High glucose decreases intracellular glutathione concentrations and upregulates inducible nitric oxide synthase gene expression in intestinal epithelial cells

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Abstract

Diabetes is associated with oxidative stress and increased concentrations of inflammatory cytokines. The aim of the study was to assess the effects of inflammatory cytokines and oxidative stress associated with increased glucose concentrations on inducible nitric oxide synthase (iNOS) promoter activity in intestinal epithelial cells. High-glucose (25 mmol/l) conditions reduced glutathione (GSH) concentrations in the human intestinal epithelial cell line, DLD-1. Addition of the antioxidant, α -lipoic acid, resulted in the restoration of GSH concentrations to normal. Upregulation of basal iNOS promoter activity was observed when cells were incubated in high glucose alone. This effect was significantly reduced by the addition of the antioxidant, α -lipoic acid, and completely blocked with inhibition of nuclear factor kappa B (NF κ B) activity. Stimulation of cytokines (interleukin-1 beta, tumour necrosis factor-alpha, interferon-gamma) induced iNOS promoter activity in all conditions and this was accompanied by an increase in nitric oxide (NO) production. Inhibition of NF κ B activity decreased, but did not completely inhibit, cytokine-induced iNOS promoter activity and subsequent production of NO. In conclusion, iNOS promoter activity induced by high concentrations of glucose is mediated in part through intracellular GSH and NF κ B.

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Introduction

Relatively little is known about the effect of diabetes on intestinal epithelium in the absence of autonomic neuropathy. It is well recognized that diabetes induces oxidative stress and that the resulting oxidative damage has a key role in the development of diabetic complications (Son *et al.* 2004). Within the small intestine, diabetes is associated with numerous changes, including hyperplasia and hypertrophy of epithelial cells (Zoubi *et al.* 1995), increased absorption of sugars and amino acids (Fedorak 1990) and increased endogenous cholesterol synthesis (Feingold *et al.* 1982). Inflammatory cytokines are also increased in diabetes (Esposito *et al.* 2002). It is believed that

increases in human histocompatibility antigens (HLA) class II, HLA-DR and HLA-DP, found in structurally normal intestine in diabetic individuals, may result from secretion of inflammatory cytokines such as interferon gamma (IFN- γ) (Savilahti *et al.* 1999). Cytokines also modulate gut permeability (McKay & Baird 1999), but there does not appear to be a consensus about the presence of altered gut permeability in diabetes. Differences in opinion may reflect differences in methodologies used by various investigators.

One of the important regulatory signalling molecules produced by the endothelium is nitric oxide (NO), which is synthesized from L-arginine by the action of three different isozymes of nitric oxide synthase (NOS) (Moncada & Higgs 1993).

NO can have either protective or deleterious effects in the gastrointestinal tract; NO generated by the action of constitutive NOS (cNOS) acts physiologically and the overproduction of NO by inducible NOS (iNOS) occurs during pathophysiological conditions. Intestinal permeability is also increased when iNOS concentrations are very high (Kilbourn & Griffith 1992). Smaller increases in iNOS are associated with epithelial repair after injury (Gookin *et al.* 2002). High concentrations of NO produced from iNOS exhibit toxic effects through nitrosative and oxidative stress; however, regulation of the induction of iNOS in diabetes remains unclear. The aim of this study was to assess the effect of inflammatory cytokines and oxidative stress associated with increased glucose concentrations on iNOS promoter activity in intestinal epithelial cells.

Materials and Methods

Materials and reagents

All chemicals were obtained from Sigma and tissue culture reagents were purchased from Invitrogen Ltd, unless otherwise stated. The cytokines were purchased from R&D Systems (Abingdon, UK). The synthetic peptidyl alpha-keto aldehyde proteasome inhibitor was synthesized on an ABI/Pioneer automated peptide synthesizer (Lynas *et al.* 1998).

Tissue culture

The human colon-carcinoma-derived epithelial cell line, DLD-1 (Dexter *et al.* 1979) was maintained in 10% Dulbecco's modified Eagle medium supplemented with antibiotics (100 µg/ml streptomycin sulphate, 60 µg/ml benzylpenicillin) and L-glutamine (2 mmol/l) in a humidified atmosphere of 5% CO₂. To determine if changes in iNOS transcription are caused by high glucose, glycation or osmotic effects, experiments were performed over a 48-h period using normal glucose (5 mmol/l; N), high glucose (25 mmol/l; H) and the non-metabolizable L-glucose (5 mmol/l D-glucose + 20 mmol/l L-glucose; L). The antioxidant, α-lipoic acid (50 µmol/l; LA), which replenishes reduced glutathione (GSH), was added to high-glucose experiments to assess the effect of GSH on cytokine-induced transcription of iNOS. The NFκB inhibitor was reconstituted in dimethyl sulphoxide

and used at a concentration of 50 µmol/l (NI) for 4 h.

Determination of intracellular glutathione

GSH determination was performed using an enzymatic recycling assay as previously described, which has been automated on the Cobas Fara centrifugal analyser (Sharpe *et al.* 1998). GSH concentrations were corrected for protein concentration.

iNOS construct

The −7.2 kb human iNOS promoter fragment (David Geller, University of Pittsburgh, USA) contains the unique human enhancer region, which includes four NFκB binding sites critical for transcriptional regulation of iNOS (Taylor *et al.* 1998). This large clone, −7.2 kb luc (sequence in Genbank AF093682), is in the parent luciferase vector, pXP2.

Transient transfection

Cells were seeded into 12-well plates and at 70% confluence were treated with Superfect (Qiagen, West Sussex, UK) containing 1–2 µg of the full-length construct described above, and 0.2–0.4 µg *pRL-TK*, a renilla luciferase gene under control of a TK promoter as an internal control. After transfection for 48 h, cells were treated for 4 h with the proinflammatory cytokine cocktail containing interleukin 1-beta (IL-1β; 2 ng/ml), tumour necrosis factor alpha (TNF-α; 10 ng/ml) and interferon gamma (IFN-γ; 1000 IU/ml) to induce iNOS gene expression. The dual-luciferase reporter assay system (Promega) was used to quantitate the luminescent signal from firefly and renilla luciferase. Luminescence was measured on a TopCount NXT microplate scintillation and luminescence counter (Packard). Transfections were performed in triplicate and normalized against the control activity from the renilla luciferase.

Nitrite/nitrate production

Nitrites and nitrates were measured spectrofluorimetrically using the 2,3-diaminonaphthotriazole (DAN) reagent. Briefly, cell culture supernatants were incubated with NADPH (67 µmol/l,

Boehringer-Mannheim) and nitrate reductase (0.14 IU/ml) for 10 min. DAN reagent (0.05 mg/ml) was added after 10 min and incubated for a further 10 min. The reaction was stopped by the addition of sodium hydroxide (2.8 mol/l). Standard curves were produced under the same experimental conditions using nitrate solutions (0.5–50 μ mol/l). The formation of DAN was measured (excitation 360 nm, emission 465 nm) using a Genios fluorescence spectrophotometer (Tecan, Austria) and corrected for protein concentration.

Statistics

Data were analysed using analysis of variance and multiple comparisons between data sets were made using modified *t*-tests only if the overall value of *P* was less than 0.05. All results are expressed as mean $\pm$ S.E.M. of at least three independent experiments performed in triplicate.

Results

Glucose-induced changes in GSH

GSH concentrations in transfected DLD-1 cells were decreased in high-glucose conditions (1.56 ± 0.07 nmol/mg protein) compared with normal-glucose conditions (2.18 ± 0.07 nmol/mg protein; $P < 0.05$, $n = 3$) and could be restored by the addition of the antioxidant, α -lipoic acid (2.10 ± 0.04 nmol/mg protein; $P < 0.05$, $n = 3$). Inhibition of NF κ B activity increased GSH concentrations to significantly above normal in glucose-treated cells (5.84 ± 0.48 nmol/mg protein; $P < 0.005$, $n = 3$).

iNOS promoter activity

High glucose (25 mmol/l) caused an increase in iNOS promoter activity to a level approximately 250% of that found with normal (5 mmol/l) glucose (Fig. 1). This effect was significantly reduced by the addition of the antioxidant, α -lipoic acid, and completely blocked with NF κ B inhibition (Fig. 1).

In all experimental conditions, the iNOS promoter activity induced by the cocktail of cytokines was above basal (Fig. 1). The restoration of GSH concentrations with α -lipoic acid (in high glucose) had no effect on the response of the iNOS promoter to cytokines; in contrast, the presence of

Figure 1 iNOS promoter activity. Cells were transfected with -7.2 kb luc construct and incubated for 48 h before the addition of a cocktail of cytokines (IL-1 β 2 ng/ml, TNF- α 10 ng/ml and IFN- γ 1000 IU/ml) for 4 h in the following conditions: N=normal glucose (5 mmol/l D-glucose); H=high glucose (25 mmol/l D-glucose); HLA=high glucose with α -lipoic acid (50 μ mol/l); HNI=high glucose with the NF κ B inhibitor (50 μ mol/l). Values are expressed as mean relative luciferase units $\pm$ S.E.M. * $P < 0.05$ compared with non-induced (no cytokine cocktail); ‡ $P < 0.05$ compared with N, † $P < 0.05$ compared with N + cytokine cocktail, # $P < 0.05$ compared with H + cytokine cocktail). Experiments were performed in triplicate and the results are representative of at least three independent experiments.

the NF κ B inhibitor significantly decreased the iNOS promoter response to cytokines in both high glucose (Fig. 1) and normal glucose (0.64 ± 0.14 relative luciferase units (RLU) for normal glucose, compared with 0.28 ± 0.07 RLU for normal glucose with NF κ B inhibitor; $P < 0.05$, $n = 3$).

Under physiological conditions, intestinal epithelial cells are exposed to a wider range of osmotic pressures than most other cells of the body. It seemed important to determine whether the effects of glucose, described above, were attributable to increased osmotic pressure. The use of the non-metabolizable analogue, L-glucose, allowed us to explore this possibility in addition to determining whether glucose metabolism was required for the effects on the iNOS promoter. iNOS promoter activity was similar in the presence of 5 mmol/l D-glucose and 5 mmol/l D-glucose plus 20 mmol/l L-glucose. Furthermore, the effect of cytokines on promoter activity was similar under these two experimental conditions (Fig. 2). Thus osmotic pressure could not explain the effect of D-glucose on

Figure 2 iNOS promoter activity. Cells were transfected with -7.2 kb luc construct and incubated for 48 h before the addition of a cytokine cocktail (IL-1 β 2 ng/ml, TNF- α 10 ng/ml and IFN- γ 1000 IU/ml) for 4 h in the following conditions: N=normal glucose (5 mmol/l D-glucose); L=combination of normal glucose (5 mmol/l D-glucose) + L-glucose (20 mmol/l L-glucose); H=high glucose (25 mmol/l D-glucose). Values are expressed as mean relative luciferase units $\pm$ S.E.M. * P <0.05 compared with non-induced (no cytokine cocktail); † P <0.05 compared with N. Experiments were performed in triplicate and the results are representative of at least three independent experiments.

iNOS promoter activity. The results clearly demonstrate that the metabolism of glucose is required in order to increase promoter activity.

Nitrite/nitrate production

The upregulation of iNOS promoter activity in high-glucose conditions was not accompanied by significant changes in NO production. In contrast, the cytokine cocktail (IL-1 β , TNF- α , IFN- γ) increased NO production in all experimental conditions tested (Table 1). As observed with iNOS promoter activity, restoration of GSH concentrations with the addition of α -lipoic acid had no significant effect on cytokine-induced production of NO. Inhibition of NF κ B activity reduced, but not significantly, the production of NO in cells treated with high concentrations of glucose (Table 1).

Discussion

This study has shown that exposure of human DLD-1 cells to high concentrations of glucose

Table 1 Nitrite/nitrate production. Cells were transfected with -7.2 kb luc construct and incubated with 5 mmol/l glucose (normal; N) or 25 mmol/l glucose (high; H), 50 μ mol/l α -lipoic acid (LA) or an NF κ B inhibitor (50 μ mol/l; NI) for 48 h before the addition of a cytokine cocktail (IL-1 β 2 ng/ml, TNF- α 10 ng/ml and IFN- γ 1000 IU/ml) for 16 h. Production of NO was quantified, corrected for protein concentration, and the results expressed as mean $\pm$ S.E.M. (μ mol/mg protein). Experiments were performed in triplicate and the results are of at least three independent experiments

	Non-induced (μ mol/mg protein)	Cytokine-stimulated (μ mol/mg protein)
N	0.87 $\pm$ 0.11	7.50 $\pm$ 0.37*
H	0.72 $\pm$ 0.10	6.89 $\pm$ 0.24*
HLA	1.44 $\pm$ 0.18	8.24 $\pm$ 1.21*
HNI	1.13 $\pm$ 0.21	4.40 $\pm$ 1.57

* P <0.05 compared with non-induced (no cytokine).

(25 mmol/l) for 48 h decreases intracellular GSH concentrations and supports the evidence that hyperglycaemia induces oxidative stress in the gastrointestinal tract (Bhor *et al.* 2004). GSH concentrations, reduced by glucose, were restored to normal by addition of the antioxidant, α -lipoic acid. A similar result has been observed in human vascular smooth muscle cells (Powell *et al.* 2001), mesangial cells (Catherwood *et al.* 2002) and human peripheral mononuclear cells of patients with non-insulin-dependent diabetes (Arnalich *et al.* 2001). α -Lipoic acid not only functions as an antioxidant, but is reduced to dihydrolipoate intracellularly, thereby increasing the availability of the rate-limiting substrate in GSH synthesis, cysteine. It is also possible that the restoration of GSH concentrations may result from the direct effects of α -lipoic acid on gene expression of the gamma-glutamylcysteine synthetase (γ -GCS) subunits (Suh *et al.* 2004). We also observed that, when NF κ B is inhibited, GSH concentrations are increased substantially above normal. This suggests that, under basal conditions, NF κ B has a negative regulatory effect on GSH. Transcription factor binding sites, including NF κ B, have been identified in the promoter region of the light subunit of γ -GCS, but the specific role of NF κ B in regulation requires further investigation.

To determine the effects of high concentrations of glucose on iNOS transcription, experiments were performed using normal glucose (5 mmol/l),

high glucose (25 mmol/l) and the non-metabolizable L-glucose (5 mmol/l D-glucose + 20 mmol/l L-glucose). Whereas Linn *et al.* (1997) were unable to show cytokine induction of an iNOS promoter in DLD-1 cells using a promoter construct less than -8.7 kb, we have shown increased promoter activity using a construct of -7.2 kb and a cytokine cocktail consisting of IL-1 β , TNF- α and IFN- γ , in the presence of both normal and high concentrations of glucose. This increase was not changed by glycation or osmotic effects and was undiminished in the presence of the antioxidant, α -lipoic acid, which restores GSH to normal concentrations in cells treated with high concentrations of glucose.

In addition, when DLD-1 cells were incubated in high-glucose conditions, there was a significant increase in iNOS promoter activity in cells that were not stimulated by cytokines. This effect may be mediated by GSH via NF κ B, as restoration of GSH concentrations with α -lipoic acid and NF κ B inhibition reduced iNOS promoter activity to near normal. High glucose also increased cytokine-induced increases in iNOS promoter activity, with the exception of cells treated with the NF κ B inhibitor. High concentrations of glucose have been shown to increase cytokine-induced increases in iNOS protein (Xu *et al.* 1999) and mRNA (Noh *et al.* 2002) in rat tissues. However, in contrast to our observation that high glucose increases iNOS promoter activity in non-cytokine-stimulated cells, it has been reported that, in bovine aortic endothelial cells, high concentrations of glucose (25 mmol/l) inhibit lipopolysaccharide-induced production of NO by decreasing the expression of iNOS and cNOS (Guo *et al.* 2000). GSH has been shown to regulate IL-1 β -induced production of NO in islets, purified β cells and insulinoma cells by modulation of iNOS gene expression (Nikulina *et al.* 2000). Miralles *et al.* (2000) observed that molecular oxygen was able to regulate expression of iNOS in rat liver at the transcriptional level, through the production of reactive oxygen species and reduction in GSH.

The effects of glucose treatment may be dependent on the duration of exposure. Stockklauser-Färber *et al.* (2000) observed that the activity of total NOS and mRNA were dependent on the duration of diabetes in rat hearts, in which there was an increase in activity after 4–6 weeks, but a reduction after 20 weeks. It is now apparent

that the mechanisms by which glucose may influence iNOS regulation may be cell-type-dependent. In rat pancreatic β cells, high concentrations of glucose are believed to enhance IL-1 β -induced production of NO via the p38 signalling pathway (Nikulina *et al.* 2000). Chronic treatment with supraphysiological concentrations of insulin (100 nmol/l) and insulin resistance induced by high glucose treatment (25 mmol/l for 12–24 h) are accompanied by marked reductions in iNOS as a result of p38 mitogen-activated protein kinase (MAPK) activation in rat aortic vascular smooth muscle cells (Begum & Ragolia 2000). Addition of a protein kinase C inhibitor, calphostin C, and an aldose reductase inhibitor suppressed the enhancement of cytokine-induced production of NO by high concentrations of glucose (30 mmol/l) in rat mesangial cells (Loven *et al.* 1986).

NF κ B, an oxidative-stress-responsive transcription factor, is activated in cells cultured in high-glucose conditions (Hattori *et al.* 2000, Du *et al.* 1999) and mediates cytokine-induced expression of iNOS in various cell types (Kwon *et al.* 1995, 1998). However, in DLD-1 cells, the inhibition of NF κ B activity appears to reduce cytokine-induced iNOS promoter activity, and to abolish completely the observed increase in iNOS promoter activity induced by glucose. The inhibition of NF κ B activity also reduces, but does not completely abolish, subsequent production of NO in cells treated with high concentrations of glucose. There are conflicting reports on the involvement of glucose, GSH and NF κ B in the regulation of iNOS (Guo *et al.* 2000, Miralles *et al.* 2000, Nikulina *et al.* 2000, Stockklauser-Färber *et al.* 2000) and this evidence, together with our findings, suggests that the regulation of iNOS by GSH and NF κ B is species- or cell-specific (or both). The exact mechanism of iNOS regulation in DLD-1 cells remains to be elucidated, but from the findings of the present study it is apparent that the mechanism by which high concentrations of glucose regulate iNOS differs from cytokine-induced activation. Cytokine-induced activation of iNOS in DLD-1 cells is independent of protein kinases A, C and G, phosphatidylinositol 3-kinase (PI3K), extracellular-regulated kinase and p38 MAPK, but requires protein tyrosine activity, especially that of IFN- γ -activated janus-activated kinase 2 (JAK 2) (Cavicchi & Whittle 1999). It is possible that protein kinase signalling pathways such as p38 MAPK and PI3K,

which are activated by high glucose concentrations in other cell types (Wilmer *et al.* 2001, Srivastava 2002), are involved in this regulation. The activation of iNOS that is induced by high concentrations of glucose may also involve other non-redox-sensitive transcription factors such as signal transducer and activator of transcription-1 (STAT-1), which can be phosphorylated by protein kinases such as JAK1/2.

In summary, this study has shown that, in DLD-1 cells, stimulation by cytokines (IL-1 β , TNF- α , IFN- γ) induces iNOS promoter activity, resulting in an increase in NO production, and that this effect is mediated in part by the redox-sensitive transcription factor, NF κ B. High concentrations of glucose alone also induce basal iNOS promoter activity through changes in intracellular GSH and NF κ B activity; therefore GSH and NF κ B represent potential targets for therapeutic intervention to alleviate the complications of long-term diabetes. Maintenance of GSH concentrations using the antioxidant, α -lipoic acid, may prove beneficial in the inhibition of hyperglycaemia-induced oxidative responses during diabetes.

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