We examined the effect of hypoxia on proliferation and osteopontin (OPN) expression in cultured rat aortic vascular smooth muscle (VSM) cells. In addition, we determined whether hypoxia-induced increases in OPN and cell proliferation are altered under hyperglycemic conditions. Quiescent cultures of VSM cells were exposed to hypoxia (3% O2) or normoxia (18% O2) in a serum-free medium, and cell proliferation as well as the expression of OPN was assessed. Cells exposed to hypoxia for 24 h exhibited a significant increase in [3H]thymidine incorporation followed by a significant increase in cell number at 48 h in comparison with respective normoxic controls. Exposure to hypoxia produced significant increases in OPN protein and mRNA expression at 2 h followed by a gradual decline at 6 and 12 h, with subsequent significant increases at 24 h. Neutralizing antibodies to either OPN or its receptor β3 integrin but not neutralizing antibodies to β5 integrin prevented the hypoxia-induced increase in [3H]thymidine incorporation. Inhibitors of protein kinase C (PKC) and p38 mitogen-activated protein (MAP) kinase also reduced the hypoxia-induced stimulation of proliferation and OPN synthesis. Exposure to high-glucose (HG) (25 mmol/l) medium under normoxic conditions also resulted in significant increases in OPN protein and mRNA levels as well as the proliferation of VSM cells. Under hypoxic conditions, HG further stimulated OPN synthesis and cell proliferation in an additive fashion. In conclusion, hypoxia-induced proliferation of cultured rat aortic VSM cells is also a key feature in the progression of atherosclerosis (4). Both systemic and local hypoxia contributes to the development of atherosclerotic lesions (5–11). Recent in vivo studies found a direct correlation of local arterial wall hypoxia, VSM cell proliferation, and atherosclerosis (12,13). However, the underlying signaling mechanisms whereby hypoxia induces VSM cell proliferation and subsequent atherosclerotic lesions remain poorly defined. Part of the problem has been in the demonstration of a mitogenic effect of hypoxia in cultured VSM cells in vitro. Hypoxia has only been shown to induce the proliferation of bovine pulmonary artery smooth muscle cells in culture when they are costimulated either with an activator of protein kinase C (PKC) or serum (3,14). To examine the effect of local hypoxia on cell proliferation, our laboratory has developed an appropriate cell culture model system in which cultured cells exhibited differentiated morphology and function by improved oxygenation (15). Using this culture model, we reported that hypoxia induces proliferation, dedifferentiation, and/or extracellular matrix synthesis in cultured renal tubular epithelial and glomerular mesangial cells (16–18). In the present study, we examined the effect of hypoxia on the proliferation of cultured rat aortic VSM cells to determine whether hypoxia directly alters their proliferative behavior.

Enhanced proliferation of VSM cells has also been demonstrated in both human and experimental models of diabetes (19,20). In addition, cultured VSM cells grown in high media glucose concentration (to mimic hyperglycemia of diabetes) have exhibited increased cell proliferation (21). The pathophysiological mechanisms responsible for accelerated VSM cell proliferation and progression into diabetic atherosclerosis are unclear. However, extensive VSM cell proliferation and atherosclerosis in association with ischemic myocardial lesions has been reported in diabetic rabbits (22). The induction of diabetes has also been shown to produce arterial wall hypoxia preceding the formation of atherosclerotic lesions (23). Together, these findings suggested a role for local hypoxia in the initiation and/or development of vascular disease in diabetes. However, it is not known whether hypoxia in arterial VSM cells...
influences their proliferative behavior in diabetes. We hypothesize that the interaction of local hypoxia and hyperglycemia causes accelerated VSM cell proliferation in diabetes.

Recently, osteopontin (OPN) has emerged as a key factor in vascular remodeling and in the development of atherosclerosis (24–26). In vitro studies have shown that OPN also promotes the proliferation of cultured rat VSM cells and human coronary artery smooth muscle cells (27,28). The OPN upregulation has also been demonstrated in the aortas of high-fat diet–induced diabetic mice and in the renal cortex of streptozotocin-induced diabetic rats (29–30). We recently reported that hypoxia-induced proliferation of cultured glomerular mesangial cells is mediated by the stimulation of OPN (31). OPN is an adhesion molecule and a growth promoter that binds to specific β3 integrin receptors, leading to stimulation of cell spreading and proliferation (32). In addition to OPN, β3 integrin receptors are also upregulated in models of atherosclerosis and are suggested to play an important role in atherosclerosis (33,34).

The present study examined the effect of hypoxia on the proliferation of rat aortic VSM cells and the role of OPN in mediating VSM cell proliferation induced by hypoxia. In addition, we compared the effects of hypoxia on the proliferation and OPN expression under normal and high-glucose (HG) media conditions to determine how local hypoxia, OPN, and VSM cell proliferation interreact in diabetes.

**RESEARCH DESIGN AND METHODS**

**Materials.** Male Sprague-Dawley rats weighing 200–250 g were obtained from Harlan Laboratories (Indianapolis, IN). [3H]thymidine and [35S]Met/Thr were purchased from ICN (San Diego, CA). MφP1B10, a rat monoclonal antibody to OPN, was obtained from the University of Iowa Developmental Studies Hybridoma Bank maintained under National Institute of Child Health and Human Development contract NO1-HD-2–3144. Neutralizing antibody to β3 integrin receptor (F11 clone) was purchased from Pharmingen, and β5 integrin receptor neutralizing antibody was obtained from Chemicon. cDNA probe for OPN, 2B7, was provided by Cecilia Giachelli (University of Washington, Seattle, WA). All other reagents were of high chemical grade.

**Cell culture.** Rat aortic VSM cells were isolated and cultured using a modification of the method described by Chamlamy-Campbell et al. (35). Briefly, aortas of Sprague-Dawley rats were resected under sterile conditions and cleaned of adventitia and connective tissue. The vessels were then minced into small pieces and suspended in sterile phosphate-buffered saline containing 6 mg/ml collagenase, 2 mg/ml elastase, and antibiotics (100 μg/ml penicillin, 100 μg/ml streptomycin, 50 mg/ml amikacin, and 2.5 μg/ml fungizone). This mixture was then incubated for 1–2 h in a shaking water bath at 37°C. After adequate dispersion, the resulting cell suspension was plated in 75-cm² flasks, and minimum essential medium (α-valine modification) containing 20% fetal bovine serum, 0.3 U/ml insulin, and antibiotics was added. Cells were kept in this medium for two passages, which permitted the growth of VSM cells while inhibiting the growth of fibroblasts.

After the second passage, cultures were grown in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s nutrient mixture F12 medium (F12) supplemented with growth medium (10% heat-inactivated fetal calf serum, 0.3 U/ml insulin, and the antibiotics). Cultures were maintained in 75-cm² flasks in growth medium at 5% CO₂/18% O₂ environment under rocked conditions as previously described (17). The purity and identity of smooth muscle cells was verified by staining with a monoclonal antibody to α-smooth muscle cell actin. Cells were passed by trypsinization after they reached 80% confluency and used between passages 5 and 10 for all the studies.

**Experimental protocol.** To assess the effect of hypoxia, VSM cells were subcultured in growth medium until the cultures reached 70–80% confluency. In some experiments, VSM cells were subcultured in DMEM growth medium containing either normal glucose (NG) (5 mM/l glucose) or HG (25 mM/l glucose) conditions to compare the effects of hypoxia under NG versus HG conditions. Cultures were allowed to become 70–80% confluent. At this point, all the cell cultures were made quiescent by incubation for 48 h in their respective insulin- and serum-free growth mediums. Quiescent cultures were then exposed either to hypoxia (3% O₂, 1.5% CO₂, 95% N₂) or normoxia (18% O₂, 3% CO₂, 87% N₂) for the indicated time. At the end of their respective incubation periods, cell proliferation and [3H]thymidine incorporation were assessed.

**Assessment of cell proliferation.** [3H]thymidine incorporation and cell number were used in the assessment of cell proliferation, which was carried out as previously described (17). Briefly, VSM cells were subcultured in six well plates as described in the experimental protocol. Quiescent cultures were then either exposed to hypoxia or maintained normoxic for 24 h. [3H]thymidine (1 μCi/ml, specific activity 20Ci/nmol) was added to one set of wells in the last 4 h of incubation. The other set of wells were processed for cell counting. For the assessment of [3H]thymidine incorporation, media was removed at the end of incubation, and cells were washed with 0.1% trichloroacetic acid 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 the total counts per minute per well.

**Western blot analysis.** Osteopontin protein levels were assessed by Western blot analysis using previously described (31). VSM cells were subcultured in 75-cm² flasks and processed for cell counting. For the assessment of [3H]thymidine incorporation, media was removed at the end of incubation, and cells were washed with 0.1% trichloroacetic acid, 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 the total counts per minute per well.

**Statistical analysis.** Statistical analyses were carried out by paired or unpaired Student’s t tests or by analysis of variance.
medium exhibited a significant amount of 66-kDa molecular weight OPN protein, as assessed by Western blot analysis. VSM cells maintained under normoxic conditions showed a progressive increase in the secretion of OPN protein for up to 12 h of incubation (Fig. 2). A similar phenomenon in the secretion of growth factors during control normoxic conditions has also been observed by others (36,37). Exposure to hypoxia produced early and late increases in OPN protein expression, as demonstrated by 167 and 76% stimulation at 2 and 24 h, respectively, when compared with corresponding normoxic controls (Fig. 2). Hypoxia had no significant effect on OPN protein levels at 6 and 12 h of incubation (Fig. 2).

Subsequent studies determined the effect of hypoxia on OPN mRNA levels in an experimental protocol similar to that of OPN protein analysis. As shown in Fig. 3, normoxic VSM cells exhibited a significant amount of OPN mRNA during 2–24 h of incubation, as determined by Northern
Role of osteopontin in hypoxia-induced proliferation of VSM cells. We next determined whether hypoxia-induced stimulation of OPN accounts for increased VSM cell proliferation. To examine a role for OPN in hypoxia-induced proliferation, quiescent VSM cells were exposed to hypoxia and normoxia for 24 h in the absence or presence of neutralizing antibodies to OPN, β3 integrin receptor, or control IgG, and then [3H]thymidine incorporation was assessed. OPN binds specifically to the β3 integrin receptor in VSM cells, whereas the β5 integrin is a preferential binding receptor for another arginine-glycine-aspartic acid-containing adhesion molecule called vitronectin (39,40). Similar to our observations in Fig. 1, hypoxia induced a significant 110% increase in [3H]thymidine incorporation (Fig. 4). Neutralizing antibodies to OPN or β3 integrin receptor had no effect on reducing the hypoxia-induced increase in [3H]thymidine incorporation (Fig. 4). Neutralizing antibodies to OPN or β3 integrin receptor had no effect on reducing the hypoxia-induced increase in [3H]thymidine incorporation under normoxic conditions (data not shown).

Role of PKC and p38 mitogen-activated protein kinase in hypoxia-induced stimulation of cell proliferation and osteopontin synthesis. Studies from our laboratory have shown that the activation of PKC and/or p38 mitogen-activated protein (MAP) kinase are involved in mediating the hypoxia-induced proliferation of cultured LLC-PK1 renal proximal tubular and mesangial cells (16, 17, 31). To examine the role of these enzymes in hypoxia-induced VSM cell proliferation and OPN synthesis, quiescent cultures were first pretreated for 30 min with different concentrations (50 nmol/l to 1 μmol/l) of the inhibitors of PKC (calphostin C) or p38 MAP kinase (SB203580) and then exposed to hypoxia or normoxia for 24 h, after which [3H]thymidine incorporation was assessed. Both calphostin C (Fig. 5A) and SB203580 (Fig. 5B) inhibited the hypoxia-induced increase in [3H]thymidine incorporation in a dose-dependent manner. The same concentrations of these inhibitors had no effect on [3H]thymidine incorporation under normoxic conditions (Fig. 5A and B).

Subsequent studies determined the role of PKC and p38 MAP kinase in hypoxia-induced stimulation of OPN expression. Quiescent cultures were preincubated for 30 min with either 1 μmol/l calphostin or 1 μmol/l SB203580 followed by exposure to hypoxia and normoxia for 24 h. At the end of the incubations, both OPN protein and mRNA levels were assessed. Exposure to hypoxia produced a 175% increase in OPN protein levels, which was significantly reduced by both calphostin C and SB203580 (Fig. 6). Hypoxia also caused a 52% increase in OPN mRNA expression, but the treatment with 1 μmol/l calphostin C or SB203580 prevented the hypoxia-induced increase in OPN mRNA levels (Fig. 7). A total of 1 μmol/l SB203580 (IC50 = 0.6 μmol/l) was required to inhibit the hypoxia-induced increase in OPN expression, although a lower concentration of calphostin C (250 nmol/l) also significantly reduced the hypoxia-induced stimulation of OPN mRNA levels (data not shown). Also, neither calphostin C nor SB203580 had any effect on OPN expression under normoxic conditions (data not shown). Taken together, these results demonstrated an important role for both PKC and p38 MAP kinase in hypoxia-induced stimulation of OPN synthesis and the proliferation of cultured VSM cells.

Effect of hyperglycemia in hypoxia-induced stimulation of cell proliferation. Because VSM cells are reported to exhibit an increased growth rate under hyperglycemic conditions (21), we examined whether hypoxia influences the proliferative behavior of these cells in NG (5 mmol/l) versus HG (25 mmol/l) media conditions. Quiescent VSM cells grown either under NG or HG media conditions were exposed to hypoxia and normoxia for 24 h, and [3H]thymidine incorporation was assessed as an index for DNA synthesis.

Figure 8 shows that VSM cells exposed to HG under normoxic conditions induced a 67% increase in [3H]thymidine incorporation in comparison with that seen in the DNA synthesis in NG medium. These results are consistent with other reports showing that HG conditions stimulate...
the proliferation of cultured VSM cells as well as mesangial cells (21,41). To determine whether the response of DNA synthesis to HG conditions was not caused by increased osmolarity, we also examined the effect of 20 mmol/l mannitol on [3H]thymidine incorporation. Cells incubated in NG medium containing 20 mmol/l mannitol for 24 h had no significant stimulatory effect on DNA synthesis compared with cells in the NG medium, confirming the specificity of the HG response (data not shown). Similar to our findings in Fig. 1, hypoxia produced an 89% increase in [3H]thymidine incorporation in NG medium when compared with corresponding normoxic controls (Fig. 8). Cells exposed to hypoxia in HG medium induced a 278% increase in [3H]thymidine incorporation in comparison with the NG normoxic controls (Fig. 8). Thus, hypoxia enhanced the effect of HG on VSM cell proliferation.

**Effect of hyperglycemia and hypoxia on osteopontin expression.** We next determined whether the alterations in VSM cell growth under NG and HG conditions were associated with the changes in OPN synthesis. Quiescent VSM cells were exposed to hypoxia and normoxia for 24 h with HG or NG in a fashion similar to that of the cell proliferation studies, and OPN protein and mRNA levels were assessed. In some experiments, HG was replaced
with 20 mmol/l mannitol to confirm whether HG-induced alterations in OPN expression are not caused by alterations in increased osmolarity. Parallel to the alterations seen in [3H]thymidine incorporation, HG and hypoxia produced 160 and 110% increases in OPN protein, respectively, as assessed by Western blot analysis (Fig. 9). The combination of hypoxia and HG resulted in a 348% increase in OPN protein levels in comparison with NG normoxic controls (Fig. 9). Qualitatively, both HG and hypoxia treatment resulted in increased expression of OPN mRNA levels, parallel to the alterations in OPN protein (Fig. 10). Cells incubated in NG medium containing 20 mmol/l mannitol had no stimulatory effect on OPN expression, confirming the specificity of HG response (data not shown). Thus, the upregulation OPN expression paralleled the increases in DNA synthesis observed under hyperglycemic and hypoxic conditions.

DISCUSSION

Our study demonstrated the development of an appropriate VSM cell culture model system in which hypoxia directly induces the proliferation of rat aortic VSM cells. In addition, we found that hypoxia caused a marked stimulation of VSM cell growth under HG conditions in comparison with NG conditions. Hypoxia and HG also stimulated OPN expression in an additive manner that paralleled the alterations in VSM cell proliferation observed under a hypoxic and hyperglycemic environment. These findings suggest a novel interaction between local hypoxia and hyperglycemia in the initiation and/or development of diabetic atherosclerosis and demonstrate an important role for OPN in mediating this process.

Systemic hypoxia has been linked to increased proliferation of pulmonary artery smooth muscle cells, which results in pulmonary hypertension (1). Systemic hypoxia produced by cigarette smoking, carbon monoxide exposure, and chronic sleep apnea is also associated with increased risk for atherosclerosis (5–8). In addition, local hypoxia has been proposed to initiate the development of atherosclerotic lesions, because occlusion or removal of the vasa vasorum in experimental animals results in intimal hyperplasia (9,10). The anoxemia theory of atherosclerosis was first suggested by Heuper in 1944 (42). Subsequently, in vitro and in situ studies found reduced pO2 levels in the arterial walls of animals with atherosclerosis (43,44). The in vivo evidence of arterial wall hypoxia has only recently been demonstrated in an experimental model of atherosclerosis (12). More importantly, a link between arterial wall hypoxia and VSM cell proliferation has now been established (13). Although the increasing body of in vivo evidence has demonstrated a role for local and systemic hypoxia in the proliferation of VSM
cells, it has been difficult to mimic the mitogenic response of hypoxia in cultured VSM cells in vitro. Earlier studies with cultured pulmonary artery smooth muscle cells required either PKC activation or serum as a prerequisite for the mitogenic response of hypoxia (2,3). However, in a recent report, hypoxia directly induced the proliferation of cultured human pulmonary artery VSM cells (45). We have now provided the evidence of a mitogenic effect of local hypoxia in a commonly studied model of cultured rat aortic VSM cells, as demonstrated by significant increases in both DNA synthesis and cell number (Fig. 1). Taken together, both in vivo and in vitro studies indicate a key role for local aortic VSM cell hypoxia as an initiating signal in VSM cell proliferation and atherosclerosis.

Increased prevalence of atherosclerotic vascular disease and cardiovascular mortality is also associated with diabetes (46). Studies in experimental animal models of diabetes and in humans demonstrated enhanced proliferation of aortic VSM cells (19,20). Also, porcine aortic VSM cells in culture exhibit increased cell proliferation under hypoglycemic conditions (21). Therefore, hyperglycemia-induced VSM cell proliferation appears to be an important cause for diabetic atherosclerosis. Consistent with in vitro studies of Natarajan et al. (21) in porcine aortic VSM cells, we found that HG also induces the proliferation of cultured rat aortic VSM cells (Fig. 7). More importantly, our studies showed a potentiating role of hypoxia in accelerated VSM cell proliferation under hypoglycemic conditions, as demonstrated by enhanced VSM cell growth under a hypoglycemic and hypoxic environment (Fig. 8). 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 (22). Moreover, diabetes has been shown to induce arterial wall hypoxia before the formation of atherosclerotic lesions in an animal model of diabetes (23). Diabetic animals exhibited significantly decreased oxygen tension throughout the arterial wall in comparison with control animals, despite no differences in the partial pressure of oxygen in the arterial blood. These previous findings in combination with the results of our present study indicate a pivotal role for local VSM cell hypoxia in accelerated VSM cell proliferation in diabetes.

A growing body of both in vivo and in vitro evidence indicates an important role for OPN, a secreted noncollagenous phosphoprotein, in VSM cell proliferation and atherosclerosis (24–28). OPN expression has been found to be upregulated in human atherosclerotic lesions, and a neutralizing antibody to OPN has been shown to inhibit neointimal thickening in rat carotid artery after balloon catheterization (24–26). Furthermore, OPN overexpression has been shown to stimulate the proliferation of cultured VSM cells, and an exogenous addition of OPN has been found to promote the proliferation of cultured human coronary artery smooth muscle cells (27,28). We recently reported that the hypoxia-induced proliferation of cultured rat mesangial cells was mediated by the increased synthesis of OPN (31). Results of our present study showed that hypoxia-induced proliferation of cultured VSM cells also involved increased expression of OPN. Hypoxia stimulated OPN protein and mRNA levels, and a neutralizing antibody to OPN or its β3 integrin receptor completely blocked the proliferation of VSM cells induced by hypoxia, suggesting a functional role of OPN in mediating VSM cell growth (Figs. 2–4). Neutralizing antibody to β5 integrin receptor, which binds preferentially to vitronectin (39,40), was unable to prevent the proliferation of VSM cells induced by hypoxia. Taken together, these results suggest that the effect on OPN is specific to hypoxia. In our studies, hypoxia caused an early and late phase of OPN stimulation, as demonstrated by significant increases in both OPN protein and mRNA expression at 2 h followed by a gradual decline at 6 and 12 h, with subsequent increases at 24 h (Figs. 2 and 3). This temporal profile of the induction of OPN by hypoxia suggests that different mechanisms may be involved in mediating the early and late phase of OPN synthesis.

The role of OPN in diabetes is not yet defined. However, the upregulation of OPN expression has recently been reported in the aortas of high-fat diet–induced diabetic mice as well as in the renal cortex of streptozotocin-induced diabetic rats (29,30). Consistent with our recent report in cultured mesangial cells (47), we found that HG also stimulated OPN expression in cultured VSM cells (Fig. 9). Increased expression of OPN by HG was also reported in a recent study with cultured VSM cells (48). In our present study, hypoxia induced a marked increase in OPN synthesis under HG conditions in parallel to the alterations in cell proliferation (Figs. 8–10). Taken together, these findings suggest that the prevalence of local hypoxia and the associated marked increases in OPN expression in diabetes may be the key events responsible for accelerated VSM proliferation and the development of diabetic atherosclerosis. Consistent with this notion, a recent study reported an increased expression of OPN in the carotid artery of streptozotocin-induced diabetic rats as well as in the forearm arteries of diabetic patients with end-stage renal disease (49). We recently reported that OPN also appears to play an important role in mediating the accelerated mesangial cell growth and collagen synthesis induced by hypoxia and high glucose (47).

The potential mechanism by which hypoxia induces OPN synthesis was also evaluated in our study. As shown in Figs. 5–7, the inhibitors of PKC and p38 MAP kinase significantly reduced the stimulation of both OPN expression and cell proliferation induced by hypoxia. In a recent study, a PKC inhibitor also prevented, in part, the HG-induced increase in OPN promoter activity (48). We showed that the activation of PKC as well as p38 MAP kinase is involved in the hypoxia-induced increase in the synthesis of OPN and growth of cultured VSM cells. We also reported similar results in cultured mesangial cells (31). Because both hypoxia and HG activate PKC as well as p38 MAP kinase (17,31,50,51), demonstration of their role in inducing OPN synthesis is of significant interest in the pathophysiology of vascular and renal complications of diabetes.

In summary, we found that local hypoxia induced the proliferation of cultured aortic VSM cells, which is mediated by the stimulation of OPN synthesis. Inhibitors of PKC and p38 MAP kinase prevented the hypoxia-induced increases in OPN and proliferation. HG also stimulated OPN expression and proliferation of VSM cells. Hypoxia enhanced the effect of HG on both OPN and cell prolifer-
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references


