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ENHANCED EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE IN MURINE MACROPHAGES AND GLOMERULAR MESANGIAL CELLS BY ELEVATED GLUCOSE LEVELS : POSSIBLE MEDIATION VIA PROTEIN KINASE C+

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SUMMARY: Increased blood flow and vascular permeability of early diabetes have been associated with increased nitric oxide formation in diabetic rats, but the specific nitric oxide synthase responsible is unknown. We examined the modulation of the induction and activity of the inducible NOS isoform by high glucose concentration in a murine macrophage cell line, RAW 264.7, and murine glomerular mesangial cells. Culturing both cell types in high glucose concentration led to significant increases in nitrite production and the mRNA encoding iNOS upon stimulation with LPS plus interferon-γ, as compared with normal glucose concentration. High glucose also modestly enhanced LPS/IFN-γ–induced stimulation of the iNOS promoter in transient transfection experiments in mesangial cells. Protein kinase C activation led to enhanced mRNA expression of iNOS, and inhibitors of protein kinase C blocked nitrite accumulation in mesangial cells. These findings suggest that high glucose in combination with stimulation by LPS plus IFN–γ enhances iNOS expression, and protein kinase C activation may be playing a role in this enhancement.

Early in the course of type I diabetes there is a characteristic increase in renal vascular flow and glomerular filtration, but the mediators of this effect have not been established. Nitric oxide (NO) is a small diffusible molecule which is a potent

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Abbreviations: iNOS, inducible nitric oxide synthase; ecNOS, endothelial constitutive nitric oxide synthase; ncNOS, neuronal constitutive nitric oxide synthase; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; LPS, lipopolysaccharide; PMA, phorbol myristate acetate; L-NNA, N^G-nitro-L-arginine; PKC, protein kinase C.

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vasodilator and has been shown to play a role in basal regulation of renal blood flow and glomerular filtration (1). The three major categories of the enzyme regulating NO production are the constitutive, calcium-dependent isoforms principally present in endothelial and neuronal cells (ecNOS and nNOS, respectively), and the inducible, calcium-independent isoform (iNOS) first described in murine macrophages (2). Expression of iNOS is upregulated by different cytokines, including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1β, as well as the gram negative-derived bacterial endotoxin, lipopolysaccharide (LPS). The iNOS pathway is also present in the glomerular mesangial cell, and production of NO by this cell type may influence glomerular hemodynamics as well as play a role in glomerular pathology (3). Mesangial cells have vascular smooth muscle-like characteristics and can control glomerular filtration by modifying the capillary surface area under the influence of a variety of vasoactive substances and cytokines. Mesangial cells not only produce NO via the iNOS pathway but can also respond to exogenous NO by relaxation, thus increasing filtration across the glomerulus (1, 3).

Recent studies have described enhanced production of NO metabolites in the urine of diabetic rats (4, 5); however, other studies have demonstrated either decreased production of NO (6) or decreased responsiveness to NO in diabetic tissues (7, 8). These studies primarily focused on the constitutive ecNOS pathway present in endothelial cells. The role of the iNOS pathway in diabetic renal disease is unknown, but it has been previously demonstrated to play a role in inflammatory glomerulonephritis, perhaps due to infiltrating macrophages (3, 9). It has recently been shown that there is enhanced production of a variety of cytokines, including TNF-α and IFN-γ, in both patients and animal models of type I diabetes (10, 11), and that macrophages infiltrate the glomeruli of rats in the earliest stages of diabetes (12). Therefore, we postulated that mesangial cell and macrophage production of NO may be increased in the diabetic state via the cytokine-inducible pathway of NO generation and that NO produced by iNOS may contribute to the altered vascular flow and glomerular pathology seen in the diabetic kidney.

In this study we examined the modulation of the iNOS pathway by high glucose concentration in murine mesangial cells and the murine monocyte-macrophage cell line, RAW 264.7, by evaluating the production of the stable end-product of NO, nitrite, the steady-state mRNA encoding iNOS, and the promoter activity of the iNOS gene under normal and high glucose conditions. In addition, we sought to identify the relevant signaling pathway that may be involved in the regulation of iNOS by high glucose, particularly the contribution of protein kinase C (PKC) activation.

MATERIALS AND METHODS

Reagents: All reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. IFN-γ was purchased from Genzyme (Cambridge, MA), acetyl CoA from Boehringer Mannheim (Indianapolis,

IN) and [^{14}C]-chloramphenicol and [^{32}P]-dCTP from Amersham (Arlington Hts, IL). All chemicals were of reagent or electrophoresis grade and all culture media were endotoxin-free as measured by the limulus-amebocyte assay.

Cell culture: Murine glomerular mesangial cells were isolated from normal SJL mouse kidneys by the technique of differential sieving applying conditions to favor mesangial cell outgrowth, and were characterized as described previously (13). Cells were studied between passages 7-20. Mesangial cells were grown in DMEM (Gibco, Grand Island, NY) with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM supplemental glutamine, and 10% fetal-calf serum (FCS) (Gibco) at 37 °C in 5% CO_2. The macrophage cell line, RAW 264.7 (ATCC #TIB 21), was studied under similar conditions as for the mesangial cells.

NO_2 accumulation: For nitrite measurements, cells were plated at a density of 10^5 cells/well in 96-well plates in phenol red-free DMEM plus 10% FCS. After adherence, fresh DMEM plus 2% FCS and varying concentrations of D-glucose were added. Twenty-four hours later the cells were stimulated with LPS (2 µg/ml) plus IFN-γ (100 U/ml). These doses were found to result in maximal stimulation of iNOS activity without cell death. After an additional 24 hours of stimulation the conditioned medium was assayed for nitrite by the Griess reagent (1% sulfanilamide/0.1% *N*-(1-napthyl)ethylenediamine dihydrochloride in 2.5% H_3PO_4) (14) as an indicator of NO production. 80 µl Griess reagent was added to 80 µl of supernatant under each condition tested (n=5 per condition). The plates were read using an ELISA microplate reader (MR 5000; Molecular Design, Menlo Park, CA) at 546 nm against a standard curve of $NaNO_2$. Parallel studies using nitrate reduction, with E. coli-derived nitrate reductase to reduce nitrate to nitrite, demonstrated qualitatively similar results (data not shown). The cell monolayer was then lysed with buffer containing 0.25 M Tris, and protein content was assessed with a protein assay kit (Biorad, Hercules, CA). Alternatively, in some experiments, the cell monolayer was harvested with trypsin and counted with a Coulter cell counter (Coulter Electronics, Hialeah, FL) and the data expressed per cell number.

Northern hybridization: 10-20 x 10^6 adherent cells in T-75 flasks were grown in DMEM plus 2% FCS with varying concentrations of D-glucose for 24 hours. Cells were then stimulated with LPS (2 µg/ml) plus IFN-γ (100 U/ml) for an additional 24 hours prior to harvesting. In separate experiments the PKC activator, PMA, was added either alone or 15 minutes prior to the addition of LPS plus IFN-γ. Total RNA was isolated and 20 µg RNA was used per lane for Northern analysis as described previously (15). All cDNA probes were separated from their plasmids in low melt agarose and labeled with 5 µCi [^{32}P]-dCTP (3000 Ci/mmol) using a random priming kit (Amersham). The cDNA probes used were: 2.26 kb 3'-fragment made by Hae II digestion of CL-3 (gift of Dr. C. Lowenstein) (16) encoding murine iNOS; a full length cDNA probe for rat neuronal NOS (gift of Dr. D. Bredt) (17) (the rat nNOS probe recognizes murine nNOS as assessed by Northern blot analysis of RNA isolated from mouse cerebellum); and a 5.8 kb EcoR1 fragment encoding human 18S ribosomal RNA (gift of Dr. N. Cooke). The labeled cDNAs were separated from unincorporated nucleotides by Sephadex G-50 spin columns (Boehringer Mannheim), and membranes were hybridized with 10^6 cpm/ml probe for 24 hours at 42 °C using hybridization and washing conditions as previously described (15). The membranes were then autoradiographed with intensifying screens (DuPont, Wilmington, DE) at -70 °C for 24-72 hours. Blots were then stripped for 30 minutes at 95 °C with 0.01x SSC and 0.01% SDS and rehybridized with a probe for human 18S ribosomal RNA to account for small loading and transfer variations. Signals on exposed films were quantitated with a scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA) and iNOS mRNA levels were calculated relative to those of 18S.

Chloramphenicol acetyl transferase (CAT) reporter gene assay for iNOS promoter activity: The transfection methods and CAT enzyme assays were essentially as previously described (18). Plasmids used for transfection were as follows: pAoCAT, containing the CAT gene without regulatory elements; pSV2CAT, containing the CAT gene linked to the SV-40 promoter and enhancer; and p1iNOS containing the complete 1.749 kb regulatory fragment for the iNOS gene (gift of Drs. Q-W. Xie and C. Nathan) (19). 10-20 µg of plasmid DNA was used to transfect mesangial cells in a 100 mm tissue culture dish using the calcium-phsosphate-DNA precipitation technique. After 16 hours incubation in 5% CO_2 at 37 °C, the monolayer was scraped off and pipetted into suspension; equal aliquots of the transfected cell population were plated onto 6-well plates and allowed to adhere and grow in 100 mg/dl glucose overnight. This maneuver assured that the transfection efficiency was equivalent among the different petri dishes before the experimental manipulation. The cells were then exposed to fresh DMEM/2% FCS with a glucose concentration of either 100 or 450 mg/dl glucose. After an additional 24 hours, half of the wells were stimulated with LPS (2 µg/ml) plus IFN-γ (100 U/ml) for 12 hours and the cell lysates were then harvested for the CAT assay using a standard protocol (19). Equal amounts of

cytosolic protein were mixed with 3.5 mg/ml acetyl CoA, 1 mCi [^{14}C]-chloramphenicol (54 mCi/mmol), and 250 mM Tris.HCl (pH 8.0), and the reaction was run at 37 °C for 60 minutes. Acetylated chloramphenicol derivatives were then attained by extraction with ethyl acetate and thin-layer chromatography (TLC) on 25-mm silica gel plates. TLC plates were then autoradiographed and bands were cut out and counted for radioactivity. CAT activity was expressed as percent [^{14}C]-chloramphenicol acetylated per hour per unit protein.

RESULTS

Enhanced production of NO by high glucose: To evaluate if the ambient glucose concentration affects iNOS activity, measurement of the stable NO metabolite, nitrite, was performed (**Figure 1**). Basal levels of nitrite were not significantly different at all levels of glucose concentration. However, stimulation of macrophages with LPS (2 μg/ml) plus IFN-γ (100 U/ml) demonstrated a dose-response effect of glucose in enhancing nitrite production. This effect was maximal at a glucose concentration of 450 mg/dl (25 mM) (3.5 fold the level attained in normal glucose). This was not an osmotic effect as the addition of mannitol to 100 mg/dl D-glucose to attain the same osmolarity as 450 mg/dl D-glucose did not reproduce the high glucose effect. A similar response was observed in mesangial cells (2-fold increase in high glucose versus normal glucose) (**Figure 2**). Addition of an L-arginine analogue, N^{G}-nitro-L-arginine (L-NNA) (2-10 mM), prior to stimulation blocked nitrite accumulation, confirming that nitrite production was via the iNOS pathway in murine mesangial cells (LPS/IFN-γ: 12 ± 2 nmoles NO_2

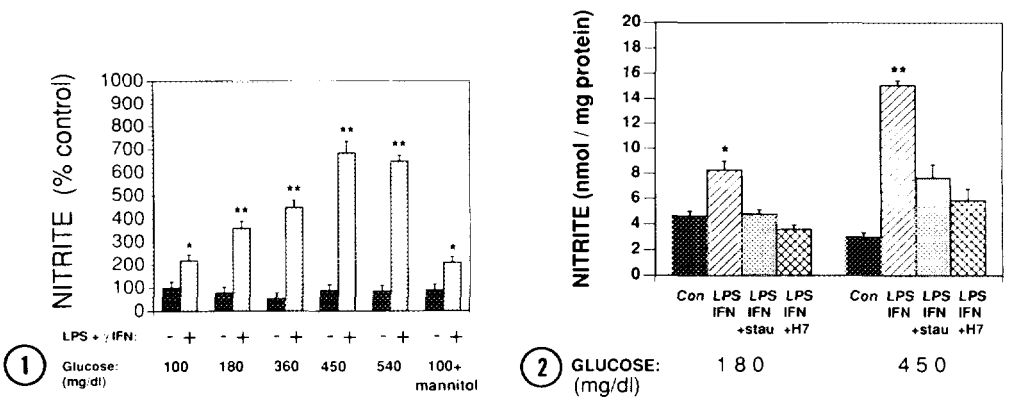

Figure 1. Concentration-dependent stimulation of nitrite production via the iNOS pathway by glucose in the monocyte-macrophage cell line RAW 264.7. Data are expressed as percent nitrite production/mg protein with the arbitrarily 100% value given to basal nitrite production with a glucose concentration of 100 mg/dl. Data are means ± SE of 5 separate experiments. *significantly different from unstimulated value, ** significantly different from stimulated value in 100 mg/dl glucose, p<.01.

Figure 2. Effects of glucose and the PKC inhibitors, staurosporine (100 nM) and H-7 (50 μM), on nitrite production in murine glomerular mesangial cells. A representative experiment of five similar experiments. Data expressed as means ± SE, n=5 per condition. *significantly different from unstimulated value. **significantly different from stimulated value in normal glucose, p<.01.

/mg protein vs stimulation in the presence of 10 mM L-NNA: 2 ± 1 nmoles NO_2 /mg protein, n=5, p<.01).

It has been reported that NOS activity may be affected by PKC activation (20) and that high glucose concentration activates PKC in mesangial cells by increasing *de novo* diacylglycerol (DAG) synthesis (21). Therefore we sought to evaluate the role of PKC in the production of NO via the iNOS pathway in mesangial cells. The addition of the PKC inhibitors, staurosporine (100 nM) or H-7 (50 μM), prior to stimulating the cells with LPS plus IFN-γ, significantly decreased cytokine-induced nitrite production via the iNOS pathway under both normal and high glucose conditions (**Figure 2**). The addition of PMA (10^{-7} M) for 15 minutes prior to addition of LPS plus IFN-γ led to enhanced nitrite production regardless of the glucose concentration (2-fold increase in NO_2 production with PMA versus stimulation without PMA, n=5, p<.05). The addition of calcium mobilizing agents, acetylcholine and bradykinin, failed to increase nitrite production over baseline in both mesangial cells and RAW 264.7 (data not shown).

High glucose enhances iNOS mRNA expression: To determine if the glucose effect was operating at the level of the steady-state message encoding iNOS, Northern analysis was performed. Growth of both mesangial cells and macrophages for 24 hours in high glucose prior to stimulation with LPS (2 μg/ml) plus IFN-γ (100 U/ml) led to a significant increase in iNOS mRNA in both macrophages and mesangial cells (**Figures 3 & 4, respectively**). There was no detectable message for iNOS in mesangial cells under non-stimulated conditions in either normal or high glucose.

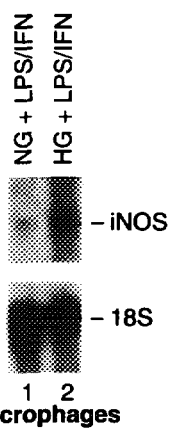

Figure 3. **Northern analysis of iNOS mRNA in RAW 264.7 cells grown under normal and high glucose conditions and probed with a murine macrophage iNOS cDNA.** Macrophages stimulated with LPS (2 μg/ml) plus IFN-γ (100 U/ml) in normal glucose, 100 mg/dl **(lane 1)** or in high glucose, 450 mg/dl **(lane 2)**. 18S ribosomal RNA detected with a human 18S cDNA probe is shown in the lower panel.

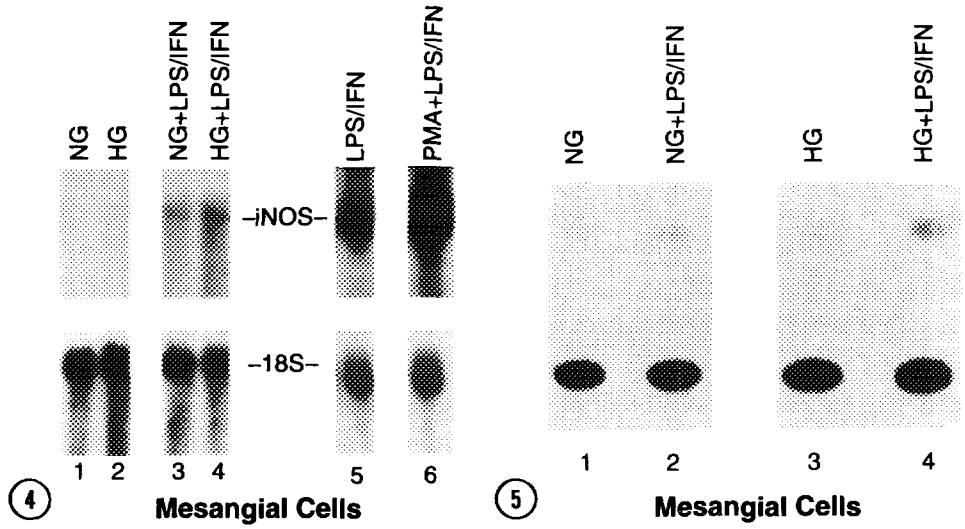

Figure 4. Northern analysis of iNOS mRNA in murine glomerular mesangial cells grown under normal and high glucose conditions and probed with a murine macrophage iNOS cDNA. Mesangial cells under non-stimulated conditions in normal glucose, 100 mg/dl **(lane 1)**, or in high glucose, 450 mg/dl **(lane 2)**. Mesangial cells stimulated with LPS (2 μg/ml) plus IFN-γ (100 U/ml) in normal glucose **(lane 3)** or high glucose **(lane 4)** demonstrate a 4.4-kb transcript. Mesangial cells stimulated with LPS plus IFN-γ in the absence **(lane 5)** or presence of PMA (10^{-7} M) **(lane 6)**. 18S ribosomal RNA detected with a human 18S cDNA probe is shown in the lower panel.

Figure 5. Modulation of iNOS promoter activity by glucose concentration in mesangial cells. The construct pIiNOS-CAT transfected into mesangial cells in normal glucose without stimulation **(lane 1)** or with stimulation by LPS (2 μg/ml) plus IFN-γ (100 U/ml) **(lane 2)**. Mesangial cells grown in high glucose without stimulation **(lane 3)** or with stimulation **(lane 4)**. High glucose in combination with LPS plus IFN-γ enhanced CAT activity by 50% versus LPS plus IFN-γ in normal glucose. Representative of 3 similar experiments.

The addition of PMA (10^{-7} M) prior to stimulating the cells caused a marked upregulation of iNOS (**Figure 4**). In separate studies the message for the constitutive nNOS was undetectable in either cell type, under either basal or stimulated conditions (data not shown).

High glucose enhances stimulated iNOS promoter activity: To determine if increased message for iNOS in high glucose was due to an enhancement in the activity of the iNOS promoter, we employed the CAT reporter assay. Mesangial cells transfected transiently with the full length 1,749 bp region of the iNOS regulatory element fused to the CAT reporter gene (19) demonstrated low activity under non-stimulated conditions in both normal and high glucose concentrations (**Figure 5**). Basal activity of iNOS was slightly higher in the high glucose condition. Upon stimulation with LPS (2 μg/ml) plus IFN-γ (100 U/ml) for 12 hours there was a 50%

greater stimulation under the high glucose condition as compared with normal glucose (**Figure 5**).

DISCUSSION

Exposure of murine mesangial cells and macrophages to high glucose for 24 hours leads to enhanced stimulation of iNOS message and iNOS activity. That the enhanced NOS activity in these cell types is due primarily to iNOS and not to constitutive isoforms is based on the lack of detectable message for nNOS under either stimulated or non-stimulated conditions, and the failure to demonstrate NO production in mesangial cells stimulated with calcium-mobilizing agents. These studies suggest that the combination of high glucose and the presence of stimulating cytokines and/or endotoxin has an additive effect to maximize iNOS gene expression and activity in mesangial cells and macrophages.

A possible mechanism of the high glucose-enhancing effect may be due to PKC activation. High glucose has previously been shown to increase *de novo* production of DAG in many cell types, including mesangial cells (21). Increased cellular DAG content is a potent activator of PKC, and diabetic tissues such as aorta, heart, and glomeruli are all in a state of chronic activation of PKC (22). We have recently reported that transcriptional activation of α1(IV) collagen gene is stimulated by high glucose, and that this effect is likely mediated by enhanced activity of PKC (23). Thus, the findings that PMA, a well-described activator of PKC, enhances iNOS mRNA expression, and that the PKC inhibitors, H-7 and staurosporine, inhibit iNOS activity are consistent with the hypothesis that the high glucose effect may be due to PKC activation. A recent study in rat macrophages has also demonstrated the dependence on PKC activation in the iNOS-stimulation of nitrite production by the activation of CD53, a member of the transmembrane 4 superfamily (24). The presence of two consensus phorbol ester-responsive AP-1 sites in the iNOS promoter (25) points to the locations in the promoter through which PKC activation may lead to enhanced transcription of iNOS. PKC activation via high glucose, LPS, or possibly IFN-γ may lead to enhanced TNF-α production (26), which can augment iNOS expression in macrophages and mesangial cells (27). PKC activation may also enhance iNOS activity independent of other factors, as has been shown in rat peritoneal macrophages (28). PKC activation may be largely a positive modulator at the pre-translational stage by increasing transcript levels, but may be inhibitory at the post-translational stage by inhibiting catalytic activity, as has been seen with isolated nNOS (20). There may also be differences in tissue and species specificity in relation to PKC activation and the iNOS system. Another signaling pathway of potential interest is the cyclic AMP (cAMP) pathway. High glucose increases cAMP in murine mesangial cells (Ziyadeh, unpublished observation) and cAMP has been implicated in mediating iNOS expression in rat mesangial cells (29), thus raising the possibility that the high glucose effect on iNOS may also be

mediated via increased cAMP. We have also advanced the hypothesis that stimulation of matrix expression in mesangial cells by high glucose is mediated by autocrine activation of transforming growth factor-β (TGF-β) (30). A possible regulatory interaction may exist between NO and TGF-β (31) which may be important in glomerular function in the diabetic state.

Recent studies also provide evidence, albeit indirect, that enhanced NO production in early diabetes is via the iNOS pathway. NO production via the constitutive isoform is actually decreased in diabetic aortas when stimulated with bradykinin or acetylcholine (7, 8), and endothelial cells incubated in high glucose produce less NO upon stimulation with acetylcholine (32), yet diabetic rats produce at least two-fold greater amounts of NO metabolites in the urine even when compared with pair-fed normal rats (4, 5). In addition, treatment with aminoguanidine, which is a relatively specific inhibitor of iNOS, attenuates the enhanced vascular permeability seen in early diabetic rats (33). Apart from the classical role of NO to cause vascular dilatation, local production of large amounts of NO in the glomerulus may play a role in cell toxicity or inhibition of growth (3). Further studies are required to determine the significance of enhanced NO production in the diabetic kidney and to evaluate if the iNOS pathway is playing a predominant role.

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