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Enhanced Stimulation of Akt-3/Protein Kinase B-γ in Human Aortic Smooth Muscle Cells

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Growth factor-induced activation of Akt (protein kinase B) is implicated in the proliferation of vascular smooth muscle cells (VSMC) in addition to antiapoptotic signaling. Although previous studies have documented increases in total Akt or Akt-1 activity in rodent VSMC, little is known about the regulation of Akt-2 or Akt-3 kinase activity in VSMC from any species. In the present study, reverse transcriptase-polymerase chain reaction revealed the expression of all three Akt isoforms in human aortic VSMC. *In vitro* kinase assays using immunoprecipitated Akt isoforms showed robust increases in Akt-3 activity after stimulation of human aortic VSMC with platelet-derived growth factor (PDGF), insulin, and insulin-like growth factor-1. In contrast, these growth factors produced modest and marginal increases in Akt-1 and Akt-2 kinase activity, respectively. Pretreatment of VSMC with a phosphoinositide-3kinase (PI-3K) inhibitor, LY294002, led to significant inhibition of growth factor(s)-induced increases in Akt-3 activity and DNA synthesis. The present findings provide the first direct evidence that the Akt-3 isoform is predominantly activated in human aortic VSMC. Moreover, these data suggest that PI-3K-dependent activation of Akt-3 may play a major role in VSMC proliferation.

Key Words: **Akt-3; protein kinase B-γ; vascular smooth muscle cells; growth factors; insulin; reverse transcriptase-polymerase chain reaction; DNA synthesis; proliferation.**

Diabetic vascular lesions, atherosclerosis and restenosis are associated with abnormal proliferation of vascular smooth muscle cells (VSMC) (1, 2). Recently, we and several other investigators have shown that Akt (protein kinase B, PKB) may play a role in VSMC proliferation, in addition to its well-characterized antiapoptotic signaling (3–6). In nonvascular, insulin-responsive tissues (liver, skeletal muscle and adipocytes), Akt mediates insulin stimulation of glucose transport and GLUT4 translocation (7–13). Akt is a 57-kDa serine/threonine protein kinase, and it exists as three different isoforms, Akt-1 (PKB-α), Akt-2 (PKB-β), and Akt-3 (PKB-γ) (14–17). All three Akt isoforms, which exhibit more than 80% sequence homology, undergo activation through sequential phosphorylation of threonine ($Thr^{308/309/305}$) and serine ($Ser^{473/474/472}$) residues in the kinase domain and carboxy-terminal domain respectively (14–17).

Several growth factors/tyrosine kinase-linked receptor agonists, including platelet-derived growth factor (PDGF), insulin, and insulin-like growth factor-1 (IGF-1), activate Akt in different cell types, including VSMC, by a phosphoinositide-3kinase (PI-3K)-dependent mechanism (3–5, 18, 19). In addition, the G protein-coupled receptor agonist, angiotensin II, activates Akt in VSMC through PI-3K pathway (20, 21). All these previous reports (3, 5, 20, 21), including our study (4), have clearly documented activation of either total Akt or Akt-1 in rodent VSMC. However, the stimulatory effects of growth factors on other Akt isoforms, Akt-2 and Akt-3, in comparison with Akt-1, have not yet been examined in VSMC from any species.

In the present study, we have used reverse-transcriptase polymerase chain reaction (RT-PCR) to assess the expression of all three Akt isoforms in human aortic VSMC. Using isoform-specific antibodies, we have performed *in vitro* kinase assays with immunoprecipitated Akt isoform(s) to determine the relative increases in the activity of all three Akt isoforms in VSMC in response to growth factors.

MATERIALS AND METHODS

Materials. Human aortic VSMC, human coronary artery VSMC, medium 231, and smooth muscle growth supplement were purchased from Cascade Biologics, Inc. (Portland, OR). Recombinant human PDGF-BB and recombinant human IGF-1 were from GIBCO BRL (Grand Island, NY). Insulin (Humulin R) was from Eli Lilly (Indianapolis, IN). Recombinant human VEGF was from R&D Systems,

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Inc. (Minneapolis, MN). Human urotensin II, endothelin-1, angiotensin II and arginine vasopressin were from American Peptide Company (Sunnyvale, CA). Anti-Akt-3 primary antibody and crosstide were from Upstate Biotechnology (Lake Placid, NY). Anti-Akt-1 and anti-Akt-2 primary antibodies, protein G Plus agarose and protein kinase A inhibitor peptide were from Santa Cruz Biotechnology (Santa Cruz, CA). LY294002 and LR-microcystin were from Calbiochem (San Diego, CA). Total RNA extracts from human adult liver, human adult skeletal muscle and human ovary were from Stratagene (La Jolla, CA). Total RNA from MCF-7 cells was from Geneka Biotechnology (Montreal, Canada). [γ-^{32}P]ATP (sp. activity: 4500 Ci/mmol) and [^{3}H]thymidine (sp. activity: 6.7 Ci/mmol) were from ICN Pharmaceuticals (Costa Mesa, CA). All other chemicals were from Fisher Scientific (Fair Lawn, NJ) or Sigma Chemical (St. Louis, MO).

Cell culture and treatments. Human aortic and coronary artery VSMC were cultured in medium 231 along with smooth muscle growth supplement (SMGS) and antibiotic antimycotic solution (AAS) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. After supplementation of medium 231 with SMGS and AAS, the final concentrations of the following components were as follows: fetal bovine serum, 5% (v/v); basic fibroblast growth factor, 2 ng/ml; epidermal growth factor, 0.5 ng/ml; penicillin, 100 U/ml; streptomycin, 100 μg/ml; and amphotericin B, 250 ng/ml. The media were changed every two days until the cells became confluent. After attainment of confluency (~80%), the cells were incubated in SMGS-free medium containing 0.5% BSA for 48 h. SMGS-starved VSMC were then exposed to the indicated agents as described in the respective figure legends. VSMC cultured between 4th and 9th passage levels were used for all experiments.

RNA extraction and RT-PCR analyses. Total RNA was extracted from human aortic VSMC and human coronary artery VSMC using the RNeasy Mini Kit according to the manufacturer's instructions (QIAGEN, Valencia, CA). The purity and concentration of RNA were determined by measurement of absorbance at 260 and 280 nm. The integrity of RNA was verified by agarose gel electrophoresis. Using the one-Step RT-PCR protocol (QIAGEN, Valencia, CA), 300 ng of RNA (from different human cells/tissues) was added to a master mix consisting of RT-PCR buffer (containing 2.5 mM $MgCl_2$), dNTP mix (400 μM of each dNTP), and RT-PCR enzyme mix (reverse transcriptases plus DNA polymerase) and the forward and reverse primers (1 μM) for human Akt-1, Akt-2, or Akt-3 (22). Fifty microliters of this RT-PCR reaction mixture was subjected to reverse transcription for 30 min at 55°C, followed by the activation step for DNA polymerase (15 min, 95°C). cDNA was amplified according to the following thermocycling parameters: 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s for a total of 30 cycles, followed by a 10-min extension at 72°C. PCR products were then subjected to agarose gel (2%) electrophoresis, and the fragments visualized by ethidium bromide staining using ultraviolet light and Kodak digital camera. The sequences for forward (F) and reverse (R) oligonucleotide primers (synthesized at the core facility, Penn State University) were as follows: Akt-1, (F) 5′-GCTGGACGATAGCTTGGA-3′ and (R) 5′-GATGAC AGATAGCTG-GTG-3′; Akt-2, (F) 5′-GGCCCCTGATCAGACTCTA-3′ and (R) 5′-TCCTCAGTC GTGGAGGAGT-3′; Akt-3, (F) 5′-GCAAGTG-GACGAGAATAAGTCTC-3′ and (R) 5′-ACA ATGGTGGGCTCAT-GACTTCC-3′; GAPDH, (F) 5′-TGAAGGTCGGAGTCAA-CGGATTTG-3′ and (R) 5′-CATGTGGGCCATGAGGTCCACCAC-3′. GAPDH was used as an internal control. A negative control without template RNA was also included in these experiments.

In vitro kinase assays with immunoprecipitated Akt isoform(s). After exposure to the indicated agents, human aortic VSMC were washed once with ice-cold PBS, and lysed using ice-cold buffer A consisting of 50 mM Tris–HCl (pH 7.5), 0.1% Triton X-100 (w/v), 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, protease inhibitor cocktail (Sigma), 0.1% β-mercaptoethanol (v/v), and 1 μM LR-microcystin. The cell lysates were centrifuged at 14,000 rpm at 4°C for 10 min. The supernatants (200 μg protein) of cell lysates were subjected to immunoprecipitation (3 h at 4°C) with 4 μg of anti-Akt-1, anti-Akt-2, or anti-Akt-3 primary antibody, which is initially preconjugated (1 h at 4°C) to protein G-plus agarose. The immunoprecipitates of Akt isoform(s) were washed thrice with buffer A containing 0.5 M NaCl, and twice with buffer B [50 mM Tris–HCl (pH 7.5), 0.03% Brij-35 (v/v), 0.1 mM EGTA, and 0.1% β-mercaptoethanol (v/v)]. Finally, Akt immunoprecipitates were washed with assay dilution buffer consisting of 20 mM MOPS (pH 7.4), 25 mM β-glycerophosphate, 1 mM sodium orthovanadate and 1 mM dithiothreitol. *In vitro* kinase assays for Akt isoforms were performed by incubating the respective Akt immunoprecipitates at 32°C for 10 min in 30 μl of assay dilution buffer (pH 7.4) in the presence of protein kinase A inhibitor peptide (1 μM) and [γ-^{32}P]ATP (10 μCi/assay), using crosstide (GRPRTSSFAEG, 30 μM) as the substrate (9, 13). The supernatants of the reaction mixture were applied onto p81 phosphocellulose papers, which were then subjected to washes with 0.75% phosphoric acid (3 times) and acetone (once). The amount of ^{32}P incorporated into crosstide was determined using a liquid scintillation counter. The observed dpm values were corrected for nonspecific binding by subtracting the background values (enzyme blank) obtained with mock immunoprecipitates.

[^{3}H]Thymidine incorporation studies. SMGS-starved human aortic VSMC were preincubated without (control) or with LY294002 (1 μM) for 30 min. Control and LY294002-treated VSMC were then exposed to PDGF, IGF-1 or insulin for 24 h. During the last 3 h incubation period, the cells were labeled with [^{3}H]thymidine (0.5 μCi/ml). The proliferation of VSMC was assessed by determination of [^{3}H]thymidine incorporation into DNA as described in our earlier studies (4).

Statistical analyses. The results are expressed as the mean ± SE of 3 or more experiments. The data were analyzed by repeated measures one-way ANOVA followed by Bonferroni t test. The differences between means were considered statistically significant when $P < 0.05$.

RESULTS AND DISCUSSION

We initially assessed the expression pattern of Akt isoform(s) mRNA in human aortic VSMC, in comparison with that expressed in several other human cells/tissues, using the RT-PCR method. As shown in Fig. 1, RT-PCR analyses revealed the expression of all three Akt isoforms in human aortic VSMC as well as in human coronary artery VSMC. Amplification of cDNA from human VSMC showed exponential increases in the formation of PCR products between 24 and 32 cycles (data not shown). In order to verify the specificity of oligonucleotide primers (22) used in the present study, we utilized the total RNA from human ovary (known to express all three Akt isoforms) and human MCF-7 estrogen receptor-positive breast cancer cell line (known to express only Akt-1 and Akt-2) as controls. Reverse transcription of the respective RNA followed by cDNA amplification revealed the expression of all three Akt isoforms in human ovary, similar to the observations of Masure *et al.* (17). Consistent with the findings of Nakatani *et al.* (23) and Okano *et al.* (22), Akt-1 and Akt-2 isoforms, but not Akt-3, were expressed in MCF-7 cells. Additionally, in the present study, all three Akt isoforms were expressed in varying

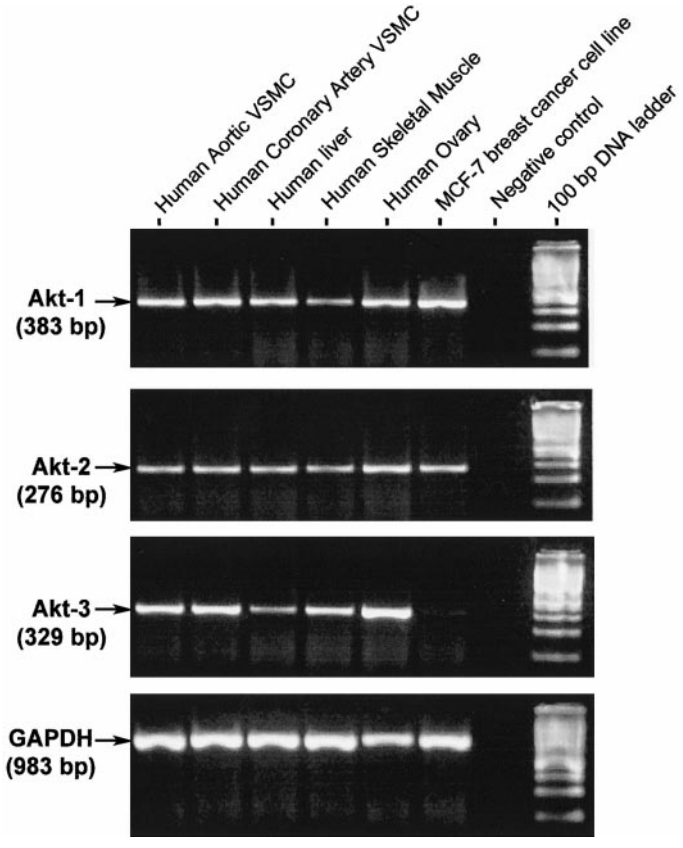

FIG. 1. RT-PCR analyses of Akt-1, Akt-2, and Akt-3 mRNA expression in human aortic VSMC in comparison with other human cells/tissues. Total RNA from different human cells/tissues was subjected to RT-PCR as described under Materials and Methods. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. GAPDH was used as internal controls. Negative controls without template RNA were also included in these experiments. The results shown are representative of 3 separate experiments.

amounts in nonvascular tissues, human liver and skeletal muscle.

We used RT-PCR method instead of Western blot analyses to determine the relative expression levels of Akt isoforms. The relative protein expression levels of Akt isoforms were not determined for the reason that the anti-Akt-3 antibody used for *in vitro* kinase assays was not suitable for Western blot analysis (9, 10). Previous studies examining the expression pattern of Akt isoforms in different tissues have revealed that Akt-1 and Akt-2 isoforms are ubiquitously expressed, whereas pronounced expression of Akt-3 is limited to tissues/cells, which include brain, heart, spleen, kidney, ovary, placenta, testis, estrogen receptor-deficient breast cancer cell lines and androgen-insensitive prostate cancer cell lines (15–17, 23). The present study shows that, in comparison with Akt-1 and Akt-2 isoforms, Akt-3 is also expressed in substantial amounts in human aortic VSMC and coronary artery VSMC, and to some extent even in human skeletal muscle.

Since the expression all three Akt isoforms were observed in human VSMC, we next compared the increases in the kinase activity of these Akt isoforms in human aortic VSMC in response to growth factors. Using isoform-specific anti-Akt primary antibodies, the respective Akt isoform(s) were immunoprecipitated and subjected to *in vitro* kinase assays to determine the activity of the Akt isoform(s). We used crosstide as the peptide substrate for these enzyme assays, since Alessi and co-workers (9) have previously demonstrated that crosstide has the same K_m value (4 μM) for all three Akt isoforms. Time-dependency experiments (Figs. 2A and 2B) revealed that exposure of human aortic VSMC to PDGF (1 nM) or insulin (300 nM) led to significant increases in Akt-3 activity, which attained maximal values between 5 and 10 min with a sustained elevation for up to 6 h. The relative maximal increases in Akt-3 activity after stimulation with PDGF and insulin were 79-fold ($P < 0.05$) and 102-fold ($P < 0.05$) respectively (basal Akt-3 activity: 2298 ± 534 dpm). In comparison, the relative maximal increases in Akt-1 activity in response to similar concentrations of PDGF and insulin were 7-fold ($P < 0.05$) and 11-fold

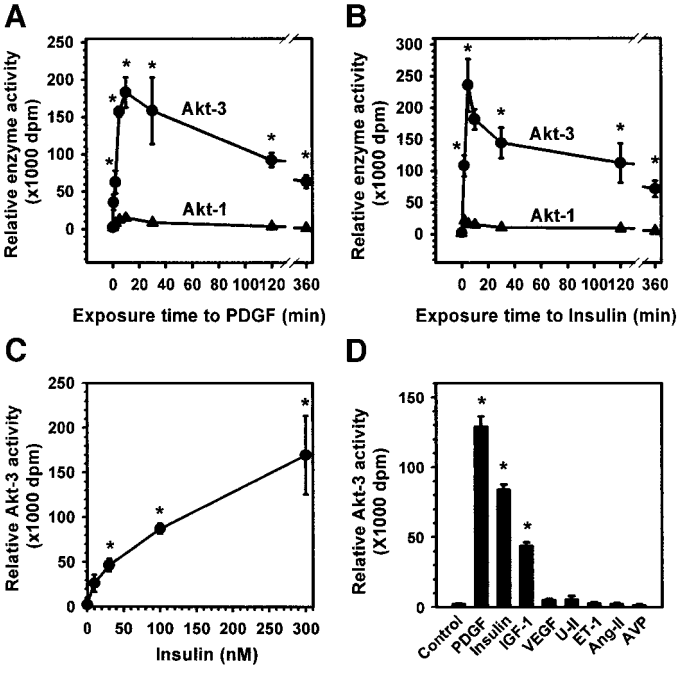

FIG. 2. Activation of Akt isoform(s) in human aortic VSMC. (A, B) Time dependency for PDGF- and insulin-induced increases in Akt-3 and Akt-1 activity. VSMC were exposed to PDGF (1 nM) or insulin (300 nM) for the indicated time intervals, after which *in vitro* kinase assays for Akt isoforms were performed (n = 3–4). (C) Concentration-dependency for insulin-induced increases in Akt-3 activity. VSMC were exposed to increasing concentrations of insulin (10–300 nM) for 5 min, after which Akt-3 activity was determined (n = 4). (D) Comparison of changes in Akt-3 activity after exposure of VSMC to tyrosine kinase-linked receptor agonists and G protein-coupled receptor agonists for 5 min (n = 3–5). *$P < 0.05$ compared with respective controls.

($P < 0.05$) respectively (basal Akt-1 activity: 1959 ± 644 dpm). The maximal increases in Akt-2 activity in response to PDGF and insulin were 3.8- and 1.8-fold respectively (basal Akt-2 activity: 1506 ± 526 dpm) ($n = 3$, data not shown in the figure).

Since pharmacological concentration of insulin (300 nM) produced marked increases in Akt-3 activity in comparison with Akt-1 and Akt-2, we examined whether physiological concentrations of insulin (≤100 nM) (24) could influence Akt-3 activity. As shown in Fig. 2C, insulin, at concentrations of 30 and 100 nM, produced significant increases in Akt-3 activity, thereby suggesting that even physiological concentrations of insulin could regulate Akt-3 activity in human aortic VSMC.

In the present study, there were no significant differences in the basal kinase activity of Akt-1, Akt-2, and Akt-3 in human aortic VSMC. However, stimulation with PDGF or insulin resulted in robust increases in Akt-3 activity with modest and marginal increases in Akt-1 and Akt-2 activity respectively in human aortic VSMC. Recent studies have shown that stimulation of estrogen receptor-deficient breast cancer cell lines or androgen-insensitive prostate cancer cell lines with insulin leads to a pronounced increase in Akt-3 activity with moderate and negligible increases in Akt-1 and Akt-2 activity respectively (23). It is interesting to note that in these cancer cell lines, which express all three Akt isoforms, there is no correlation between the expression levels of Akt isoforms and stimulated increases in the activity of the respective Akt isoforms (23). Similarly, in the present study, although the expression of Akt-3 was marginally higher than that of Akt-1 isoform, growth factor(s)-induced increases in Akt-3 enzyme activity were several orders of magnitude higher than that of Akt-1. Such a lack of correlation between Akt isoform expression and stimulated enzyme activity has also been observed in insulin-responsive, nonvascular tissues, rat hepatocytes and skeletal muscle (9). These tissues express similar levels of Akt-1 and Akt-2 but show marked increases in Akt-1 activity in comparison with Akt-2 after exposure to insulin (9). Furthermore, the findings of Masure *et al.* (17) and the present data demonstrate low to modest expression of Akt-3 isoform in human liver and skeletal muscle, and those of Roth and co-workers (10) have shown modest expression of Akt-3 in rodent adipocytes. However, studies using both human and rodent nonvascular tissues (liver, skeletal muscle, and adipocytes) have provided evidence for marked increases in Akt-1 and/or Akt-2 activity with negligible or relatively less increases in Akt-3 activity (9–11, 13).

Since PDGF and insulin are tyrosine kinase-linked receptor agonists, we also verified whether other tyrosine kinase-linked receptor agonists, insulin-like growth factor (IGF-1) (19) and vascular endothelial growth factor (VEGF) (25) could influence Akt-3 activity in human aortic VSMC (Fig. 2D). In comparison, we examined the effects of G protein-coupled receptor agonists/vasoconstrictor peptides (26, 27), urotensin II (UII), endothelin-1 (ET-1), angiotensin II (Ang II), and arginine vasopressin (AVP) on Akt-3 activity (Fig. 2D). Similar to the predominant stimulation of Akt-3 by PDGF and insulin, exposure of VSMC to IGF-1 (1 nM) led to robust increases in Akt-3 activity (Fig. 2D), with modest and marginal increases in Akt-1 and Akt-2 activity, respectively (data not shown). These data suggest that stimulation of multiple tyrosine kinase-linked receptors leads to activation of Akt-3. However, another tyrosine kinase-linked receptor agonist, VEGF (10 ng/ml) did not produce significant increases in Akt-3 activity in human aortic VSMC, thereby suggesting that VEGF may not regulate VSMC proliferation. It is pertinent to point out that VEGF-induced activation of Akt in endothelial cells, where VEGF receptors are predominantly expressed, leads to endothelial cell proliferation and migration (25, 28). Previously, we and other investigators have demonstrated that vasoconstrictor peptides, at 100 nM concentrations, produce maximal increases in cytosolic free calcium and contractility in VSMC (26, 27). Hence, in the present study, we used a similar concentration of vasoconstrictor peptide(s) to determine its effects on Akt-3 activity. Figure 2D shows that, although PDGF, insulin and IGF-1 stimulated Akt-3 activity, none of the four vasoconstrictor peptides, UII (100 nM), ET-1 (100 nM), Ang II (100 nM), and AVP (100 nM), produced significant increases in Akt-3 activity in human aortic VSMC. Recently, it has been reported that Ang II stimulates total Akt or Akt-1 in rat VSMC (20, 21). However, the present study, using human aortic VSMC, suggests that activation of Akt-3 is subject to regulation by tyrosine kinase-linked receptor agonists but not by G protein-coupled receptor agonists.

The role of PI-3K to mediate growth factor(s)-induced stimulation of Akt-3 activity and DNA synthesis was then investigated. As shown in Fig. 3A, preincubation of human aortic VSMC with the PI-3K inhibitor, LY294002, led to a concentration-dependent inhibition of PDGF-stimulated Akt-3 activity. The percentage inhibitions of PDGF-induced increases in Akt-3 activity by LY294002 at 10, 100, 300, and 1000 μM were 27.8 ± 1.3 ($P < 0.05$), 72.6 ± 0.6 ($P < 0.05$), 93.6 ± 2.1 ($P < 0.05$) and 99.8 ± 0.1 20 ($P < 0.05$) respectively. In addition to inhibition of PDGF-stimulated Akt-3, insulin- and IGF-1-induced increases in Akt-3 activity were also significantly attenuated by LY294002 (Fig. 3B), thus further establishing that PI-3K mediates growth factor(s)-induced activation of Akt-3 in human aortic VSMC. [^{3}H]thymidine incorporation studies revealed that PDGF (1 nM), insulin (100–300 nM) and IGF-1 (1 nM) produced significant increases in DNA synthesis, and that these were significantly inhibited ($P < 0.05$) by LY294002 (Fig.

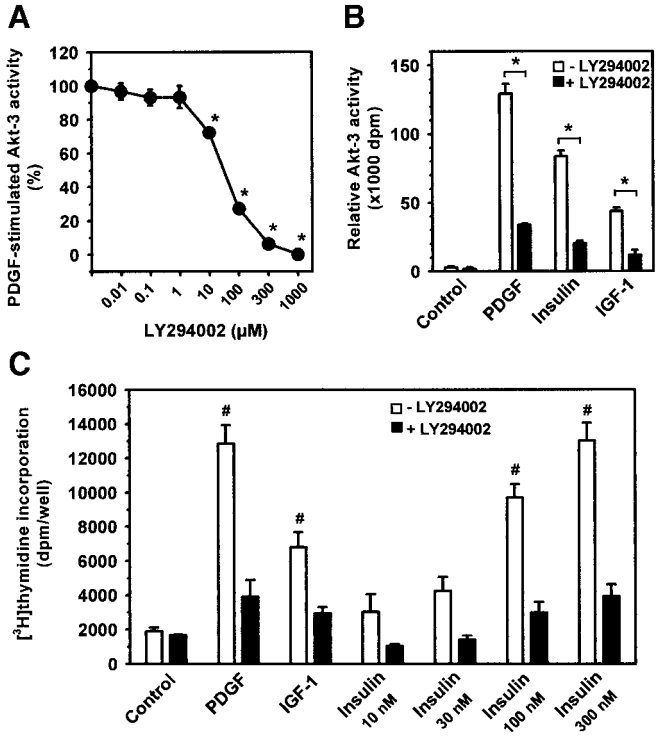

FIG. 3. Effects of PI-3K inhibitor, LY294002, on growth factor(s)-induced increases in Akt-3 activity and DNA synthesis in human aortic VSMC. (A) VSMC were preincubated without or with increasing concentrations of LY294002 for 30 min, exposed to PDGF (1 nM) for 5 min, and Akt-3 activity was determined ($n = 4$). (B) Control and LY294002-pretreated (100 μM, 30 min) VSMC were exposed to PDGF (1 nM), insulin (100 nM), or IGF-1 (1 nM) for 5 min, after which Akt-3 activity was determined ($n = 3–5$). C, Control and LY294002-pretreated (1 μM, 30 min) VSMC were exposed to PDGF (1 nM), IGF-1 (1 nM), or insulin (10–300 nM) for 24 h, after which [^{3}H]thymidine incorporation into DNA was determined ($n = 4–6$). *$P < 0.05$ compared with respective treatments; #$P < 0.05$ compared with control.

3C). Previously, it has been reported that PI-3K mediates activation of Akt-1 and Akt-2 in different tissues (18, 29). The present study using human aortic VSMC in culture and recent studies using human embryonic kidney 293 cells transfected with Akt-3 constructs (16, 17) demonstrate that activation of the Akt-3 isoform is also dependent on the upstream effector, PI-3K. Additionally, [^{3}H]thymidine incorporation studies suggest that the activation of PI-3K/Akt-3 signaling pathway may lead to proliferation of human aortic VSMC.

In conclusion, the present findings suggest that PI-3K -dependent activation of Akt-3 may play a major role in growth factor(s)-induced proliferation of VSMC. Increases in the concentrations of PDGF, insulin and IGF-1 have been observed in diabetic vascular complications, atherosclerosis and restenosis (1, 2). Enhanced activation of PI-3K/Akt-3 signaling pathway under these conditions may contribute to exaggerated VSMC proliferation. Therapeutic strategies aimed at selective inhibition of Akt-3 may prevent abnormal vascular growth in these diseased states. Furthermore, the design of specific Akt-3 inhibitors may spare insulin-induced activation of Akt-1 and/or Akt-2 isoforms required for glucose transport (7–13, 17), and thus may not predispose the human subjects to diabetes or complicate the preexisting diabetic state.

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