine.

AA stimulates DNA synthesis and migration through H_2O_2 production

Secondly, to confirm the involvement of H_2O_2, we treated VSMCs with catalase or aminotriazole (ATZ), a catalase inhibitor, before the addition of AA. If H_2O_2 participates in the AA-stimulated proliferation and migration, catalase would potentially decrease the cellular response to AA. In contrast, ATZ would potentiate the cellular response to AA. As shown in Figures 2 and 3, AA elicited a dose-dependent increase in [^{3}H]-thymidine incorporation and migration. AA-induced [^{3}H]-thymidine incorporation and migration were reduced in the presence of catalase (3000 u ml^{-1}). AA-induced [^{3}H]-thymidine incorporation and migration were enhanced in the presence of ATZ (50 mM). Thus, increasing the catalase activity effectively decreased the cellular response to AA and decreasing the catalase activity effectively enhanced the cellular response to AA. These results strongly suggest the involvement of H_2O_2 in AA-induced [^{3}H]-thymidine incorporation and migration.

Phenylephrine-stimulated DNA synthesis and chemotaxis through AA production

We investigated the involvement of AA in phenylephrine-stimulated proliferation and migration of VSMCs. Pretreatment of VSMCs with aristolochic acid (50 μM), a specific inhibitor of phospholipase A_2 (PLA_2), blocked phenylephrine-stimulated VSMCs [^{3}H]-thymidine incorporation and migration VSMCs. Also, the enhancement of the cellular response by phenylephrine was inhibited by BSA (1 mg ml^{-1}), an inhibitor of reuptake of released AA. This PLA_2 inhibitor-induced inhibition of the cellular response was ameliorated by adding AA in a dose-dependent manner (Figures 3 and 4). These results substantiate the hypothesis that AA release at least partly mediates phenylephrine-stimulated VSMC proliferation and migration (Figures 3 and 4).

Phenylephrine-stimulated DNA synthesis and migration through H_2O_2 production

Further, to investigate the involvement of H_2O_2 in phenylephrine-stimulated SMC proliferation and migration, we pretreated VSMCs with catalase and ATZ. Catalase inhibited the cellular response while ATZ enhanced the cellular response to phenylephrine (Figures 3 and 5). These results suggest that H_2O_2 participates in phenylephrine-stimulated VSMC [^{3}H]-thymidine incorporation and migration.

Effect of AA and phenylephrine on the ROS content of VSMCs

For ROS to fulfill the role of a signalling intermediate for AA and phenylephrine, AA and phenylephrine must be able to induce the production of ROS. We measured the relative concentration of ROS in SMCs, by use of DCFH-DA and FACS. DCFH-DA is oxidized to membrane-impermeable, fluorescent DCFH-DA in the presence of H_2O_2 and possibly

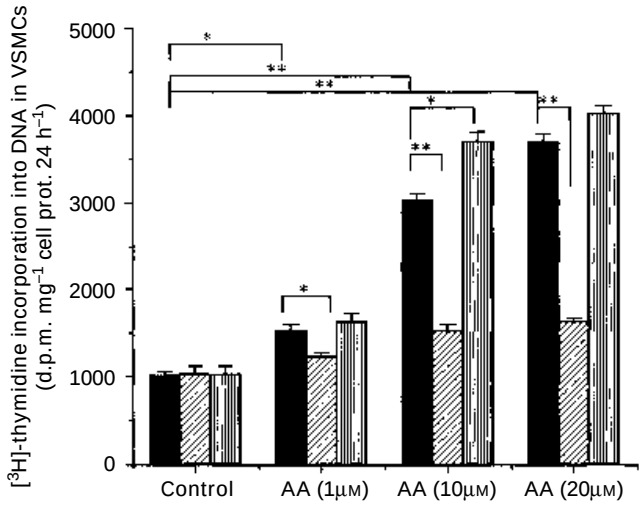

Figure 2 Effects of AA on [^{3}H]-thymidine incorporation in the absence (solid columns) and presence of catalase (diagonally hatched columns) or aminotriazole (ATZ), vertically hatched columns. Growth-arrested VSMC were treated with AA at varying concentrations in the absence or presence of catalase (3000 u ml^{-1}) or ATZ (50 mM). [^{3}H]-thymidine incorporation was measured over a 24 h period after the cells had been stimulated, as described in Methods. Each value represents the mean ± s.d. for three independent experiments in duplicate. *$P<0.05$ and **$P<0.01$.

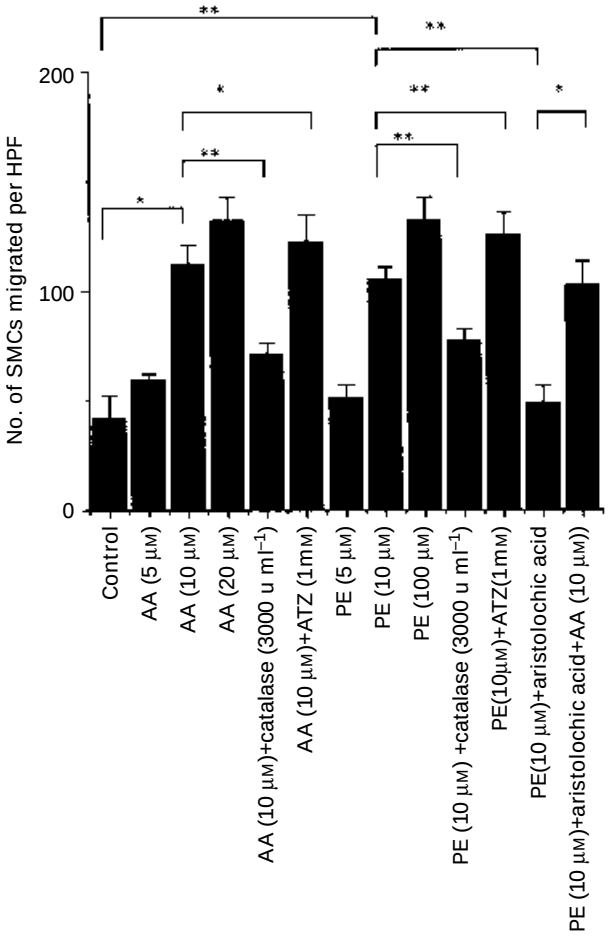

Figure 3 Effect of the AA and phenylephrine (PE) on migration. VSMCs were suspended in DMEM in the upper chamber. The lower chamber was filled with DMEM with the indicated migration factor. The migration of the cells was determined as described in Methods. Results were from three independent experiments in duplicate and are expressed as the number (mean ± s.d.) of migrated VSMC per high-power field (HPF). *$P<0.05$ and **$P<0.01$, respectively.

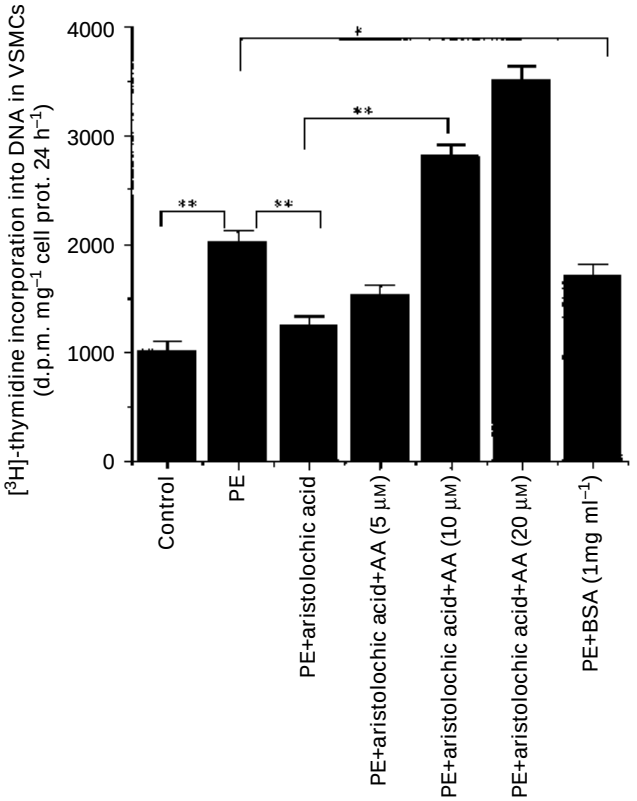

Figure 4 Effects of inhibition of phospholipase A_2 on [^{3}H]-thymidine incorporation. Growth-arrested VSMCs were pretreated with aristolochic acid (50 μM) a PLA_2 inhibitor, and the pretreated cells were stimulated with phenylephrine PE (10 μM) in the presence of various concentrations of AA or BSA (1 mg ml^{-1}). [^{3}H]-thymidine incorporation was measured over a 24 h period after the cells had been stimulated, as described in Methods. Results are the mean of three independent experiments in duplicate. $^*P<0.05$ and $^{**}P<0.01$.

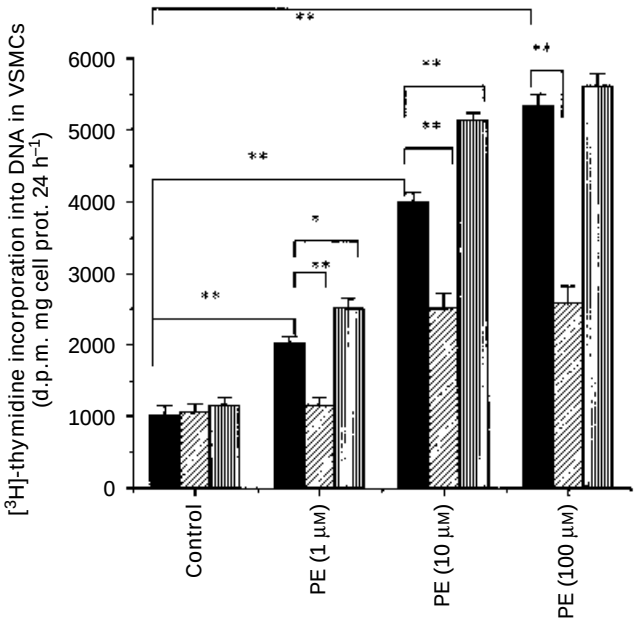

Figure 5 Effects of phenylephrine (PE) on [^{3}H]-thymidine incorporation in the absence (solid columns) and presence of catalase (diagonally hatched columns) or ATZ (vertically hatched columns). Growth-arrested VSMCs were treated with phenylephrine at varying concentrations and with catalase (3000 u ml^{-1}) or ATZ (50 mM). [^{3}H]-thymidine incorporation was measured over a 24 h period after the cells had been stimulated, as described in Methods. Each value represents the mean$\pm$s.d. for three independent experiments in duplicate. $^*P<0.05$ and $^{**}P<0.01$.

ROS derived from it. Incubation of VSCMs with AA and phenylephrine resulted in a concentration-dependent increase in fluorescent intensity (Figure 6, Table 1). Thus, AA and phenylephrine are capable of generating ROS in VSMCs.

Phenylephrine stimulated VSMC DNA synthesis through α_1-adrenoceptors

Lastly, to determine the specificity of α_1-adrenoceptors in phenylephrine-stimulated proliferation, VSMCs were stimulated with phenylephrine in the presence of the α_1-AR specific antagonist, prazosin or the α_2-AR specific antagonist, yohimbine. Figure 7 shows the effects of antagonists on [^{3}H]-thymidine incorporation induced by 10 μM phenylephrine in VSMCs. Prazosin 100 μM completely inhibited the phenylephrine-stimulated response. The IC_{50} value of prazosin was about 1 μM in VSMCs. Under the same experimental conditions, yohimbine showed a weak inhibition of 10 μM-phenylephrine-stimulated VSMC proliferation even at 100 μM. These results indicate that phenylephrine-induced VSMC proliferation is mediated by the stimulation of α_1-AR in the cells.

Discussion

Proliferation and chemotaxis of VSMCs is a key event, pathologically, in the development of atherosclerotic lesions and vascularization of tumours. As mentioned in the Introduction, α_1-adrenoceptors stimulate the proliferation of the cells. Previously, it was found that phenylephrine-stimulated VSMC proliferation was blocked by pertussis toxin, which indicated that pertussis toxin-sensitive G proteins exerted a modulatory effect on the proliferative responses of VSMCs to α_1-AR stimulation (James & Michael, 1994). However, no studies have investigated a further mechanism for phenylephrine-induced VSMC function.

In a previous paper, we demonstrated that phenylephrine stimulated AA release through pertussis toxin-sensitive G protein in VSMCs (Nishio *et al*., 1996b). There have been many publications about the ability of AA and its metabolites to modulate the proliferation of VSMCs *in vitro*. Prostaglandins (PG) of the E series (PGE_1 and PGE_2) (Loesberg *et al*., 1985) as well as of the A and J series inhibit VSMC proliferation (Sasaguri *et al*., 1992), while $PGF_{1\alpha}$ and $PGF_{2\alpha}$ stimulate proliferation (Cornwell *et al*., 1979). Metabolites of the lipoxygenase pathway, such as 12-hydroxyeicosatetraenoic acid, stimulate the migration of VSMCs (Nakao *et al*., 1982). Gadiparthi *et al*. (1994) showed that AA caused the activation of mitogen-activating protein kinase in VSMCs in a time- and dose-dependent manner. But there is no other clear explanation concerning the underlying mechanism.

H_2O_2 is the product of superoxide dismutase and several oxidases in the cells. H_2O_2 can diffuse freely across the membrane and give rise to the highly reactive hydroxyl radical. H_2O_2 has been shown to act as an intracellular second messenger. For example, H_2O_2 was demonstrated to act as a signal-transducing molecule in VSMCs stimulated by platelet-derived growth factor (Maitrayee *et al*., 1995). Therefore, we investigated whether AA and H_2O_2 are involved in phenylephrine-stimulated VSMC function.

The two observations that the proliferation and migration of VSMCs were enhanced by phenylephrine and that this phenomenon was abolished by catalase and PLA_2 inhibitor pretreatment may be closely related to H_2O_2 and AA production caused by the addition of phenylephrine. The addition of catalase even in large amounts (3000 u ml^{-1}) and the addition of a PLA_2 inhibitor for the duration of the experiment produced no visible effects on VSMCs and did not affect viability, as assessed by trypan blue exclusion. In addition, al-

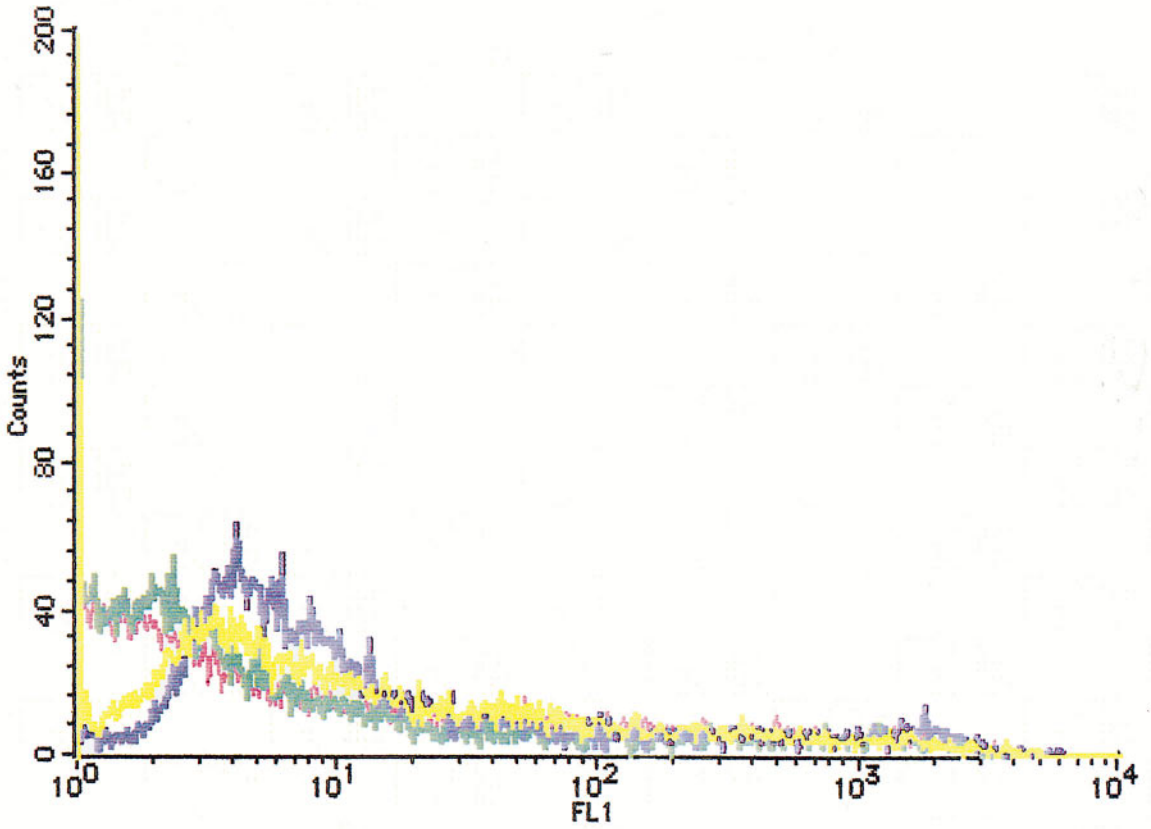

Figure 6 Effects of arachidonic acid (AA) on relative levels of intracellular H_2O_2 in VSMCs. Growth-arrested VSMCs were incubated for 30 min with 5 mM DCFH-DA, followed by a 5 min incubation with AA at various concentrations. The fluorescent intensities of 10,000 cells were analysed. The data are representative of three independent experiments. Red: control, green: AA (5 μM), yellow: AA (10 μM), purple: AA (20 μM).

Table 1 Effect of arachidonic acid (AA) and phenylephrine on the H_2O_2 content in vascular smooth cells

Treatment	*H_2O_2 content*
Control	100±17
AA (5 μM)	139±15
AA (10 μM)	150±21*
AA (20 μM)	171±23**
PE (1 μM)	112±18
PE (10 μM)	141±15*
PE (100 μM)	151±12*
PE (10 μM)+PLA_2 inhibitor (50 μM)	105±8

Growth-arrested VSMCs were incubated for 30 min with 5 mM DCFH-DA in the absence or presence of aristolochic acid (50 μM), followed by a 5 min incubation with AA or phenylephrine (PE) at the indicated concentrations. The level of intracellular H_2O_2 was estimated by FACS analysis. Data are expressed as the mean±s.d. of 10,000 independent measurements. *$P<0.05$, **$P<0.01$ vs control. Repeated three times with similar results.

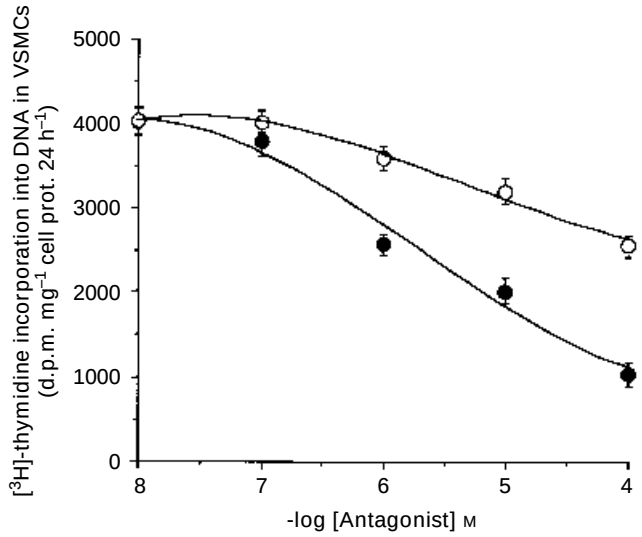

Figure 7 Effect of prazosin (●) and yohimbine (○) on the phenylephrine (10 μM)-stimulated [^{3}H]-thymidine incorporation. Growth-arrested VSMCs were incubated with phenylephrine and indicated antagonists. [^{3}H]-thymidine incorporation was measured over a 24 h period after the cells had been stimulated as described in Methods. Each point represents the mean±s.d. (vertical lines) of three independent experiments performed in triplicate.

though catalase-loaded cells had attenuated responses to phenylephrine and AA, the NO-apoptotic activity did not change, when a NO donor (SNAP) was added to the medium (Nishio *et al*., 1996a). This indicates that the H_2O_2 and NO pathways are separable and that increased intracellular catalase does not have a global effect on cell signal transduction pathway.

In summary, data presented in this study demonstrate that phenylephrine-stimulated DNA synthesis and migration are at least partly mediated by AA and ROS products, and also suggest that AA, which was released from phenylephrine-stimulated VSMCs, produces ROS containing H_2O_2. Indeed, phenylephrine-stimulated proliferation and migration may be mediated by H_2O_2 production by AA, because the H_2O_2 content of the cells decreased when the cells were pretreated with a PLA_2 inhibitor before treatment with phenylephrine when

compared to the H_2O_2 content of VSMCs that had been treated with phenylephrine alone (Table 1). Nevertheless, other routes of H_2O_2 generation were not excluded and there may be another pathway independent of AA. For example, another phospolipid such as platelet-activating factor (PAF, Honda *et al*., 1994), phosphatidic acid (Inui *et al*., 1994), or lysophosphatidylcholine (Ohara *et al*., 1994) may be involved. It remains to be elucidated whether the AA-dependent component is the main signal transduction system or whether there is another component, such as a protein kinase C-dependent component. But, these observations also raise the possibility that oxidative stress may predispose to the early onset of atherosclerosis. If these findings in cell culture are confirmed *in vivo*, then the antioxidants may provide an important therapeutic approach to atherosclerosis induced by hypertension.

Abbreviations

AA, arachidonic acid; VSMCs, vascular smooth muscle cells; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; PLA_2, phospholipase A_2; α_1-AR, α_1-adrenergic receptor; DCFH-DA, 2′-7′-Dichlorofluorecin diacetate; S-PBN, N-tert-butyl-2-sulphophenylnitorne; NAC, N-acetyl-L-cysteine; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorting; PG, Prostaglandin; ATZ, aminotriazole.

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