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Insulin-Like Growth Factor-1 Increases Endothelin Receptor A Levels and Action in Cultured Rat Aortic Smooth Muscle Cells

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Abstract Insulin is known to cause an increase in endothelin-1 (ET-1) receptors in vascular smooth muscle cells (SMCs), but the effect of insulin-like growth factor 1 (IGF-1) on ET-1 receptor expression is not known. We therefore carried out the present study to determine the effect of IGF-1 on the binding of ET-1 to, and ET type A receptor (ETAR) expression and ET-1-induced ^{3}H-thymidine incorporation in, vascular SMCs. In serum-free medium, IGF-1 treatment increased the binding of ^{125}I-ET-1 to SMC cell surface ET receptors from a specific binding of 20.1% $\pm$ 3.1% per mg of protein in control cells to 45.1% $\pm$ 8.6% per mg of protein in cells treated with IGF-1 (10 nM). The effect of IGF-1 was dose-related, with a significant effect (1.4-fold) being seen at 1 nM. The minimal time for IGF-1 treatment to be effective was 30 min and the maximal effect was reached at 6 h. Immunoblotting analysis showed that ETAR expression in IGF-1-treated cells was increased by 1.7-fold compared to controls. Levels of ETAR mRNA measured by the RT-PCR method and Northern blotting were also increased by 2-fold in IGF-1-treated SMCs. These effects of IGF-1 were abolished by cycloheximide or genistein. Finally, ET-1-stimulated thymidine uptake and cell proliferation were enhanced by IGF-1 treatment, with a maximal increase of 3.2-fold compared to controls. In conclusion, in vascular SMCs, IGF-1 increases the expression of the ET-1 receptor in a dose- and time-related manner. This effect is associated with increased thymidine uptake and involves tyrosine kinase activation and new protein synthesis. These findings support the role of IGF-1 in the development of atherosclerotic, hypertensive, and diabetic vascular complications. J. Cell. Biochem. 94: 1126–1134, 2005.
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Endothelin-1 (ET-1), synthesized and secreted by vascular endothelial cells, is the most potent endogenous vasoconstrictor [Yanagisawa et al., 1988]. It also stimulates DNA synthesis in vascular smooth muscle cells (SMCs) [Nakaki et al., 1989] and is therefore thought to play an important role in the development of various circulatory disorders, including hypertension and atherosclerosis. After secretion from endothelial cells, ET-1 binds to specific receptors on the adjacent vascular SMCs and perhaps at other distal sites [Simonson and Dunn, 1991; Clozel et al., 1989]. Two distinct types of endothelin (ET) receptor have been cloned and sequenced from bovine and rat tissues [Arai et al., 1990; Sakurai et al., 1990]. ET type A receptors (ETAR) are expressed predominantly

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in vascular SMCs and mediate vasoconstriction and cellular proliferation [Eguchi et al., 1992; Ihara et al., 1992]. Recently, we demonstrated overexpression of the vascular ETAR in a fructose-induced hypertensive rat model [Juan et al., 1998], providing further support for the role of ET-1 and its receptors in hypertension. Insulin-like growth factor-1 (IGF-1) is a 7.6 kDa peptide, which is highly homologous to proinsulin in structure and function [Ullrich et al., 1986], and is involved in the structural changes associated with atherosclerosis and related conditions [Hansson et al., 1987; Khorsandi et al., 1992]. A genetic epidemiological study on 502 Caucasian individuals found that genetic polymorphism of IGF-1 is related to fat mass [Sun et al., 1999]. Furthermore, in patients with essential hypertension, serum levels of IGF binding protein 1 and the [binding protein 1]/[binding protein 3] ratio are both inversely correlated with the insulin resistance index [Laviades et al., 1998], indicating that tissue availability of IGF-1 is a determining factor in insulin sensitivity in patients with essential hypertension. Unlike insulin, which is only produced in the pancreas, IGF-1 is produced by various cell types, including vascular endothelial cells [Hansson et al., 1987] and SMCs [Khorsandi et al., 1992].

Several studies have shown that insulin treatment increases the number of ET-1 receptors in vascular SMCs [Frank et al., 1993; Kwok et al., 1993a; Hopfner et al., 1998], but we are not aware of any study on the effect of IGF-1 on the ET-1 receptor. We therefore carried out the present study to determine the effect of IGF-1 on ET-1 binding to, and ETAR expression and ET-1-induced ^{3}H-thymidine incorporation in, vascular SMCs.

MATERIALS AND METHODS

Materials

Tissue culture plastic-ware was from Costar (Cambridge, MA), Dulbecco's modified Eagle medium (DMEM) from Hyclone (Logan, UT), fetal bovine serum (FBS) from Gibco (Grand Island, NY), IGF-1 from Peninsula Laboratories, Inc. (Delmont, CA), ET-1 from the Peptide Institute (Osaka, Japan), ^{125}I-insulin (specific activity 2,000 Ci/mmol) from Amersham (Chicago, IL), PVDF membranes from MSI (Westboro, MA), anti-endothelin-1 receptor antibodies from BD Transduction Laboratories (Lexington, KY), anti-β-actin antibodies from Chemicon (Temecula, CA), Western Blot Chemiluminescence Reagent Plus Kits from NEN (Boston, MA), and insulin, cycloheximide, genistein, bovine serum albumin (BSA), and other reagents were from Sigma (St. Louis, MO).

Culture of SMCs

This was performed as described previously [Ross, 1971]. Briefly, male Sprague–Dawley rats (150–200 g body weight) were decapitated, the thoracic aortas sterilely dissected out, and the adventitia and intima completely removed. The medial layers were then sliced into 2×2 mm squares which were transferred to gelatin-coated p60 tissue culture dishes and incubated in DMEM containing 10% FBS in a 37°C humidified incubator with an atmosphere of 95% air and 5% CO_2. Between days 5 and 7 after explant isolation, SMCs started to migrate out of the explants and to proliferate. At day 14, the explants were removed using fine forceps and the cells trypsinized and subcultured at a ratio of 1:3 into other dishes. Confluent SMC cultures showed the characteristic "hill and valley" growth pattern and were able to form multilayers on cultures. The cells were then harvested by treatment with 0.05% trypsin in 0.2% EDTA and seeded in gelatin-coated tissue culture dishes. Cells from passages 5–15 were used in these studies.

ET-1 Binding Assay

SMCs in 6-well plates were incubated in medium containing various concentrations of IGF-1 for 0.5, 1, 2, 4, 6, and 24 h, then the cells were washed three times to remove residual IGF-1 and incubated in medium without IGF-1 for the remainder of the total 24 h incubation period, then ET-1 binding was measured. ET-1 binding was assayed as described previously [Kwok et al., 2000]. Briefly, cells were washed twice with 50 mM HEPES buffer, pH 7.4, containing 0.2% BSA (HBSA), then incubated for 16 h at 4°C in 1 ml of HBSA containing 0.01 pmol of ^{125}I-ET-1 and various amounts of unlabeled ET-1. The cells were then washed twice with phosphate-buffered saline (PBS), pH 7.4, solubilized with 0.1% sodium dodecyl sulfate (SDS), and counted in a gamma counter (LKB) with 75% efficiency. Non-specific binding, determined using 1 μM of unlabeled ET-1, was 3%–20% of total binding. The binding data were

expressed as the percentage of the total ^{125}I-ET-1 (0.01 pmol) bound in the test normalized to 1 mg of protein, measured using the Lowry assay [Lowry et al., 1951].

Western Blots

After treatment with 0, 10^{-8}, or 10^{-7} M IGF-1 for 24 h, the cells were washed twice with PBS, then lysed with 0.5 ml of lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 0.5% Nonidet P-40). Protein concentrations of lysates were measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA), then 50 μg of lysate proteins was separated by SDS–PAGE using a 10% polyacrylamide gel and electroblotted onto a PVDF membrane. After blockage of nonspecific binding sites for 1 h with 5% nonfat milk in PBS containing 0.05% Tween 20 (TPBS), the membrane was incubated at room temperature for 1 h with mouse monoclonal antibodies against the ETAR or β-actin, washed six times with TPBS, incubated with horseradish peroxidase-conjugated goat anti-mouse IgG at room temperature for 1 h, then washed six times with TPBS. Bound antibody was visualized using a Western Blot Chemiluminescence Reagent Plus Kit and the intensity of the bands quantified using a densitometer (Molecular Dynamics, Sunnyvale, CA).

RNA Extraction

Total RNA was extracted from the treated SMCs using a Tri Reagent Kit (Molecular Research Center, Inc., Cincinnati, OH). The integrity of the extracted total RNA was examined by 1% agarose gel electrophoresis and its concentration determined by UV light absorbance at 260 nm. All RNA samples were incubated for 30 min at 37°C with RNase-free DNase I, then for 10 min at 100°C to inactivate the DNase.

RT-PCR Analysis

One microgram of total RNA was reverse-transcribed for 1 h at 42°C using poly(dT)12-18 primers and SUPER RT reverse-transcriptase in a 50 μl reaction volume. Five microliters of this reaction solution was then used for PCR in a 50 μl volume of reaction mixture using the following conditions: 1 cycle of 95°C × 5 min; 35 cycles of 95°C × 30 s, 55°C × 1 min, 72°C × 1 min; and a final 7 min extension period. The primers used were: for rat ETAR, sense primer, 5′-CAGAT CCACA TTAAG ATGGG-3′, and antisense primer, 5′-CAATG ACCAC GTAGA TAAGG-3′; for rat β-actin, sense primer, 5′-GAGAA GATTT GGCAC CACAC-3′, and antisense primer, 5′-CATCA CAATG CCAGT GGTAC-3′. These combinations would amplify fragments of 412 bp for ETAR cDNA and 213 bp for β-actin cDNA. Ten microliters each of the ETAR and β-actin PCR products amplified using the same RT template solution were combined and electrophoresed on a 2% agarose gel, then, after ethidium bromide staining, the ETAR and β-actin cDNA bands were measured by densitometry and the ETAR mRNA/β-actin mRNA ratio measured.

Northern Blotting

Plasmids containing ETAR cDNA probe was given to us by Y.F. Chen (University of Alabama, Birminingham, Alabama). Northern blotting was carrier out according to the procedure previously described [Juan et al., 1998]. For hybridization, the inserted cDNA probes were cut out and radiolabeled using a random primer labeling system [Feinberg and Vogelstein, 1983] with reagents purchased from Promega (Madison, WI). After hybridization, membranes were washed and autoradiographed with an intensifying screen at −80°C. Hybridization signals in autoradiogram were expressed in arbitrary densitometric units based on the ratios over their corresponding G3PDH mRNA bands and for comparison.

Thymidine Uptake

Confluent cells were maintained for 24 h in DMEM medium or DMEM medium containing 10 nM of IGF-1, then thymidine uptake was measured as described previously [Kwok et al., 1989]. Briefly, the growth medium was replaced for 24 h with DMEM containing 0.5% BSA, then various amounts of ET-1 were added to each well and the cells were incubated for 16 h before pulsing with 1 μCi of ^{3}H-thymidine for 2 h at 37°C. The cells were washed twice with PBS and solubilized in 0.1% SDS, then the proteins were precipitated with 10% cold trichloroacetic acid, washed, and counted in a scintillation counter.

Cell Proliferation

Cell number was quantified on a hemocytometer after trypsinization.

Statistics

All studies were performed in triplicate in each experiment. Experiments were repeated separately at least three times. Differences between control and experimental groups were evaluated using the two-tail Student's *t*-test. Difference were reported as significant if $P < 0.05$.

RESULTS

^{125}I-ET-1 binding to cell surface ET receptors was increased in SMCs after IGF-1 treatment. At tracer concentrations of ET-1, control SMCs exhibited a maximal ET-1 binding of 20.1% ± 3.1% per mg of protein, whereas cells treated with IGF-1 (10 nM, 2 h) exhibited a ET-1 binding of 45.1% ± 8.6% per mg protein (Fig. 1, $P < 0.05$). In 125I-ET-1 competitive binding studies, the IC_{50} was similar in the control and IGF-1-treated groups (1.9 and 2.1 nM, respectively). The effect of IGF-1 was dose-related, with a significant effect (1.4-fold) being seen at a concentration of 1 nM (Fig. 2). Using 10 nM IGF-1, the minimal period of IGF-1 treatment for an effect to be seen was 30 min, and the maximal effect was seen after 6 h of treatment (Fig. 3). Note that, in all cases, ET-1 binding was measured 24 h after the start of IGF-1 treatment.

The effect of IGF-1 on ETAR expression was further investigated using Western blots. As shown in Figure 4, the size of the band in both the control and IGF-1-treated groups is consistent with the reported molecular weight of the ETAR [Hayzer et al., 1992]. As evaluated by densitometry, the ETAR levels in SMCs incubated with 10^{-7} M IGF-1 for 24 h were increased by 1.7-fold (Fig. 4).

The ETAR mRNA/β-actin mRNA ratio in rat aortic SMCs treated with different doses of IGF-1 was measured using the RT-PCR method. Compared to the control group, the ETAR mRNA levels in SMCs treated with 10^{-7} M IGF-1 for 24 h were increased by 1.9-fold. (Fig. 5).

Results of Northern blotting indicated that the ETAR cDNA probe detected two bands of 5.2 and 4.2 kb, respectively (Fig. 6), which were identical to those of authentic ETAR mRNA previous described [Lin et al., 1991; Hori et al.,

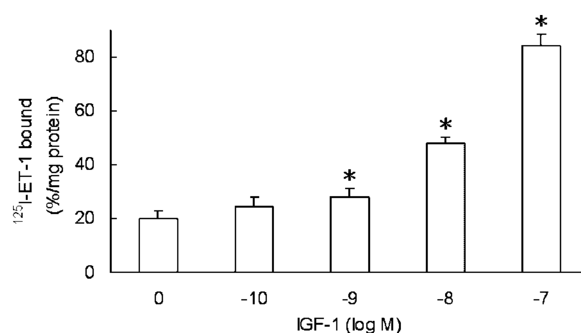

Fig. 2. Dose-response of the effect of IGF-1 on ET-1 binding in SMCs. After 2 h treatment in serum-free medium containing the indicated concentrations of IGF-1, the cells were washed three times, maintained in medium without IGF-1 for 24 h, then ET-1 binding was measured as described in the ''Materials and Methods'' section. Each point represents the mean ± SEM for three separate experiments using SMCs from different passages (5–15). *$P < 0.05$ compared to control.

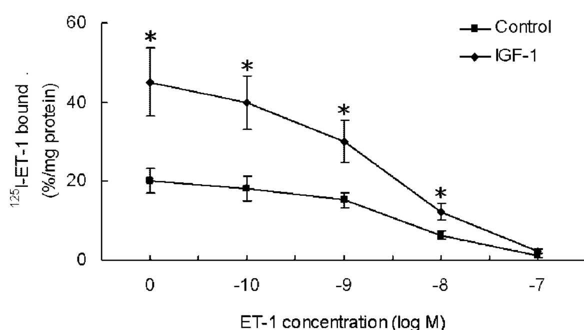

Fig. 1. Endothelin-1 (ET-1) binding competitive inhibition curves for control and insulin-like growth factor 1 (IGF-1)-treated aortic smooth muscle cells (SMCs). The cells were incubated in serum-free medium with or without 10 nM IGF-1 for 2 h, then ET-1 binding was measured as described in the ''Materials and Methods'' section. Each point represents the mean ± SEM for three separate experiments using SMCs from different passages (5–15). *$P < 0.05$ compared to control.

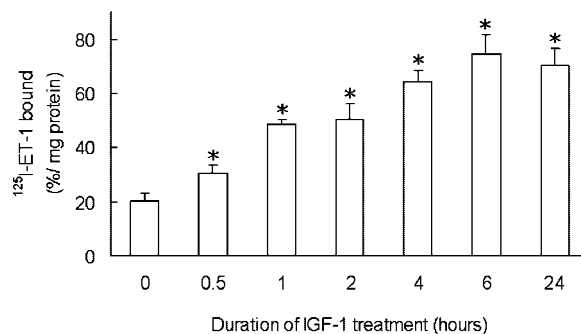

Fig. 3. Time-course of the effect of IGF-1 on ET-1 binding in aortic SMCs. After treatment with 10 nM IGF-1 in serum-free medium for 0.5, 1, 2, 4, 6, or 24 h, the cells were washed three times to remove IGF-1, then placed in medium without IGF-1 for the remainder of the 24 h total incubation period, before ET-1 binding was measured as described in the ''Materials and Methods'' section. Each point represents the mean ± SEM for three separate experiments using SMCs from different passages (5–15). *$P < 0.05$ compared to control (0 h).

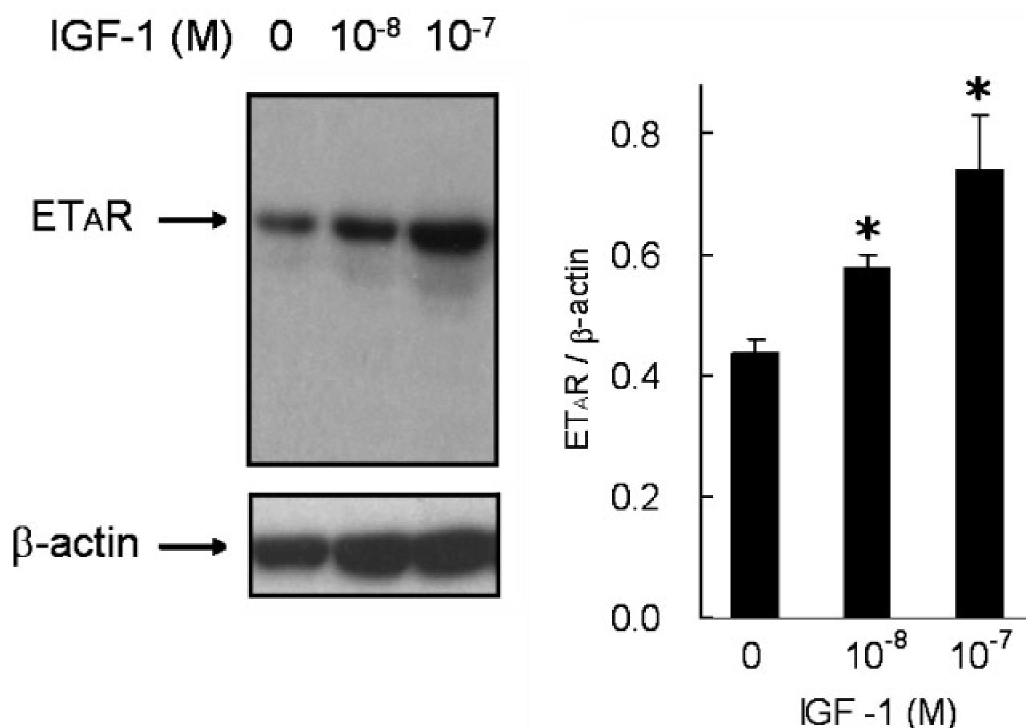

Fig. 4. Western blot analysis of ET-1 receptor (ET-A) levels in aortic SMCs incubated for 24 h in serum-free medium with or without IGF-1. Lysates (50 μg) were subjected to electrophoresis on 10% polyacrylamide gels and analyzed. **Lane 1**, control; **lane 2**, 10^{-8} M IGF-1; **lane 3**, 10^{-7} M IGF-1. The data represent the mean ± SEM for three separate experiments using SMCs from different passages (5–15). *$P < 0.05$ compared to IGF-1 alone.

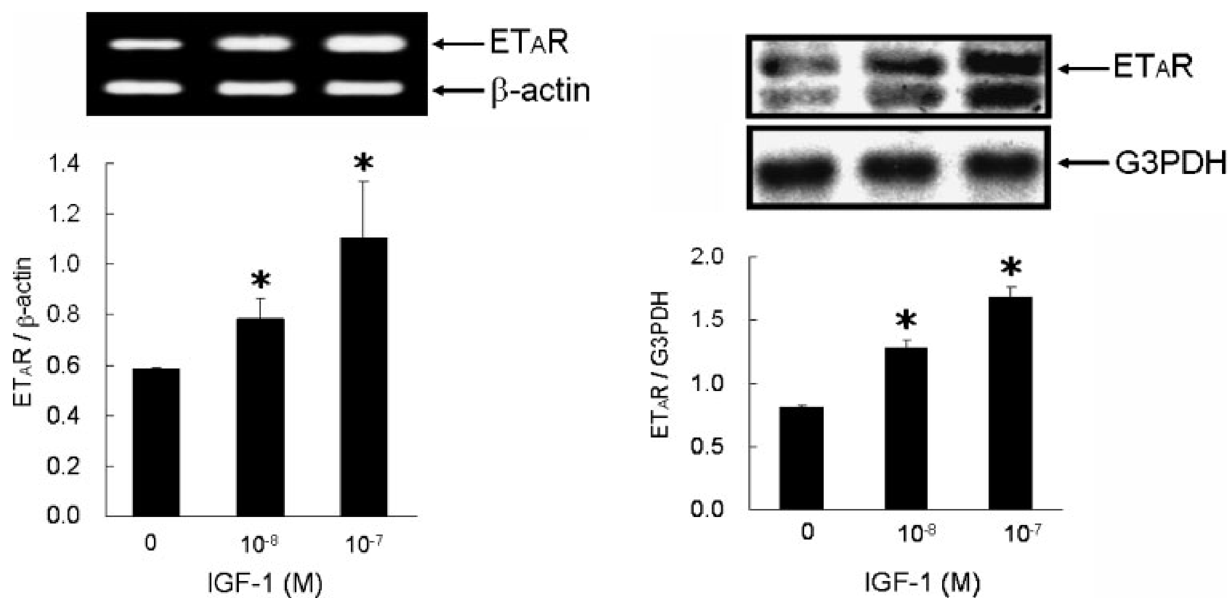

Fig. 5. ET type A receptor (ETAR) and β-actin mRNA levels in rat SMC incubated for 24 h with different doses of IGF-1 in serum-free medium measured by RT-PCR. The data shown are the mean ± SEM for three separate experiments using SMCs from different passages (5–15). **Lane 1**, control; **lane 2**, 10^{-8} M IGF-1; **lane 3**, 10^{-7} M IGF-1. *$P < 0.05$ compared to control.

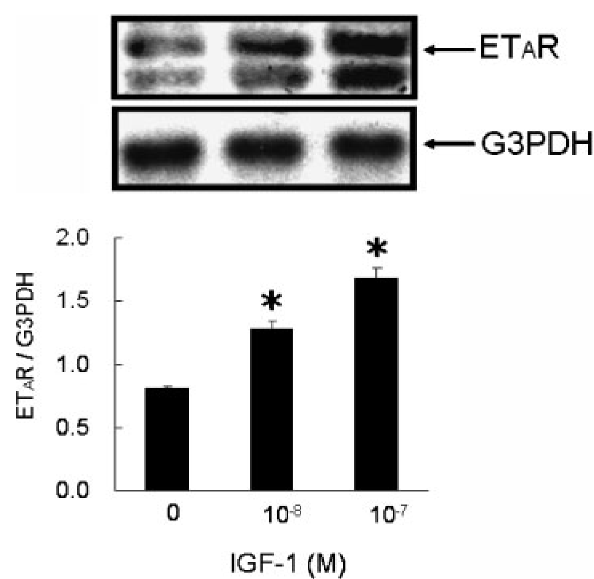

Fig. 6. ETAR and β-actin mRNA levels in rat SMC incubated for 24 h with different doses of IGF-1 in serum-free medium measured by Northern blotting. **Lane 1**, control; **lane 2**, 10^{-8} M IGF-1; **lane 3**, 10^{-7} M IGF-1. The data represent the mean ± SEM for three separate experiments using SMCs from different passages (5–15). *$P < 0.05$ compared to IGF-1 alone.

1992]. Compared to the control group, rat aortic SMC treated for 24 h with IGF-1 showed a dose-dependent increase in ETAR mRNA levels (Fig. 6).

To determine whether the increase in ET-1 receptor levels involved tyrosine kinase, we used genistein, a tyrosine kinase inhibitor, and found that it blocked the IGF-1-induced increase in ET-1 binding (Fig. 7). Similar results were obtained using cycloheximide, a protein synthesis inhibitor (Fig. 7).

To determine whether this increase in ET-1 binding was associated with any biological effect, ET-1-stimulated thymidine uptake was measured after 24 h treatment with 10 nM IGF-1 and was found to be increased by about 3.2-fold compared to in control cells (Fig. 8A, n = 3, $P < 0.05$). Results of cell counting showed that addition of 10^{-8} M ET-1 alone slightly increased cell number by 1.37-fold compared with the control (Fig. 8B, n = 3, $P < 0.05$). IGF-1 alone has little effect on cell proliferation. After 24 h pretreatment with 10 nM IGF-1, addition of 10^{-8} M ET-1 also caused cell number to increase by 3.1-fold compared with the control (Fig. 8B, n = 3, $P < 0.05$).

DISCUSSION

In this report, we found that IGF-1 was able to increase the amount of ETAR. The mechanism of this effect is probably through stimulation of net receptor synthesis, as it was abolished by cycloheximide, a protein synthesis inhibitor. The IGF-1 receptor is a tyrosine kinase and the binding of IGF-1 to its receptor results in activation of a series of steps involving tyrosine phosphorylation. In this report, we also found that genistein, a tyrosine kinase inhibitor, abolished the effect of IGF-1 on ET-1 binding, confirming the involvement of tyrosine kinase in the action of IGF-1. Besides the increase in ETAR levels, IGF-1 also enhanced the mitogenic action of ET-1 on aortic SMCs, as measured by thymidine incorporation and cell number counting. Both IGF-1 and ET-1 are believed to play significant roles in the development of atherosclerosis and of hypertensive and diabetic vascular complications. Our report lends further support to this notion and suggests that at least part of the effect of IGF-1 may be through the increase in ETAR levels.

Several lines of evidence indicate that insulin increases the number of ET-1 receptors in

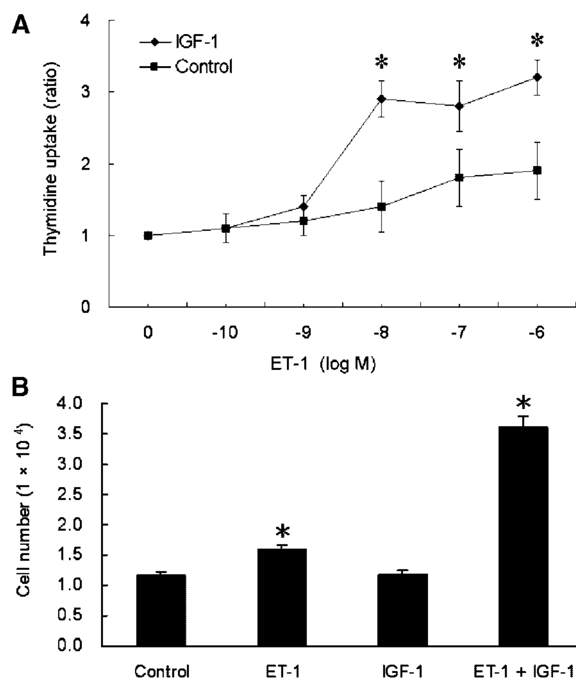

Fig. 8. ET-1-stimulated (**A**) thymidine uptake and (**B**) cell proliferation in IGF-1-treated aortic SMC. A: Confluent cells were maintained for 24 h in control medium or medium containing 10 nM of IGF-1, then thymidine uptake was measured as described in the "Materials and Methods" section. B: Confluent cells were maintained for 24 h in control medium or medium containing 10 nM of IGF-1. Then 10^{-8} M ET-1 or vehicle was added to each well and the cells were incubated for another 24 h. The cell number was counted as described in the "Materials and Methods" section. The data shown are the mean ± SEM for three separate experiments using SMCs from different passages (5–15). *$P < 0.05$ compared to control.

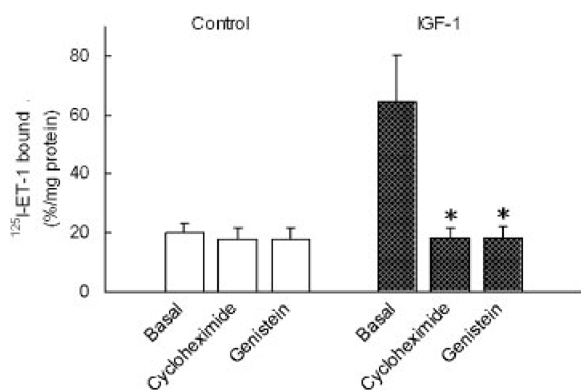

Fig. 7. Effects of cycloheximide and genistein on the IGF-1-induced increase in ET-1 binding in aortic SMC. In our study, confluent cells were pretreated with cycloheximide (10^{-5} M) or genistein (10^{-4}) for 2 h, and then incubated with or without IGF-1 for 24 h. At the end of incubation period, ^{125}I-ET-1 binding capacity to the SMCs was evaluated as described in the "Materials and Methods" section. The data represent the mean ± SEM for three separate experiments using SMCs from different passages (5–15). *$P < 0.05$ compared to IGF-1 alone.

vascular SMCs [Frank et al., 1993; Kwok et al., 1993a; Hopfner et al., 1998]. Recently, Nagai et al. [2003] also demonstrated that ET-1 production in the endothelium is activated by insulin via the IGF-1 receptor. On the basis of Nagai's findings, it is very likely that insulin also stimulates an increase in ETAR levels via the IGF-1 receptor. These studies highlight the significant role of IGF-1 signaling in the regulation of the vascular ET-1 system.

IGF-1 is a progression factor for SMCs and plays an important role in SMC proliferation [Liu et al., 2001]. In addition, an increase in IGF-1 mRNA levels has been reported after balloon denudation [Cercek et al., 1990; Khorsandi et al., 1992]. IGF-1 has also been shown to play a role in SMC migration [Bornfeldt et al., 1994], which is another important step in both the development of atherosclerosis and restenosis after angioplasty. Furthermore, treatment of rats with the long-acting somatostatin analog, octreotide, which acts primarily by inhibiting growth hormone secretion and, thus, IGF-1 gene expression in liver and peripheral tissues, evokes a dose-dependent decrease in neointima/media ratios after balloon injury of femoral arteries, and femoral artery IGF-1 mRNA levels are also markedly decreased and fail to increase after arterial injury [Yumi et al., 1997]. Hayry et al. [1995] demonstrated that treatment of rats with a stable peptide analog of IGF-1 designed to act as an IGF-1 receptor antagonist decreases ^{3}H-thymidine labeling and intimal thickness after carotid injury. Taken together, these studies indicate that IGF-1, acting as an autocrine/paracrine growth factor, may be a significant mediator of SMC proliferation after arterial injury. In addition, ET-1 is secreted locally by the endothelium and reaches adjacent SMCs by diffusion and therefore may induce SMC proliferation, which is one of the major histological changes of the atherosclerotic process and the vascular hypertrophy associated with hypertension [Mulvany et al., 1978; Ross, 1986; Banskota et al., 1989b]. Under pathogenic conditions, highly expressed IGF-1 may cause a dramatic increase in ETAR levels and subsequent SMC proliferation and migration. Our findings suggested that ETAR may be involved in the association between IGF-1 and atherosclerosis.

IGF-I has a profound impact on the vascular system. IGF-I can be produced by endothelial and vascular SMCs, and specific receptors for IGF-I are abundant in the vascular wall [Sarzani et al., 1989; Delafontaine and Lou, 1993; LeRoith et al., 1995]. Furthermore, it has been suggested that, due to the presence of increased amounts of IGF-1 receptor and more IGF-1 transcytosis in the aortic endothelial cells of diabetic rats [Bar et al., 1990; Kwok et al., 1993b], the sub-endothelium of the arterial walls may be exposed to a higher concentration of IGF-1. Taken together with these reports, our findings indicate that diabetes may result in enhanced ET-1 action on sub-endothelial SMCs and thus contribute to the acceleration of atherosclerosis in diabetes.

Raised levels of ET-1 have also been detected in the human coronary sinus following percutaneous transluminal angioplasty [Suzuki et al., 2000] and balloon angioplasty causes a significant increase in medial ET binding and pronounced neointima formation in pig coronary arteries [Dashwood et al., 1999]. Administration of an ETAR antagonist markedly inhibits the increase in neointima formation and medial ET binding caused by balloon angioplasty [Dashwood et al., 1999]. An ETAR antagonist has also been reported to inhibit intimal SMC proliferation in human saphenous veins [Maguire et al., 2002]. Consequently, it was suggested that vascular SMC proliferation and subsequent neointima formation is mediated predominantly via the ETAR. In the present study, we found that IGF-1 induced ETAR overexpression and subsequent SMC proliferation. Thus, it is possible that the high expression of IGF-1 caused by vascular injuries directly induces overexpression of ET-1 and ETAR, leading to neointima formation and SMC proliferation. Taken together previous evidence with our present observations, IGF-1 may play a functional role, through ETAR overexpression, in neointima formation and SMC proliferation after vascular injury.

IGF-1 also cooperates with other growth factors, such as platelet-derived growth factor (PDGF), to induce aortic SMC proliferation [Banskota et al., 1989a]. Using embryonic fibroblast cell lines derived from mice with targeted disruption of the type 1 IGF receptor (IGF-1R), Sell et al. [1994] observed that about 60% of the proliferative capacity of serum or PDGF is dependent on autocrine production of IGF-1. Furthermore, the integrity of the IGF-1R is obligatory for the PDGF-dependent increase in proliferating cell nuclear antigen mRNA

levels in replicating cells [Miura et al., 1994]. In our study, we also found that the increase in ET-1 binding to IGF-1-treated cells was significantly higher in the presence of 10% FBS (growth factor-rich) (data not shown).

In conclusion, we have found that, in vascular SMCs, IGF-1 increases ET-1 receptor levels in a dose- and time-related manner. This effect involves tyrosine kinase activation and new protein synthesis and is associated with enhancement of thymidine uptake and cell proliferation. Our findings support a role of IGF-1 in the development of atherosclerosis and hypertensive and diabetic vascular complications. Further studies to confirm its role in vivo are warranted.

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