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The regulation of mitogenesis and apoptosis in response to the persistent stimulation of α_1-adrenoceptors: a possible role of 15-lipoxygenase

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1 Activation of α_1-adrenoceptor stimulation regulates eicosanoid metabolism and growth in vascular smooth muscle cells (VSMCs). The purpose of this study was to investigate the functional implications of lipoxygenase pathway in α_1-adrenoceptor-stimulated VSMCs growth through mutually exclusive biological functions, that is cell proliferation and cell death.

2 Phenylephrine (10 μM), a specific α_1-adrenoceptor agonist, enhanced [^{3}H]-thymidine incorporation by 300% above basal. Nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor, caused 36 and 50% decrease in phenylephrine (10 μM)-stimulated [^{3}H]-thymidine incorporation at concentrations of 1 μM and 10 μM respectively.

3 Inversely, treatment of phenylephrine (10 μM)-stimulated VSMCs with NDGA induced DNA fragmentation in a dose-dependent fashion. The level of induction of DNA fragmentation by NDGA was 225, 319 and 406% above the phenylephrine (10 μM)-level at concentrations of 0.1 μM, 1 μM and 10 μM, respectively. This induction of DNA fragmentation was partially prevented by exogenous 15-hydroxyeicosatetraenoic acid (15-HETE). The inhibition of apoptosis was 53 and 63% at concentrations of 5 μM and 10 μM of 15HETE, respectively, as compared with phenylephrine (10 μM) in the presence of NDGA (10 μM).

4 Furthermore, we performed the time-course analysis of Bcl-2 protein expression in phenylephrine (10 μM)-stimulated VSMCs. The expression of Bcl-2 protein disappeared after a 2 h incubation in the presence of NDGA (10 μM), but remained stable after a 2 h incubation period in the absence of NDGA (10 μM).

5 These results suggest that the lipoxygenase pathway is involved in cell proliferation by preventing apoptosis through the level of Bcl-2 protein expression.

Keywords: Vascular smooth muscle cells; α_1-adrenoceptors; apoptosis; mitogenesis; lipoxygenase

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), it has been demonstrated that, apart from acute effects on vascular smooth muscle tone (Kenneth & Timothy, 1994), activation of α_1-adrenoceptors regulates gene expression (Mark *et al*., 1990), eicosanoid metabolism (Julius *et al*., 1988), and proliferation of VSMCs (James & Michaei, 1994). Thus it has been suggested that α_1-adrenoceptor-mediated regulation of cell growth may play an important role in the development of atherosclerotic vascular disease.

Apoptosis, or programmed cell death, plays an indispensible role in embryonic development (Martin *et al*., 1993), maturation of the immune system (David *et al*., 1995), and maintenance of tissue and organ homeostasis. The mechanism whereby apoptosis is induced has recently gained attention as one for possible treatments for a variety of diseases, including excessive cell proliferation (Thompson, 1995). For example, it should be possible to develop therapeutic agents to increase or decrease the susceptibility of particular cells to apoptosis. Drugs that promote apoptosis could amplify the effects of cancer chemotherapeutic agents on resistant cells. Examples of such agents might include inhibitors of Bcl-2 expression. Such pharmacological manipulation of apoptosis may be less harmful to the patient since apoptotic cells do not release their proinflammatory contents into the surrounding environment.

Previous studies indicated that apoptosis was observed in human atherosclerosis and restenosis (Jeffrey *et al*., 1995). Clowes *et al*. (1983) have suggested that there may be evidence of cell death and proliferation of SMCs in baloon-injured lesions. These authors indicated that in the baloon-injured rat carotid artery proliferative activity of VSMCs persisted at relatively high levels (3.8%) for up to 12 weeks after injury. In the absence of cell death, these authors calculated that the level of ongoing proliferation should have led to a 36% increase in number. However, total cell number was unchanged at 12 weeks. The authors concluded that cell death must account for their finding (Clowes *et al*., 1983).

The present study addressed the hypothesis that α_1-adrenoceptor stimulation modulates SMC numbers, through its pleiotropic biological action inducing two mutually exclusive cellular functions, that is cell proliferation and cell death.

Evidence is also accumulating that polyunsaturated fatty acid metabolites, synthesized by the smooth muscle cell itself, appear to play an important role in controlling the response of these cells to mitogens. The stimulation of α_1-adrenoceptors causes arachidonic acid release (Nishio *et al*., 1996c) and metabolites produced via the lipoxygenase pathway may be involved in the proliferation of VSMCs (Cornwell *et al*., 1979; Bailey *et al*., 1982).

We have found that the mitogenic responses caused by the α_1-adrenoceptor agonist are inhibited by a non-selective lipoxygenase inhibitor and that apoptotic responses caused by α_1-adrenoceptor agonists are increased by a non-selective lipoxygenase inhibitor. We also have found that α_1-adrenoceptor agonists increased lipoxygenase activity. These results suggest that the persistant stimulation of α_1-adrenoceptors results in the induction of mitogenesis and the prevention of apoptosis through the activation of lipoxygenase.

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Method

Cell culture

Aortic VSMCs were obtained from thoracic aorta of the Japanese white rabbit by the method described previously (Nishio *et al*., 1996b). 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 fifth passage. Cells were grown to confluence, at which time they were rendered quiescent by serum deprivation and maintained in serum-free medium for 36 h before experimentation.

In situ *labelling of nuclear DNA fragments*

A total of 3×10^5 cells/well were plated onto 4-chamber slides, cultured with DMEM containing 10% FBS. After confluence cells were cultured with DMEM only for 24 h. Thereafter cells were treated with phenylephrine (PE, 10 μM) and nordihydroguaiaretic acid (NDGA; 10 μM) in combination for 24 h. Then slides were fixed at room temperature for 20 min with 4% formaldehyde in PBS. Terminal deoxytransferase (TDT) and digoxigenin-dUTP in TDT buffer were then added to cover the slide, and slides incubated in a humidified chamber at 37°C for 2 h. The reaction was terminated by adding the stop/wash buffer for 30 min at 37°C, and incubated in the anti-digoxigenin-peroxidase solution for 50 min at room temperature. The slides were then rinsed with PBS for 15 min at room temperature and stained with 3,3′-diaminobenzidine tetrahydrochloride substrate solution containing 0.02% hydrogen peroxidase for 30 min at room temperature. Then slides were counterstained with methyl green for 30 s, washed in 100% butanol (Nishio *et al*., 1996a).

DNA fragmentation assay

VSMCs (1×10^5) were lyzed at 37°C for 60 min in a buffer containing 0.5% sodium N-lauroyl-sarcosinate, 50 mM Tris buffer (pH 7.8) and 10 mM EDTA. The lysate was then incubated in RNase (100 μg ml^{-1}) for 30 min at 50°C. After proteinase (100 μg ml^{-1}) treatment for 1 h at 50°C, DNA was extracted with a equal volume of phenol/chloroform, 1 : 1 and precipitated with 1/10 th vol 7 M ammonium acetate and 2.5 vol ice-cold ethanol at room temperature for 1 h (Herrmann *et al*., 1994), and 20 μg of DNA was run on a 1.2% agarose gel.

Quantification of fragmented DNA

To quantify DNA fragmentation, VSMCs labelled with [^{3}H]-thymidine (1 μCi ml^{-1}) were treated for 24 h in DMEM only. Then the cells were rinsed twice with PBS. Subsequently, the cells were incubated with phenylephrine and various inhibitors in combination for 24 h. Thereafter, lysis buffer (20 mM Tris, 4 mM EDTA (pH 7.4), 0.4% Triton X-100) was added to each culture well and the suspension was transferred to an Eppendorf tube, incubated on melting ice for 10 min, and centrifuged at 8,000 *g* for 5 min at 4°C. Subsequently, fragmented radiolabelled DNA in the supernatant was counted by liquid scintillation counting. Fragmented DNA results are expressed as a percentage of total DNA.

Immunoblotting

Cell lysates were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred electrophoretically to PVDF membranes. The membranes were probed with anti-bcl-2 mouse antibodies. After the membrane had been treated with peroxidase-conjugated goat anti-mouse secondary antibodies, peroxidase activity was detected by use of ECL reagents (Bennett *et al*., 1995).

[^{3}H]-thymidine incorporation

VSMCs were seeded at a density of 5×10^4 cells/dish and synchronized at the G_0/G_1 phase of the cell cycle by incubation in DMEM containing 0.5% (v/v) foetal bovine serum for 3 days. The medium was then removed and the cells were stimulated to proliferate in DMEM containing phenylephrine with or without NDGA, and [^{3}H]-thymidine was added (5 μCi ml^{-1}). After 24 h incubation, the incorporation of [^{3}H]-thymidine into acid-insoluble materials was measured (Hoshina *et al*., 1990).

Assay of 15-lipoxygenase activity

VSMCs, which were treated with an appropriate amount of phenylephrine for 24 h, were lyzed on ice with 0.1% (v/v) final concentration of Triton X-100 and stored on ice. Activity of 15-lipoxygenase was measured based on its ability to oxidize linoleic acid to 13(**S**)-hydroperoxy-octadecadienoate (13(**S**)-HPODE), which is further reduced in a reaction to the more stable corresponding 13(**S**)-hydroxy-octadecadienoate (13(**S**)-HODE) (Rapoport *et al*., 1979). This product was detected on a C18 reverse phase column at 234 nm and quantified by comparing the peak areas of individual samples versus standards (Biomol, Plymouth Meeting, PA). The reaction was started by addition of 10 μl linolenic acid (in ethanol, final concentration of 581 μM) to 100 μM sonicated cell extract and incubated for 10 min at 4°C. Addition of 100 μl of mobile phase (acetonitrile 350 : water 250 : methanol 150 : acetic acid 1) and 10 μl of trimethylphosphate stops the reaction and reduces the sample, which is then analysed (Rapoport *et al*., 1979).

Lactate dehydrogenase (LDH) assay

VSMCs (5×10^4/well) were cultured for 24 h in 96-well culture plates with appropriate amounts of NDGA in the presence of phenylephrine (10 μM). LDH activity was measured in cell lysates and culture supernatants by use of a calorimetric kit. The percentage of LDH release was calculated as: % LDH release = LDH released in culture supernatant/(LDH released in culture supernatant + LDH in cell lysate) (Mosmann, 1983).

Materials

Nordihydroguaiaretic acid (NDGA), indomethacin (preferentially inhibiting cyclo-oxygenase-1 (COX-1) over COX-2), ibuprofen (with comparable inhibition for both COX-1 and COX-2), 15-HETE, 12-HETE, 5-HETE, and 5, 8, 11-eicosatreinoic acid were obtained from Sigma [^{3}H]-thymidine and ECL Western blotting detection kit were from Amersham. Baicalein and caffeic acid were from Funakoshi Pharmaceutical, Tokyo. All cell culture materials from Life Technologies.

Statistical analysis

Results are expressed as mean ± s.d. The data were first analysed by use of two-way ANOVA, and the post hoc LAD analysis was used to compare the differences between intergroups. P values less than 0.05 were taken to be significant.

Results

NDGA inhibits phenylephrine-induced [^{3}H]-thymidine incorporation and induces cell cytotoxicity

The addition of NDGA into the culture medium caused inhibition of phenylephrine-stimulated VSMCs [^{3}H]-thymidine incorporation. The growth-inhibitory effect was obtained at 0.1 μM of NDGA and was dose-dependent (Figure 1a). Conversely, the addition of NDGA induced a dose-dependent increase in LDH release in phenylephrine-stimulated VSMCs (Figure 1b). In contrast to the results obtained with NDGA,

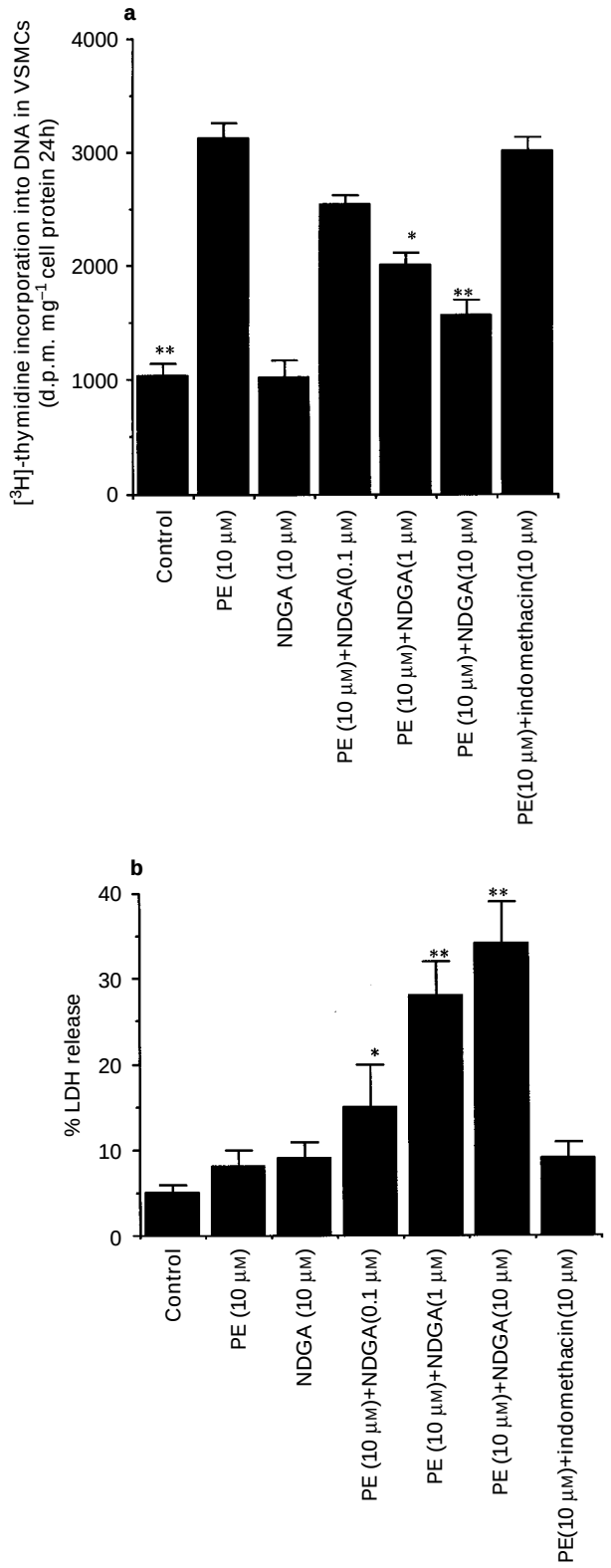

Figure 1 (a) Effect of NDGA or indomethacin on the [^{3}H]-thymidine incorporation into DNA of phenylephrine-treated VSMCs. Synchronized VSMCs were incubated with the inhibitors in the presence of phenylephrine (PE, 10 μM) and [^{3}H]-thymidine. After 24 h, the [^{3}H]-thymidine incorporation was determined. Results are expressed as the mean$\pm$s.d. for three independent experiments in duplicate. *$P<0.05$ and **$P<0.01$, compared with PE alone. (b) Effect of NDGA or indomethacin on the release of LDH from phenylephrine-treated VSMCs. VSMCs were cultured in 96-well plates with various inhibitors in the presence of phenylephrine (10 μM) for 24 h. LDH activity was measured as described in Methods. Each value represents the mean$\pm$s.d. for three independent experiments in duplicate. *$P<0.05$ and **$P<0.01$, compared with PE alone.

the cyclo-oxygenase inhibitors, indomethacin and ibuprofen did not induce the growth-inhibitory effect at the doses tested (5–100 μM) (Figure 1a and data not shown).

NDGA induces apoptosis in phenylephrine-treated VSMCs

The growth inhibition induced by NDGA was accompanied by induction of apoptosis, as revealed by *in situ* apoptosis labels by use of the TUNEL method (Figure 2), electrophoresis (Figure 3), and DNA fragmentation assay (Figure 4). NDGA and 5,8,11-eicosantrienoic acid (ETI), which are non-selective lipoxygenase inhibitors, induced potent cell death in a dose-dependent manner as assessed by the DNA fragmentation assay (Figure 4). However, baicalein, a selective inhibitor of 12-lipoxygenase, and caffeic acid, a selective inhibitor of 5-lipoxygenase, did not induce apoptosis in phenylephrine-treated VSMCs at 100 μM (Figure 4b). Furthermore, apoptosis in phenylephrine-treated VSMCs by NDGA was partially prevented by 15-HETE addition (Figure 4a), but only by 12-HETE or 5-HETE (Figure 4b). Neither phenylephrine, NDGA nor ETI alone induced apoptosis (Figure 4).

The effect of phenylephrine on the lipoxygenase activity in VSMCs

Figure 5a shows that 15-lipoxygenase activity measured with linoleic acid as substrate increased with time, reaching its peak 24 h after phenylephrine addition and remaining constant at 36 h. Therefore, the 24 h time point for 15-lipoxygenase activity was chosen for further experiments. Figure 5b demonstrates that 15-lipoxygenase activity increased in VSMCs treated with phenylephrine and was inhibited by the addition of NDGA in a dose-dependent fashion.

The effect of NDGA on the expression on Bcl-2 protein in VSMCs

The role of Bcl-2 protein in the apoptosis of phenylephrine-stimulated VSMCs by lipoxygenase inhibitor was examined. Blotting experiments revealed that NDGA treatment in the phenylephrine-stimulated VSMCs led to a decrease in Bcl-2 protein in a time- and dose-dependent manner (Figure 6), while phenylephrine alone caused no change in the level of Bcl-2. The decrease of Bcl-2 expression was prevented by 15-HETE, but not by 12-HETE or 5-HETE (Figure 6).

Discussion

Apart from their classical acute effects on vascular smooth muscle tone, the stimulation of α_1-adrenoceptors also causes proliferation of VSMCs (James *et al*., 1994) and induces metabolism of arachidonic acid in VSMCs (Nishio *et al*., 1996c). The findings of the present study provide new information demonstrating that stimulation of α_1-adrenoceptors exerts two opposing actions in regulating the number of VSMCs, that is cell proliferation and apoptosis.

Recently, immunohistochemical studies indicated the expression of a 15-lipoxygenase in human atheroma (Yla-Herttula *et al*., 1991) and in lesions of cholesterol-fed rabbits (Sparrow *et al*., 1988; Isabelle *et al*., 1995), but its absence in normal vessel wall. Furthermore, there is overwhelming evidence implicating 15-lipoxygenase in the oxidative process (Hartmut *et al*., 1997). And several lines of experimental evidence suggest that oxidative modification of low density lipoprotein is an important process for atherosclerosis (Holvot & Collen, 1994). However, only few experimental data are available as to the role of 15-lipoxygenase for cell growth.

Firstly, we investigated the role of lipoxygenase in VSMC growth caused by the stimulation of α_1-adrenoceptors

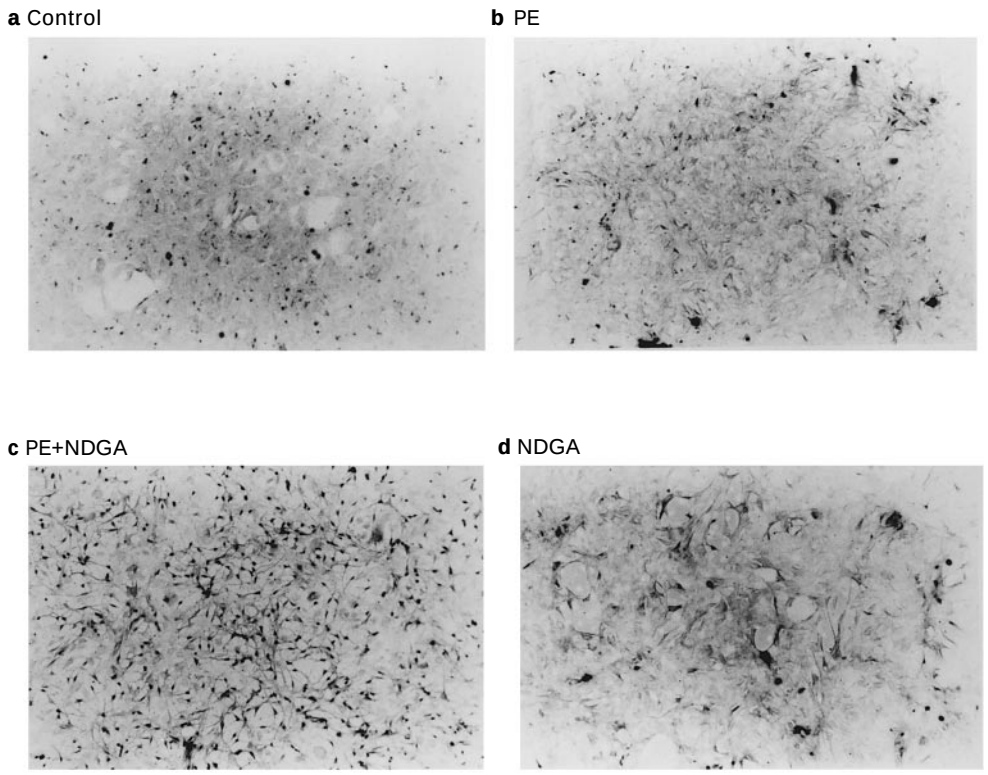

Figure 2 Apoptosis induction in SMCs exposed to phenylephrine (PE) and NDGA in combination. Synchronized VSMCs were incubated for 24 h with phenylephrine (10 μM) and NDGA (10 μM) alone and in combination. Thereafter, the cells were stained by the TdT-mediated dUTP nick end-labelling (TUNEL) method and imaged with Nikon optics. Apoptotic VSMCs exhibit positive nuclei staining. The results are representative of three independent experiments.

through proliferation and apoptosis. The non-selective NDGA decreased DNA synthesis and increased apoptosis in VSMCs by stimulating α_1-adrenoceptors in a dose-dependent manner. Indeed, NDGA is an indiscriminant, non-selective lipoxygenase inhibitor that also possesses many lipoxygenase-unrelated effects, such as blocking voltage-dependent Ca^{2+} currents (Stephen & Richard, 1990), inhibiting P450 monooxygenase activity (Agarwal *et al*., 1991), and working as an antioxidant (William, 1988). However, several lines of evidence suggest that NDGA-induced cell apoptosis occurs primarily through its effects on lipoxygenase. Firstly, at low concentrations (i.e., <10 μM), NDGA is known to have a preferential inhibitory effect on lipoxygenase activity (Goodman *et al*., 1994). In this study, NDGA at $1-10$ μM induced apoptosis of phenylephrine-treated VSMCs (Figure 4a). Secondly, the NDGA-induced DNA fragmentation could be inhibited by exogenous 15-HETE, which appeared to be one of the major lipoxygenase products of phenylephrine-treated VSMCs found in the h.p.l.c. assay (Figure 5). The apoptosis-blocking effects of 15-HETE could not be mimicked by its precursor, 15-HPETE (data not shown) or other lipoxygenase products (e.g. 12-HETE) (Figure 4b). Finally, NDGA is widely known as an antioxidant. However, from the preliminary data, apoptosis of phenylephrine-treated VSMCs triggered by NDGA appeared to involve free radical generation, since the process could be effectively inhibited by N-acetyl-L-cysteine (5 mM), the glutathione precursor/radical scavenger, which is thought to prevent apoptosis as an antioxidant (data not shown) (Tsai *et al*., 1996). These findings suggest that apoptosis in VSMCs occurs through a pathway unrelated to its antioxidant activity.

Secondly, we investigated whether the stimulation of α_1-adrenoceptors activates 15-lipoxygenase, to confirm the involvement of 15-lipoxygenase in α_1-adrenoceptor-stimulated DNA synthesis. Figure 5a shows that the stimulation of α_1-adrenoceptors activated the 15-lipoxygenase in a time-dependent manner. We used linoleic acid as substrate to assess for 15-lipoxygenase activity, because linoleic acid is the predominantly essential fatty acid in the vessel walls (Simon *et al*., 1989). Furthermore, Figure 5b demonstrates that 15-lipoxygenase activity increased in VSMCs treated with phenylephrine

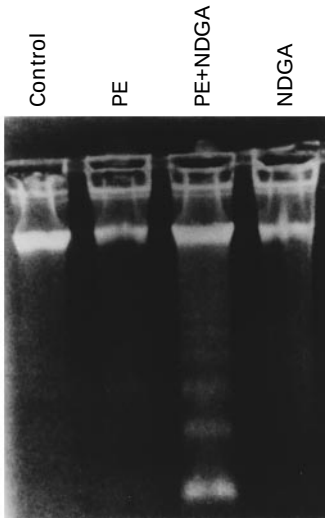

Figure 3 Effect of NDGA on DNA fragmentation in phenylephrine (PE)-treated VSMCs was assessed by electrophoresis. DNA, isolated from phenylephrine-treated VSMCs with the appropriate amount of NDGA for 12 h, was subjected to electrophoresis in 2% agarose and visualized with ethidium bromide. Lanes $1-4$ are control, phenylephrine (10 μM) alone, phenylephrine ((10 μM) + NDGA (10 μM) and NDGA (10 μM) alone. The results are a representative of three independent experiments.

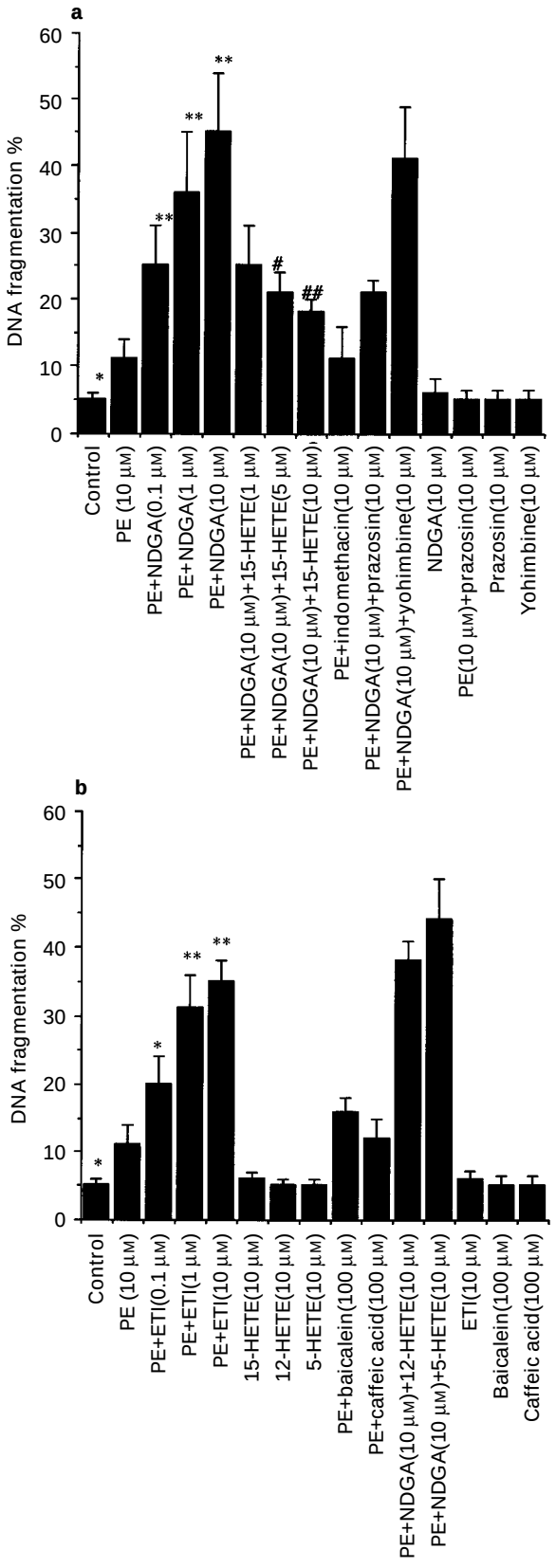

Figure 4 (a) Effects of NDGA or an α-blocker on DNA fragmentation in phenylephrine (PE)-treated VSMCs were assessed by DNA fragmentation assay. Synchronized VSMCs were treated with different concentrations of NDGA or α-blocker in the presence of phenylephrine (10 μM) for 12 h. DNA fragmentation is expressed as described in Methods. Each value represents the mean ± s.d. for three independent experiments in duplicate. $^{*}P<0.05$ and $^{**}P<0.01$, compared with PE alone. $^{\#}P<0.05$ and $^{\#\#}P<0.01$, compared with PE + NDGA (10 μM). (b) Effects of various lipoxygenase inhibitors on DNA fragmentation in phenylephrine-treated VSMCs were assessed by DNA fragmentation assay. Each value represents the mean ± s.d. for three independent experiments in duplicate. $^{*}P<0.05$ and $^{**}P<0.01$, compared with PE alone.

and was inhibited by the addition of NDGA in a dose-dependent fashion.

In the present study, in spite of complete inhibition of lipoxygenase activity by NDGA at 10 μM, phenylephrine induced-mitogenesis was not completely inhibited by NDGA at 10 μM (Figure 1a). This suggests that phenylephrine-induced mitogenesis is partly independent of the lipoxygenase pathway. Furthermore, the DNA fragmentation assay (Figure 3) demonstrated that phenylephrine alone without NDGA induced apoptosis compared to the control. Previously, we found that

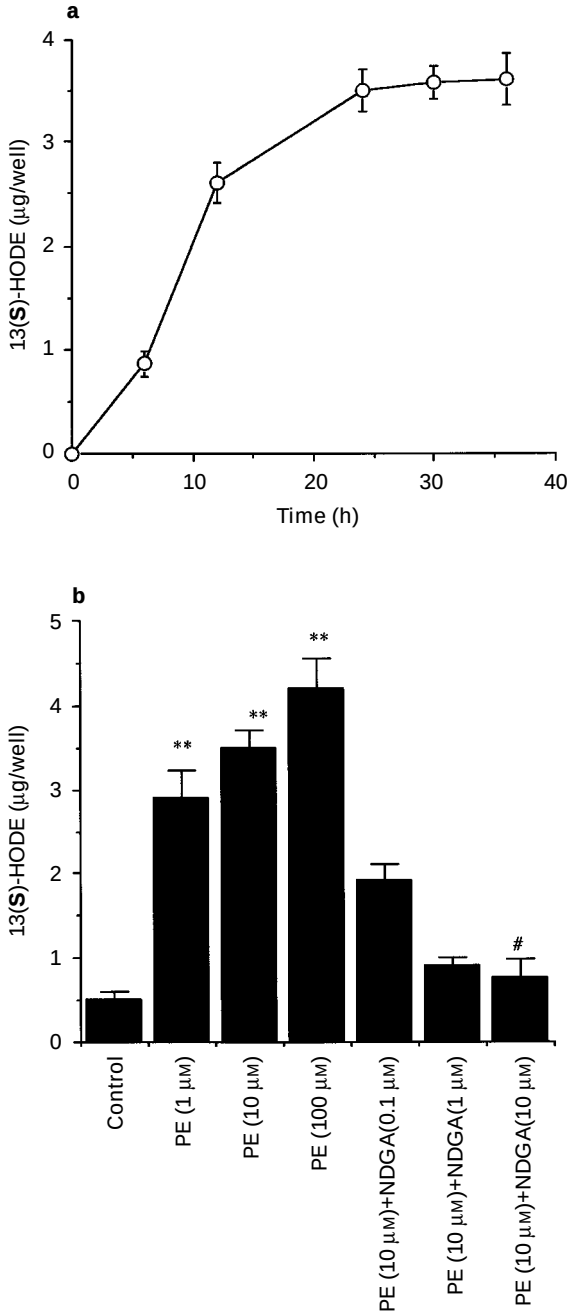

Figure 5 (a) The time course of phenylephrine (PE, 10 μM)-induced (13(**S**)-hydroxy-octadecadienoate (13(**S**)-HODE) production. Extract from cells at indicated times after phenylephrine (10 μM) stimulation were assayed for 13(**S**)-HODE production with linoleic acid as a substrate. Each value represents the mean and s.d. (vertical lines) for three independent experiments as amount of 13(**S**)-HODE per well. (b) Effect of phenylephrine on the activity of 15-lipoxygenase of VSMCs. The 15-lipoxygenase activity was assessed as described in Methods. Each of the values represents the mean ± s.d. for three independent experiments in duplicate. $^{**}P<0.01$, compared with control, $^{\#}P<0.05$ compared with PE (10 μM) alone.

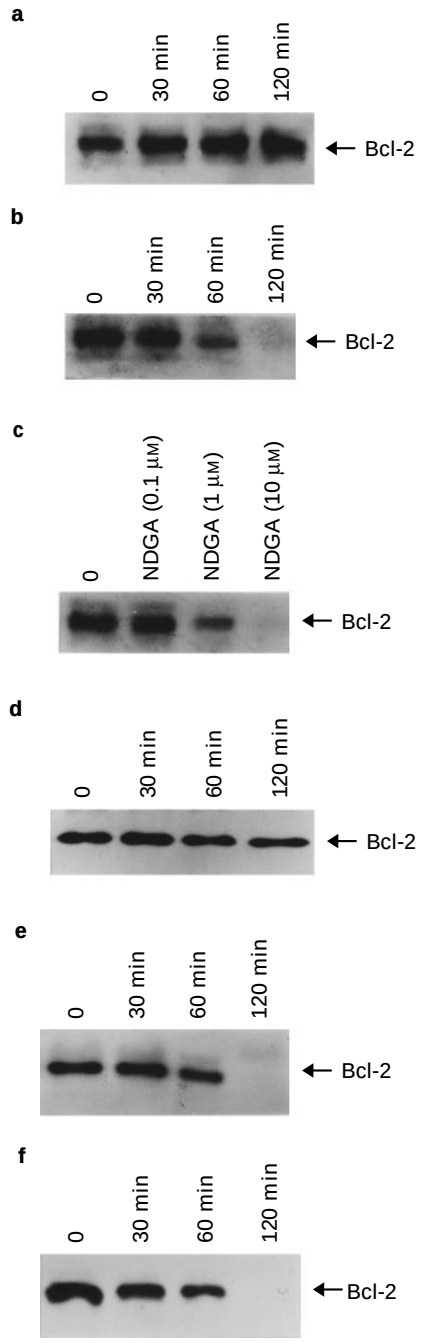

Figure 6 The effect of NDGA on the expression of Bcl-2 in VSMCs. Whole cell lysates of VSMCs treated with phenylephrine (10 μM) and the indicated amount of NDGA for the time intervals indicated were separated on SDS-PAGE. The membrane was probed and reprobed as described in Methods. The results are a representative of three independent experiments. (a) VSMCs with phenylephrine (10 μM) alone for the time intervals indicated. (b) VSMCs with phenylephrine (10 μM) and NDGA (10 μM) in combination for the time intervals indicated. (c) VSMCs with phenylephrine (10 μM) and NDGA at the indicated concentrations for 2 h. (d) VSMCs with phenylephrine (10 μM), NDGA (10 μM) and 15-HETE (10 μM) in combination for the time intervals indicated. (e) VSMCs with phenylephrine (10 μM), NDGA (10 μM), and 5-HETE (10 μM) in combination for the time intervals indicated. (f) VSMCs with phenylephrine (10 μM), NDGA (10 μM) and 12-HETE (10 μM) in combination for the time intervals indicated.

activation of protein kinase C by culturing with PMA (100 nM) for 8 h induced apoptosis in VSMCs (unpublished data) and the activation of α_1-adrenoceptors led to arachidonic acid release from VSMCs through pertussis toxin-sensitive G-protein (Nishio *et al*., 1996c). It is known that the α_1-adrenoceptor mainly couples to one class of G-protein, that is G_q. The activation of $G_q\alpha$ activates phospholipase C_β, which hydrolyzes PtdIns to produce Ins(1,4,5) P_3 and diacylglycerol (Berrige, 1993). The production of diacylglycerol subsequently activates the isotypes of protein kinase C. These results imply that there are at least two pathways to apoptosis in VSMCs, that is, one mediated by protein kinase C activation and the other mediated by arachidonic acid production.

Thirdly, we investigated the potential biochemical mechanisms involved in lipoxygenase-regulated apoptosis, particularly about Bcl-2 protein. The Bcl-2 proto oncogene is a 24–26 kDa protein which has been localized to the inner mitochondrial membrane. The major function of Bcl-2 appears to be an inhibitor of apoptosis (Reed, 1994). The molecular basis for its death-sparing activity has been proposed to be an antioxidant function (David *et al*., 1993). However, the findings that Bcl-2 still protects cells from apoptosis under anaerobic conditions suggest yet other molecular functions of Bcl-2 (Jacobson & Raff, 1995). Figure 6 demonstrates that NDGA treatment in the phenylephrine-treated VSMCs led to a dramatic decrease in Bcl-2 in a time- and dose-dependent manner, while phenylephrine alone induced no change in the level of Bcl-2. The mechanism for the rapid decrease of Bcl-2 is unknown. The decrease of Bcl-2 may be the result of rapid turnover rates induced by the addition of NDGA, since Bcl-2 is a long-lived protein with a half-life of 10–14 h (Yang *et al*., 1995). The decrease of Bcl-2 expression by both phenylephrine and NDGA was prevented by 15-HETE, but not 12-HETE or 5-HETE. This result would be expected from the effects observed in the DNA fragmentation assay (Figure 4a).

Lastly, there is the possibility that the inhibition of DNA synthesis by NDGA and ETI could be attributed to their α_1-adrenoceptor blocking properties. However, we found that NDGA and ETI did not inhibit α_1-adrenoceptor-stimulated MAP-kinase activation (data not shown). Therefore, it is unlikely that NDGA and ETI produce a general, non-specific inhibition of the response induced by the stimulation of α_1-adrenoceptors.

In conclusion, the present study suggests that, in phenylephrine-treated VSMCs, the lipoxygenase pathway is involved in cell mitogenesis and the cellular apoptotic machinery. In particular, although the lack of a specific 15-lipoxygenase inhibitor did not allow us to assess directly the involvement of 15-lipoxygenase in VSMC mitogenesis and apoptosis, 15-lipoxygenase may have such a regulatory role. The precise mechanism whereby the lipoxygenase pathway is coupled to apoptosis is unknown. However, intriguingly, it was observed in this study that after treatment of VSMCs with lipoxygenase inhibitor, there was a rapid decrease in the level of Bcl-2 protein. Further studies are required to dissect the role and molecular mechanisms of individual lipoxygenases in regulating cell mitogenesis and apoptosis, and to elucidate the cause-and-effect relationship between the lipoxygenase activity and important apoptosis-regulatory molecules such as Bcl-2 protein.

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