Hypoxia and high glucose upregulate AT$_1$ receptor expression and potentiate ANG II-induced proliferation in VSM cells

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Sodhi, Chhinder P., Yashpal S. Kanwar, and Atul Sahai. Hypoxia and high glucose upregulate AT$_1$ receptor expression and potentiate ANG II-induced proliferation in VSM cells. Am J Physiol Heart Circ Physiol 284: H846–H852, 2003. First published November 14, 2002; 10.1152/ajpheart.00625.2002.—We examined the effect of hypoxia and high glucose (HG) on ANG II type 1 (AT$_1$) receptor expression and proliferation in cultured vascular smooth muscle (VSM) cells. Exposure of quiescent cells to hypoxia in a serum-free DME-Ham’s F-12 medium for 6–24 h induced a progressive increase in AT$_1$ mRNA expression. Exposure of cells to 24 h of hypoxia also resulted in a significant increase in ANG II receptor binding as assessed with $^{125}$I-labeled ANG II. Treatment with ANG II (1 μM) for 24 h under normoxic conditions caused an ~1.5-fold increase in both DNA synthesis and cell number, which was enhanced to ~3.0-fold under hypoxic conditions. An AT$_1$ receptor antagonist (losartan, 10 μM) blocked the ANG II-induced increase in DNA synthesis under both normoxic and hypoxic conditions. Incubations in HG medium (25 mM) for 12–24 h under normoxic conditions induced an ~2.5-fold increase in AT$_1$ mRNA levels, which was markedly enhanced by hypoxia to ~5.5-fold at 12 h and ~8.5-fold at 24 h. ANG II under HG-normoxic conditions caused a complete downregulation of AT$_1$ expression, which was prevented by hypoxia. These results demonstrate an upregulation of AT$_1$ receptor expression by hypoxia and HG in cultured VSM cells and suggest a mechanism for enhanced ANG II-induced VSM cell proliferation and the development of atherosclerosis in diabetes.

vascular smooth muscle cells; diabetes; chronic hypoxia; cell growth; angiotensin II receptor

ARTERIAL WALL HYPOXIA and the associated vascular smooth muscle (VSM) cell proliferation have been implicated in the development of atherosclerosis (7, 23). Moreover, we recently reported (43) that hypoxia directly induces the proliferation of cultured rat aortic VSM cells. Others have shown that hypoxia is also mitogenic to cultured pulmonary artery smooth muscle and endothelial cells (12, 13, 25). Prevalence of tissue hypoxia and increased VSM cell proliferation is also reported in experimental models of diabetes and hypertension (1, 10, 28, 35, 40, 45). Elevated glucose concentrations in medium have been shown to produce both hypertrophic and hyperplastic effects in cultured porcine aortic smooth muscle cells (32). We found (43, 44) that high medium glucose also induces the proliferation of cultured VSM cells as well as renal glomerular mesangial cells. In addition, hypoxia potentiates the effect of high medium glucose on the proliferation of both VSM and mesangial cells (43, 44). Together, these results strongly suggest an important role for local hypoxia in accelerated VSM cell proliferation in diabetes. The mechanisms responsible for the accelerated VSM cell growth and progression into cardiovascular disease in diabetes remain to be clearly defined.

Activation of the renin-angiotensin system (RAS) plays an important role in the pathogenesis of vascular complications of diabetes (16). The primary active component of the RAS, ANG II, causes hypertrophy, hyperplasia, and the deposition of extracellular matrix proteins in VSM cells (19, 21, 36, 49). Interestingly, ANG II-induced proliferation of cultured rat aortic VSM cells has been shown to be enhanced under hypoglycemic conditions (4). The ANG II type 1 (AT$_1$) receptor is a G protein-coupled receptor that mediates most of the known biological effects of ANG II (30, 47). Both local production of ANG II and AT$_1$ receptor expression are increased in cardiac myocytes and preglomerular vessels in streptozotocin-induced diabetic rats (3, 42). Also, AT$_1$ receptor expression has been shown to be increased in the kidney and/or VSM cells in experimental models of hypertension, cyclosporin nephrotoxicity, and myocardial infarction (5, 6, 9, 15, 18, 26, 27). Of interest, both diabetes and hypertension, including cyclosporin administration, have been shown to produce tissue hypoxia (40, 50, 52). These findings suggest a link between cellular hypoxia and AT$_1$ receptor upregulation in cardiovascular injury. However, a definitive role of hypoxia and high glucose in AT$_1$ receptor regulation and their interaction with ANG II-induced proliferation remain poorly defined.

Because arterial wall hypoxia appears to be associated with increased AT$_1$ receptor expression and proliferation of VSM cells in various experimental models, we directly examined the effect of hypoxia and high glucose on AT$_1$ receptor expression in cultured aortic...
VSM cells. In addition, we determined the role of hypoxia in ANG II-induced regulation of VSM cell AT₁ expression and proliferation under hyperglycemic conditions.

RESEARCH DESIGN AND METHODS

Materials. Male Sprague-Dawley rats weighing 200–250 g were obtained from Harlan Laboratories (Indianapolis, IN). [³H]thymidine and [³²P]dCTP were purchased from ICN (San Diego, CA). cDNA probe for the rat AT₁ receptor was generously provided by Dr. Thomas Thekkumkara (University of Colorado, Denver, CO; Ref. 48). All other reagents were of high chemical grade.

Cell culture. Rat aortic VSM cells were isolated and cultured as previously described (43). Briefly, aortas of Sprague-Dawley rats were resected under sterile conditions, subjected to collagenase digestion, and cultured in MEM (d-valine modification) containing 20% fetal bovine serum, 0.3 U/ml insulin, and antibiotics. Cells were kept in this medium for two passages, which permitted the growth of VSM cells while the growth of fibroblasts was inhibited. After the second passage, cultures were grown in DMEM-Ham's F-12 medium supplemented with 10% heat-inactivated fetal calf serum, 0.3 U/ml insulin, and antibiotics (growth medium). Cultures were maintained in 75-cm² flasks in growth medium and a 5% CO₂–95% air environment under rocked conditions as previously described (43). The purity and identity of smooth muscle cells were verified by staining with an anti-α-smooth muscle cell actin. When the cells reached ~80% confluence, they were passed by trypsinization and used between passages 3 and 10 for all studies.

Experimental protocol. To assess the effect of hypoxia, VSM cells were subcultured in growth medium until the culture reached 70–80% confluence. In some experiments, VSM cells were subcultured in DME growth medium containing either normal glucose (NG, 5 mM glucose) or high glucose (HG, 25 mM glucose), and the effects of hypoxia under NG versus HG conditions were compared. Cultures were made quiescent by incubation for 30 h in their respective insulin- and serum-free growth media. Quiescent cultures were then exposed to either hypoxia (3% O₂, medium PO₂ = 30–40 mmHg) or normoxia (95% air, medium PO₂ = 140–150 mmHg) in the serum-free medium (0.1% serum) for the indicated time and the expressions of AT₁ receptor and cell proliferation were assessed.

Assessment of cell proliferation. [³H]thymidine incorporation and cell number were used to assess cell proliferation as previously described (43). Briefly, VSM cells were subcultured in six-well plates as described in Experimental protocol. Quiescent cultures were exposed to hypoxia or normoxia for 24 h in the absence or presence of ANG II (1 μM). [³H]thymidine (1 μCi/ml, specific activity 20 Ci/mmol) was added for the last 4 h of incubation. At the end of incubation, medium was removed, and cells were washed with 10% TCA and digested with 0.5 N NaOH. Radioactivity in the cell digest was counted in a Beckman scintillation counter. [³H]thymidine incorporation is expressed as total counts per minute per well. In a similar experimental protocol, cultures were also trypsinized, and cell numbers were counted in a Coulter counter. In some experiments, [³H]thymidine incorporation was assessed in cultures incubated with the AT₁ receptor antagonist losartan (10 μM) in the absence or presence of ANG II.

Assessment of ANG II receptor binding. VSM cells were subcultured in 12-well plates and exposed to hypoxia or normoxia as described in Experimental protocol. At the end of 24 h of incubation, an ANG II receptor binding assay was performed by the procedure of Thekkumkara et al. (48). At the end of each respective incubation, cells were washed twice with ice-cold PBS. Thirty microliters of binding buffer containing unlabeled ANG II (1 pM–10 μM) were added to each well followed by 270 μl of binding buffer containing 0.04–0.06 nM [¹²⁵I]–[Sar¹,Ile⁸]ANG II or [¹²¹I]–[Sar¹,Ile⁸]ANG II. Binding was performed for 1 h at room temperature. Reactions were terminated by quickly removing the incubation medium and adding ice-cold PBS. Cells in plates were dissolved in 200 μl of 0.2 N NaOH. Solutions were transferred in 12 × 75-mm disposal tubes, and radioactivity was determined with a Packard Auto-Gamma Counter. Results are expressed as specific binding defined as total binding minus nonspecific binding (in the presence of unlabeled ANG II, 1 pM). Scatchard plot analysis was performed with GraphPad Prism software, and maximum binding capacity (Bmax) and Kd values were calculated.

Northern blot analysis. For the assessment of AT₁ mRNA levels, VSM cells were subcultured in 75-cm² flasks and processed as described in Experimental protocol. Quiescent cultures were exposed to hypoxia or normoxia for 6–24 h followed by the assessment of mRNA expression of the AT₁ receptor by Northern blot analysis. At the end of each respective incubation, cultures were harvested and total RNA was isolated with TRizol (GIBCO-BRL, Grand Island, NY). Total RNA (10–15 μg) was subjected to 1% agarose gel electrophoresis, transferred to nitrocellulose membranes, and hybridized with [³²P]-labeled AT₁ receptor cDNA probe (kindly provided by Dr. Thomas Thekkumkara; Ref. 48). Briefly, the cDNA probe was 257 bp corresponding to the coding sequence was amplified by reverse transcriptase with forward (5'CAAGGAGCAATCGAAGTATGCG-3') and reverse (5'-GGTAGCCTTCTTCTTCTT-3') primers. The hybridization signals were normalized to those of 18S RNA. Quantitations of Northern blots were performed by densitometric analysis with an Eagle Eye II video system.

Statistical analysis. Statistical analyses were carried out by paired or unpaired Student’s t-test or by ANOVA.

RESULTS

Effect of hypoxia on AT₁ expression. We first examined the effect of hypoxia on AT₁ receptor expression in cultured VSM cells grown in regular DME-F-12 growth medium containing 17.5 mM glucose. Quiescent cultures were exposed to hypoxia or normoxia for 6, 12, and 24 h in serum-free medium followed by assessment of AT₁ mRNA levels by Northern blot analysis as described in RESEARCH DESIGN AND METHODS. As shown in Fig. 1, VSM cells maintained under normoxic conditions over 6–24 h exhibited significant AT₁ mRNA expression. Exposure to hypoxia induced a progressive increase in AT₁ mRNA levels beginning at 6 h of examination with a maximal 146% (~2.5-fold) stimulation observed at 24 h of incubation compared with respective normoxic controls (Fig. 1).

Effect of hypoxia on AT₁ receptor binding. Figure 2 shows the competition binding curves of ANG II receptor antagonist [¹²⁵I]–[Sar¹,Ile⁸]ANG II in cultured VSM cells exposed for 24 h to either normoxia or hypoxia. Scatchard plot analysis was performed, and the specific binding coefficients Kd and Bmax were calculated.

Bmax of [¹²¹I]–[Sar¹,Ile⁸]ANG II binding was increased from 280 ± 43 fmol/mg protein in normoxia to 762 ± 97...
[Thymidine incorporation, which was significantly increased in normoxic controls (Fig. 3A)]. Treatment of VSM cells with conditions similar to Fig. 1 induced a significant increase in thymidine incorporation and cell number. Exposure of VSM cells to 24 h of hypoxia under experimental conditions is mediated by selective stimulation of the AT1 receptor. Cultures were incubated in the absence or presence of ANG II and the AT1 receptor antagonist losartan (10 μM) under both normoxic and hypoxic conditions, and [3H]thymidine incorporation was assessed. As shown in Fig. 4, treatment with losartan caused a marked inhibition of DNA synthesis induced by ANG II under both normoxic and hypoxic conditions (Fig. 4).

Effect of ANG II on AT1 expression under normoxic and hypoxic conditions. To further examine the potential mechanism of enhanced ANG II-induced VSM cell growth under hypoxic conditions, the effect of ANG II on AT1 receptor expression was determined. VSM cells were incubated for 24 h with 1 μM ANG II under normoxic and hypoxic conditions, and AT1 mRNA expression was assessed by Northern blotting. Figure 5 shows that exposure of VSM cells to ANG II markedly reduced AT1 mRNA levels under normoxic conditions. However, ANG II had no significant inhibitory effect on AT1 expression under hypoxic conditions (Fig. 5). Similar to our observations in Fig. 1, hypoxia alone increased AT1 mRNA expression compared with normoxic controls (Fig. 5).

Effect of HG on AT1 expression under normoxic and hypoxic conditions. Subsequently, the effect of HG on AT1 expression was assessed under both normoxic and hypoxic conditions. Cultures were exposed for 12 and 24 h in serum-free DME medium containing either NG (5 mM) or HG (25 mM) under both normoxic and hypoxic conditions. AT1 mRNA expression was assessed by Northern blot analysis. As shown in Fig. 6, A and B, exposure to HG under normoxic conditions produced ~100% (~2-fold) and 170% (~2.7-fold) increases in AT1 mRNA levels at 12 and 24 h, respectively, compared with respective NG controls. NG cultures exposed to hypoxia resulted in 70% (~1.7-fold) and 100% (~2-fold) increases in AT1 expression at 12 and 24 h, respectively, compared with their expression observed under normoxic conditions (Fig. 6, A and B). Cultures incubated in an hypoxic-HG environment caused marked increases in AT1 mRNA levels to 446% (~5.5-fold) at 12 h and 763% (~8.5-fold) at 24 h compared with baseline AT1 expression with NG-normoxia (Fig. 6, A and B).

Fig. 1. Time course of the effect of hypoxia (Hyp) on ANG II type 1 (AT1) receptor expression in cultured vascular smooth muscle (VSM) cells. Quiescent cultures were exposed in a serum-free DME-F-12 medium to hypoxia or normoxia (Norm) for 6–24 h, followed by assessment of AT1 mRNA levels by Northern blot analysis. A: blot representative of 4 different experiments. B: average densitometric analysis as % increase in AT1/18s mRNA expression by hypoxia.

Fig. 2. Competition binding curves of 125I-labeled [Sar1,Ile8]ANG II ([125I]ANG II) in cultured VSM cells exposed to normoxia and hypoxia. Quiescent cultures were exposed to hypoxia or normoxia in a serum-free DME-F-12 medium for 24 h, and specific binding was studied at 22°C for 30 min in the presence of increasing concentrations of unlabeled ANG II (1 PM–10 μM) as competitor. Scatchard plot analysis was performed, and maximum binding capacity (Bmax) and Kd were calculated. Values are means ± SE of 3 separate determinations. CPM, counts per minute.
To determine whether the HG response to AT1 expression was due to increased osmolarity, we examined the effect of 20 mM mannitol on AT1 mRNA levels under NG conditions. We found that mannitol had no significant stimulatory effect on AT1 mRNA levels when compared with the effect observed under HG conditions (Fig. 6C).

Effect of ANG II on hypoxia and HG-induced AT1 expression. We further examined the regulation of AT1 expression by ANG II under HG and hypoxic conditions. Quiescent VSM cells were treated with 1 μM ANG II and incubated under NG and HG medium conditions for 24 h in both a normoxic and hypoxic environment. Similar to our observations in Fig. 6, HG and hypoxia increased AT1 expression, and the combination of both caused a dramatic 420% (≈5-fold) increase in AT1 mRNA expression compared with NG-normoxic controls (Fig. 7). Treatment with ANG II under normoxic conditions completely inhibited the expression of AT1 with both NG and HG (Fig. 7). However, in contrast to normoxic-HG conditions, ANG II had no significant inhibitory effect on AT1 mRNA levels under hypoxic-HG conditions (Fig. 7). ANG II-induced inhibition of AT1 expression persisted in an hypoxic-NG environment, suggesting the specificity of hypoxic response under hyperglycemic conditions (Fig. 7).

DISCUSSION

The present study demonstrated the upregulation of AT1 receptor expression by hypoxia and HG in cultured VSM cells. In addition, the combination of hypoxia and HG induced a marked increase in AT1 expression. Hypoxia also prevented the downregulation of AT1 expression by ANG II under HG conditions and potentiated the effect of ANG II on VSM cell growth. These findings suggest an important role for hypoxia and associated AT1 receptor upregulation in enhanced ANG II-induced signaling in HG conditions.

The current findings are the first direct demonstration of a role for hypoxia in the upregulation of ANG II receptor AT1 in VSM cells. Exposure to hypoxia for 24 h induced a significant increase in AT1 mRNA expression (Fig. 1) that paralleled the increases in ANG II receptor binding (Fig. 2), indicating a stimulating effect of
hypoxia on both AT1 expression and receptor density in VSM cells. Chronic systemic hypoxia has been shown to activate RAS in a number of tissues, including the kidney, lung, heart, and pancreas (11, 29, 34). Leung et al. (24) also recently reported an upregulation of AT1 receptor expression and function in the carotid body by isobaric hypoxia. It has been documented in many cell types, including smooth muscle cells, endothelial cells, and cardiac myocytes, that AT1 mediates the vasoconstrictor and growth-promoting effect of ANG II (8, 46, 47). In our present study, hypoxia induced the proliferation of VSM cells, which is consistent with our previous observations (38, 39, 43) in cultured VSM cells as well as renal mesangial and tubular epithelial cells. Moreover, hypoxia potentiated the effect of ANG II on VSM cell proliferation, which was blocked by the AT1 receptor antagonist losartan. These findings suggest that the proliferative response of ANG II is mediated by the selective stimulation of the AT1 receptor. AT1 receptor expression has also been shown to be increased in the kidneys of spontaneously hypertensive rats (SHR), which also exhibit renal tissue hypoxia (15, 50). In addition, cyclosporin A, which causes renal tissue hypoxia and hypertension, also induces the upregulation of AT1 receptors in VSM cells (5, 18, 52). Similarly, erythropoietin, which is induced by hypoxia and may cause hypertension, also stimulates AT1 receptor expression in cultured VSM cells (6). In view of these findings of the prevalence of tissue hypoxia and associated AT1 receptor upregulation, the hypoxia-induced increase in AT1 expression observed in the present study appears to account for enhanced VSM cell proliferation and potentially the development of atherosclerosis.

Our results showed that incubations with HG medium also induce an upregulation of AT1 expression compared with NG medium (Fig. 6). Furthermore, the effect of HG on AT1 expression was markedly enhanced in an hypoxic environment (Fig. 6). This potentiating effect of hypoxia on HG-induced AT1 expression paralleled the increases in VSM cell growth previously observed in our laboratory under similar experimental conditions (43). Increased prevalence of atherosclerotic vascular disease and cardiovascular mortality is associated with diabetes, and enhanced proliferation of VSM cells has been demonstrated in both humans and experimental models of diabetes (1, 35, 45). This may increase the predisposition of myocardial dysfunction in diabetes, even in the absence of coronary artery disease, systemic hypertension, or valvular heart disease (37). Interestingly, a high incidence of ischemic myocardial lesions and prominent VSM cell proliferation has been found to be associated with the development of atherosclerosis in diabetes (28). Moreover, the induction of diabetes has been shown to produce arterial wall hypoxia preceding the formation of atherosclerotic lesions in an animal model of diabetes (40). The abnormalities in both circulating and local tissue RAS have been well described in diabetic rats (3, 42). Significant increases in AT1 receptor expression in the heart have also been reported in streptozotocin-induced diabetic rats (20, 42). Therefore, the enhanced upregulation of AT1 receptor synthesis observed in our studies in an hypoxic and hyperglycemic environment may be the key mechanism for augmented ANG II-

![Image](http://ajpheart.physiology.org/)

**Fig. 6.** Effect of high glucose (HG) on AT1 mRNA levels under normoxic or hypoxic conditions in cultured VSM cells. Quiescent cultures were exposed for 12 and 24 h to HG (25 mM) or normal glucose (NG, 5 mM) under either normoxic or hypoxic conditions followed by assessment of AT1 mRNA expression by Northern blot analysis. In some experiments, HG was replaced with 20 mM mannitol in NG medium and cultures were exposed for 24 h to normoxia (C). A: blot of AT1 mRNA levels representative of 3 separate experiments. B: corresponding average of densitometry analysis of AT1/18S mRNA as %increase over NG-normoxia.

![Image](http://ajpheart.physiology.org/)

**Fig. 7.** Effect of ANG II on AT1 mRNA levels under normoxic and hypoxic conditions incubated with NG or HG. Quiescent cultures were exposed to NG or HG medium for 24 h under normoxic and hypoxic conditions and in the absence or presence of ANG II (1 mM). A: blot representative of 3 separate experiments. B: densitometric analysis of AT1/18S mRNA as %change over NG-normoxia.
produced proliferation in VSM cells. Consistent with this notion, the induction of hypoxia and the associated AT1 receptor upregulation may also account for the enhancement in ANG II-induced increases in VEGF and specific protein kinase activities reported under prolonged HG conditions (4, 31, 33).

The potential mechanism by which hypoxia causes enhanced ANG II-induced VSM cell proliferation was also evaluated in our studies. As shown in Figs. 5 and 7, ANG II at a concentration of 1 μM markedly downregulated AT1 mRNA expression under normoxic conditions at either NG (5 mM) or HG (both at 17.5 and 25 mM). These results were consistent with other studies in which ANG II has been shown to cause a downregulation of AT1 in VSM cells (22, 41). In contrast to a normoxic environment, however, ANG II-induced inhibition of AT1 mRNA expression was completely prevented by hypoxia at both 17.5 and 25 mM medium glucose concentrations (Figs. 5 and 7). Because ANG II has been shown to increase the expression of AT1 in cardiac tissue in rats with myocardial infarction and streptozotocin-induced diabetes (27, 42), our observations strongly suggest a role for hypoxia-induced sustained AT1 expression in enhanced ANG II signaling in cardiovascular disease in a HG environment. Of interest, sustained AT1 receptor stimulation with ANG II is required for enhanced DNA synthesis in VSM cells from SHR (17), which also exhibits tissue hypoxia (50).

In summary, we found that both hypoxia and HG stimulate ANG II receptor AT1 expression in cultured VSM cells and the combination of hypoxia and HG produces a marked increase in AT1 expression. In addition, hypoxia potentiates the effect of ANG II on VSM cell proliferation and prevents the downregulation of AT1 receptor expression with ANG II, suggesting an important role for hypoxia in enhanced ANG II-induced VSM cell growth and in the development of atherosclerosis in diabetes.

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REFERENCES


27. Mento PF, Pica ME, Hilepo J, Chang J, Hirsch L, and Wilkes BM. Increased expression of glomerular AT1 receptors in...
Regulation of AT1 expression by hypoxia and high glucose.


