Hypoxia and high glucose cause exaggerated mesangial cell growth and collagen synthesis: role of osteopontin

Sodhi, Chhinder P., Sarojini A. Phadke, Daniel Batlle, and Atul Sahai. Hypoxia and high glucose cause exaggerated mesangial cell growth and collagen synthesis: role of osteopontin. Am J Physiol Renal Physiol 280: F667–F674, 2001.—The effect of hypoxia on the proliferation and collagen synthesis of cultured rat mesangial cells was examined under normal-glucose (NG, 5 mM) and high-glucose (HG, 25 mM)-media conditions. In addition, a role for osteopontin (OPN) in mediating these processes was assessed. Quiescent cultures were exposed to hypoxia (3% O2) and normoxia (18% O2) in a serum-free medium with NG or HG, and cell proliferation, collagen synthesis, and OPN expression were assessed. Cells exposed to hypoxia in NG medium resulted in significant increases in [3H]thymidine incorporation, cell number, and [3H]proline incorporation, respectively. HG incubations also produced significant stimulation of these parameters under normoxic conditions, which were markedly enhanced in cells exposed to hypoxia in HG medium. In addition, hypoxia and HG stimulated the mRNA levels of type IV collagen, and the combination of hypoxia and HG resulted in additive increases in type IV collagen expression. Hypoxia and HG also stimulated OPN mRNA and protein levels in an additive fashion. A neutralizing antibody to OPN or its β3-integrin receptor significantly blocked the effect of hypoxia and HG on proliferation and collagen synthesis. In conclusion, these results demonstrate for the first time that hypoxia in HG medium produces exaggerated mesangial cell growth and type IV collagen synthesis. In addition, OPN appears to play a role in mediating the accelerated mesangial cell growth and collagen synthesis found in a hyperglycemic and hypoxic environment.

chronic hypoxia; hyperglycemia; mesangial cells; cell proliferation; extracellular matrix; diabetes

AN IN VIVO LINK BETWEEN THE PROLIFERATION OF MESANGIAL CELLS AND THE SUBSEQUENT DEVELOPMENT OF MESANGIAL MATRIX EXPANSION HAS BEEN ESTABLISHED IN MODELS OF BOTH IMMUNE AND NONIMMUNE GLomerULAR INJURY (7, 10, 11, 15). EVOLUTION OF DIABETIC GLOMERULOPATHY IS ALSO CHARACTERIZED BY EARLY MESANGIAL CELL PROLIFERATION, FOLLOWED BY THE ACCUMULATION OF EXTRACELLULAR MATRIX PROTEINS (21, 38). Moreover, high media glucose concentration has been shown to cause the proliferation of cultured mesangial as well as distal tubular epithelial and vascular smooth muscle cells (24, 35, 37). In addition, high glucose (HG) stimulates extracellular matrix synthesis in cultured mesangial cells (3). However, hyperglycemia in diabetes appears to be a necessary but not a sufficient factor in the development of diabetic nephropathy (5). The specific factor(s) and the pathophysiological mechanisms that initiate and/or accelerate the progression of diabetic glomerulosclerosis remain poorly defined.

Studies from our laboratory have recently shown that exposure of cultured mesangial cells to hypoxia induces proliferation and stimulates the expression of extracellular matrix proteins (29, 30). Others have shown that chronic hypoxia also induces matrix production in cultured human proximal tubular epithelial cells and interstitial fibroblasts (8, 26). A growing body of evidence indicates that the prevalence of tissue hypoxia is associated with the development of diabetic retinopathy, neuropathy, and atherosclerosis (4, 20, 31). A recent study found that moderate renal ischemia accelerates the progression of nephropathy in a streptozotocin rat model of diabetes (23). These findings suggest that hypoxia may also play an important role in the initiation and/or progression of diabetic nephropathy. Although a link between local hypoxia and hyperglycemia in the vascular complications of diabetes has been shown, it is not known whether hypoxia can influence the effect of hyperglycemia on cell growth and matrix synthesis, which is a hallmark of both renal and vascular complications of diabetes.

Recently, osteopontin (OPN) has emerged as an important growth-promoting factor for vascular smooth muscle cells both in vivo and in vitro and is implicated in the development of atherosclerosis (1, 12, 19). Renal cortical expression of OPN is also upregulated in various models of glomerulonephritis and renal fibrosis, where OPN is suggested to play a role in progressive renal disease (13, 16, 39). Moreover, a recent study found increased expression of OPN in the kidneys of streptozotocin-induced diabetes, suggesting a role for OPN in the progression of diabetic nephropathy (9). We recently reported that the mitogenic effect of hypoxia...
on mesangial cells is mediated by the stimulation of OPN synthesis (32). OPN is a secreted adhesion molecule and a phosphoprotein that exerts its effect by binding to a specific β3-integrin receptor on the cell surface (28). In our studies, hypoxia stimulated OPN expression, and neutralizing antibodies to OPN or its β3-integrin receptor blocked the mesangial cell proliferation induced by hypoxia (32). These results suggested a key role for OPN in hypoxia-induced proliferation of mesangial cells. Recently, exogenous addition of OPN has been reported to promote the proliferation of human coronary artery smooth muscle cells and prostate epithelial cells (6, 27). However, the role of OPN in the regulation of matrix synthesis remains undefined.

In the present study, we examined the effect of hypoxia on cultured mesangial cell proliferation and collagen synthesis under normal- and high-media-glucose conditions and determined the role of OPN in mediating these processes.

**METHODS**

**Materials.** Male Sprague-Dawley rats weighing 200–250 g were obtained from Harlan Laboratories, Indianapolis, IN. [3H]thymidine, [14C]leucine, [3H]proline, and [32P]dCTP were purchased from ICN, San Diego, CA. A monoclonal mouse anti-α2 type IV collagen antibody (MPl1B10) 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. The neutralizing antibody to β3-integrin receptor (F11 clone) was received from Pharmingen, Los Angeles, CA. cDNA probe for osteopontin, B7, was generously provided by Cecilia Giachelli, University of Washington, Seattle, Washington. cDNA probe for α-type IV collagen was obtained from American Type Culture Collection (ATCC), Bethesda, MD. All other reagents were of high chemical grade.

**Cell culture.** Primary cultures of glomerular mesangial cells were established from the kidneys of healthy Sprague-Dawley rats as previously described (29). Cultures were grown in DME/F-12 medium supplemented with 10% heat-inactivated FCS, 0.3 U/ml insulin, and the antibiotics and is referred to as “growth medium.” Cultures were maintained in 75-cm² flasks in growth medium at 5% CO2-18% O2 environment under rocked conditions as previously described (29). Cells were passed by trypsinization after they reached 80% confluency and utilized between passages 3 and 15 for all the studies.

**Experimental protocol.** To assess the effect of hypoxia on all the parameters described in this article, mesangial cells were subcultured in DME growth medium containing either normal glucose (NG, 5 mM glucose) or HG (25 mM glucose). In some experiments the HG medium was replaced with 20 mM mannitol to ensure the specificity of a HG response. Cultures were grown until they reached 70–80% confluency. At this time, cells were made quiescent by incubation for 48 h in their respective insulin- and serum-free growth medium. Quiescent cultures were then exposed to either hypoxia (3% O2, medium P O2 = 30–40 mmHg) or normoxia (18% O2, medium P O2 = 140–150 mmHg) for 2–72 h in the same NG or HG medium containing 0.1% serum, which is referred to as “serum-free medium” throughout the present study. At the end of respective incubations, cell proliferation, collagen synthesis, and the expression of type IV collagen and OPN were assessed.

**Assessment of cell proliferation.** [3H]thymidine incorporation and cell number were used as indexes of cell proliferation and carried out as previously described (29). Briefly, mesangial cells were subcultured in 6-well plates as described in the experimental protocol. Quiescent cultures were then either exposed to hypoxia or maintained normoxic in serum-free NG or HG medium for 24 h. [3H]thymidine (1 μCi/ml, specific activity 20 Ci/mmol) was added to one set of wells in the last 4 h of incubation. The other set of wells was processed for cell counting. For the assessment of [3H]thymidine incorporation, media was removed at the end of incubation and cells were washed with 10% trichloroacetic acid (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.

**Assessment of collagen synthesis.** Measurement of total collagen synthesis was assessed by [3H]proline incorporation as described (29). Mesangial cells were subcultured in 12-well plates as described in Experimental protocol. Quiescent cultures were then exposed to hypoxia or normoxia for 24–72 h with NG or HG medium in the presence of 4 μCi/ml [3H]proline and 50 μg/ml ascorbic acid. At the end of the respective incubation times, conditioned media proteins were precipitated with an equal volume of 12% TCA. The TCA-precipitated proteins were centrifuged at 1,000 g for 10 min. The resulting pellets were washed three times with 6% TCA and then solubilized in 0.2 N NaOH. Aliquots from each sample were counted in a Beckman scintillation counter. The remaining samples were adjusted to contain (in mM) 100 NaCl, 50 HEPES, and 3 CaCl2, pH 7.0. Collagenase (type III, 100 U/ml) was then added to each sample, followed by incubation for 16 h at room temperature. After collagenase digestion, the proteins were again precipitated as described above, washed three times with 6% TCA, and solubilized in 0.2 N NaOH, and subjected to liquid scintillation counting. Collagenase-sensitive [3H]proline incorporation was calculated as the difference between TCA precipitable counts before and after collagenase digestion, which reflects total collagen synthesis.

**Western blot analysis.** OPN protein levels were assessed by Western blot analysis as previously described (32). Mesangial cells were subcultured in 75-cm² flasks under NG or HG media conditions and exposed to hypoxia or normoxia as described in Experimental protocol. At the end of 24–72 h of incubation, conditioned medium was removed and centrifuged at 1,000 rpm for 5 min to remove any cell debris. Supernatants were then mixed with SDS-sample buffer, boiled for 5 min and subjected to 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nylon membrane and blotted for OPN by using 1:10 dilution (5 μg/ml) of MPl1B10 OPN monoclonal antibody. The bound primary antibody was detected with a horseradish peroxidase-conjugated secondary antibody and visualized with enhanced chemiluminescence method (Amersham, Arlington Heights, IL). Quantitations of Western blots were performed by densitometric analysis by using an Eagle Eye II video system.

**Northern blot analysis.** For the assessment of the mRNA levels of type IV collagen and OPN, mesangial cells were subcultured in 75-cm² flasks and processed in a manner similar to Western blotting analysis. Quiescent cultures were exposed to either hypoxia or normoxia under NG and HG conditions for 24–72 h followed by the assessment of mRNA expression by Northern blot analysis. At the end of respective incubations, cultures were harvested, and total RNA was isolated by TRIzol reagent method (GIBCO-BRL, Grand Is-
land, NY). Total RNA (5–10 µg) was electrophoresed in a 1% formaldehyde agarose gel, transferred to nitrocellulose membranes, and hybridized with [32P]-labeled cDNA probe of mouse pro-a-type IV collagen and rat OPN (2B7) by random priming. The cDNA probe used for type IV collagen recognizes both pro-a1 and -a2 type IV collagen. Therefore, mRNA expressions were combined to reflect total alterations in type IV collagen mRNA levels. The hybridization signals were normalized to those of 18s RNA to correct for differences in loading. Quantitations of Northern blots were performed by densitometric analysis by using an Eagle Eye II video system.

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

**RESULTS**

**Effect of hypoxia and HG on cell proliferation.** As shown in Fig. 1, quiescent cells exposed to hypoxia for 24 h under NG (5 mM) conditions resulted in a significant 80% increase in [3H]thymidine incorporation compared with normoxic cells. Exposure of cells to HG (25 mM) under normoxic conditions induced a 114% increase in [3H]thymidine incorporation compared with normoxic NG incubations. However, [3H]thymidine incorporation was significantly enhanced to 264% in cells incubated with HG under a hypoxic environment (Fig. 1). In a similar experimental condition, the number of cells at the end of respective incubations was also counted. Parallel to the alterations in DNA synthesis, hypoxia and HG individually stimulated cell number and produced a marked increase in cell replication under hypoxic/HG conditions (Fig. 2). To determine whether the HG response to cell proliferation was not due to increased osmolarity, we examined the effect of 20 mM mannitol on [3H]thymidine incorporation. We found that mannitol had no significant stimulatory effect on [3H]thymidine incorporation compared with the effect observed under NG conditions (data not shown).

We next determined the effect of hypoxia on [14C]leucine incorporation under NG or HG conditions as an index of total protein synthesis. Cultures were exposed to hypoxia or normoxia in a fashion similar to the assessment of DNA synthesis except 0.5 µCi/ml [14C]leucine was added in the incubating medium. We found that neither hypoxia nor HG had any significant effect on [14C]leucine incorporation, suggesting that hypertrophy is not a feature of increased mesangial cell growth (Fig. 3).

**Effect of hypoxia and HG on collagen synthesis.** Cultures maintained in NG under normoxic conditions showed a progressive increase in total collagen synthesis from 24 to 72 h as assessed by [3H]proline incorporation (Fig. 4). Exposure to hypoxia with NG medium...
produced significant 32, 133, and 48% increases in collagen synthesis at 24, 48, and 72 h of incubation, respectively, compared with their normoxic counterparts (Fig. 4). Cultures exposed to HG in a normoxic environment also caused a significant stimulation of collagen synthesis as HG induced 280, 129, and 150% increases in collagen production at 24, 48, and 72 h vs. respective collagen secretion observed with NG (Fig. 4). Hypoxia enhanced the effect of HG on collagen synthesis as HG-induced alterations in collagen production were increased to 369, 275, and 181% during 24–72 h of incubation compared with respective NG normoxic conditions (Fig. 4). Thus hypoxia and HG appear to cause an additive stimulatory effect on collagen synthesis.

We next examined the mRNA levels of type IV collagen under the experimental conditions similar to those in Fig. 4 to determine whether hypoxia and HG alter its expression. Exposure of cells to hypoxia under NG conditions produced 50 and 140% increases in mRNA levels at 24 and 72 h of incubation, respectively (Fig. 5). Cells exposed to hypoxia for 48 h in NG had no effect on type IV collagen expression. Incubations with HG under normoxia caused 73, 76, and 480% stimulation of mRNA levels at 24, 48, and 72 h of exposure, respectively, compared with corresponding normoxic controls (Fig. 5). The combination of hypoxia and HG resulted in a marked stimulation of mRNA levels of type IV collagen. Mesangial cells coincubated in hypoxia and HG induced 300–600% increases in the type IV collagen expression during 24–72 h of incubation, maximal being observed at 72 h (Fig. 5).

**Effect of hypoxia and HG on OPN expression.** In the present study, we first determined the effect of hypoxia on OPN mRNA and protein expression, under NG and HG conditions. Under similar experimental conditions, we then assessed the role of OPN in mediating mesangial cell growth and matrix synthesis. Cells exposed to hypoxia under NG conditions resulted in a 90% increase in OPN mRNA levels at 24 h, followed by no change at 48 h, and a subsequent 210% increase at 72 h of examination (Fig. 6). Similarly, HG incubations under normoxic conditions produced 230, 180, and 700% increases in the mRNA expression of OPN at 24, 48, and 72 h, respectively (Fig. 6). The effect of HG on OPN expression was further enhanced under hypoxic conditions as the exposure of cells to hypoxia with HG media induced marked increases in OPN mRNA levels to 390,
360, and 1,150% at 24, 48, and 72 h of incubation, respectively (Fig. 6).

In an experimental protocol similar to that in Fig. 6, the effect of hypoxia and HG on OPN protein levels was assessed at 24–72 h of incubation by Western blotting procedure using a monoclonal antibody to OPN (MPIIIIB10). Exposure to hypoxia in NG media produced 110 and 80% increases in OPN protein levels at 24 and 72 h, respectively, compared with corresponding normoxic controls (Fig. 7). HG incubations resulted in 90% stimulation of OPN at 24 h and 142% stimulation at 72 h of incubation, which were enhanced to 237 and 231%, respectively, by hypoxia (Fig. 7).

Role of OPN in hypoxia- and HG-induced stimulation of mesangial cell growth. To determine a role for increased OPN synthesis in hypoxia- and HG-induced stimulation of mesangial cell growth, neutralizing antibodies to OPN and its β3-integrin receptor were used. Cultures were exposed to hypoxia and normoxia with NG or HG for 24 h in the absence or presence of either neutralizing antibody to OPN or β3-integrin receptor, and [3H]thymidine incorporation was assessed. As shown in Fig. 8, under NG conditions, hypoxia induced a 73% stimulation of DNA synthesis compared with respective normoxic controls. Addition of neutralizing antibody to OPN or β3-integrin receptor produced a 50 and 38% inhibition of a hypoxia-induced increase in DNA synthesis, respectively (Fig. 8). Similar to our results in Fig. 1, HG stimulated DNA synthesis by 91% under normoxic conditions. Treatment with OPN antibody resulted in a 67% inhibition of HG-induced increase in DNA synthesis, whereas β3-integrin antibody reduced the DNA synthesis by 29% (Fig. 8). Hypoxia under HG conditions enhanced mesangial cell proliferation to 182%. The enhanced effect of hypoxia on DNA synthesis under HG environment was completely prevented by either OPN or β3-integrin receptor antibody (Fig. 8). Both OPN and β3-integrin-neutralizing antibodies did not have any effect on DNA synthesis under NG normoxic control conditions (Fig. 8).

Role of OPN in hypoxia- and HG-induced stimulation of matrix synthesis. We subsequently examined the role of OPN in the stimulation of collagen production as an index for matrix synthesis. Cultures were exposed to hypoxia and normoxia with NG or HG for 48 h; in the absence or presence of neutralizing antibodies to either OPN or β3-integrin receptor, [3H]proline incorporation was assessed. Similar to our observations in Fig. 4, exposure to hypoxia for 48 h under NG conditions produced a 165% increase in collagen synthesis (Fig. 9). Addition of OPN or β3-integrin antibody caused 79 and 36% inhibition of a hypoxia-induced increase in collagen synthesis, respectively (Fig. 9). HG incubation also stimulated collagen production by 130% compared with NG under normoxic conditions. Incubation with either OPN or β3-integrin antibody resulted in ~50% reduction in collagen synthesis induced by HG in a normoxic environment (Fig. 9). Hypoxia augmented the HG-induced increase in collagen secretion to 311% compared with the effect observed in NG normoxic conditions. Treatment with OPN antibody caused an 80% inhibition of collagen synthesis compared with the synthesis of collagen observed under HG normoxic conditions (Fig. 9). β3-integrin antibody also inhibited the marked increase in collagen synthesis by 46% under similar experimental conditions. Both OPN and β3-integrin-neutralizing antibodies did not have any significant effect on collagen synthesis under NG normoxic control conditions (Fig. 9).
mean incorporation was assessed as described in Fig. 4. Each value is the
mean ± SE of 4 separate experiments.

**DISCUSSION**

Our studies demonstrate for the first time that the combination of hypoxia and HG causes marked stimulation of mesangial cell growth and matrix synthesis. Because enhanced mesangial cell proliferation and the accumulation of glomerular extracellular matrix synthesis have been reported in the development of diabetic nephropathy (14, 21, 22, 38), our findings suggest an important role for hypoxia in the initiation and/or acceleration of diabetic glomerulosclerosis. Recent studies in experimental models of diabetes have linked the prevalence of local hypoxia to the development of diabetic retinopathy, neuropathy, nephropathy, and atherosclerosis (4, 20, 23, 31). Measurements of PO2 in the eyes, nerves, and the arterial wall of diabetic animals by using the oxygen electrode method have found decreased PO2 levels, which preceded the vascular and neuronal complications of diabetes (4, 20, 31). However, this invasive procedure has its limitations in assessing local hypoxia deeper in the tissue, such as inner and outer cortex of the kidney. Recently, a noninvasive procedure for examining PO2 in the kidney is being developed, where, in a preliminary study, an angiotensin-converting enzyme inhibitor, enalapril, is found to prevent the slow decline in PO2 within the kidney cortex (25).

In our studies, hypoxia induced marked increases in mesangial cell proliferation and type IV collagen synthesis under hyperglycemic conditions. Stimulation of mesangial cell proliferation by hypoxia in suboptimal glucose levels (11 mM) has already been demonstrated in our previous studies (29, 32). In addition, HG concentrations have been shown to cause the proliferation of cultured mesangial, Madin-Darby canine kidney distal tubular epithelial, and vascular smooth muscle cells (24, 35, 37). In the experimental conditions used in our studies, neither hypoxia nor HG had any significant effects on total protein synthesis (Fig. 3), suggesting that hypertrophy is not a feature of increased mesangial cell growth. Experiments with mannitol excluded the possibility that the stimulus provided by HG is a function of hyperosmolarity. The ability of hypoxia to produce exaggerated mesangial matrix synthesis under hyperglycemic conditions paralleled the accumulation of matrix expansion seen in both human and animal models of diabetic nephropathy.

The signaling mechanisms by which hypoxia and HG produce profound stimulation of mesangial cell growth and collagen synthesis were evaluated in subsequent studies. We recently reported that hypoxia induces mesangial cell proliferation by the sustained activation of protein kinase C (PKC) (29). Sustained activation of PKC has also been demonstrated in diabetic animals and mesangial cultures exposed to HG (17). We and others have now shown that PKC activation causes the stimulation of OPN release in cultured rat mesangial and aortic smooth muscle cells and human glioma cells (32–34). In our studies, hypoxia-induced activation of PKC, as well as p38 mitogen-activated protein kinase, induced the stimulation of OPN synthesis in mesangial cells, which plays a key role in cell proliferation under a hypoxic environment (32). Because OPN is also up-regulated in the kidney cortex of streptozotocin-induced diabetic rats (9), we examined the role of OPN in hypoxia-induced stimulation of proliferation and collagen synthesis under normal and hyperglycemic conditions. We found that HG incubations under normoxic conditions stimulate the expression of OPN at 24–72 h of examination (Figs. 6 and 7). Consistent with our observations, HG has been recently shown to increase OPN expression in cultured aortic smooth muscle cells (33). In our studies, the effect of HG on OPN synthesis displayed an additive response under hypoxic conditions, which paralleled the alterations in both cell proliferation and collagen synthesis. OPN has been shown to contain an arginine-glycine-aspartate (RGD) motif that binds to a specific β3-integrin receptor, leading to stimulation of spreading and cell proliferation (28). We found that a neutralizing antibody to either OPN or its β3-integrin receptor partially but significantly blocks the effect of HG on DNA and collagen synthesis (Figs. 8 and 9). These results suggest that both OPN-dependent and -independent mechanisms, such as other potential growth factors, (36), account for increased mesangial cell growth and matrix synthesis under normoxic HG conditions. However, the enhanced response of the combination of hypoxia and HG on DNA and collagen synthesis was prevented by the same antibodies, suggesting a key role for OPN in accelerated mesangial cell growth and matrix synthesis in diabetes. The ability of OPN antibodies to prevent both the synthesis of DNA and collagen under hypoxic/HG conditions further suggests a link between mesangial cell proliferation and matrix synthesis in the pathogenesis of diabetic nephropathy.
In conclusion, results of our studies demonstrate that the combination of cellular hypoxia and hyperglycemia produces exaggerated mesangial cell growth and matrix synthesis. In addition, HG stimulates OPN expression, which is markedly enhanced under hypoxic conditions. Both hypoxia- and HG-induced increases in OPN appear to play an important role in accelerated mesangial cell proliferation, as well as collagen synthesis, which may have important implications in the development of diabetic nephropathy.

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