Modulation of Basal Nitric Oxide-dependent Cyclic-GMP Production by Ambient Glucose, *Myo*-Inositol, and Protein Kinase C in SH-SY5Y Human Neuroblastoma Cells

Hideo Shindo,*‡ Thommey P. Thomas,* Dennis D. Larkin,* Anil K. Karihaloo,* Hidetoshi Inada,‡ Toshimasa Onaya,‡ Martin J. Stevens,* and Douglas A. Greene*

**Division of Endocrinology and Metabolism, Department of Internal Medicine, and Michigan Diabetes Research and Training Center, University of Michigan, Ann Arbor, Michigan 48109: and ‡Third Department of Internal Medicine, University of Yamanashi Medical School, Tamaho, Yamanashi-ken 409-38, Japan*

Abstract

Defective tissue perfusion and nitric oxide production and altered *myo*-inositol metabolism and protein kinase C activation have been invoked in the pathogenesis of diabetic complications including neuropathy. The precise cellular compartmentalization and mechanistic interrelationships of these abnormalities remain obscure, and nitric oxide possesses both neurotransmitter and vasodilator activity. Therefore the effects of ambient glucose and *myo*-inositol on nitric oxide–dependent cGMP production and protein kinase C activity were studied in SH-SY5Y human neuroblastoma cells, a cell culture model for peripheral cholinergic neurons. D-Glucose lowered cellular *myo*-inositol content, phosphatidylinositol synthesis, and phosphorylation of an endogenous protein kinase C substrate, and specifically reduced nitric oxide–dependent cGMP production a time- and dose-dependent manner with an apparent IC_{50} of $\sim$ 30 mM. The near maximal decrease in cGMP induced by 50 mM D-glucose was corrected by the addition of protein kinase C agonists or 500 μM *myo*-inositol to the culture medium, and was reproduced by protein kinase C inhibition or downregulation, or by *myo*-inositol deficient medium. Sodium nitroprusside increased cGMP in a dose-dependent fashion, with low concentrations (1 μM) counteracting the effects of 50 mM D-glucose or protein kinase C inhibition. The demonstration that elevated D-glucose diminishes basal nitric oxide–dependent cGMP production by *myo*-inositol depletion and protein kinase C inhibition in peripheral cholinergic neurons provides a potential metabolic basis for impaired nitric oxide production, nerve blood flow, and nerve impulse conduction in diabetes. (*J. Clin. Invest.* 1996. 97:736–745.) Key words: diabetes • diabetic neuropathy • *myo*-inositol • protein kinase C • nitric oxide

Introduction

Nitric oxide (NO) is a highly reactive, rapidly diffusible gas synthesized from L-arginine by tissue- and cell-specific nitric oxide synthases (NOS)[1] (1, 2). The calcium-calmodulin–dependent "constitutive" NOS isoforms in neuronal (ncNOS) and vascular endothelial cells (ecNOS) produce low levels of NO which specifically interact with and activate heme-containing soluble guanylyl cyclase in neighboring neuronal and smooth muscle cells respectively in a paracrine fashion (1, 2). Fold higher levels of NO produced by the calcium-calmodulin–independent, cytokine-"inducible" isoform (iNOS) in macrophages and vascular smooth muscle cells also nonspecifically interact with and chemically modify proteins and DNA, thereby mediating cytokine-induced cytotoxicity (3). In both concentration ranges, NO functions as a potent vasodilator, at low levels modulating vascular tone as the "endothelial-dependent relaxing factor" (4), and at high levels mediating endotoxic shock (1–3). Similarly, in the nervous system, low levels of NO mediate neurotransmission while high levels mediate neurotoxicity (3, 5). Cholinergic neurons employ NO as a retrograde inhibitory neurotransmitter (5), which, in sympathetic ganglia, could importantly modulate blood flow in tissues devoid of local vascular autoregulation such as peripheral nerve (6).

Both NO deficiency (7–13) and excess (14) have been invoked in the pathogenesis of chronic diabetic complications. Glomerular NO deficiency has been linked to glomerular hypertension in the diabetic rat (11) and ascribed to activation of protein kinase C (PKC) by D-glucose–derived diacylglycerols (DAGs) and/or to overproduction of thromboxanes (12). In diabetic rat peripheral nerve, NO deficiency has been hypothesized to reflect competition for NADPH cofactor by NOS and aldose reductase (AR), which is activated by D-glucose substrate in hyperglycemic states (13). Alternatively, inhibition of nerve PKC activity, which is attributed to D-glucose–induced depletion of *myo*-inositol (MI) and arachidonyl DAG,

Address correspondence to Douglas A. Greene, 3920 Taubman Center, Box 0354, University of Michigan Medical Center, 1500 E. Medical Center Drive, Ann Arbor, MI 48109-0354. Phone: 313-936-5505; FAX: 313-936-9240; E-mail: dgreene@uv1.im.med.umich.edu

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1. *Abbreviations used in this paper:* AR, aldose reductase, CDP-DG, cytidine-diphosphodiglyceride; DAG, diacylglycerol; DiC_8, dioctanoylglycerol; ecNOS, endothelial constitutive nitric oxide synthase (NOS isoform III); IBMX, 3-isobutyl-1-methylxanthine; iNOS, inducible nitric oxide synthase (NOS isoform II); L-NAME, N^{ω}-nitro-L-arginine; MARCKS, myristoylated alanine-rich C-kinase substrate; MI, *myo*-inositol; NOS, nitric oxide synthase (EC 1.14.13.39); nc-NOS, neural constitutive nitric oxide synthase (NOS isoform I); PI, phosphatidylinositol; PKC, protein kinase C; RPE, retinal pigment epithelial; SHTE, selenium hydrocortisone, transferrin, and estradiol; SNP, sodium nitroprusside; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

could underlie NO deficiency and/or reduced blood flow in diabetic nerve (15–20). Cultured mouse neuroblastoma cells exhibit D-glucose–induced MI depletion (21, 22) and NO-mediated cGMP production (23, 24). Hence, human SH-SY5Y neuroblastoma cells provide a well-characterized, readily available cholinergic neuronal model to investigate further the relationship between ambient D-glucose concentration, MI depletion, PKC activity, and NO in human nerve cells. The experiments reported in this communication suggest that D-glucose inhibits neuronal NO in SH-SY5Y cells and does so at least in part through MI depletion and inhibition of PKC, thereby implicating this putative metabolic pathway of neural glucose toxicity in NO-mediated neural dysfunction and/or impaired neurally regulated nerve blood flow.

Methods

Dulbecco's minimal essential medium (DMEM), HBSS, trypsin-EDTA, and commercial assay-kits for PKC and cGMP were purchased from GIBCO BRL (Grand Island, NY) and Amersham International Corp. (Arlington Heights, IL), respectively. Bovine calf serum (BCS) was purchased from Hyclone Labs (Logan, UT). D-Glucose, L-glucose, mannitol, 3-*O*-methyl-glucose, N^{ω}-nitro-L-arginine (L-NAME), sodium nitroprusside (SNP), bicinchoninic acid, copper sulfate, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), dioctanoylglycerol (DiC_8), ATP, Triton X-100, saponin, SDS, dexamethasone, LPS, recombinant IFN-γ, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Chemical Co. (St. Louis, MO). Acrylamide, bis-acrylamide, ammonium persulfate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were from Bio-Rad (Melville, NY), and γ-^{32}P-ATP was from ICN (Costa Mesa, CA). Tissue culture supplies were purchased from Corning Glass Works (Corning, NY) and Costar Corp. (Cambridge, MA). Calphostin C was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA). Griess Reagents were obtained from a commercial nitrite assay kit (Cayman Chemical Co., Ann Arbor, MI). GIBCO, Baker Chemical Co. (Phillipsburg, NJ), and Fisher Scientific Inc. (Fair Lawn, NJ) supplied other reagent grade chemicals.

Cell culture. SH-SY5Y cells (passage 70–85 kindly provided by Dr. Eva Feldman at the University of Michigan, Ann Arbor, MI) were grown in DMEM containing 10% BCS, 100 U/ml penicillin, and 100 μg/ml streptomycin in humidified 10% CO_2 with media changed every 2–3 d. Cells were plated in 10-cm, or for PKC assay, 15-cm dishes for 1 d, and then exposed to various experimental conditions as indicated in text and figures. 5 mM D-glucose served as the reference control. In selected experiments exploring the effect of MI-deficient medium, cells were cultured in serum-free DMEM supplemented with SHTE (30 nM selenium, 10 nM hydrocortisone, 100 μg/ml transferrin, and 10 nM β-estradiol [25]) containing 5 mM D-glucose and normal (40 μM) or reduced (0.1 μM) MI.

Analytical techniques. Sorbitol and MI were quantitated by gas-liquid chromatography of aldonitrile derivatives of cell extracts (26) and normalized to cell protein content. cGMP was measured in cells rinsed with PBS and homogenized in 65% ethanol by scintillation proximity RIA using a commercially available RIA kit and normalized to cell protein. Cytidine-diphosphodiglyceride (CDP-DG) was measured by liquid scintillation spectrometry after equilibrium labeling with [5-^{3}H]-cytidine, and extraction and separation by thin layer chromatography (24). Soluble and 0.5% Triton X-100–solubilized membrane-associated PKC activity was measured in cytosolic and particulate fractions ($10^5 \times g \times 0.5$ h) as incorporation of [^{32}P]-ATP (~ 0.2 μCi/nmol) into a synthetic PKC-specific peptide substrate (Ac-MBP:4–14) using PKC:19–36 (a PKC pseudosubstrate inhibitor) to correct for nonspecific phosphorylation (27). Phosphorylation of the myristoylated, alanine-rich C-kinase substrate protein (MARCKS) was assessed in cells labeled with ^{32}P-orthophosphate for 2 h and lysed in buffer containing 0.5% Triton X-100 and 0.5 mg/ml saponin (28). Nuclei were removed by centrifugation (9000 $g \times 2$ min), and proteins from the detergent soluble supernatant (0.8 ml) collected at the interface of a methanol-chloroform extraction (4:1), solubilized in buffer containing 2% SDS, quantitated using bicinchoninic acid (29), and incubated with 40% acetic acid. The MARCKS-containing supernatant was separated by SDS-PAGE and assayed for ^{32}P in a phosphorimager. NO_2 content was measured in 100 μl aliquots of phenol red–free culture media by the addition of 50 μl each of Greiss Reagent-1 and Greiss Reagent-2 as absorbency at 540 nm after 15 min, with a standard curve generated by 0–50 μM $NaNO_2$ (30). Total cell protein was measured using bicinchoninic acid (29). The presence of ncNOS and iNOS expression in SH-SY5Y cells was explored by reverse-transcriptase (RT)-PCR using specific oligonucleotide primers for human ncNOS (sense 5′-TGTGGTCACCAGCACCTTTG-3′, antisense 5′-GGTGGCGTGGTGATGTCCAG-3′, spanning bases 2424–3326 of the coding region) and iNOS (sense 5′-TCCAAATCT-TGCCTGGGGTC-3′, antisense 5′-TCAACGACAGCCTGGTCT-TTCC-3′, spanning bases 540–1435 of the coding region) (31–34). First-strand cDNA was reverse transcribed at 40°C for 1 h from 1 μg total SH-SY5Y RNA in 50 μl using 3 μg oligo-dT, 1 × reverse transcription buffer, RNAsin (Promega, Madison, WI), and avian myoblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, IN). PCR was performed on 5 μl of reverse transcribed product in a 100 μl volume using Amplitaq (Perkin Elmer, Branchburg, NJ) and the products resolved on a 5% polyacrylamide gel and visualized by ethidium bromide.

Statistics. Results are mean±SE. As specified in the text and figure legends, when statistical analysis was performed on replicate measures within a single experiment, the results were confirmed in one or more independent experiments. Statistical significance of differences among groups was analyzed by Student-Neuman-Keuls test, with significance defined as $\alpha < 0.05$.

Results

The presence of 5–50 mM D-glucose in the culture medium had no readily apparent effect on cell growth rate or attained cell density after 4–6 d, e.g., SH-SY5Y cells seeded at an initial density of 0.55×10^6/10-cm dish and grown in the presence of 5 or 50 mM D-glucose for 4 d attained total protein masses of 1.18±0.05 and 1.09±0.04 mg/dish respectively ($n = 5$, P = NS) with no obvious morphological differences. Growth was noticeably slower at 100 or 300 mM D-glucose, with initially higher plating densities of 0.7×10^6 and 2.9×10^6/10-cm dish yielding nearly identical protein masses at 4 d of 1.31±0.05 and 1.38±0.06 mg/dish ($n = 5$, P = NS).

Effect of D-glucose on sorbitol, MI, phosphoinositide synthesis, and PKC. SH-SY5Y cells cultured in 50–100 mM D-glucose for 6 d exhibited osmotically trivial increases in sorbitol content (35) (Fig. 1 *A*), and modest decreases in MI content (to 82 and 62% of the 5 mM D-glucose control for 50 and 100 mM D-glucose, respectively, $P < 0.05$) (Fig. 1 *B*). Cellular MI depletion was neither produced by equimolar concentrations of nonmetabolized glucose analogues, e.g., mannitol (Fig. 1, *shaded bars*), nor prevented by the AR inhibitor sorbinil (Fig. 2). The MI depletion induced by D-glucose rendered MI rate limiting for phosphatidylinositol (PI) synthesis as evidenced by accumulation of CDP-DG, the cosubstrate with MI for PI-synthase (Fig. 1 *C*). These findings are considered indicative of PI synthase inhibition by mass action secondary to MI depletion in other cells and tissues exposed to elevated ambient D-glucose levels (17, 19, 26). Exposure to 50 mM D- (but not L-) glucose for 6 d decreased both membrane-associated PKC

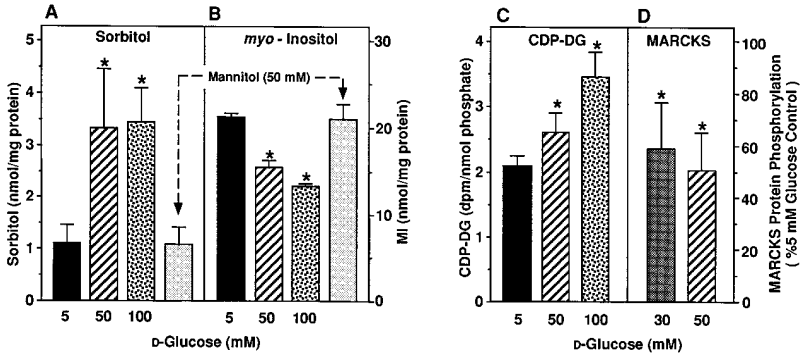

Figure 1. Effect of D-glucose on sorbitol, MI, CDP-DG, and MARCKS phosphorylation in SH-SY5Y cells. Cells were cultured in 5–100 mM D-glucose (or 50 mM mannitol as an osmotic control) for 5–6 d and processed for determination of sorbitol, MI, CDP-DG ($n = 4$ replicate measures within a single experiment, confirmed in an independent experiment), and endogenous MARCKS phosphorylation ($n = 3$ independent experiments; L-glucose results not shown) as described in Methods. $^*P < 0.05$ vs. respective 5 mM D-glucose controls.

activity[2] and the phosphorylation of MARCKS, a specific endogenous PKC substrate (Fig. 1 *D*) (28). Diminished MARCKS phosphorylation after 6 d could not be attributed to PKC downregulation after a putative transient initial increase in PKC activity induced by D-glucose (as described in various nonneural cells [36–41]) since MARCKS phosphorylation was not increased 2 h and 24 h after exposure to 50 mM D-glucose (data not shown). Thus in SH-SY5Y cells cultured in elevated levels of D-glucose, flux through AR was low relative to sorbitol clearance, MI depletion was primarily non–AR mediated perhaps by competitive inhibition by D-glucose of Na-MI cotransport (42–44), depletion of MI limited basal PI synthesis (7, 19, 26, 45), and basal PKC-mediated MARCKS phosphorylation was reduced (19).

Effect of D-glucose on cGMP production. The cGMP content of SH-SY5Y cells increased progressively over 7 d when subcultured in 5 mM (Fig. 3, *solid circles*) but not 50 mM (Fig. 3, *open triangles*) D-glucose, leaving cGMP levels significantly lower in 5 vs. 50 mM D-glucose after 2, 4, and 7 d. This effect of D-glucose on cGMP was concentration dependent, with an apparent IC_{50} of $\sim$ 30 mM and near maximal inhibition at 50 mM (Fig. 4, *open circles*); it was not reproduced by equimolar concentrations of the nonmetabolizable glucose analogue L-glucose, 3-*O*-methylglucose, or mannitol (Fig. 4), or by the alternate glycolytic substrate fructose (data not shown). This D-glucose effect was demonstrable at hyperglycemic concentrations found in experimental diabetes (e.g., 30 mM D-glucose [13]) and, at the near maximal D-glucose inhibitory concentration of 50 mM, reflected altered cGMP production rather than degradation since it persisted in the presence of 500 μM IBMX, a phosphodiesterase inhibitor (46) (Fig. 4, *inset*). These data suggest that altered metabolism of D-glucose after brief exposure to hyperglycemic ($\sim$ 30 mM [13]) levels impairs basal cGMP production.

Effects of NOS inhibition and NO agonists on cGMP. The NOS inhibitor L-NAME was used to determine if basal levels of cellular cGMP reflected tonic cellular NO activity. L-NAME (10^{-8}–10^{-6} M) decreased basal cGMP in a concentration-dependent fashion in 5 mM D-glucose (Fig. 5, *open circles*) and exhibited additivity with 50 mM D-glucose at 10^{-8} M and 10^{-7} M (Fig. 5, *open triangles*). Both 100 (Fig. 5, *open squares*) and 300 mM D-glucose (Fig. 5, *closed squares*) lowered cGMP similar to the highest tested concentration of L-NAME (10^{-6} M) without exhibiting additivity. The effect of L-NAME on cGMP was evident in 20 min (data not shown), overcome by the addition of 10 mM L-arginine (the NOS substrate for which L-NAME is a stereospecific competitive inhibitor, Fig. 5, *inset*), and reversed in 10 min by 1 μM SNP, which chemically generates NO independent of NOS (cGMP in 5 mM D-glucose, 5 mM D-glucose + 1 μM L-NAME, 5 mM D-glucose + 1 μM SNP, and 5 mM D-glucose + 1 μM L-NAME + 1 μM SNP, were, respectively, 132.0±10.1, 69.9±10.6, 142.2±27.0, and 105.2±5.6 fmol/min per mg protein, all $P < 0.05$ vs. 5 mM D-glucose + 1 μM L-NAME only). The relationship between D-glucose metabolism, NOS, and cGMP was further explored by testing whether SNP could acutely ($\leq$ 10 min) reverse the near maximal effect of D-glucose. SNP (1 μM) had no effect on

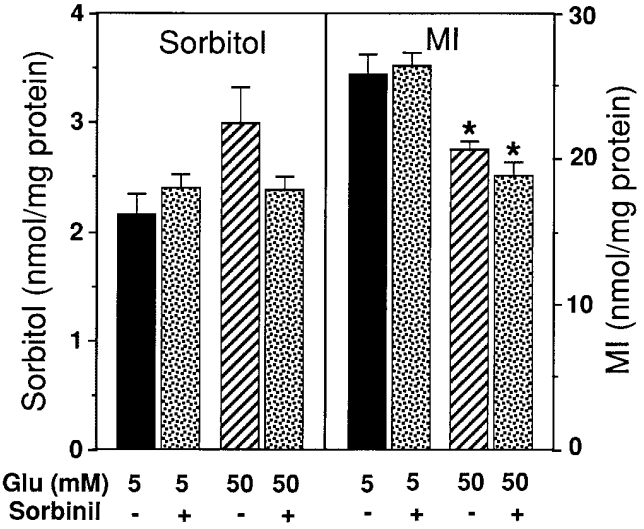

Figure 2. Effect of AR inhibitor sorbinil on sorbitol and MI in SH-SY5Y cells. Cells were cultured in 5 or 50 mM D-glucose (*Glu*)±10 μM sorbinil for 6 d ($n = 3$ replicate measures within a single experiment). Results were confirmed in an independent experiment except that the sorbitol content in 50 mM D-glucose was statistically significantly greater than in the 5 mM D-glucose. $^*P < 0.05$ vs. respective 5 mM D-glucose control.

2. Cytosolic and membrane-associated PKC activities were measured in cells cultured in 5 and 50 mM D-glucose for 6 d. Cytosol activity was unaffected by 50 mM D-glucose (96.9±9.2% of control, $n = 9$, P = NS) and > 90% of measurable activity was associated with the cytosolic fraction. Indeed, no membrane-associated activity was detected in the cells exposed to 5 mM D-glucose in five of nine experiments, but in four of nine experiments when membrane-associated activity was detected in the 5 mM D-glucose sample, membrane-associated PKC activity was reduced in cells exposed to 50 mM D-glucose (55.4±3.1% control, $n = 4$, $P < 0.05$) but not in cells exposed to 50 mM L-glucose (82.0±56.5% control, $n = 4$, P = NS).

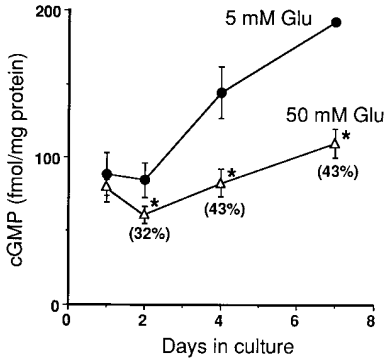

Figure 3. Time course for glucose-induced changes in cGMP in SH-SY5Y cells cultured in 5 or 50 mM D-glucose (*Glu*) for 1–7 d. The results are the mean of three independent experiments. The values in parentheses represent the percent reductions compared to respective 5 mM D-glucose controls. $^*P < 0.05$ vs. 5 mM D-glucose control.

cGMP in the presence of 5 mM D-glucose (Fig. 6, *solid bars*), but acutely raised cGMP in 50 mM D-glucose (Fig. 6, *hatched bars*) to levels approximating the 5 mM D-glucose control. Moreover, 10 μM SNP raised cGMP levels above the 5 mM D-glucose control in cells cultured in 5 and 50 mM D-glucose (Fig. 6, *stippled bars*). These data are consistent with the hypothesis that NO acutely modulates cGMP production in SH-SY5Y cells, and that elevated levels of D-glucose progressively decrease cGMP production predominantly through an indirect NO-mediated effect on guanylyl cyclase. This contention is further supported by the observation that D-glucose decreased NO_2 appearance in the cell culture medium in a dose-dependent fashion. SH-SY5Y cells were cultured for 7 d in 5, 30, or 50 mM D-glucose. NO_2 appearance in the medium over the last 24 h was 5.28±0.43, 4.11±0.41, and 2.97±0.39 nmol/mg protein in 5, 30, and 50 mM D-glucose, respectively ($n = 3$, $P < 0.05$ for 5 vs. both 30 and 50 mM D-glucose, with similar results replicated in two additional independent experiments).

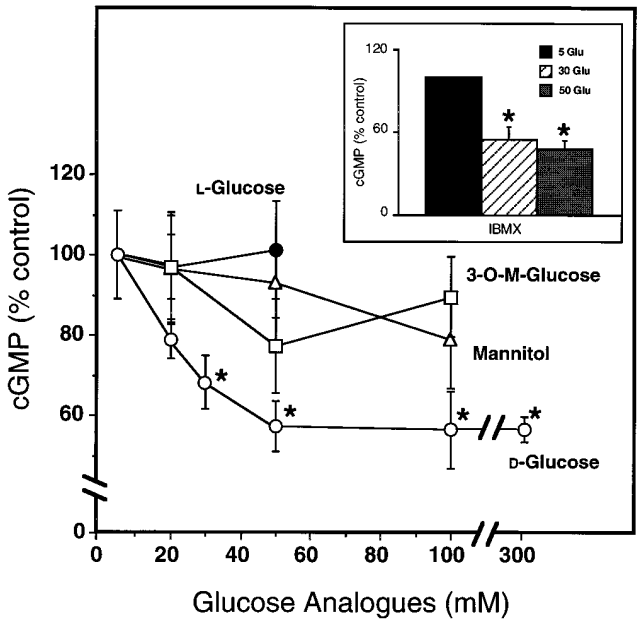

Figure 4. Concentration dependency of effects of D-glucose and non-metabolized glucose analogues on cGMP levels in SH-SY5Y cells. Cells were cultured for 4 d in 5–300 mM D-glucose (*open circles*) or 5 mM D-glucose media containing 15, 45, and 95 mM L-glucose, 3-*O*-methylglucose or mannitol. The results are 10 replicate measurements from two independent experiments. (*Inset*: Effect of IBMX on D-glucose–induced reduction in cGMP. Cells were cultured in 5, 30, or 50 mM D-glucose for 5 d before transfer to new medium±500 μM IBMX for 15 min [$n = 4$ replicate measures within a single experiment with confirmation in an independent experiment]). $^*P < 0.05$ vs. 5 mM D-glucose control.

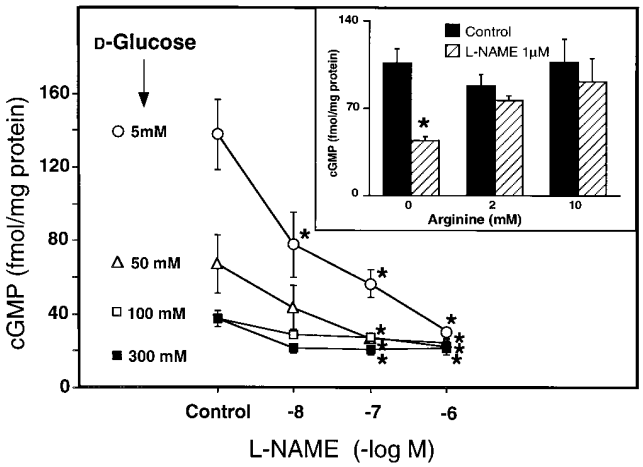

Figure 5. Effect of L-NAME on the D-glucose–induced decrease in cGMP levels in SH-SY5Y cells cultured for 4 d in 5–300 mM D-glucose and 0–10^{-6} M L-NAME ($n = 4$ replicate measures within a single experiment with confirmation in an independent experiment.) (*Inset*: Effect of L-arginine on the L-NAME–induced decrease in cGMP. Cells were cultured in 5 mM D-glucose and 1 μM L-NAME for 5 d±2 or 10 mM L-arginine for the last 2 d; the results were confirmed in an independent experiment). $^*P < 0.05$ vs. respective controls without L-NAME.

Role of PKC and MI depletion in the depression of cGMP by D-glucose. The near maximal depression of basal cGMP by 50 mM D-glucose was acutely reversed by the PKC agonists DiC_8 (50 μM × 2 h) and TPA (100 nM × 10 min or 2 h) (Fig. 7, *hatched bars*), which did not affect cGMP in 5 mM D-glucose (Fig. 7, *solid bars*) despite translocation of PKC to the membrane fraction (that raised membrane-associated PKC > five-fold; data not shown). Conversely, exposure to 1 μM TPA for 24 h (which depressed cytosolic and membrane-associated PKC activity to undetectable levels; data not shown) decreased cGMP in 5 mM (Fig. 7, compare *open bar* with *solid*

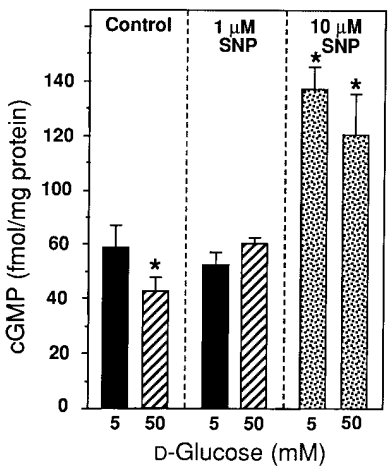

Figure 6. Effect of SNP on D-glucose–induced decrease in cGMP in SH-SY5Y cells cultured for 4 d in 5 or 50 mM D-glucose and incubated±1 or 10 μM SNP for 10 min ($n = 4$ replicate measures within a single experiment with confirmation in an independent experiment). The results were confirmed in an independent experiment. $^*P < 0.05$ vs. 5 mM D-glucose controls.

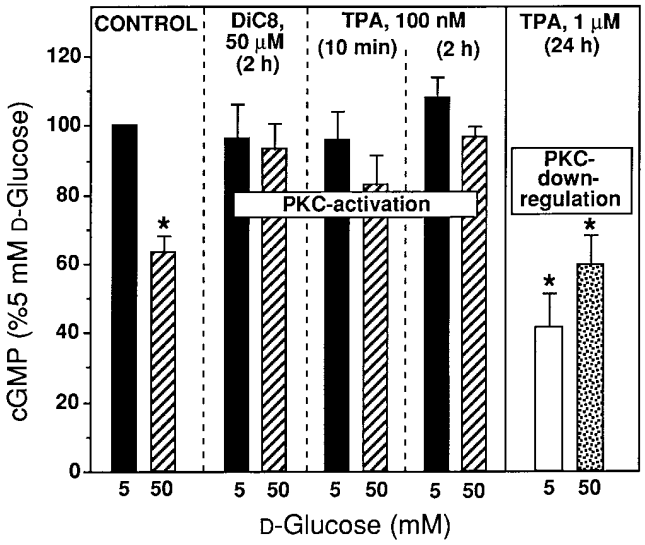

Figure 7. Effect of PKC-activation and -downregulation on D-glucose–induced decrease in cGMP. Cells were cultured for 4 d in 5 or 50 mM D-glucose±DiC_8 or TPA as indicated ($n = 4$ replicate measures within a single experiment with confirmation in an independent experiment). *$P < 0.05$ vs. 5 mM D-glucose controls.

bar on left) but not 50 mM (Fig. 7, compare *stippled bar* with *hatched bar* on left) D-glucose. Calphostin C, a specific PKC inhibitor, decreased cGMP levels in 5 mM (Fig. 8, *solid bars*) but not 50 mM (Fig. 8, *hatched bars*) D-glucose, while SNP (1 μM × 10 min) reversed the effect of 50 mM D-glucose or calphostin C, acutely returning cGMP levels to that of the 5 mM D-glucose control (Fig. 8, *open bars*). Higher concentrations of SNP (10 μM) elevated cGMP to the same higher levels in both 5 mM and 50 mM D-glucose (Fig. 8, *stippled bars*). These studies suggest that the near maximal effect of 50 mM D-glucose on cGMP is mediated by one or more D-glucose–sensitive fractions of PKC (38) that, within a critical range of activity, modulate NOS.

Supplementation of the DMEM–10% BCS culture medium with 500 μM MI partially corrected PI synthase inhibition (expressed as CDP-DG accumulation [Fig. 9, *left*]) and completely corrected near maximal cGMP depression (Fig. 9, *right*) in 50 mM D-glucose without affecting either parameter in 5 mM D-glucose (data not shown) strongly implicating MI depletion as a mediator of these D-glucose effects. Conversely, MI-depleted medium reproduced the near maximal effect of 50 mM D-glucose on cGMP: cGMP levels after 7 d culture in serum-free DMEM-SHTE (25) and 5 mM D-glucose with normal (40 μM) or reduced (0.1 μM) MI were 49.3±4.5 and 33.2±3.4 fmol/mg protein, respectively, ($n = 4$, $P < 0.05$, with identical results in a second independent experiment). The reduction of cGMP induced by MI-deficient DMEM-SHTE was acutely reversed by TPA: cGMP in 40 μM and 0.1 μM MI for 7 d were, after exposure to 100 nM TPA for 10 min, 49.0±7.3 and 47.4±6.5 fmol/mg protein, respectively, (both P = NS vs. 40 μM MI without TPA). On the other hand, 10 μM sorbinil, which did not affect D-glucose–induced MI depletion (see above), did not alter cGMP content in either 5 mM or 50 mM D-glucose (respectively, 112.0±7.6 and 86.3±5.7 fmol/mg protein [$P < 0.05$] in the absence of sorbinil; and 107.5±2.5 and 92.5±6.9 fmol/mg protein [$P < 0.05$] in the presence of sorbinil, P = NS±sorbinil). These data support the view that the effect of elevated D-glucose on cGMP production in SH-SY5Y cells is mediated by MI depletion and inhibition of PI synthase by mass action, leading to decreased PI turnover and a reduction in a critical component of basal PKC activation that modulates basal NOS activity. However, in contrast to rat peripheral nerve (13, 15, 16), SH-SY5Y cells exhibit low AR activity, and, accordingly, neither D-glucose–induced MI depletion nor its consequences were corrected by AR inhibition (19, 35).

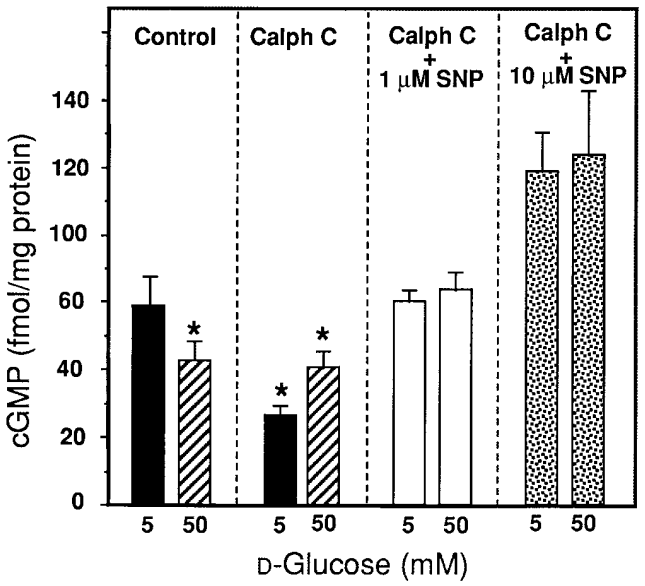

Figure 8. Effect of calphostin C and SNP on D-glucose–induced decrease in cGMP in cells cultured for 4 d in 5 or 50 mM D-glucose. Calphostin C or SNP was added for the last 2 h or 10 min, respectively ($n = 4$ replicate measures within a single experiment with confirmation in an independent experiment). *$P < 0.05$ vs. 5 mM D-glucose control.

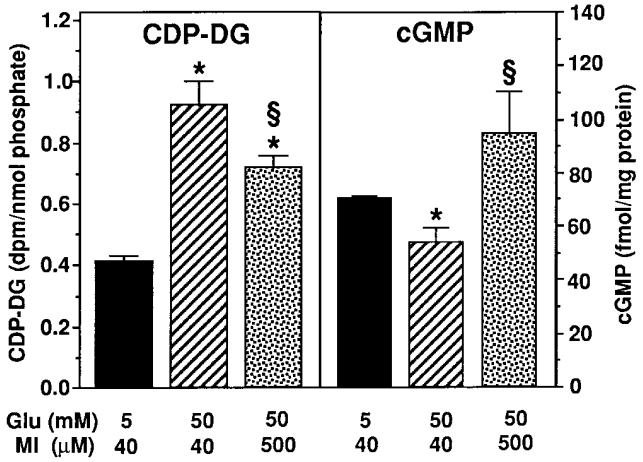

Figure 9. Effect of MI supplementation on D-glucose–induced changes in CDP-DG and cGMP. Cells were cultured for 5 d in 5 or 50 mM D-glucose (*Glu*) with 40 μM or 500 μM MI. These results from four replicate samples were confirmed in an independent experiment of the same size. *$P < 0.05$ vs. respective 5 mM D-glucose controls. §$P < 0.05$ versus respective 50 M D-glucose controls.

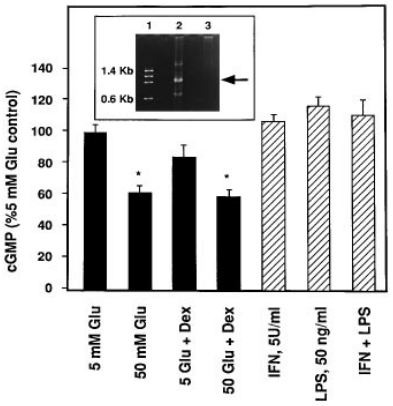

Figure 10. Effect of dexamethasone (*Dex*), interferon-γ (*IFN*), and LPS on ncNOS-dependent cGMP levels. Cells were cultured for 5 d in 5 or 50 mM D-glucose (*Glu*)±2.5 μM Dex for the last 2 d (*solid bars*), and in 5 mM D-glucose with 5 U/ml IFN and 50 ng/ml LPS alone or in combination (*hatched bars*) for the last 2 d. These results from four replicate samples were confirmed in an independent experiment of the same size. $*P < 0.05$ vs. respective 5 mM D-glucose controls. (*Inset*: Ethidium bromide stained PAGE of RT-PCR products amplified from total RNA from SH-SY5Y cells cultured in 5 mM D-glucose using primers specific for human ncNOS [lane *2*] or human iNOS [lane *3*]. Lane *1* contains molecular weight standards and *arrow* indicates the predicted ~ 900 bp size cDNA for ncNOS and iNOS).

Characterization of NOS in SH-SY5Y cells. The presence of ncNOS but not iNOS mRNA in SH-SY5Y cells was implied by an ~ 900 bp product amplified from SH-SY5Y reverse-transcribed total RNA using primers specific for human nc-NOS (Fig. 10, *inset*, lane *2*). A number of contaminating minor bands (some of which are shown) were of the predicted size for misprimed products. No products were observed after RT-PCR using the iNOS primers (Fig. 10, *inset*, lane *3*). Consistent with the absence of iNOS mRNA, SH-SY5Y cells also exhibited little iNOS-like enzymatic activity since dexamethasone (2.5 μM for 2 d), which suppresses iNOS induction (47), failed to modify basal or maximally D-glucose–inhibited cGMP levels (Fig. 10), and the addition of IFN-γ (5 U/ml), LPS (50 ng/ml), or their combination, which induce iNOS (47), failed to increase cGMP levels (Fig. 10).

Discussion

A recent report from this laboratory implicating NO deficiency in the slowing of nerve conduction in the acutely streptozotocin-diabetic rat (13) combined with other recent reports of NO defects in diabetes (7–14, 48, 49) prompted the present examination of the effect of D-glucose on NO-dependent cGMP levels in cultured human SH-SY5Y neuroblastoma cells. These well-differentiated neuronal cells selectively subcloned from the SKN-SH neuroblastoma (50) are commonly employed to study neuronal metabolism and signal transduction (51), in this case the relationship of D-glucose and MI metabolism to PKC and NO-dependent cGMP.

The progressive rise in cGMP after subculturing in 5 mM D-glucose most likely represents progression of SH-SY5Y cells from G_0 through G_1 (52). The effects of elevated ambient D-glucose levels on cGMP were time- and dose-dependent, with an IC_{50} of ~ 30 mM and near maximal inhibition at ~ 50 mM D-glucose. These parameters lie within the general concentration range and duration of physiologic hyperglycemia that produces NO-related nerve conduction slowing in acute experimental diabetes (13). The effects of 30–50 mM D-glucose were physiologically and metabolically specific since they were unaccompanied by obvious changes in cell viability, growth, or morphology and were not reproduced by equimolar concentrations of nonmetabolized glucose analogues. Production rather than degradation of cGMP was altered since the effect of 50 mM D-glucose persisted in the presence of the phosphodiesterase inhibitor IBMX. The D-glucose effect on cGMP appears to be mediated by NO, a known regulator of neuronal guanylyl cyclase (1, 2): (*a*) L-NAME, a stereospecific L-arginine analogue that competitively inhibits NOS, reproduced the effect of D-glucose in a dose-dependent, L-arginine–sensitive manner without exhibiting additivity at maximal concentrations; (*b*) SNP, a chemical NO source, reversed the effects of 50 mM D-glucose and L-NAME on cGMP, arguing against direct effects of either agent on guanylyl cyclase (53); and (*c*) D-glucose decreased NO_2 appearance in the cell culture medium in a dose-dependent fashion. The magnitude of the maximal reduction of basal NO-dependent cGMP by 50 mM D-glucose was virtually identical to that invoked in glomerular hypertension in diabetes (12).

The effect of D-glucose on cGMP in SH-SY5Y cells appears to reflect MI depletion, impaired PI synthesis, and PKC inhibition. D-glucose decreased MI and raised CDP-DG in a dose-dependent fashion (17, 26). Culture in MI-deficient SHTE medium reproduced and supplementation of DMEM with 500 μM MI overcame the effect of 50 mM D-glucose on cGMP. Biochemical and pharmacological results support PKC as an important mediator of the effects of D-glucose and MI depletion on NO-dependent cGMP through regulation of ncNOS. Firstly, 50 mM D-glucose appeared to decrease the very low basal level of membrane-associated PKC activity (54) and reduced PKC-dependent MARCKS phosphorylation. Short-term stimulation of PKC with TPA or DiC_8 (55, 56) overcame the effect of 50 mM D-glucose on cGMP. Conversely, downregulation of PKC with TPA (57, 58) or its inhibition by calphostin C (59) reproduced, but did not add to, the effect of 50 mM D-glucose. Similarly, SNP, which reversed the reduction of cGMP by 50 mM D-glucose, also overcame the effect of calphostin C. Finally, the effect of MI depleted media on cGMP was reversed by PKC activation with TPA (Thomas, T.P., H. Shindo, and D.A. Greene. manuscript in preparation). These results do not, however, exclude the existence of alternative or parallel mechanisms (e.g., inositol-phosphate–regulated calcium or eicosanoid signaling) linking MI depletion to alterations in ncNOS activity, nor do they preclude a parallel subtle effect of PKC on guanylyl cyclase (53). Indeed, MI depletion has been shown to alter intracellular calcium (Thomas, T.P., H. Shindo, and D.A. Greene, manuscript in preparation) and D-glucose has been shown to alter eicosanoid production (Stevens, M.J., unpublished data) in SH-SY5Y cells. Thus, PKC signaling might represent only one of several redundant mechanisms by which ncNOS and NO action might be metabolically regulated in SH-SY5Y cells by D-glucose and phosphoinositide (60).

The observation that D-glucose impairs basal NO activity in SH-SY5Y cells via MI and PKC add to a growing chain of evidence linking D-glucose to DAG and PKC regulation (16–19, 36–41, 61), on the one hand, and PKC activation to NOS regulation (47, 62–70) on the other. However, these views are at best complex and fragmentary, if not at times paradoxical and contradictory. Diabetes and/or elevated D-glucose levels have been reported to decrease MI and phosphoinositide-derived

arachidonyl-DAG (18), PKC activity (16, 39), and/or PKC-related cellular functions (20, 61) in some but not all tissues prone to diabetic complications. Yet detailed patterns of response may vary even within the same tissue. For example, sciatic nerve from untreated streptozotocin-diabetic rats exhibits diminished total and arachidonyl-DAG (17, 18), and increased activation (71) and phosphorylation (20) of (Na,K)-ATPase by exogenous PKC agonists, consistent with a diminished basal level of PKC-mediated phosphorylation of (Na,K)-ATPase. In separate studies, diminished activation of PKC (72) and decreased total and cytoplasmic intrinsic PKC activity have been noted in streptozotocin-diabetic rat sciatic nerve (16), perhaps reflecting the reported discordance between measures of cellular PKC activity and its translocation (73, 74). In other tissues equally prone to diabetic complications, D-glucose appears to increase rather than decrease total DAG and PKC activation (36, 38, 41, 62). Recent studies in cultured human retinal pigment epithelial (RPE) cells suggest that diametrically opposite effects of D-glucose on phosphoinositide and DAG metabolism reflect varying levels of expression of AR and AR-induced MI depletion within the same cell type (19). In RPE cells with high intrinsic AR gene expression (75), the predominant effect of elevated ambient D-glucose is AR-mediated sorbitol accumulation, reciprocal MI depletion (35), inhibition of PI synthase by mass action (26), diminished phosphoinositide turnover, and depletion of basal and agonist-stimulated arachidonyl-DAG (19). In RPE cells with low-grade intrinsic AR expression (75), D-glucose increases phosphoinositide turnover and DAG levels (19). Analogously divergent effects of D-glucose on DAG and PKC signal transduction have been reported in retinal microvascular endothelial cells (76) and pericytes (39), that also differ in AR expression (77). Although not the focus of this report, the relationship between AR pathway activity and MI in SH-SY5Y cells may be different from either high or low AR-expressing RPE cells. Sorbitol accumulation in SH-SY5Y cells is low compared to high AR-expressing RPE cells (19), yet MI depletion nevertheless occurs albeit by an AR inhibitor–insensitive mechanism. It is tempting to speculate that MI depletion in SH-SY5Y cells may reflect competitive inhibition or another AR-independent interaction between D-glucose or a D-glucose metabolite with the Na-MI cotransporter (42, 78, 79), which is now under investigation.

The relationship between PKC and NOS is equally complex. PKC activation has been reported to increase (62) or attenuate (63) ncNOS activity. The studies reported in this communication suggest that normal basal PKC activity is sufficient to promote normal basal ncNOS activity, but, within a critical range, inhibition of PKC by D-glucose-induced MI-depletion limits basal ncNOS activation. (A parallel relationship between basal PKC activity and basal [Na,K]-ATPase activity was reported in peripheral nerve from diabetic animals [80], and NO has also been reported recently to stimulate vascular [Na,K]-ATPase independent of cGMP [81]). Given that attenuation of ncNOS by PKC activation has been noted primarily when ncNOS was stimulated by other agonists (47, 69, 70), the studies in this report suggest that PKC could tonically stimulate basal ncNOS activity yet nevertheless dampen the activation of ncNOS by other agonists. (PKC may also attenuate agonist-induced iNOS gene expression [82]). This relationship would complement the reported inverse bidirectional interactions between NO and PKC described in platelets, where NO inhibits agonist-induced phosphoinositide turnover, calcium mobilization, and PKC substrate phosphorylation while enhancing PKC substrate phosphorylation with calcium ionophore (83).

The effect of D-glucose on ncNOS could have significant implications for the pathogenesis of diabetic complications, particularly diabetic neuropathy (Fig. 11). Metabolic (84) and vascular (13, 76, 85–87) factors have been invoked in the pathogenesis of diabetic neuropathy, but the interrelationships among these factors are poorly understood. In the diabetic rat, impairment of nerve blood flow and conduction velocity have been ascribed in part to activation of the AR pathway (85, 86, 88). Moreover, NO deficiency has been implicated as a possible link between AR activation, vascular dysfunction (49), and slowing of nerve conduction (11). As a putative postganglionic, postsynaptic inhibitory neurotransmitter (5), neuronal NO would normally dampen sympathetic tone and endoneurial vascular resistance (which does not locally autoregulate [6]) in the maintenance of normal endoneurial perfusion (88), which accordingly would be impaired by postganglionic NO deficiency (Fig. 11). This contention is consistent with the recently reviewed evidence (88, 89) indicating that chemical sympathectomy essentially normalizes nerve blood flow in experimental diabetes to the same extent as peripheral vasodilators.[3] A parallel inhibition by D-glucose of ncNOS in somatic myelinated peripheral neurons might also impair nerve conduction independent of ischemia through such potentially NO-regulated processes as (Na,K)-ATPase activity (Fig. 11) (81, 92). Whether similar or different regulation of ecNOS or iNOS by PKC is also relevant to neuropathy or other diabetic complications remains to be determined (Fig. 11).

Possible mechanisms linking AR activation to NO deficiency include competition between AR and NOS for NADPH cofactor (13), and/or D-glucose–induced alterations in PKC activity, which in turn appears to modulate NOS. The present studies favor linkage by PKC rather than NADPH, although the latter is not entirely excluded in diabetic peripheral nerve in the rat where nerve sorbitol accumulation is more robust, where MI depletion also decreases nerve PKC activity (16), and where vascular as well as neural NOS could be involved. The apparent discrepancy in the potency of AR inhibition to correct D-glucose–induced, MI-related NO abnormalities in vivo and in vitro could simply reflect different mechanisms underlying MI depletion. Thus, AR-mediated MI depletion would predominate in intact nerve, which rapidly accumulates sorbitol in diabetes, while modest sorbitol accumulation in SH-SY5Y cells would unmask competitive inhibition of Na-MI cotransport by D-glucose as the predominant cause of MI depletion (42, 78, 79).

In summary, hyperglycemia diminishes basal NO-dependent cGMP levels in human SH-SY5Y neuroblastoma cells in a dose-dependent fashion, mediated by a D-glucose–induced

3. A recent report (90) that dietary MI supplementation improved neither nerve conduction nor blood flow in streptozotocin-diabetic rats employed an unusually high MI dose previously shown to be nontherapeutic for nerve conduction in diabetic rats and neurotoxic to nondiabetic rats (15, 91), thereby explaining the apparent discrepancy with recently published and currently unpublished data demonstrating improved nerve conduction (13) and laser doppler blood flow (Stevens M.J., and D.A. Greene manuscript in preparation) in MI supplemented streptozotocin-diabetic rats.

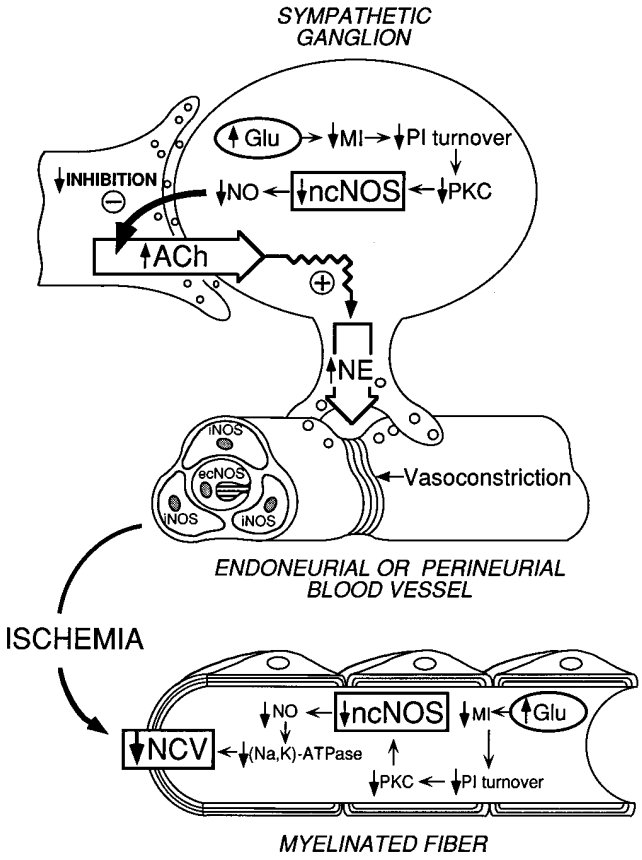

Figure 11. Pictorial model illustrating D-glucose (*Glu*)-induced inhibition of ncNOS in postganglionic sympathetic or myelinated peripheral somatic neurons leading to slowing of nerve conduction velocity (*NCV*) in acute experimental diabetes. In this model, ncNOS is tonically regulated by PKC in both postganglionic sympathetic neurons (*top*) and in myelinated peripheral somatic neurons (*bottom*). In postganglionic sympathetic neurons, ncNOS sustains NO-mediated inhibition of pre-synaptic acetylcholine (*ACh*) release (*top left*) to tonically dampen sympathetic tone (5). In large myelinated somatic neurons (*bottom*), ncNOS regulates putative NO-dependent functions such as (Na,K)-ATPase activity (81). This communication reports that human SH-SY5Y neuroblastoma cells cultured in D-glucose levels approximating those in the plasma of diabetic rats undergo MI depletion, inhibition of basal PI turnover, diminished PKC-mediated protein phosphorylation, and a reduction in NO synthesis by ncNOS. Extrapolation of these results in vivo would imply a decrease in NO-mediated inhibition of presynaptic acetylcholine release potentiating postganglionic norepinephrine (*NE*) release, vasoconstriction of non-autoregulating endoneurial or perineurial microvessels (*center*), and endoneurial ischemia, which could decrease nerve conduction. At the same time, D-glucose–mediated inhibition of NO synthesis in peripheral somatic neurons (*bottom*) might additionally (or alternatively) slow nerve conduction by interfering with NO-dependent intraneuronal functions such as (Na,K)-ATPase (13, 81, 92). Whether analogous D-glucose–mediated inhibition of endothelial ecNOS or smooth muscle iNOS (*center*) also contributes to ischemia and/or slowing of nerve conduction in diabetes remains to be established. This model provides a potential mechanistic link between metabolic responses of peripheral nerve to diabetes (e.g., MI depletion) through NO to endoneurial ischemia and slowed nerve conduction velocity, which now needs further exploration and clarification in vivo.

decrease in MI content and PKC activity that in turn causes decreased ncNOS activity. A similar decrease in peripheral neuronal NO in vivo could at least in part explain impaired nerve conduction in diabetes through alterations in neurally regulated nerve blood flow, or by direct impairment of other NO and/or cGMP-regulated neuronal functions, e.g., (Na,K)-ATPase activity (35, 81, 92) (Fig. 11). By extrapolation, D-glucose–induced perturbations in other NOS isoforms could extend to diabetic complications other than neuropathy, including diabetic glomerulopathy, where thromboxane-mediated PKC activation impairs NO generation and action (12), macrovascular disease (49), where glucose-mediated NO-deficiency appears to inhibit (Na,K)-ATPase activity (81, 93), and diabetic retinopathy, where NO has been implicated in pericyte relaxation (94) and where NO deficiency could reduce retinal blood flow (95).

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