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European Journal of Pharmacology 336 (1997) 267–273

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# Role of the lipoxygenase pathway in phenylephrine-induced vascular smooth muscle cell proliferation and migration

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Received 30 January 1997; revised 5 August 1997; accepted 8 August 1997

**Abstract**

We studied the effects of phenylephrine-stimulated proliferation and migration of vascular smooth muscle cells and the role of 12-lipoxygenase-mediated pathways under normal as well as high glucose conditions. Phenylephrine-induced increases in cellular proliferation and migration were attenuated by the specific 12-lipoxygenase inhibitor baicalein. In contrast, neither of the cyclo-oxygenase inhibitors, indomethacin or ibuprofen, had any effect. Direct addition of the 12-lipoxygenase product, 12-*S*-hydroxyeicosatetraenoic acid (12-HETE), increased the proliferation and migration of vascular smooth muscle cells treated with both phenylephrine and nordihydroguaiaretic acid. Furthermore, we observed that phenylephrine induced greater increases in the proliferation and migration of vascular smooth muscle cells and also that the 12-lipoxygenase inhibitor prevented the enhancement of proliferation and migration of vascular smooth muscle cells induced by phenylephrine in the presence of high glucose (25 mmol/l). These results suggest that 12-lipoxygenase activation plays a key role in phenylephrine-induced responses of vascular smooth muscle cells under normal and hyperglycemic conditions. 12-lipoxygenase may be a good pharmacological target for treatment of vascular disease of hypertension and diabetes mellitus. © 1997 Elsevier Science B.V.

*Keywords:* Smooth muscle cell; Lipoxygenase; Proliferation; Migration

## 1. Introduction

Enhancement of the proliferation and migration of vascular smooth muscle cells is thought to be one of the main events implicated in the pathogenesis of atherosclerosis. Migration of these cells from media to intima is considered to be the first event following endothelial injury. Upon reaching the intima, they proliferate, incorporate lipids and biosynthesize and release connective tissue proteins. Repetition of these events causes the formation of a neointima of atheromatous plaque (Ross, 1993).

Stimulation of adrenoreceptors regulates the proliferation of cultured vascular smooth muscle cells, gene expression (Majesky et al., 1990) and eicosanoid metabolism (Axelrod et al., 1988). Indeed, in this context, it has been suggested that adrenoreceptor-mediated regulation of cell growth may play an important role in the development of vascular smooth muscle cell hyperplasia (Noveral and Grunstein, 1994). These processes are thought to play a pivotal role in the development of atherosclerosis and in restenosis after angioplasty of human coronary arteries (Hamon et al., 1995). However, the mechanism responsible for these pathological changes is still incompletely understood.

Diabetes mellitus is associated with the development of several vascular complications as well as with a substantially increased prevalence of hypertension and atherosclerotic cardiovascular disease (Colwell et al., 1983). Previous evidence demonstrated that vascular smooth muscle cells cultured in high glucose have an increased growth rate (Natarajan et al., 1992). It has also been recently demonstrated that vascular smooth muscle cells cultured in elevated glucose showed increased activity and expression of 12-lipoxygenase (Natarajan et al., 1993). However, the effects of high glucose conditions on phenylephrine-induced vascular smooth muscle cell responses, particularly proliferation and migration, have not been studied previously.

In this report, we investigated the role of the 12-lipoxygenase pathway in the phenylephrine-induced acceleration of proliferation and migration. Furthermore, these

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responses were compared in vascular smooth muscle cells cultured in the presence of normal glucose or high glucose concentrations. The results suggest that 12-lipoxygenase activation plays a key role in phenylephrine-induced acceleration of proliferation and migration and that under conditions of phenylephrine are enhanced in vascular smooth muscle cells cultured under conditions of high glucose.

## 2. Materials and methods

### 2.1. Materials

Nordihydroguaiaretic acid, indomethacin, ibuprofen, 15-*S*-hydroxyeicosatetraenoic acid (15-HETE), 12-HETE, 5-HETE, and 5,8,11-eicosatrienoic acid were obtained from Sigma. [$^3$H]thymidine was obtained from Amersham. Baicalein and caffeic acid were from Funakoshi Pharmaceutical, Tokyo. All cell culture materials were from Life Technologies.

### 2.2. Cell culture

Aortic vascular smooth muscle cells were obtained from the rabbit thoracic aorta by the method previously described (Nishio et al., 1996a). Briefly, the sections of aorta were rinsed with Dulbecco's Modified Eagle's Medium (DMEM) containing penicillin (100 U/ml)/streptomycin (100 μg/ml). The endothelial layer was peeled off and the underlying smooth muscle layers were placed on petri dishes. Vascular smooth muscle cells grew out from the explants within 3–4 days and confluence was achieved. Cells were passaged by using trypsin–EDTA and used for experiments from passages 2–6. The identity of the vascular smooth muscle cells in these explant cultures was confirmed by immunofluorescence with anti-alpha smooth muscle cell actin antibody.

### 2.3. Assay for DNA synthesis by vascular smooth muscle cells

The assay was performed by measuring the incorporation of [$^3$H]thymidine into acid-insoluble materials (Hayashi et al., 1992). Vascular smooth muscle cells were seeded at a density of $5 \times 10^4$ cells into 35 mm diameter dishes in 2 ml of DMEM containing 10% fetal bovine serum and incubated at 37°C in humidified atmosphere 5% $CO_2$/95% air for 24 h. Cells were synchronized in the $G_0/G_1$ phase of the cell cycle by incubating the cells with DMEM containing 0.5% (v/v) fetal bovine serum for 3 days. The medium was then removed and the cells were stimulated to proliferate in DMEM containing 0.5% (v/v) fetal bovine serum, phenylephrine and [$^3$H]thymidine (5 μCi/ml). [$^3$H]thymidine incorporation was measured over a 24 h period after the cells had been stimulated.

### 2.4. Cell migration assay

Vascular smooth muscle cell migration was monitored in a Transwell cell culture chamber by using a polycarbonate membrane with pores of 8 μm, as reported previously (Biro et al., 1993). Briefly, smooth muscle cells were suspended in DMEM at a concentration of $2.5 \times 10^6$ cells per ml. In the standard assay, 200 μl of cell suspension was placed in the upper compartment of the chamber. The lower compartment contained 600 μl of DMEM supplemented with 0.5% (v/v) fetal bovine serum and phenylephrine. Incubation was at 37°C in an atmosphere of 95% air/5% $CO_2$ for 24 h. After incubation, nonmigrated cells on the upper surface were scraped off gently and the filters were fixed in methanol and stained with 10% Giemsa stain. The number of vascular smooth muscle cells that had migrated to the lower surface of the filters was determined microscopically.

### 2.5. Assay of lipoxygenase activity

Vascular smooth muscle cells, which were treated with an appropriate amount of phenylephrine for 10 min, were lysed on ice using 0.1% (v/v) final concentration of Triton X-100 and stored on ice. The lysate was diluted with 50 mmol/l Tris–HCl buffer, pH 7.4, on ice and transferred in 50 μl samples to an ice-cold 96-well plate. The assay was initiated by the addition of 50 μl arachidonic acid (final concentration, 70 μmol/l) in 50 mmol/l Tris–HCl buffer, pH 7.4 and incubated at 37°C for 10 min. The assay was terminated by the addition of 100 μl Fox reagents: sulfuric acid (25 mmol/l), xylenol orange (100 μmol/l), iron(II) sulfate (100 μmol/l), methanol:water (9:1). Absorbance of the sample at 620 nm was measured on a plate reader (Waslidge and Hayes, 1995).

### 2.6. Measurement of lipoxygenase products 12- and 15-HETE

Serum starved confluent cells in normal glucose (5.5 mmol/l) or high glucose (25 mmol/l) in 100 mm dishes were placed in medium containing 0.2% bovine serum albumin and preincubated for 20 min at 37°C, with nordihydroguaiaretic acid (10 μmol/l) added during the last 10 min. Phenylephrine was then added and the cells were incubated for an additional 10 min. The reaction was terminated by cooling on ice. The HETE(s) in the supernatants was extracted as described (Natarajan et al., 1990). Cell pellets were first deacylated to release the cell-associated esterified HETEs by treating them with 1.5 ml of 0.2

M NaOH in methanol and 50 μmol/l *n*-propyl gallate under $N_2$ for 45 min. The solution was then diluted to 15% methanol and extracted. The lipoxygenase products in the supernatants as well as cell extracts were quantitated by a specific RIA (Advanced Magnetics, Cambridge, MA, USA).

### 2.7. Statistical analysis

Unless indicated otherwise, all experiments were carried out three or four times on different days and each was performed in duplicate. The results were expressed as mean ± S.D. mean. Statistical analysis was performed by the use of one-way analysis of variance followed by a Bonferroni *t*-test for multiple comparisons with a *P*-value $< 0.05$ being considered statistically significant.

## 3. Results

### 3.1. The effects of phenylephrine on the proliferation in vascular smooth muscle cells cultured in normal glucose and high glucose

Phenylephrine produced a dose-dependent increase in DNA incorporation in cells cultured under both normal glucose and high glucose conditions (Fig. 1). To determine the α-adrenoreceptor subtype involved in the changes in DNA incorporation, we performed additional experiments using the selective $\alpha_1$-adrenoreceptor antagonist prazosin and the $\alpha_2$-adrenoreceptor antagonist yohimbine. As shown in Fig. 2, prazosin but not yohimbine blocked the phenylephrine-induced increase in DNA incorporation in a dose-dependent manner.

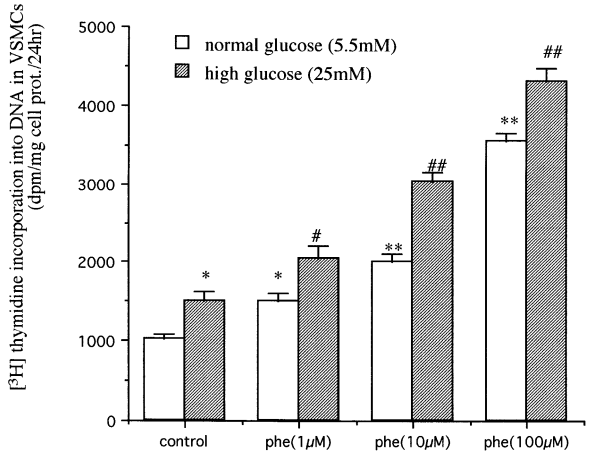


Fig. 1. Effect of phenylephrine on the [3H]thymidine incorporation into DNA of VSMCs. Synchronized VSMCs were incubated with phenylephrine at the indicated concentrations and [3H]thymidine in DMEM with 0.5% FBS and glucose. After 24 h, the [3H]thymidine incorporation was determined. Results are expressed as the mean ± S.D. mean for four independent experiments in duplicate. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, compared with the control under normal glucose. # and ## indicate $P < 0.05$ and $P < 0.01$ respectively, compared with the control under high glucose.

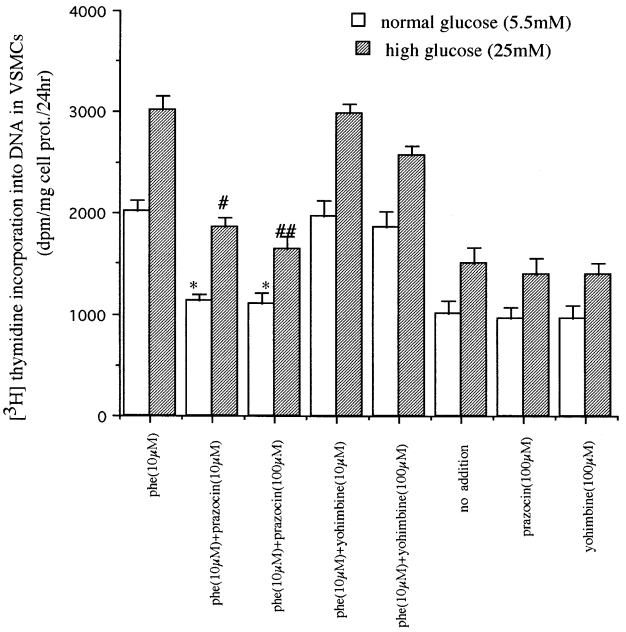


Fig. 2. Effect of prazosin or yohimbine on the phenylephrine (10 μmol/l)-stimulated [3H]thymidine incorporation. Growth-arrested VSMCs were incubated with phenylephrine and indicated antagonists. [3H]thymidine incorporation was measured over a 24 h period after the cells had been stimulated, as described in Section 2. Each point represents the mean ± S.D. mean of four independent experiments performed in triplicate. * indicates $P < 0.01$ compared with phe (10 μmol/l) alone under normal glucose. # and ## indicate $P < 0.05$ and $P < 0.01$ respectively, compared with phe (10 μmol/l) alone under high glucose.

### 3.2. Effects of lipoxygenase and cyclo-oxygenase inhibition on phenylephrine-induced DNA incorporation

Fig. 3 demonstrates the effects of the lipoxygenase inhibitor and specific cyclo-oxygenase inhibitors ibuprofen and indomethacin on phenylephrine-induced DNA incorporation. Neither inhibitor alone significantly altered basal DNA incorporation (data not shown). However, the general lipoxygenase inhibitors, nordihydroguaiaretic acid and 5,8,11-eicosatrienoic acid and the 12-lipoxygenase inhibitor, baicalein, significantly blocked phenylephrine-induced DNA incorporation both under normal and high glucose conditions. In marked contrast, the cyclo-oxygenase inhibitors indomethacin (preferentially inhibiting cyclo-oxygenase-1 over cyclo-oxygenase-2) and ibuprofen (with comparable inhibition for both cyclo-oxygenase-1 and cyclo-oxygenase-2) had no effect on phenylephrine action at the doses tested (5–100 μmol/l) (Fig. 3 and data not shown).

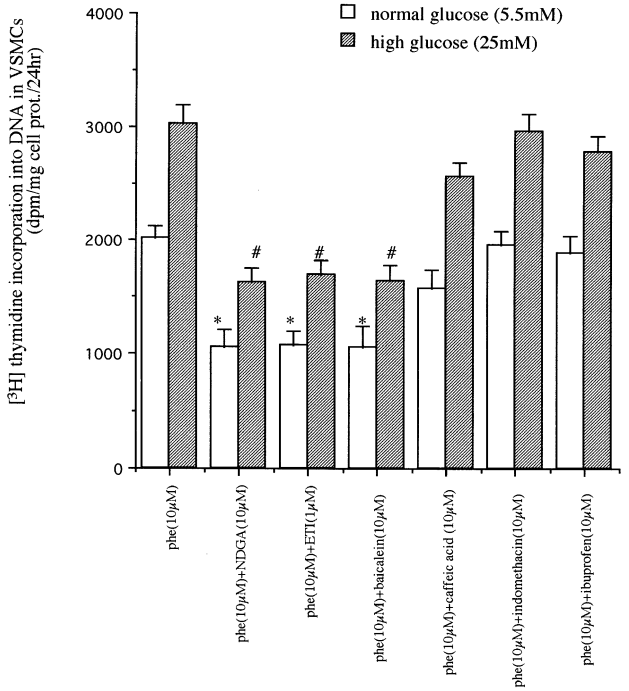


Fig. 3. Effect of lipoxygenase inhibitor and cyclo-oxygenase inhibitor on the [$^3$H]thymidine incorporation into DNA of phenylephrine-treated VSMCs. Synchronized VSMCs were incubated with the inhibitors in the presence of phenylephrine (10 μmol/l) and [$^3$H]thymidine under normal and high glucose conditions. After 24 h, the [$^3$H]thymidine incorporation was determined. Results are expressed as the mean ± S.D. mean for four independent experiments in duplicate. * indicate $P < 0.01$ compared with phe (10 μmol/l) only under normal glucose. # indicate $P < 0.01$ compared with phe (10 μmol/l) only under high glucose.

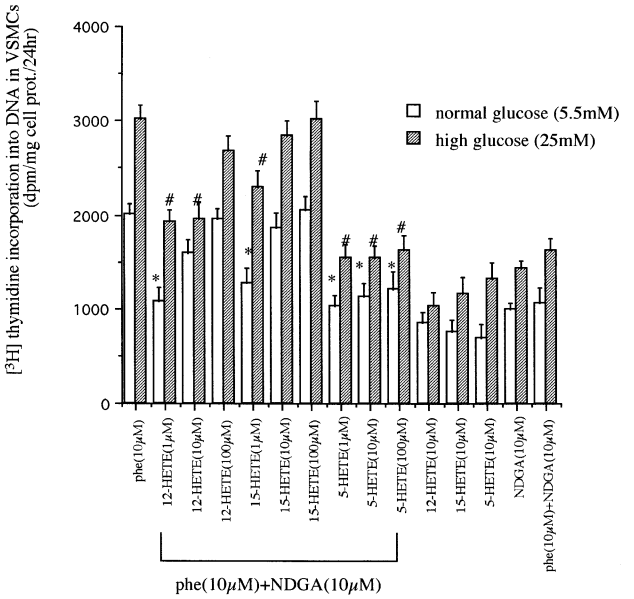


Fig. 4. Effect of lipoxygenase products on the [$^3$H]thymidine incorporation into DNA of both phenylephrine and NDGA-treated VSMCs. Synchronized VSMCs were incubated with the inhibitors in the presence of phenylephrine (10 μmol/l) and [$^3$H]thymidine and indicated lipoxygenase products. After 24 h, the [$^3$H]thymidine incorporation was determined. Results are expressed as the mean ± S.D. mean for three independent experiments in duplicate. * indicates $P < 0.01$ compared with phe (10 μmol/l) only under normal glucose. # indicate $P < 0.01$ compared with phe (10 μmol/l) only under high glucose.

### *3.3. Direct effects of 5-, 12- and 15-lipoxygenase products on DNA incorporation*

Vascular smooth muscle cells cultured in high glucose are reported to have an increased activity and expression of 12-lipoxygenase (Natarajan et al., 1992; Natarajan et al., 1993). We therefore evaluated the direct effect of addition of these lipoxygenase products on DNA incorporation in vascular smooth muscle cells cultured with normal glucose and high glucose. Fig. 4 shows that the addition of 12-HETE or 15-HETE reversed in a dose-dependent manner the inhibitory effects of nordihydroguaiaretic acid on the phenylephrine induced [$^3$H]thymidine incorporation in both cases. However, 5-HETE did not stimulate [$^3$H]thymidine incorporation in vascular smooth muscle cells treated with both phenylephrine and nordihydroguaiaretic acid in the presence of high glucose and normal glucose. These results suggest that a lipoxygenase, most probably 12-, 15-lipoxygenase and certainly not 5-lipoxygenase, may be involved in the regulation of phenylephrine-treated vascular smooth muscle cell proliferation. Both under normal glucose and high glucose conditions, 12-HETE, 15-HETE and

Table 1
Dose-dependent effects of phenylephrine on released 12-HETE and 15-HETE levels in VSMCs grown in the presence of normal glucose or high glucose concentrations

| | 12-HETE (pg/well) | | 15-HETE (pg/well) | |
|---|---|---|---|---|
| | Normal glucose | High glucose | Normal glucose | High glucose |
| Basal | 110 ± 12 | 130 ± 21 | 118 ± 10 | 138 ± 11 |
| Phe (1 μM) | 118 ± 16 | 138 ± 17 | 120 ± 13 | 135 ± 13 |
| Phe (10 μM) | 161 ± 19 [a] | 215 ± 18 [b] | 151 ± 14 [a] | 213 ± 11 [b] |
| Phe (100 μM) | 213 ± 12 [a] | 250 ± 13 [b] | 201 ± 13 [a] | 252 ± 19 [b] |

Note: 12- and 15-HETE levels were measured by a specific RIA of cell supernatants after 10 min treatment with or without phenylephrine. Results are expressed as means ± S.D. from three independent experiments in duplicate.
[a] $P < 0.01$ versus basal.
[b] $P < 0.01$ versus normal glucose.

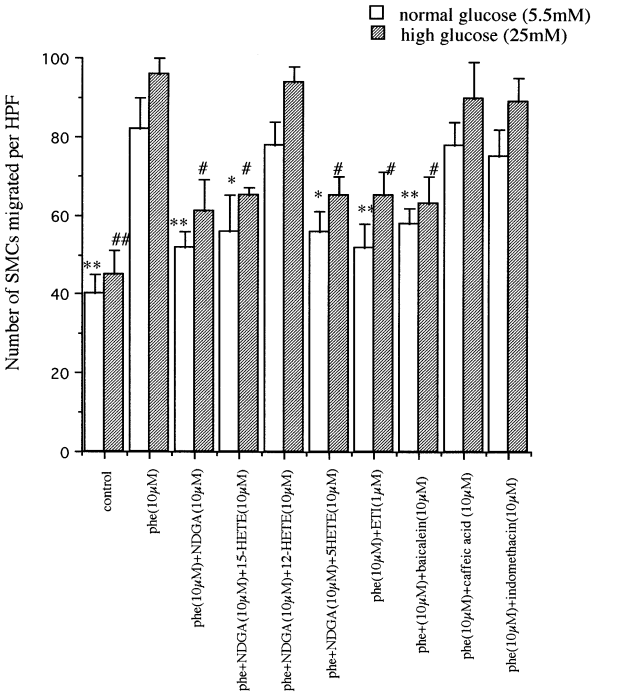


Fig. 5. Effect of the phenylephrine on the migration. Each of the VSMCs were suspended in DMEM in the upper chamber. The lower chamber was filled with DMEM with the indicated migration factor. The migration of the cells was determined as described in Section 2. Results were from three independent experiments in duplicate and are expressed as the number (mean ± S.D. mean) of migrated VSMC per high-power field (HPF). * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, compared with phe (10 μmol/l) only under normal glucose. # and ## indicate $P < 0.05$ and $P < 0.01$ respectively, compared with phe (10 μmol/l) only under high glucose.

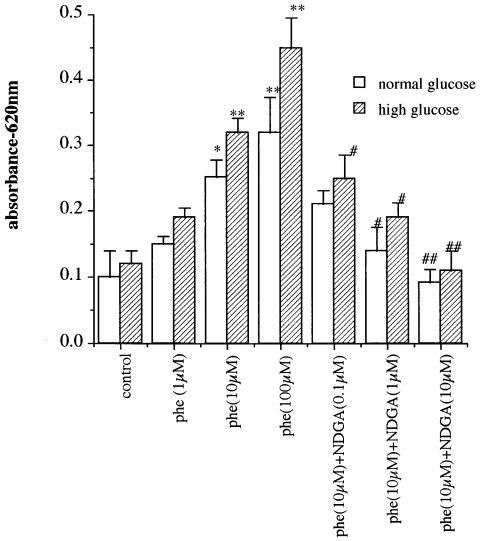


Fig. 6. Effect of phenylephrine on the activity of lipoxygenase of VSMCs under normal and high glucose. The lipoxygenase activity was assessed as described in Section 2. Each of the values represents the mean ± S.D. for three independent experiments in duplicate. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, compared with each control. # and ## indicate $P < 0.05$ and $P < 0.01$, respectively, compared with each phe (10 μmol/l) alone.

5-HETE had inhibitory effects on DNA incorporation. Furthermore, 12-HETE (10 μmol/l) alone did not increase [$^3$H]thymidine incorporation in the high glucose condition as compared with the normal glucose condition. This result suggests that 12-lipoxygenase is mainly involved in phenylephrine-stimulated vascular smooth muscle cells under conditions of high glucose.

### *3.4. Effect of phenylephrine and 12-HETE on migration of vascular smooth muscle cells*

Phenylepheine stimulated vascular smooth muscle cell mobility under both normal glucose or high glucose conditions. These enhancements of vascular smooth muscle cell mobility were inhibited by the general lipoxygenase inhibitors nordihydroguaiaretic acid and 5,8,11-eicosatrienoic acid and by the 12-lipoxygenase specific inhibitor baicalein. In marked contrast, the 5-lipoxygenase inhibitor, caffeic acid and the cyclo-oxygenase inhibitor had no effect on phenylephrine-stimulated vascular smooth muscle cell mobility (Fig. 5). Furthermore, the decrease of phenylephrine-induced mobility in vascular smooth muscle cells treated with nordihydroguaiaretic acid was partially

Table 2
Effect of lipoxygenase inhibitor on phenylephrine-induced HETE release in VSMCs grown in the presence of normal glucose or high glucose concentrations

| | 12-HETE (pg/well) | | 15-HETE (pg/well) | |
|---|---|---|---|---|
| | Normal glucose | High glucose | Normal glucose | High glucose |
| Basal | 110 ± 12 | 130 ± 21 | 118 ± 10 | 138 ± 11 |
| Phe (10 μM) | 161 ± 19 | 215 ± 18 | 151 ± 14 | 213 ± 11 |
| Phe (10 μM) + baicalein (10 μM) | 121 ± 15 [a] | 158 ± 23 [a] | 122 ± 15 [a] | 156 ± 12 [a] |
| Baicalein (10 μM) | 113 ± 15 [N.S.] | 143 ± 15 [N.S.] | 120 ± 13 [N.S.] | 135 ± 16 [N.S.] |

Note: 12- and 15-HETE levels were measured by a specific RIA of cell supernatants after 10 min treatment with phenylephrine in the presence or absence of baicalein. Results are expressed as means ± S.D. from three independent experiments in duplicate.
[a] $P < 0.01$ versus phenylephrine alone.
[N.S.] versus basal.

ameliorated by 12-HETE but not by 15-HETE. These results suggest that a lipoxygenase, most probably 12-lipoxygenase, may be involved in phenylephrine-treated vascular smooth muscle cell mobility.

### *3.5. The effect of phenylephrine on the lipoxygenase activity in vascular smooth muscle cells*

To further confirm the involvement of lipoxygenase, we treated vascular smooth muscle cells with phenylephrine under normal or high glucose conditions and assessed the lipoxygenase activity. Fig. 6 demonstrates that lipoxygenase activity was activated in vascular smooth muscle cells treated with phenylephrine and inhibited by the addition of nordihydroguaiaretic acid in a dose-dependent fashion under normal and high glucose conditions. The colorimetric assay used in these studies originally was based on a source of high lipoxygenase activity (platelets), but it may also be used with stimulated-vascular smooth muscle cells.

### *3.6. Effect of glucose on phenylephrine-released HETE levels*

To validate the finding concerning lipoxygenase activity, we measured the 12-HETE and 15-HETE released under normal and high glucose conditions. Table 1 shows that phenylephrine treatment for 10 min caused a dose-dependent increase in 12- and 15-HETE levels under conditions of normal and high glucose. Furthermore, the enhancement of 12-HETE and 15-HETE levels was greater higher in the high glucose as compared to the normal glucose conditions. Table 2 shows that the specific lipoxygenase inhibitor, baicalein (10 μmol/l), significantly reduced the phenylephrine-induced release of 12- and 15-HETE under normal or high glucose conditions without altering basal HETE release.

## 4. Discussion

The present study suggests that phenylephrine induces increases in DNA synthesis and migration of vascular smooth muscle cells through the lipoxygenase pathway. The lack of specific 15-lipoxygenase inhibitor did not allow us to directly assess the involvement of 15-lipoxygenase in vascular smooth muscle cell proliferation and migration. But the increase in vascular smooth muscle cell proliferation may be mediated predominantly through the 12-, 15-lipoxygenase pathway, because baicalein, but not indomethacin nor ibuprofen, prevented phenylephrine-induced increases in DNA synthesis and 12-HETE, the 12-lipoxygenase product, and 15-HETE, the 15-lipoxygenase product, directly ameliorated the decrease in phenylephrine-induced DNA synthesis in vascular smooth muscle cells treated with nordihydroguaiaretic acid. The increase in vascular smooth muscle cell migration may be mediated through the 12-lipoxygenase pathway. This is supported by results showing that a relatively selective 12-lipoxygenase inhibitor, baicalein, inhibited phenylephrine-stimulated mobility. These chemicals had no toxic effect at the concentrations used in the present study.

Baicalein is a specific 12-lipoxygenase inhibitor that also possesses many lipoxygenase-unrelated effects such as blocking calcium mobilization (Nyby et al., 1996) and acting as an antioxidant (Hanasaki et al., 1994). However, several lines of evidence suggest that the baicalein inhibition of cell proliferation and migration is primarily mediated via its effects on 12-lipoxygenase. First, at low concentrations (i.e. $< 10$ μmol/l), baicalein is known to demonstrate a preferential inhibitory effect on 12-lipoxygenase activity (Sekiya and Okuda, 1982). In this study, baicalein at 1–10 μmol/l inhibited phenylephrine-stimulated vascular smooth muscle cell proliferation and migration. Secondly, the baicalein-induced inhibition of smooth muscle cell proliferation and migration could be reversed by exogenous 12-HETE. Finally, baicalein decreased phenylephrine-stimulated 12- and 15-HETE release.

We reported previously that phenylephrine stimulated prelabelled arachidonic acid release from membrane phospholipids in vascular smooth muscle cells through a pertussis-toxin sensitive protein (Nishio et al., 1996b). In other words, phenylephrine is an activator of the arachidonic acid cascade in vascular smooth muscle cells. However, as yet no reports have been published concerning the effects of phenylephrine on the lipoxygenase pathway of arachidonic acid. It has been demonstrated that cultured aortic vascular smooth muscle cells convert arachidonic acid to HETEs (Brinkman et al., 1991). We observed that phenylephrine increased lipoxygenase activity and particularly 12- and 15-HETE production. The present study clearly demonstrates that the endogenous lipoxygenase product is at least one of the mediators of phenylephrine actions on vascular smooth muscle cell proliferation and migration. In the current study, the precise mechanism whereby the lipoxygenase pathway is coupled to the enhancement of vascular smooth muscle cell proliferation and migration is unknown. However, previous studies have shown that 12-lipoxygenase products can activate protein kinase C activity (Shearman et al., 1989) and oncogene expression (Haliday et al., 1991). Further studies will be needed to determine the underlying mechanism.

Another major finding of the present study is the demonstration that high glucose potentiates the stimulatory effect of phenylephrine on vascular smooth muscle cell proliferation and migration. The effect of glucose on the phenylephrine-induced increase in vascular smooth muscle cell proliferation and migration is not simply due to hyperosmolar effects, as we have the data showing that similar concentrations of mannose did not alter the phenylephrine-induced increase in proliferation and migration (data not shown). Further study will be needed to deter-

mine the precise mechanism by which glucose can potentiate the phenylephrine-induced enhancement of vascular smooth muscle cell proliferation and migration. However, it has been reported that elevated glucose can lead to de novo synthesis of diacylglycerols and activate protein kinase C in vascular smooth muscle cells (Tesfamariam et al., 1991). Furthermore, high glucose can increase the activity and expression of the 12-lipoxygenase pathway (Natarajan et al., 1992; Natarajan et al., 1993). Therefore, increases in 12-HETE with a subsequent increase in specific isoforms of protein kinase C may be involved in the upregulation of these responses. However, many additional mechanisms are possible, including changes among receptors, GTP-binding protein and effectors. It is also unclear how different 5-, 12- and 15-lipoxygenase pathways are related to vascular smooth muscle cell proliferation and migration.

Diabetes mellitus and hypertension are associated with atherosclerosis a cardiovascular disease. The biochemical changes induced by prolonged hyperglycemia have been associated with diabetes-specific vascular disease (Ruderman et al., 1992; Nishio and Watanabe, 1996c). It has been shown that co-existence of diabetes mellitus and hypertension stimulates atherosclerotic disease (Paris et al., 1996). The present study supports the hypothesis that phenylephrine-induced responses of vascular smooth muscle cells may be accentuated in diabetes by activation of a 12-lipoxygenase pathway in vascular smooth muscle cells.

## Acknowledgements

This work was supported in part by grants from the Smoking Research Foundation to Y.W. and by ONO Medical Research Foundation to E.N.

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