Acta Medica et Biologica
Vol. 43, No. 1, 11-16, 1995

The Effect of Angiotensin II on DNA Synthesis in Cultured Rat Aortic Vascular Smooth Muscle Cells with Various Glucose Concentrations

Masahiko IGARASHI, Kiyoshi ISHIYAMA, Makoto DAIMON, Norio FUKASE, Yoshihiro MORITA, Keiichi YAMATANI, Makoto TOMINAGA and Hideo SASAKI

The Third Department of Internal Medicine, Yamagata University School of Medicine, Yamagata, Japan

Received October 17 1994; accepted January 18 1995

Summary. **To determine the relationship between angiotensin II (Ang II) and glucose concentrations on cultured rat aortic vascular smooth muscle cells (VSMC), we examined 20 kinds of culture conditions for VSMC culture and measured [^{3}H]-DNA contents in all groups. Rat VSMC were obtained from the thoratic aorta of Wistar rats by the explant technique and cultured. Passages 3-5 were used for this experiment. VSMC were cultured for 7 days with four concentrations of additional glucose: 0 mM, 5.5 mM, 11 mM or 16.5 mM. The cells were then maintained in a serum-free medium and various amounts (0 M, 10^{-9}M, 10^{-8}M, 10^{-7}M or 10^{-6}M) of Ang II were replaced for 24 h. Afterward, 1 μCi of [^{3}H]-thymidine was added to each well for 18 h, and the radioactivities were counted. Compared to the group cultured without Ang II and glucose, the other 19 groups cultured with various amounts of glucose or Ang II were significantly higher in the levels of [^{3}H]-thymidine incorporation (TI). With Ang II from 10^{-9}M to 10^{-7}M, TI increased in a dose-dependent manner in each glucose concentration. In all Ang II concentrations used, the rates of TI on cultured VSMC in 11 mM glucose were significantly higher than in other glucose concentrations. These results suggest that the concomitant increase in Ang II and glucose concentrations synergistically stimulates the proliferation of VSMC.**

INTRODUCTION

The mechanisms of atherosclerosis associated with diabetes mellitus are still unclear. Prolonged hyperglycemia and hyperinsulinemia are considered to be the main causes for diabetic vascular complications, since they can regulate the biochemical pathways related to glucose matabolism.[1-5] The abnormal growth of vascular smooth muscle cells (VSMC) is an early event in the formation of atherosclerosis.[6] Elucidation of the mechanism regulating VSMC proliferation is important to understand the enhanced risk of cardiovascular disease in diabetes. Recent studies have shown that the expression of some autocrine growth factors, such as platelet-derived growth factor (PDGF),[7] basic fibroblast growth factor (bFGF)[8] and transforming growth factor β1(TGF-β1)[9], stimulates the proliferation of VSMC in rats. Furthermore, it has been demonstrated that vasoactive substances, such as angiotensin II (Ang II)[10] and endothelin[11] play important roles in the regulation of VSMC proliferation. As for Ang II, Powell et al.[10] reported that angiotensin converting enzyme (ACE) inhibitors suppressed the abnormal VSMC proliferation induced by hypertension and vascular injury. Moreover, Ang II is an important local factor leading to VSMC hypertrophy and neointimal VSMC proliferation.[12] Interestingly, Ang II induces an increase in the expression of proto-oncogenes[13-15] and the mRNA of some growth factors in VSMC.[15,16] Based on these reports, the question arises whether an interaction affecting VSMC growth exists between Ang II and glucose. The effect of Ang II might play important roles in the development of diabetic vascular complications.

In this study, we investigated the influence of glucose and Ang II on cultured rat aortic VSMC growth under 20 kinds of culture conditions.

Correspondence: Masahiko Igarashi, The Third Department of Internal Medicine, Yamagata University School of Medicine, 2-2-2 Iida-nishi, Yamagata 990-23, Japan.

MATERIALS AND METHODS

Cell cultures

VSMC were obtained from the thoratic aorta of male Wistar rats (10 weeks old, weighing approximately 250 g, Funabashi Farm, Chiba, Japan) by the media explant technique described previously with minor modifications.[17] Briefly, the rats were anesthetized with ethyl ether (Nakalai Tesque, Inc., Kyoto, Japan) and the thoratic aorta was aseptically removed and placed in a Petri dish (Falcon, Becton Dickinson, Heidelberg, Germany) containing ice-cold Medium 199 (M199, GIBCO, Eggenstein, Germany). The intima and adventitial tissues were carefully removed with a sharp scalpel blade. The VSMC tissue layers were cut into approximately 1-mm^2 blocks using fine forceps, and these samples were transferred into a tissue culture dish (Primaria, Falcon, Becton Dickinson, Heidelberg, Germany), to which M199 supplemented with antibiotics (penicillin 10,000 μ/ml-streptomycin 10 mg/ml-fungizone 25 μg/ml mixture, Whittaker Bioproducts, Walkersville, MD, U.S.A.) and 10% fetal bovine serum (FBS), Whittaker Bioproducts, Walkersville, MD, U.S.A.) were added. The blocks of tissue were cultured in a humidified incubator containing 5% CO_2 at 37°C for 7-10 days. VSMC were allowed to grow out from the blocks, and then the tissues were removed. After reaching confluence, the cells were harvested by a brief exposure to 0.25% trypsin (Wako Pure Chemical Industries, Osaka, Japan) and 0.02% EDTA (Wako Pure Chemical Industries, Osaka, Japan), and routinely subcultured with the medium in tissue culture flasks (Falcon, Becton Dickinson, Heidelberg, Germany).

The purity of VSMC was estimated by typical growth morphology, and immunoexpression for monoclonal anti-α smooth muscle actin (Sigma, St. Louis, MO, U.S.A.)[18] and acetylated low density lipoprotein labeled with 1, 1′-dioctadecyl-1-3, 3, 3′, 3′-tetramethyl-indocarbocyanineperchlorate (Dil-Ac-LDL, Biomedical Technologies Inc., Stoughton, MA, U.S.A.)[19] with Lab-Tek chamber slide (Nunc, Inc., Naperville, IL, U.S.A.). Also, cell viability was more than 98% as determined by the exclusion of 0.2% Trypan blue (Nakalai Tesque, Inc., Kyoto, Japan).

DNA synthesis

Passages 3-5 were used for this experiment. VSMC were plated at a density of 100,000 cells per dish on 1% gelatin (Iwaki Glass, Tokyo, Japan)-coated 24 multiwell dishes (Falcon, Becton Dickinson, Heidelberg, Germany) and cultured with M199 supplemented with 5% FBS. After 2 days, M199 supplemented with 5% FBS containing four concentrations of additional glucose, 0 mM, 5.5 mM, 11 mM or 16.5 mM, were replaced every 2 days during culture for a further 7 days. The cells were then maintained to reach quiescence in serum-free M199 for 24 h,[20] and various amounts (0 M, 10^{-9}M, 10^{-8}M, 10^{-7}M or 10^{-6}M) of human Ang II (Peninsula Laboratories, Inc., Belmont, CA, U.S.A.) were replaced for another 24 h. The experimental procedures for measuring the incorporation of [^{3}H]-thymidine to DNA were based on the method of Stiles et al.[21] with modifications. One μCi of [6-^{3}H]-thymidine (Amersham lnternational plc, Buckinghamshire, England) was added to each well for 18 h. At the end of the incubation, VSMC were washed three times with PBS, and then twice with 10% trichloroacetic acid (TCA, Wako Pure Chemical Industries, Osaka, Japan) for 5 min at 4°C. They were solubilized in 250 μl of 2 N NaOH for 15 min, neutralized with 50 μl of 6 N HCl for 5 min, and precipitated with 250 μl of 30% TCA for 30 min. The total sample was transferred from each well and filtered in a vacuum using glass fiber filters (Advantec, Tokyo, Japan). The filters were washed three times with 2 ml of 5% TCA and 95% ethanol (Wako Pure Chemical Industries, Osaka, Japan). Then, the radioactivities were counted in a liquid scintillation counter (Tricarb 1500, Packard Instrument Company, Downers Grove, IL, U.S.A.). All studies were done in triplicate.

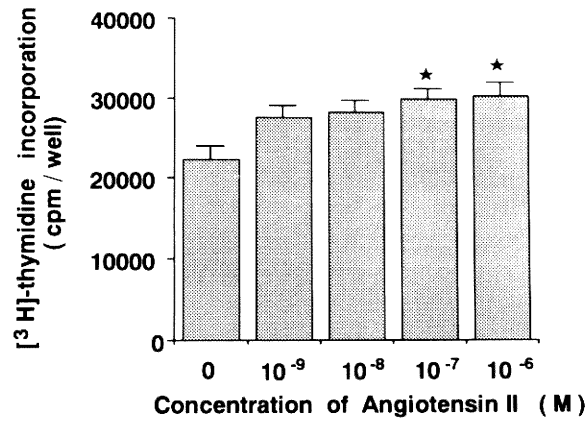

Fig. 1. Dose-response effect of Angiotensin II (Ang II) on DNA synthesis in rat aortic vascular smooth muscle cells (VSMC) before changing to a medium containing various amounts of glucose. Each bar represents the mean ± SEM (n=8) of triplicate assays. ★ $p<0.05$, when compared to the value unstimulated with Ang II.

Statistical methods

Values are given as ±SEM Statistical analysis of data was performed using non-paired Student's *t*-test. Statistical significance was determined using an analysis of variance with $p<0.05$ accepted as significant.

RESULTS

1) Cultured cells

The cultured cells exhibited the "hill and valley" pattern of typical growth morphology on cultured smooth muscle cells. They were characterized by a positive reactivity to monoclonal anti-α smooth muscle actin and a negative reactivity to Dil-Ac-LDL (Data not shown). Also, the viability of these cells was indicated to be more than 98%.

2) Effects of Ang II on DNA synthesis

Dose-response effects of Ang II on DNA synthesis in VSMC are shown in Fig. 1. When VSMC were exposed to Ang II before changing the medium cotaining various amounts of glucose, the incorporation of [^{3}H]-thymidine into DNA increased in a dose-dependent manner. At higher concentrations of Ang II (10^{-7}M and 10^{-6}M), the levels of DNA synthesis were significantly increased compared with the control (unstimulated with Ang II) (+34.4% and +36.1%, respectively, $p<0.05$).

3) Effects of glucose on DNA synthesis

Fig. 2 shows DNA synthesis in VSMC cultured with the four concentrations of additional glucose: 0 mM, 5.5 mM, 11 mM or 16.5 mM, for 7 days. The levels of DNA synthesis increased in all cultures with either Ang II or glucose more than the cultures without Ang II or glucose. In the absence of Ang II, the levels of DNA synthesis were significantly enhanced in proportion to glucose concentrations compared with that obtained in the absence of glucose (5.5 mM; +95.3%, 11 mM; +116.8%, 16.5 mM; +138.8%, respectively, $p<0.05$).

4) Combined effects of Ang II and glucose

In the presence of 10^{-9}M Ang II, the levels of DNA synthesis were significantly enhanced in a dose-dependent manner with the addition of glucose concentrations up to 11 mM, compared with that obtained in

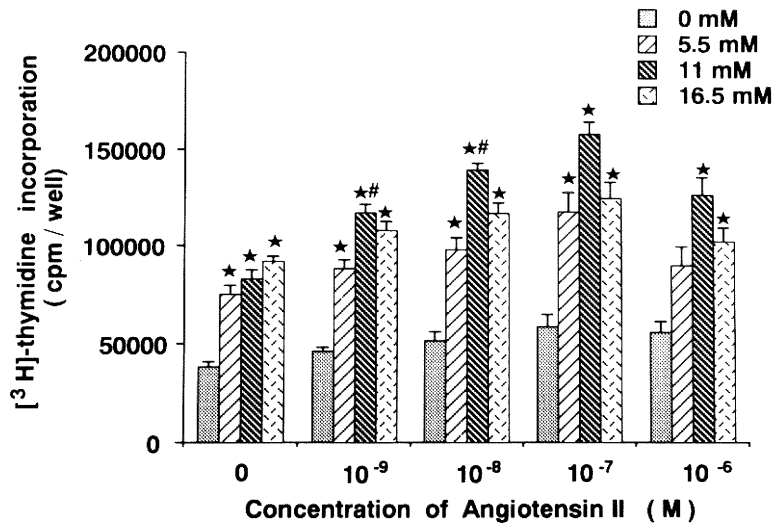

Fig. 2. Effect of Angiotensin II (Ang II) on DNA synthesis in rat aortic vascular smooth muscle cells (VSMC) cultured with Medium 199 (M199) containing 5% fetal bovine serum (FBS), and four concentrations of Ang II with or without the addition of glucose for 7 days. Each bar represents the mean±SEM (n=6) of triplicate assays. ★ $p<0.05$, when compared to the value at each Ang II concentration without the addition of glucose. # $p<0.05$, when compared to the value at each Ang II concentration in the presence of 5.5 mM glucose.

the absence of glucose (5.5 mM;+92.1%, 11 mM;+153.6%, 16.5 mM;+133.4%, respectively, $p<0.05$). Regarding the levels of DNA synthesis, similar but higher results were obtained using higher concentrations of Ang II from 10^{-8}M to 10^{-7}M. These results indicated that the combined stimulation of Ang II and glucose enhances VSMC proliferation considerably more than individual stimulation. Computed from the results shown in Fig. 2, the levels of DNA synthesis by different concentrations of Ang II with the addition of various concentrations of glucose were shown in Fig. 3. The levels of DNA synthesis at 10^{-7}M Ang II in all cultures with the addition of glucose were significantly increased compared with those unstimulated with Ang II ($p<0.05$), which did not significantly increase in the culture without the addition of glucose. The combination of 11 mM glucose and 10^{-7}M Ang II had the strongest effect on DNA synthesis of VSMC proliferation.

DISCUSSION

The renin-angiotensin system is generally thought to play a key role in the pathogenesis of hypertension and atherosclerosis, and may also contribute to the progression of diabetic cardiovascular and renal complications.[1,5,22] The universal underlying abnormality in these pathologic process is mainly concerned with alterations in VSMC structure and function, which are enhanced by circulating and locally generated Ang II through its effects on contractility, growth and sympathetic nervous system. Ang II by itself has also been implicated in both hypertrophic as well as hyperplastic growth effects on VSMC.[23] Recent data obtained with VSMC have demonstrated that Ang II causes a number of short term effects on some signalling events, leading to cell proliferation in response to growth factors: the activation of tyrosine-specific protein kinases,[24,25] induction of the proto-oncogenes c-fos or c-myc,[13-15] and stimulation of the mRNA of some growth factors.[15,16] However, the exact mechanism underlying the intracellular signalling pathway with Ang II remains obscure.

The present study demonstrates that the combination of Ang II and glucose results in the synergistic enhancement of DNA synthesis in rat VSMC. Previous studies reported that Ang II appears to be a relatively weak mitogen for VSMC and increases DNA synthesis ranging from zero to fourfold.[10,12,26-30] Our

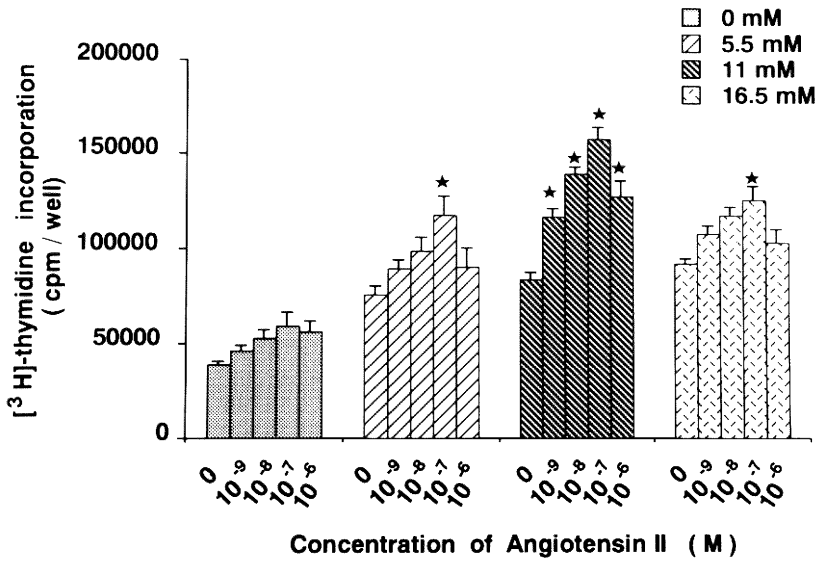

Fig. 3. Effect of Angiotensin II (Ang II) on DNA synthesis in rat aortic vascular smooth muscle cells (VSMC) cultured with Medium 199 (M199) containing 5% fetal bovine serum (FBS), and four concentrations of Ang II with or without the addition of glucose for 7 days. Each bar represents the mean ± SEM ($n=6$) of triplicate assays. ★ $p<0.05$, when compared to the value at each glucose concentration in the absence of Ang II.

experimental results revealed that treatment with Ang II alone leads to a slight dose-response enhancement in DNA synthesis of VSMC (Fig. 1), being consistent with other findings. Low et al.[31] have reported that Ang II rapidly stimulates the rate of transcription of the GLUT-1 gene in VSMC. According to their investigation, 2-deoxy-D-glucose uptake in VSMC was stimulated by Ang II from 10^{-10}M to 10^{-6} M in proportion to its concentration, and the maximal induction was three times higher than the basal activity at 10^{-6}M because of a rapid increase in GLUT-1 mRNA. This finding may be related to Ang II-stimulated VSMC proliferation.

However, in the presence of various amounts of glucose, we observed a synergistic effect on Ang II-induced DNA synthesis (Figs. 2 and 3). The levels of DNA synthesis were increased in all cultures with either Ang II or glucose more than the cultures without Ang II or glucose. In cultures with the addition of Ang II, the levels of DNA synthesis were significantly enhanced in a dose-dependent manner in relation to addition of glucose concentrations up to 11 mM compared with 0 mM, and DNA synthesis was elevated in proportion to Ang II concentrations up to 10^{-7}M. These results demonstrated that the combination of Ang II and glucose resulted in a much greater enhancement of VSMC growth than either individual stimulation factors alone. High glucose concentrations such as found in diabetes mellitus can stimulate the transcription of the genes for growth factors in VSMC. Similar to our data, McClain et al.[32] reported that mRNAs of transforming growth factor α(TGF-α) and bFGF were increased by glucose. According to their data, VSMC cultured in a medium containing 30 mM glucose exhibited a 2-fold increase in TGF-α mRNA and a 3-fold increase in bFGF mRNA, compared with cells grown in normal (5.5 mM) glucose. In our experiments, the maximal DNA synthesis of VSMC was obtained by 10^{-7}M Ang II, which was higher than that with 16.5 mM glucose. The discrepancy between the findings of these studies may be due to differences in the culturing period. Before the experiment, we cultured VSMC for 7 days to examine the chronic hyperglycemic effect, while McClain et al. cultured for 3 days. In another study, Williams et al.[33] reported that elevated extracellular glucose concentrations activate protein kinase C, a serine-threonine protein kinase that is relatively abundant in vascular tissue and plays important roles in the regulation of VSMC growth and contraction, and disturbs the physiological regulation of VSMC function. In this way, VSMC cultured in high glucose concentrations, such as that in diabetes mellitus, may proliferate beyond physiological regulation by the induction of autocrine growth factors or the dysfunction of cellular mechanisms. In addition, Natarajan et al.[34] suggested that Ang II can activate a dose-dependent increase in the total cellular protein content in VSMC by the activation of a 12-lipoxygenase pathway. It is well known that 12-lipoxygenase products can increase protein kinase C activity and oncogene expression. According to their studies, it is reasonable to assume that Ang II further activates the pathological proliferation of VSMC in high glucose concentrations by the 12-lipoxygenase pathway.

In diabetic patients, VSMC might be exposed both to hyperglycemia and Ang II, but in this study, the effect of Ang II was studied in the transformed cells by glucose. Therefore, the result of the present study might be applicable to only a limited situation. The effect of hyperglycemia on DNA synthesis in cultured cells exposed to Ang II warrants a further study.

In conclusion, these results demonstrate that the concomitant increase in Ang II and glucose concentrations synergistically stimulates the pathological growth of VSMC, suggesting that hypertension-associated diabetic mellitus may potentially contribute to the development of arteriosclerotic cardiovascular and renal complications.

REFERENCES

1) Santen RJ, Willis PW, Fajans SS: Atherosclerosis in diabetes mellitus. Correlations with serum lipid levels, adiposity, and serum insulin level. *Arch Inter Med* **130:** 833-843, 1972.
2) Kannel WB, McGee DL: Diabetes and glucose tolerance as risk factors for cardiovascular disease: the Framingham study. *Diabetes* **241:** 2035-2038, 1979.
3) Reaven GM: Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes* **37:** 1595-1607, 1988.
4) Stout RW: Insulin and atheroma. *Diabetes* **13:** 631-654, 1990.
5) Schwartz CS, Kelly JL, Valente AJ, Cayatte AJ, Sprague EA, Rozek MM: Pathogenesis of the atherosclerotic lesion. Implication for diabetes mellitus. *Diabetes* **15:** 1156-1167, 1992.
6) Ross R: The pathogenesis of atherosclerosis: an update. *N Eng J Med* **314:** 488-500, 1986.
7) Barrett TB, Benditt EP: Platelet-derived growth factor gene expression in human atherosclerotic plaques and normal artery wall. *Proc Natl Acad Sci USA* **85:** 2810-2814, 1988.
8) Lindner V, Lappi DA, Baird A, Majack RA, Reidy MA: Role of basic fibroblast growth factor in vascular lesion formation. *Circ Res* **68:** 106-113, 1991.
9) Majesky MW, Lindner V, Twardzik DR, Schwartz SM, Reidy MA: Production of transforming growth

factor b1 during repair of arterial injury. *J Clin Invest* **88:** 904-910, 1991.
10) Powell JS, Clozel JP, Muller KM, Kuhn H, Hefti F, Hosang M, Baumgartner HR: Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. *Science* **245:** 186-188, 1989.
11) Hirata Y, Takagi Y, Fukuda Y, Maruma F: Endothelin is a potent mitogen for rat vascular smooth muscle cells. *Atherosclerosis* **78:** 225-228, 1989.
12) Daeman MJAP, Lombardi DM, Bosman FT, Schwartz SM: Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall. *Circ Res* **68:** 450-456, 1991.
13) Naftilan AJ, Pratt RE, Eldridge CS, Lin HL, Dzau VJ: Angiotensin II induces c-*fos* expression in smooth muscle via transcriptional control. *Hypertension (Dallas)* **13:** 706-711, 1989.
14) Itoh H, Pratt RE, Dzau VJ: Interaction of atrial natriuretic polypeptide and angiotensin II on protooncogene expression and vascular cell growth. *Biochem Biophys Res Commun* **176:** 1601-1609, 1991.
15) Naftilan AJ, Pratt RE, Dzau VJ: Induction of platelet-derived growth factor A-chain and c-*myc* gene expressions by angiotensin II in cultured rat vascular smooth muscle cells. *J Clin Invest* **83:** 1419-1424, 1989.
16) Gibbons GH, Pratt RE, Dzau VJ: Vascular smooth muscle specific cell hypertrophy vs. hyperplasia. Autocrine transforming growth factor expression determines growth response to angiotensin II. *J Clin Invest* **90:** 456-461, 1992.
17) Ross RJ: The smooth muscle cell II. Growth of smooth muscle in culture and formation of elastic fiber. *J Cell Biol* **50:** 172-186, 1971.
18) Diglio CA, Grammas P, Giacomelli F, Wiener J: Angiogenesis in rat aorta ring explant cultures. *Lab Invest* **60:** 523-531, 1989.
19) McGuire PG, Orkin RW: Methods in laboratory investigation. Isolation of rat aortic endothelial cells by primary explant techniques and their phenotypic modulation by defined substrata. *Lab Invest* **57:** 94-105, 1987.
20) Ko Y, Haring R, Stiebler H, Wieczorek AJ, Vetter H, Sachinidis A: High-density lipoprotein reduces epidermal growth factor—induced DNA synthesis in vascular smooth muscle cells. *Atherosclerosis* **99:** 253-259, 1993.
21) Stiles CD, Capone GT, Scher CD, Antoniades HN, Van Wky JJ, Pledger WJ: Dual control of cell growth by somatomedins and platelet-derived growth factor. *Proc Natl Acad Sci USA* **76:** 1279-1283, 1979.
22) Hsueh WA: Effects of renin-angiotensin system in the vascular disease of type II diabetes mellitus. *Am J Med* **92** (Suppl. 4B): 13S-19S, 1992.
23) Owens GK: Determinants of angiotensin II-induced hypertrophy versus hyperplasia in vascular smooth muscle. *Drug Develop Res* **29:** 83-87, 1993.
24) Molley CJ, Taylor DS, Weber H: Angiotensin II stimulation of rapid protein tyrosine phosphorylation and protein kinase activation in rat aortic smooth muscle cells. *J Biol Chem* **268:** 7338-7345, 1993.
25) Butcher RD, Schollmann C, Marme D: Angiotensin II mediates intracellular signaling in vascular smooth muscle cells by activation of tyrosine-specific protein kinase and c-raf-1. *Biochem Biophys Res Commun* **196:** 1280-1287, 1993.
26) Millar JA, Harris EL, Cassie NJ: Mitogenesis in cultured vascular smooth muscle cells from two rat models of hypertension in response to fetal calf serum and angiotensin II. *J Carduovasc Pharmacol* **16** (Suppl. 7): S14-S16, 1990.
27) Araki S, Kawahara Y, Kariya K, Sunako M, Tsuda T, Fukuzaki H, Yoshimi T: Stimulation of platelet-derived growth factor—induced DNA synthesis by angiotensin II in rabbit vascular smooth muscle cells. *Biochem Biophys Res Commun* **168:** 350-357, 1990.
28) Parrott DP, Lockey PM, Bright CP: Comparison of the mitogenic activity of angiotensin II and serotonin on porcine arterial smooth muscle cells. *Atherosclerosis* **88:** 213-218, 1991.
29) Bunkenburg B, van Amelsvoort T, Rogg H, Wood JM: Receptor—mediated effects of angiotensin II on growth of vascular smooth muscle cells from spontaneously hypertensive rats. *Hypertension (Dallas)* **20:** 746-754, 1992.
30) Stouffer GA, Owens GK: Angiotensin II-induced mitogenesis of spontaneously hypertensive rat-derived cultured smooth muscle cells is dependent on autocrine production of transforming growth factor-beta. *Circ Res* **70:** 820-828, 1992.
31) Low BC, Ross IK, Grigor MR: Angiotensin II stimulates glucose transport activity in cultured vascular smooth muscle cells. *J Biol Chem* **267:** 20740-20745, 1992.
32) McClain DA, Paterson AJ, Roos MD, Wei X, Kudlow JE: Glucose and glucosamine regulate growth factor gene expression in vascular smooth muscle. *Proc Natl Acad Sci USA* **89:** 8150-8154, 1992.
33) Williams B, Schrier RW: Characterization of glucose-induced *in situ* protein kinase C activity in cultured vascular smooth muscle cells. *Diabetes* **41:** 1464-1672, 1992.
34) Natarajan R, Gonzales N, Lanting L, Nadler J: Role of the lipoxygenase pathway in angiotensin II-induced vascular smooth muscle cell hypertrophy. *Hypertension* **23** (Suppl. 1): I-142-I-147, 1994.