Epidermal growth factor receptor transactivation by endogenous vasoactive peptides contributes to hyperproliferation of vascular smooth muscle cells of SHR

Yuan Li, Louis-Olivier Lévesque, and Madhu B. Anand-Srivastava

Department of Physiology, Faculty of Medicine, University of Montreal, Montréal, Québec, Canada

Submitted 2 June 2010; accepted in final form 19 July 2010

Li Y, Lévesque LO, Anand-Srivastava MB. Epidermal growth factor receptor transactivation by endogenous vasoactive peptides contributes to hyperproliferation of vascular smooth muscle cells of SHR. Am J Physiol Heart Circ Physiol 299: H1959–H1967, 2010. First published September 17, 2010; doi:10.1152/ajpheart.00526.2010.—We showed previously that vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHR) exhibit increased proliferation. The present study was undertaken to examine whether the enhanced levels of endogenous angiotensin (ANG) II and endothelin (ET)-1 contribute to the enhanced proliferation of VSMC from SHR and to further investigate the underlying mechanisms responsible for this response. The enhanced proliferation of VSMC from SHR compared with Wistar-Kyoto (WKY) rats was attenuated by losartan, BQ-123, BQ-788, and AG-1478, inhibitors of AT₁, ET₁, ET₂, and epidermal growth factor (EGF-R) receptors, respectively. In addition, BQ-123 and BQ-788 also attenuated the enhanced production of superoxide anion (O₂⁻) and NADPH oxidase activity. Furthermore, diphenyleneiodonium (DPI, inhibitor of NADPH oxidase activity), N-acetyl-L-cysteine (NAC, O₂⁻ scavenger), and PP2 (inhibitor of c-Src) also inhibited the augmented proliferation of VSMC from SHR to WKY levels. In addition, the enhanced phosphorylation of EGF-R in VSMC from SHR compared with WKY was also attenuated by inhibitors of AT₁, ET₁, ET₂, and EGF-R but not by inhibitors of platelet-derived growth factor receptor or insulin-like growth factor receptor. Furthermore, the enhanced phosphorylation of ERK1/2 in VSMC from SHR was also attenuated by AT₁, ET₁, ET₂, and EGF-R inhibitors. The phosphorylation of c-Src was significantly augmented in VSMC from SHR compared with WKY and was attenuated by DPI and NAC. These data suggest that endogenous vasoactive peptides, through increased oxidative stress and resultant activation of c-Src, transactivate EGF-R, which through mitogen-activated protein (MAP) kinase signaling may contribute to the hyperproliferation of VSMC from SHR.

oxidative stress; extracellular signal-regulated kinase 1/2; vascular smooth muscle cell proliferation; spontaneously hypertensive rat

EXCESSIVE VASCULAR smooth muscle cell (VSMC) proliferation contributes to vascular remodeling that occurs in several vascular disease states including atherosclerosis, hypertension, and diabetes (42). It is widely recognized that vasoactive peptides such as angiotensin (ANG) II and endothelin (ET)-1 regulate a variety of physiological functions including blood pressure, VSMC proliferation, cell differentiation, and apoptosis (40). We recently demonstrated (20) that ANG II, ET-1, and arginine-vasopressin (AVP) increased the proliferation of A10 VSMC through the mitogen-activated protein (MAP) kinase/phosphatidylinositol 3-kinase (PI3-kinase) pathway.

ANG II elicits its physiological effects by interacting with at least two distinct receptor subtypes, designated as AT₁ and AT₂ (41), which are G protein-coupled receptors (GPCR). The presence of the AT₁ receptor subtype has been shown in rat vascular tissues; however, a small proportion of AT₂ receptors are also present in rat aorta (6). Most of the physiological effects of ANG II are mediated through the activation of AT₁ receptors that are coupled to several signaling pathways including adenyl cyclase/cAMP inhibition through G α proteins (1, 2), MAP kinase (10), and phosphatidylinositol turnover through Gα₁₁α (10). On the other hand, ET-1 interacts with two other GPCR subtypes, ETₐ and ETₐ. ETₐ is coupled to Gα₁₁, G₁₂/G₁₃, and Gi proteins, leading to stimulation of the phospholipase C/protein kinase C pathway, small RhoA, and inhibition of adenyl cyclase, respectively (44, 45), whereas ETₐ is coupled to Gi and Gi (5, 11); however, in endothelial cells its activation releases nitric oxide (NO) and results in vasorelaxation (38). In addition, the activation of ETₐ receptor also stimulates intracellular signaling pathways including MAP kinase/PI3-kinase, which are similar to those activated by growth factor receptors that possess intrinsic protein tyrosine kinase activity (5). The activation of GPCR ETₐ/ETₐ or AT₁ by ET-1 and ANG II, respectively, has also been reported to enhance the activation of growth factor receptors such as epidermal growth factor receptor (EGF-R), platelet-derived growth factor (PDGF-R), and insulin-like growth factor (IGF-R) in a variety of cell types (12, 21), the phenomenon known as transactivation (7).

Hypertension is associated with enhanced cell proliferation (27, 32). We (27) and others (32) have demonstrated that VSMC from spontaneously hypertensive rats (SHR) exhibit enhanced proliferation compared with age-matched Wistar-Kyoto (WKY) rats, which was shown to be attributed to the enhanced expression of Gα₁ proteins. In addition, the levels of ANG II and ET-1 as well as growth factors have also been shown to be increased in hypertension (25, 36). In addition, VSMC from SHR have also been reported to exhibit enhanced levels of ET-1 and ANG II (16, 30). Furthermore, endogenous ANG II has also been reported to augment the levels of several growth factors including transforming growth factor (TGF)-β1, PDGF-AA, and basic fibroblast growth factor (bFGF) proteins in VSMC from SHR (37). Thus, in light of these findings, it may be possible that the enhanced levels of endogenous vasoactive peptides and growth factors may contribute to hyperproliferation of VSMC from SHR. The present study was therefore undertaken to investigate the role of endogenous vasoactive peptides and growth factors in the enhanced prolif-
eration of VSMC from SHR and to examine whether the transactivation of growth factor receptor by endogenous vasoactive peptides through activation of MAP kinase signaling contributes to the enhanced proliferation of VSMC from SHR.

We showed that endogenous ANG II, ET-1, and growth factor EGF may be responsible for the enhanced proliferation of VSMC in SHR and the transactivation of EGF-R by endogenous ANG II and ET-1 through AT₁, ETA as well as ETB receptors, respectively, and oxidative stress may contribute to the enhanced cell proliferation observed in SHR.

MATERIALS AND METHODS

ETₐ receptor antagonist BQ-123, ETₐ receptor antagonist BQ-788, EGF-R antagonist AG-1478, IGF-R antagonist AG-1024, and PDGF-R antagonist AG-1295 were purchased from Calbiochem (La Jolla, CA). The antibodies pEGF-R (Tyr 1173), EGF-R (1005), p-ERK (E-4) (monoclonal phosphospecific-Tyr1173), and ERK 2 (C-14) were from Santa Cruz Biotechnology (Santa Cruz, CA). N-acetyl-l-cysteine (NAC) was from BDH (Toronto, ON, Canada). All other chemicals used in these experiments were purchased from Sigma (St. Louis, MO).

Cell culture. VSMC from 12-wk-old SHR and age-matched WKY rats were cultured from aortas as described previously (3). The purity of the cells was checked by immunofluorescence technique using α-smooth muscle-specific actin as described previously (29). These cells were found to contain high levels of smooth muscle-specific actin. The cells were plated in 75-cm² flasks and incubated at 37°C in 95% air-5% CO₂ humidified atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) (with glucose, 1-glutamine, and sodium bicarbonate) containing antibiotics and 10% heat-inactivated fetal bovine serum (FBS). Cells were passaged upon reaching confluence with 0.5% trypsin-containing 0.2% EDTA and utilized between passages 5 and 15. Confluent cells were starved by incubation for 24 h in DMEM without FBS at 37°C to reduce the interference by growth factors present in the serum. All animal procedures used in this study were approved by the Comité de Déontologie de l’Expérimentation sur les Animaux (CDEA) of the University of Montreal (University of Montreal (99050)). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Pub. No. 85-23, revised 1996).

Cell lysis and immunoblotting. VSMC from SHR and WKY rats were incubated in the absence or presence of vasoactive peptide antagonists or growth factor receptor inhibitors (10⁻⁴ M) for 30 min at 37°C. After treatment, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed by using lysis buffer. The cell lysates were centrifuged at 12,000 g for 10 min at 4°C, and supernatants were used for immunoblotting as described previously (26) by using monoclonal phospho-specific-Tyr²⁰⁴-ERK1/2 antibody for pERK1/2, phospho-specific-Tyr¹¹⁷³-p-EGFR antibody for pEGF-R, and phospho-specific-Tyr¹⁴⁸ antibody for p-c-Src.

Determination of cell proliferation. Cell proliferation was quantified by DNA synthesis and by directly counting the cell number.

DNA synthesis was evaluated by incorporation of [³H]thymidine into cells as described previously (20). Subconfluent aortic VSMC were plated in six-well plates for 24 h and were serum deprived for 24 h to induce cell quiescence. VSMC from SHR and WKY rats were incubated in the absence or presence of EGF-R inhibitor AG-1478 (10⁻⁶ M), vasoactive peptide antagonists losartan, BQ-123, or BQ-788 (10⁻⁶ M), NADPH oxidase inhibitor diphenyleneiodonium (DPI; 10⁻⁶ M), superoxide (O₂⁻) scavenger NAC (20 mM), or c-Src inhibitor PP2 (10⁻⁶ M) for 24 h. [³H]thymidine (1 μCi) was added and further incubated for 4 h before the cells were harvested. The cells were rinsed twice with ice-cold PBS and incubated with 5% trichloroacetic acid for 1 h at 4°C. After being washed twice with ice-cold water, the cells were incubated with 0.4 Na hydroxide solution for 30 min at room temperature, and radioactivity was determined by liquid scintillation counter. Cell viability was checked by the Trypan blue exclusion technique, which indicated that >90–95% cells were viable. Cell counts were determined by counting the viable cells in a hemocytometer with the Trypan blue dye exclusion assay.

Superoxide anion measurements. Basal O₂⁻ production in VSMC was measured by the lucigenin-enhanced chemiluminescence method with a low concentration (5 μM) of lucigenin as described previously (26). The cells after treatment with losartan (10⁻⁵ mM) were washed in oxygenated Krebs-HEPES buffer, scraped, and placed in scintillation vials containing lucigenin solution, and the emitted luminescence was measured with a liquid scintillation counter (Wallaam 1409; Perkin Elmer Life Science, St Laurent, QC, Canada) for 5 min. The average luminescence value was estimated and the background value subtracted, and the result was divided by the total weight of proteins in each sample.

NADPH oxidase activity determination. The activation of NADPH oxidase activity in the samples was assessed by adding 10⁻⁴ M NADH (Sigma) in the vials before counting. Basal O₂⁻-induced luminescence was then subtracted from the luminescence value induced by NADH (26).

Statistical analysis. Results are expressed as means ± SE. Comparisons between groups were made with one-way analysis of variance (ANOVA) followed by Newman-Keuls test. Results were considered significant at a value of P < 0.05.

RESULTS

Implication of endogenous vasoactive peptides in enhanced proliferation of VSMC from SHR. To investigate whether the enhanced levels of vasoactive peptides reported in VSMC from SHR (16, 30) may contribute to the enhanced proliferation of VSMC in SHR, we examined the effects of ANG II and ET-1 receptor antagonists on cell proliferation of VSMC from SHR and WKY rats, and the results are shown in Fig. 1. As reported previously (27), VSMC from SHR exhibited enhanced proliferation compared with WKY (∼650%) as determined by [³H]thymidine incorporation (Fig. 1A), which was significantly attenuated by ∼50% by the AT₁ receptor antagonist losartan. On the other hand, losartan did not have any effect on the proliferation of VSMC from WKY. In addition, endothelin receptor ETA and ETB antagonists BQ-123 and BQ-788, respectively, also attenuated the enhanced proliferation of VSMC from SHR by 65% and 55%, respectively, whereas these antagonists did not reduce the proliferation of VSMC from WKY rats. In addition, the cell number as determined by Trypan blue dye exclusion assay was also increased by ∼550% in VSMC from SHR, which was attenuated to control WKY levels by ETA and ETB antagonists BQ-123 and BQ-788 (Fig. 1B).

Implication of endogenous growth factor in enhanced proliferation of VSMC from SHR. Since the levels of endogenous growth factors have been reported to be increased in SHR (36), it was of interest to examine the contribution of EGF-R in the enhanced proliferation of VSMC from SHR. To investigate this, the effect of EGF-R inhibitor AG-1478 on proliferation of VSMC from SHR and WKY was examined. The results shown in Fig. 2 indicate that the enhanced proliferation (∼550%) as determined by [³H]thymidine incorporation of VSMC from SHR (Fig. 2A) and enhanced cell number (∼450%) (Fig. 2B) compared with WKY was restored toward control WKY levels by AG-1478 and suggest the implication of EGF-R in the enhanced proliferation of VSMC from SHR. In addition, AG-
1478 also inhibited the basal proliferation of VSMC from WKY rats.

Role of vasoactive peptides in transactivation of EGF-R in VSMC from SHR. Since both endogenous vasoactive peptides such as ET-1 and ANG II and EGF-R contribute to the enhanced proliferation of VSMC from SHR, it was of interest to examine whether the enhanced proliferation induced by endogenous vasoactive peptides is mediated through the transactivation of EGF-R. To test this, the effect of losartan, BQ-123, and BQ-788 on the phosphorylation of EGF-R was examined in VSMC from SHR. As shown in Fig. 3, phosphorylation of EGF-R was enhanced by ~80% in VSMC from SHR compared with VSMC from WKY rats. However, the levels of EGF-R were not different between the two groups (66 vs. 70 in arbitrary units). The enhanced phosphorylation was restored to control WKY levels by losartan, BQ-123, and BQ-788. These results suggest that endogenous ET-1- and ANG II-induced enhanced proliferation of VSMC of SHR may be mediated through the transactivation of EGF-R. In addition, AG-1478, an EGF-R inhibitor, also restored the enhanced phosphorylation to control levels, whereas the inhibitors of PDGF-R and IGF-R did not have any effect on the phosphorylation of EGF-R (Fig. 4). These results suggest the specificity of the EGF-R inhibitor.

Role of oxidative stress in enhanced proliferation of VSMC from SHR. We showed previously (26) that VSMC from SHR exhibited enhanced O$_2$ production, which was attributed to endogenous levels of ANG II. To investigate whether endogenous ET-1 also contributes to the enhanced oxidative stress in SHR, the effects of ETA and ETB receptor antagonists BQ-123 and BQ-788, respectively, on O$_2$ production and NADPH oxidase activity was examined in VSMC from SHR and WKY rats, and the results are shown in Fig. 5. As reported previously, both production of O$_2$ (Fig. 5A) and NADPH oxidase activity (Fig. 5B) were significantly augmented in VSMC from SHR compared with WKY and were attenuated by ETA and ETB receptor antagonists, suggesting the implication of endogenous ET-1 in enhanced oxidative stress in SHR.

To further investigate whether the enhanced proliferation of VSMC from SHR is due to the enhanced oxidative stress, the effect of DPI, an inhibitor of NADPH oxidase that has been shown to inhibit both the production of O$_2$ and NADPH oxidase activity in VSMC from SHR (26, 34), and NAC, a

Fig. 1. Effect of vasoactive peptide antagonists on thymidine incorporation and cell number in vascular smooth muscle cells (VSMC) from 12-wk-old spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Confluent VSMC from SHR and WKY rats were incubated in the absence or presence of losartan (10$^{-6}$ M), BQ-123 (10$^{-6}$ M), or BQ-788 (10$^{-6}$ M) for 24 h. Thymidine incorporation (A) and cell number (B) were determined as described in MATERIALS AND METHODS. Results are expressed as % of WKY control (taken as 100%). Values are means ± SE of 6 separate experiments. ***P < 0.001 vs. WKY rats; §§§P < 0.001 vs. SHR.

Fig. 2. Effect of growth factor receptor inhibitors on thymidine incorporation and cell number in VSMC from 12-wk-old SHR and WKY rats. Confluent VSMC from SHR and WKY rats were incubated in the absence or presence of AG-1478 (10$^{-6}$ M), for 24 h. Thymidine incorporation (A) and cell number (B) were determined as described in MATERIALS AND METHODS. Results are expressed as % of WKY control (taken as 100%). Values are means ± SE of 3 separate experiments. *P < 0.05, ***P < 0.001 vs. WKY rats; §§§P < 0.001 vs. SHR.

Fig. 3. Effect of vasoactive peptide antagonists on epidermal growth factor receptor (EGF-R) phosphorylation (pEGF-R) in VSMC from 12-wk-old SHR and WKY rats. Confluent VSMC from SHR and WKY rats were incubated in the absence or presence of losartan (10$^{-6}$ M), BQ-123 (10$^{-6}$ M), or BQ-788 (10$^{-6}$ M) for 24 h. Cell lysates were used for immunoblotting as described in MATERIALS AND METHODS. Blots are representative of 5 separate experiments. Results are expressed as % of WKY control (taken as 100%). Values are means ± SE of 5 separate experiments. ***P < 0.001 vs. WKY rats; §§§P < 0.001 vs. SHR.

Fig. 4. Effect of AG-1478 (10$^{-6}$ M) on EGF-R phosphorylation (pEGF-R) in VSMC from 12-wk-old SHR and WKY rats. Confluent VSMC from SHR and WKY rats were incubated in the absence or presence of AG-1478 (10$^{-6}$ M), for 24 h. Cell lysates were used for immunoblotting as described in MATERIALS AND METHODS. Results are expressed as % of WKY control (taken as 100%). Values are means ± SE of 5 separate experiments. *P < 0.05, ***P < 0.001 vs. WKY rats; §§§P < 0.001 vs. SHR.
scavenger of O$_2^-$, on cell proliferation was examined. As shown in Fig. 6, proliferation as determined by thymidine incorporation (Fig. 6A) and cell number (Fig. 6B) was significantly augmented by about fivefold and sevenfold, respectively, in SHR compared with WKY, and both DPI and NAC inhibited the enhanced proliferation of VSMC from SHR toward control WKY levels; however, these antioxidants also inhibited the proliferation of VSMC from WKY rats by ~50%.

**Role of c-Src in enhanced proliferation of vascular smooth muscle cells from SHR.** To investigate whether c-Src, which has been shown to be activated by reactive oxygen species (ROS) in aortic VSMC (9), is also implicated in enhanced proliferation of VSMC from SHR, we examined the effect of PP2, an inhibitor of c-Src, on proliferation of VSMC from SHR and WKY rats. The results shown in Fig. 7 indicate that the increased proliferation of VSMC from SHR determined by thymidine incorporation (Fig. 7A) or cell number (Fig. 7B) was restored to control WKY levels by PP2. On the other hand, treatment of cells with PP3, an inactive structural analog of PP2, did not have any significant effect on the proliferation of VSMC from SHR or WKY rats.

**Role of enhanced oxidative stress in activation of c-Src in VSMC from SHR.** To further examine whether VSMC from SHR exhibit enhanced activation of c-Src and the implication of enhanced oxidative stress in this activation, the phosphorylation of c-Src was determined. The results shown in Fig. 8 indicate that VSMC from SHR exhibited augmented phosphorylation of Tyr418 on c-Src by ~60% compared with control cells, which was attenuated by DPI, NAC, as well as PP2, suggesting the implication of oxidative stress in increased phosphorylation of c-Src in VSMC from SHR.

**Implication of endogenous vasoactive peptides in enhanced ERK1/2 phosphorylation.** To further investigate whether the enhanced phosphorylation of ERK1/2 in VSMC from SHR (26, 27) that has been shown to be implicated in cell proliferation (20) is attributed to the enhanced levels of endogenous vasoactive peptides, we examined the effect of losartan, BQ-123, and BQ-788 on ERK1/2 phosphorylation in VSMC from SHR, and the results are shown in Fig. 10. As reported previously (26), ERK1/2 phosphorylation was significantly enhanced (~65%) in VSMC from SHR compared with WKY control rats, and this enhanced phosphorylation was attenuated by ~60% by losartan and by ~85% by BQ-123 and BQ-788, suggesting the implication of AT$_1$, ET$_A$, and ET$_B$ receptors in enhanced ERK1/2 phosphorylation in VSMC from SHR. On
the other hand, total ERK was not different between WKY and SHR (42 vs. 44 in arbitrary units).

In addition, to implicate the role of EGF-R and c-Src in enhanced ERK1/2 phosphorylation in SHR, we also examined the effect of EGF-R inhibitor AG-1478 and c-Src inhibitor PP2 on ERK1/2 phosphorylation in VSMC from WKY and SHR. The results shown in Fig. 11 indicate that AG-1478 (Fig. 11A) and PP2 (Fig. 11B) were also able to inhibit the enhanced phosphorylation of ERK1/2 to below control levels. On the other hand, AG-1478 did not have any effect on the ERK1/2 phosphorylation in VSMC from WKY rats, whereas PP2 significantly inhibited (~50%) ERK1/2 phosphorylation in VSMC from WKY rats. In addition, total ERK was not different between WKY and SHR (42 vs. 44 in arbitrary units).

**DISCUSSION**

Vascular remodeling that contributes to the pathophysiology of vascular diseases including hypertension is associated with alteration in VSMC growth, hypertrophy, etc. We (27) and others (32) reported previously that VSMC from SHR exhibit exaggerated cell growth (proliferation) compared with VSMC from WKY rats. The enhanced proliferation of VSMC from SHR was shown to be attributed to the enhanced levels of G\(\alpha\) proteins, because the treatment of VSMC from SHR with pertussis toxin that inactivates G\(\alpha\) proteins resulted in the restoration of enhanced proliferation to control WKY levels (27). However, in the present study we report for the first time that endogenous vasoactive peptides such as ANG II and ET-1 through the transactivation of growth factor receptors may contribute to the enhanced proliferation of VSMC from SHR.

The role of vasoactive peptides in the proliferation of VSMC has been well documented (4, 20). We recently reported (20) that ANG II, ET-1 and AVP increased the proliferation of A10 VSMC via G\(\alpha/\beta\)/MAP kinase pathways. In addition, ANG II treatment of VSMC from SHR was shown to enhance the proliferation to a greater extent compared with VSMC from WKY, which was attenuated by the ANG II AT1 receptor antagonist losartan, indicating the implication of AT1 receptor in the enhanced proliferation of VSMC from SHR (14).

We demonstrate that blockade of AT1, ETA, and ETB receptors by losartan, an AT1 receptor antagonist, BQ-123, an ETA receptor antagonist, and BQ-788, an ETB receptor antagonist, attenuated the enhanced proliferation of VSMC from SHR determined by thymidine incorporation as well as by counting the cell number. These results suggest that the autocrine effects of ANG II and ET-1 through the activation of AT1 and ETA and ETB receptors, respectively, may contribute to the hyperproliferation of VSMC from SHR. Our results are in agreement with the studies of other investigators who also reported the role of endogenous vasoactive peptides in the enhanced proliferation of VSMC from SHR (18, 23, 24, 31, 33) and suggest that vasoactive peptides promote the proliferation in an autocrine manner. In this regard, Kubo et al. (24) showed that DNA synthesis in aortic VSMC from 10-wk-old SHR that was significantly enhanced compared with VSMC from WKY was attenuated by an AT1 receptor antagonist (CV-11974) as well as by delapril, an angiotensin-converting enzyme (ACE) inhibitor. Furthermore, Fukuda et al. (16) have also reported the production of ANG II in VSMC from SHR. In addition, the autocrine role of ET-1 in the regulation of proliferation was...
also shown by the studies of various investigators (18, 23, 31, 33). Lu et al. (31) reported that VSMC in addition to endothelial cells could also synthesize and secrete ET-1. However, VSMC from SHR release more ET-1 and have a higher growth rate than VSMC from WKY when cultured in a serum-free medium (31). In addition, Lu et al. (31) showed that anti-ET-1 antibody as well as BQ-123 and BQ-788, ET<sub>A</sub> and ET<sub>B</sub> receptor antagonists, respectively, attenuated the enhanced proliferation of VSMC from SHR and suggested the contribution of endogenous ET-1 through ET<sub>A</sub> as well as ET<sub>B</sub> receptors in hyperproliferation of VSMC from SHR. The intracellular signaling mechanism by which endogenous ANG II and ET-1 increased the cell proliferation of VSMC from SHR appears to be activation of the MAP kinase pathway, because losartan, BQ-123, and BQ-788, antagonists of AT<sub>1</sub>, ET<sub>A</sub>, and ET<sub>B</sub> receptors, respectively, which inhibited enhanced proliferation, also attenuated the enhanced phosphorylation of ERK1/2. In this regard, the implication of MAP kinase signaling in the regulation of cell proliferation by vasoactive peptides is well documented (28, 39).

VSMC have been shown to express various growth factors that promote cell proliferation (19, 37). Hamet et al. (19) showed the implication of TGF-β in increased DNA synthesis in VSMC from SHR. Furthermore, studies showing that the deletion of TGF-β by antisense oligonucleotide complementary to TGF-β mRNA inhibited the enhanced proliferation of VSMC from SHR further support the involvement of TGF-β in augmented growth of VSMC from SHR. The enhanced expression of various growth factors including TGF-β, PDGF-A chain, and bFGF has been shown in VSMC from SHR compared with VSMC from WKY (37). Fukuda et al. (15) have reported the increased expression of TGF-β type II receptor in VSMC from SHR compared with cells from WKY. However, we report that the activation but not the expression of EGF-R was augmented in VSMC from SHR. The fact that the inhibition of EGF-R by AG-1478 attenuated the enhanced proliferation of VSMC from SHR further suggests that the endogenous EGF that may be upregulated in VSMC from SHR may also contribute to the hyperproliferation of VSMC from SHR through the activation of EGF-R. In addition, the results showing that an EGF-R inhibitor also inhibited the basal proliferation of VSMC from WKY further suggest that VSMC express EGF-R that may contribute to the proliferation of VSMC.

The transactivation of growth factor receptors by exogenous ANG II and ET-1 has been shown by several investigators (8, 12, 21). Eguchi et al. (13) have shown that the transactivation of EGF-R by ANG II through AT<sub>1</sub> receptor induced protein synthesis through the activation of MAP kinase signaling in cultured VSMC. Furthermore, infusion of ANG II in rats was also shown to increase the levels of EGF-R protein in the heart (22). However, we demonstrate for the first time that endoge-
ous ANG II and ET-1 through the activation of AT₁ and ETₐ receptors, respectively, could also transactivate but not enhance the expression of EGFR in VSMC from SHR because the enhanced tyrosine phosphorylation of EGFR exhibited by VSMC from SHR was attenuated by losartan, BQ-123, and BQ-788, the inhibitors of AT₁, ETₐ, and ETₐ receptors, respectively. The fact that the enhanced phosphorylation of ERK₁/2 in VSMC from SHR was also attenuated by the inhibitor of EGFR as well as by the inhibitors of AT₁, ETₐ, and ETₐ receptors suggests that endogenous ANG II and ET-1 through the transactivation of EGFR may activate MAP kinase signaling that contributes to the enhanced cell growth in SHR. In addition, the implication of EGFR in ANG II- and ET-1-induced enhanced proliferation of VSMC was further supported by our recent study (17) showing that ANG II-evoked increased proliferation of A10 VSMC was attenuated by AG-1478, an EGFR inhibitor. In addition, the implication of growth factor receptor transactivation in ANG II-induced enhanced activation of MAP kinase has been shown by various studies (8, 13, 17).

We also examined the intracellular signaling mechanisms that lead to the transactivation of EGFR in VSMC from SHR. Our results showing that antioxidants DPI and NAC as well as PP2, a c-Src inhibitor, attenuated the enhanced activation of EGFR as well as the hyperproliferation of VSMC from SHR suggest the implication of oxidative stress and c-Src in the transactivation of EGFR and resultant hyperproliferation of VSMC from SHR. In this regard, a role of Src kinases in the transactivation of EGFR and PDGF-R has been reported (35, 43). Furthermore, the fact that DPI and NAC also attenuated the enhanced phosphorylation of c-Src suggests that oxidative stress is the upstream signaling molecule of c-Src. Our results are in accordance with the studies of Ushio-Fukai et al. (43), who have shown the contribution of oxidative stress in ANG II-induced phosphorylation of c-Src in VSMC. Thus, in light of these findings, it may be suggested that oxidative stress through the activation of c-Src transactivates EGFR that contributes to the enhanced proliferation of VSMC from SHR.

In conclusion, we demonstrate for the first time that endogenous ANG II and ET-1 in VSMC from SHR, through the activation of AT₁, ETₐ, and ETₐ receptors, increase the oxidative stress that by activating c-Src results in the transactivation of EGFR, which by augmenting the activity of MAP kinase contributes to the enhanced proliferation of VSMC from SHR. From these studies, it may be suggested that the inhibitors of AT₁, ETₐ, ETₐ, and EGFR that attenuate the enhanced proliferation of VSMC from SHR may contribute to the amelioration of vascular remodeling and may thus be used as potential therapeutic agents for the treatment of vascular complications of hypertension.

ACKNOWLEDGMENTS

We thank Christiane Laurier for her valuable secretarial help.

GRANTS

This study was supported by a grant from the Canadian Institutes of Health Research (MOP 53074)
REFERENCES


