Interaction between NO and COX pathways in retinal cells exposed to elevated glucose and retina of diabetic rats

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Du, Yunpeng, V. P. Sarthy, T. S. Kern. Interaction between NO and COX pathways in retinal cells exposed to elevated glucose and retina of diabetic rats. Am J Physiol Regul Integr Comp Physiol 287: R735–R741, 2004; 10.1152/ajpregu.00080.2003.—A nonselective inhibitor of cyclooxygenase (COX; high-dose aspirin) and a relatively selective inhibitor of inducible nitric oxide synthase (iNOS; aminoguanidine) have been found to inhibit development of diabetic retinopathy in animals, raising a possibility that NO and COX play important roles in the development of retinopathy. In this study, the effects of hyperglycemia on retinal nitric oxide (NO) production and the COX-2 pathway, and the interrelationship of the NOS and COX-2 pathways in retina and retinal cells, were investigated using a general inhibitor of NO [Nω-nitro-arginine methyl ester (L-NAME)], specific inhibitors of iNOS [L-Nω-(1-iminoethyl)lysine (L-NIL)] and COX-2 (NS-398), and aspirin and aminoguanidine. In vitro studies used a transformed retinal Müller (glial) cell line (rMC-1) and primary bovine retinal endothelial cells (BREC) incubated in 5 and 25 mM glucose with and without these inhibitors, and in vivo studies utilized retinas from experimentally diabetic rats (2 mo) treated or without aminoguanidine or aspirin. Retinal rMC-1 cells cultured in high glucose increased production of NO and prostaglandin E2 (PGE2) and expression of iNOS and COX-2. Inhibition of NO production with L-NAME or L-NIL inhibited all of these abnormalities, as did aminoguanidine and aspirin. In contrast, inhibition of COX-2 with NS-398 blocked PGE2 production but had no effect on NO or iNOS. In BREC, elevated glucose increased NO and PGE2 significantly, whereas expression of iNOS and COX-2 was unchanged. Viability of rMC-1 cells or BREC in 25 mM glucose was significantly greater than normal amounts of NO and PGE2 and of iNOS and COX-2. Oral aminoguanidine and aspirin significantly inhibited all of these increases. The in vitro results suggest that the hyperglycemia-induced increase in NO in retinal Müller cells and endothelial cells increases production of cytotoxic prostaglandins via COX-2. iNOS seems to account for the increased production of NO in Müller cells but not in endothelial cells. We postulate that NO and COX-2 act together to contribute to retinal cell death in diabetes and to the development of diabetic retinopathy that inhibition of retinopathy by aminoguanidine or aspirin is due at least in part to inhibition of this NO/COX-2 axis.

PROSTAGLANDINS AND NITRIC OXIDE (NO) are important mediators of inflammation, cell growth, and homeostasis. A possible role of these molecules in the pathogenesis of diabetic retinopathy has been suggested by findings that the development of diabetic retinopathy in animals was inhibited by a nonselective inhibitor of cyclooxygenase (COX; high-dose aspirin) and by a relatively selective inhibitor of inducible NO synthase (iNOS; aminoguanidine) (13, 14, 18, 20).

Both COX and NOS have constitutive and inducible isoforms. The inducible isoforms (COX-2 and iNOS) have been reported to contribute to cytotoxicity in some cell types via production of proinflammatory prostaglandins, NO, superoxide, and peroxynitrite (1, 15, 44). Moreover, there is evidence that these two enzymes can regulate the activity of each other (31, 35). Whether this occurs in retina in diabetes is not clear.

In this study, we used diabetic rats in vivo, as well as transformed retinal Müller (glial) cells (rMC-1) and bovine retinal endothelial cells (BREC) in culture, to investigate 1) the diabetes-induced alterations of the NO and prostaglandin pathways in the retina, 2) the interrelation of NO and COX pathways, and 3) the contribution of these pathways to death of retinal cells induced by elevated glucose concentration. In addition, we investigated the effect of therapies previously reported to inhibit retinopathy in diabetic animals (aminoguanidine and aspirin) on iNOS and COX-2.

MATERIALS AND METHODS

Aminoguanidine, aspirin, L-Nω-(1-iminoethyl)lysine (L-NIL), L-arginine methyl ester (L-NAME), HEPES, penicillin-streptomycin solution, and trypsin-EDTA solution were purchased from Sigma Chemicals (St. Louis, MO). COX-2 inhibitors NS-398 and meloxicam (ME) were purchased from Calbiochem (San Diego, CA).

Müller Cell Culture

Transformed retinal Müller (glial) cells (rMC-1 cell line) (39) were cultured and passaged in DMEM medium containing 5 mM glucose and 10% FBS. When cells were 50% confluent, the proliferation rate was slowed by reducing the concentration of fetal calf serum in 5 or 25 mM glucose media to 2%, and the experiments were immediately conducted. Cells were incubated in 5 or 25 mM glucose, with or without aminoguanidine (10 μg/ml, 90 μM), aspirin (2 mM), L-NIL (30 μM), L-NAME (1 mM), NS-398 (50 μM), or meloxicam (50 μM). L-NIL is a selective iNOS inhibitor, having an IC50 value for iNOS of 3.3 μM compared with IC50 values for neuronal NOS (nNOS) of 92 μM and endothelial NOS (eNOS) of 138 μM, respectively (12, 30). L-NAME is a nonselective inhibitor of nitric oxide synthetases; it exhibits IC50 values of 15 nM, 39 nM, and 4.4 μM for nNOS (bovine), eNOS (human), and iNOS (murine), respectively (9, 11). NS-398 and meloxicam are selective COX-2 inhibitors, having...
IC₅₀ values for COX-2 of 0.32 and 4.7 μM, respectively, compared with IC₅₀ values for COX-1 of >100 and 36.6 μM, respectively (4, 33). Media were changed every other day for up to 5 days. Cells were harvested by treating with a trypsin-EDTA solution [0.5% and 0.02%, respectively (wt/vol)].

Endothelial Cell Culture

Primary bovine retinal endothelial cells (BREC; between 5th and 10th passages) were grown on fibronectin-coated dishes (Iwaki Glass, Tokyo) containing EBM (Clonetics, Santa Rosa, CA) with 5 mM glucose, 10% plasma-derived horse serum, 50 mg/l heparin, and 50 mg/l endothelial cell growth factor (Boehringer Mannheim, Indianapolis, IN). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ and grown until 50% confluent. Cells were incubated in 5 or 25 mM glucose as well as inhibitors as described above, and media were changed every other day for up to 5 days. Cells were harvested by treating with a trypsin-EDTA solution [0.5% and 0.02%, respectively (wt/vol)]. The cells were identified as endothelial cells on the basis of their cobblestone morphology and by the presence of von Willebrand factor, shown by immunofluorescence staining.

Animals

Male Sprague-Dawley rats (225–250 g) were randomly assigned to become diabetic or remain as nondiabetic group for 2 mo. Diabetes was induced by intraperitoneal injection of freshly prepared solution of streptozotocin in citrate buffer (pH 4.5) at 55 mg/kg body wt. Diabetic rats randomly were assigned to receive aminoguanidine (3.0 g/kg diet) or aspirin (0.19 g/kg diet) with their diet or to remain as untreated diabetic control. These concentrations yielded daily intakes similar to those found previously by us to inhibit the development of lesions of early retinopathy in diabetic rats and to inhibit the apoptotic death of retinal capillary cells in diabetes (Ref. 20 and unpublished observations). Insulin was given as needed to achieve slow weight gain without preventing hyperglycemia and glucosuria (0.02%, respectively (wt/vol)). The cells were identified as endothelial cells on the basis of their cobblestone morphology and by the presence of von Willebrand factor, shown by immunofluorescence staining.

Measurement of NO

NO was determined by measuring the stable metabolites of NO (nitrate + nitrite) using a fluorimetric assay kit (Cayman Chemical, Ann Arbor, MI) as reported previously by us (6). This method has been shown to be specific and sensitive down to as little as 4 pmol in 0.12 ml according to manufacturer’s instruction (26). In each experiment, the three experimental groups (5 mM, 25 mM, and 25 mM + therapy for cultured cells; or normal, diabetic, and diabetic + therapy for in vivo experiments) were assayed simultaneously.

Western Blot Analysis

Retinas or cells were homogenized in buffer containing protease inhibitors (leupeptin 1 μg/ml; aprotinin 1 μg/ml), and 50 μg of protein from each sample were loaded on 8% PAGE-SDS and transferred onto nitrocellulose membrane (Schleicher and Schuell, Keene, NH). Membranes were blocked overnight at 4°C with 5% nonfat dry milk. Membranes were incubated with anti-iNOS polyclonal antibody (1:1,000 dilution; Transduction Laboratories, Lexington, KY) or COX-2 polyclonal antiserum (1:500 dilution; Cayman Chemical, Ann Arbor, MI) for 1 h at room temperature. Both blots were washed and incubated with anti-rabbit IgG antibody coupled to horseradish peroxidase (Bio-Rad) at a dilution of 1:3,000 for another hour. After another extensive washing, protein bands detected by the antibodies were visualized by enhanced chemiluminescence (Amersham) and evaluated by densitometry (Molecular Dynamics). Prestained protein markers (Bio-Rad) were used for molecular mass determinations, and purified standards of iNOS (mouse macrophage; Transduction Laboratories, Lexington, KY) and COX-2 (ovine; Cayman Chemical) were used to confirm the identity of iNOS and COX-2 in the gels. To ensure equal loading among lanes, the membranes were stained with Ponceau S (Sigma, St. Louis, MO) and the intrinsic protein actin (mouse monoclonal anti-β-actin antibody; Sigma) before and after, respectively, staining for iNOS and COX-2 (6). Protein concentration of tissue and/or cell lysates was measured by the Bradford procedure using the protein dye reagent from Bio-Rad Laboratories and BSA as a standard.

Measurement of PGE₂

PGE₂ in homogenized samples of retina (n = 6 per group), Müller (6 observations in each group), and BREC cells (12 observations in each group) was measured by ELISA using a commercial kit (Cayman Chemical) following the instructions of the manufacturer. The retina tissue and/or cell culture medium (50 μl) was assayed for immuno-detectable PGE₂ using an enzyme immunoassay with rabbit antiserum specific for PGE₂. All assays were done in duplicate and at two different dilutions of homogenate. The lower limit of sensitivity for this assay is 31 pg/ml, and all values reported were within the linear range of the assay. The amounts of PGE₂ in medium were corrected by the total amount of protein in the corresponding cell extracts. The protein content of each sample was determined by the Bradford procedure, and the PGE₂ content of each sample was calculated as picograms per milligram protein.

Cell Death

rMC-1 cells were incubated in 5 or 25 mM glucose, with or without l-NAME (30 μM), l-NIL (1 mM), and NS-398 (50 μM) and a combination of NS-398 (50 μM) plus l-NIL (30 μM). Media were changed every other day for up to 5 days. BREC cells were incubated in 5 or 25 mM glucose as well as inhibitors as described above for 5 days. Cell death was determined by light microscopy using a hemocytometer and a 0.4% trypan blue dye exclusion method. The number of cells that did not exclude the dye was expressed per 1,000 total cells. A minimum of 800 cells was counted per assay (8 dishes, >100 cells counted per dish), and the assay was replicated three times on different days.

Statistical Analysis

Data are expressed as means ± SD. Statistical analysis was performed using the ANOVA followed by Fisher’s test to correct for multiple comparisons. Similar conclusions were reached using non-parametric Kruskal Wallis test followed by Mann Whitney U-test.

RESULTS

Interrelationship of NOS and COX

In vitro studies using Müller cells. Incubation of rMC-1 cells in 25 mM glucose significantly increased cellular NO (as estimated by nitrite plus nitrate) compared with that measured at 5 mM glucose (P < 0.05), and iNOS expression also was increased (P < 0.001; Fig. 1, A–C). A nonspecific inhibitor of NO production (l-NAME) as well as a selective inhibitor of iNOS (l-NIL, 30 μM) significantly inhibited NO accumulation (both P < 0.05) and iNOS expression, whereas an inhibitor of COX-2 (NS-398) had no effect on either (Fig. 1, A and...
B). A different inhibitor of COX-2 (meloxicam) likewise had no effect on NO production or iNOS expression (not shown).

PGE$_2$ production and COX-2 expression also were measured in rMC-1 cells incubated at both glucose concentrations. Glucose at 25 mM increased PGE$_2$ released into the medium as well as expression of COX-2 compared with that at 5 mM glucose (Fig. 1, D and E). Inhibition of either NO production with l-NAME, or activities of iNOS or COX-2 with l-NIL or NS-398, respectively, significantly inhibited these glucose-induced increases of PGE$_2$ and COX-2 expression (all $P < 0.05$; Fig. 1, D and E).

**CELL VIABILITY.** Incubation of rMC-1 cells in 25 mM glucose for 5 days significantly increased cell death compared with that in 5 mM glucose ($P < 0.001$; Fig. 2), and this increase was significantly inhibited by l-NAME, l-NIL, or NS-398. The combination of NS-398 plus l-NIL did not inhibit cell death significantly better than either agent alone, although it tended to do so.

In summary, incubation of rMC-1 cells in diabetic-like concentration of glucose increased NO production via iNOS, and this NO regulated the COX-2-mediated production of PGE$_2$. Conversely, inhibition of COX-2 had no effect on NO production.

**In vitro studies using retinal endothelial cells.** In BREC, the concentration of nitrite plus nitrate in elevated 25 mM glucose increased above that in 5 mM glucose ($P < 0.05$), but l-NIL
had no significant effect on its production. Inhibition of COX-2 with NS-398 had no significant effect on NO production (Fig. 3A). The expression of iNOS did not change in elevated glucose (Fig. 1C), and inhibitors of iNOS or COX-2 had no effect on its expression (not shown).

PGE2 production by these vascular cells in 25 mM glucose likewise significantly increased above that in 5 mM glucose ($P < 0.05$). The COX-2 inhibitor (NS-398) significantly decreased the hyperglycemia-induced increase in production of PGE2. Inhibition of total NOS activity with L-NAME significantly inhibited PGE2 production ($P < 0.05$), but L-NIL had no effect (Fig. 3B), suggesting that NO increased production of PGE2 also in this cell type but that iNOS was not responsible for the NO production. Elevated glucose concentration did not alter the expression of COX-2 in endothelial cells (Fig. 1C), and neither L-NIL nor NS-398 affected the expression of COX-2 (data not shown).

Incubation of BREC cells in 25 mM glucose for 5 days significantly increased cell death compared with that in 5 mM glucose ($P < 0.001$), and this increase was partially, but significantly, inhibited by NS-398 or L-NAME ($P < 0.05$; Fig. 3C).

In summary, production of NO by retinal endothelial cells incubated in 25 mM glucose increased compared with that in 5 mM glucose, but iNOS was not responsible for the increase. The NO generated in BREC incubated in 25 mM glucose does regulate COX-2 activity, and a product of COX-2 does contribute to endothelial death.

Effect of Aminoguanidine and Aspirin on iNOS, COX-2, and Their Products

In vitro studies. Aminoguanidine and aspirin significantly inhibited the glucose-induced upregulation of NO production and iNOS expression in rMC-1 cells ($P < 0.05$) (Fig. 1, A and B). The two therapies also significantly inhibited the increase in PGE2 production and partially inhibited the induction of COX-2 in 25 mM glucose (Fig. 1, D and E). In contrast to results in the glial cells, aminoguanidine had no significant effect on production of either NO or PGE2 by BREC exposed to 25 mM glucose (Fig. 3, A and B).

In vivo studies. Diabetic rats were hyperglycemic and failed to gain weight at a normal rate. Body weight at 8–10 wk of study averaged $234 \pm 56$, $290 \pm 28$, $266 \pm 58$, and $448 \pm 78$ g for diabetic control, aminoguanidine-treated diabetic, aspirin-treated diabetic, and nondiabetic rats, respectively. Serum glucose levels of the diabetic, aminoguanidine-treated, and aspirin-treated diabetic animals were greater than normal ($301 \pm 45$, $315 \pm 33$, $313 \pm 98$, and $57 \pm 7$ mg/dl, respectively), and glycated hemoglobin levels likewise were greater than normal in the diabetic groups ($9.0 \pm 1.4$, $9.3 \pm 3.2$, $11.5 \pm 1.5$, and $3.3 \pm 0.4\%$, respectively).

The concentration of NO and PGE2 in retinas of diabetic control rats was elevated more than threefold above that seen in retinal tissue from nondiabetic animals ($P < 0.001$), and treatment with aminoguanidine or aspirin significantly inhibited the diabetes-induced increases (both $P < 0.05$, respectively). The expression of iNOS and COX-2 proteins in retina of diabetic rats likewise was significantly increased compared with that in nondiabetic rats ($P < 0.001$), and administration of aminoguanidine or aspirin to the diabetic rats significantly inhibited the expression of the enzymes (both $P < 0.05$, respectively) (Table 1).
NOS and COX in Diabetic Retina

Effect of diabetes and subsequent therapy on retinal content of NO or PGE2 and retinal expression of iNOS and COX-2 in rats

<table>
<thead>
<tr>
<th></th>
<th>NO, mmol/mg protein (n = 7)</th>
<th>iNOS Expression, % of normal (n = 6)</th>
<th>PGE2, pg/mg protein (n = 6)</th>
<th>COX-2 Expression, % of normal (n = 6)</th>
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<tbody>
<tr>
<td>Nondiabetic</td>
<td>1.72 ± 0.51</td>
<td>100</td>
<td>70 ± 20</td>
<td>100</td>
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<tr>
<td>Diabetic</td>
<td>7.5 ± 2.48</td>
<td>173 ± 29*</td>
<td>617 ± 17*</td>
<td>196 ± 19*</td>
</tr>
<tr>
<td>Diabetic + AMG</td>
<td>1.73 ± 1.17</td>
<td>152 ± 36†</td>
<td>243 ± 122†</td>
<td>107 ± 39†</td>
</tr>
<tr>
<td>Diabetic + Asp</td>
<td>2.92 ± 1.48†</td>
<td>117 ± 40†</td>
<td>235 ± 63†</td>
<td>135 ± 33†</td>
</tr>
</tbody>
</table>

Values are means ± SD. Diabetes increases retinal content of nitric oxide (NO) or prostaglandin E2 (PGE2), and these are inhibited by either aminoguanidine (AMG) or aspirin (Asp). Likewise, retinal expression of inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 is increased in diabetes and is inhibited by either AMG or Asp. *P < 0.001, diabetes vs. nondiabetic control. †P < 0.05, diabetes + therapy vs. diabetes control (n = 6 observations).

DISCUSSION

Diabetic retinopathy has been found in animals to be inhibited by therapies that, among other actions, inhibit production of NO (aminoguanidine) and prostaglandins (aspirin). Thus increased production of NO and prostaglandins is at least closely associated with the development of diabetic retinopathy, raising a question regarding their role in its pathogenesis. The inducible isoform of NOS (iNOS) has been found previously to be induced in the retina of diabetic animals (2, 6, 40), but the isoform of COX responsible for the increase in prostaglandin production has not been assessed. The present work demonstrates that both iNOS and COX-2 are induced in the retina in diabetes and that they or their products are related to hyperglycemia-induced death of retinal cells.

There is evidence that iNOS and COX-2 can regulate each other in some cell types. NO generated by iNOS increased PGE2 release in astroglial cells, macrophages, osteoblasts, and air pouch, and this increase was at least partially attenuated by L-NAME, L-NIL, aminoguanidine, or NS-398 (17, 29, 35, 36, 38, 43). Consistent with this, the hyperglycemia-induced increase in PGE2 production was found to have been regulated by NF-κB and to activate the nuclear transcription factor NF-κB and to upregulate LPS-induced release of NO in rat Kupffer cells (10), and to activate the nuclear transcription factor NF-κB and expression of NOS (34). In this study, we found that selective inhibition of COX-2 had no significant effect on the glucose-induced increase of NO in cultured retinal Müller or endothelial cells, thus providing no evidence that products of COX-2 play a major role in the regulation of NO production in retinal cells in diabetes.

Cell death plays an important role in the development of diabetic retinopathy (5). Accelerated death of retinal capillary cells by an apoptotic-like process was found by us to be demonstrable in retinal endothelial cells and pericytes from diabetic humans and diabetic and experimentally galactosemic rats (28) before any other retinal histopathology is evident. Therapies that inhibit retinal capillary cell apoptosis in diabetes also inhibit the development of lesions characteristic of the early stages of the retinopathy (20), suggesting that the capillary cell death plays a role in the ensuing development of acellular capillaries and retinal microvascular disease. Data presented herein suggest that NO and COX-2 play a role in hyperglycemia-induced death of retinal glial and endothelial cells in diabetes. A major source of the hyperglycemia-induced increase in NO in the Müller cells comes from iNOS, whereas other isoforms of NOS apparently account for the increase in NO produced in retinal endothelial cells. High concentrations of glucose also have been reported to induce cell death via NO production in differentiated PC12 cells (22), and activation of NO and COX pathways increased cell death in cultured osteoarthritis synovial fibroblasts (16).

Aminoguanidine (13, 14, 18) and high-dose aspirin (18) have been found to inhibit development of lesions of diabetic retinopathy in animals. We now demonstrate that these therapies also significantly inhibit the diabetes-induced increase in retinal NO and PGE2 and in expression of iNOS and COX-2. Aminoguanidine is a relatively selective inhibitor of iNOS, inhibiting this inducible isomerase better than either the endothelial or neural isoforms (IC50 values for inhibition of murine iNOS and rat nNOS are 5.4 and 160 μM, respectively) (25). The evidence that aspirin, but not COX-2 inhibitors, blocked the hyperglycemia-induced increase in expression of iNOS in rMC-1 cells suggests that the effect of aspirin on iNOS expression was not mediated via prostaglandins. iNOS expression is regulated by NF-κB, a transcription factor whose activity is known to be regulated by aspirin (21). Neither aminoguanidine nor aspirin is a totally specific inhibitor of iNOS or COX-2 so other isoforms of NOS and COX might also be inhibited by these therapies (8, 41).

We conclude that the increase in prostaglandin production detected in retinal cells incubated under diabetic-like conditions is secondary to increased production of NO. Whether PGE2 itself directly kills cells is controversial (27, 32), but our data suggest that COX-2 or its products play a role in the cell death. The source of the NO appears to differ among different retinal cell types, since iNOS contributes to NO production in Müller cells exposed to high glucose but not in endothelial cells. The available evidence suggests that the observed inhibition of early diabetic retinopathy by aminoguanidine or aspirin likely is due in part to inhibition of inflammatory mediators such as iNOS, COX-2, and production of nitric oxide and prostaglandins.

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NOS and COX in Diabetic Retina

GRANTS
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