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The Enhancement of Endotoxin-Induced Nitric Oxide Production by Elevation of Glucose Concentration in Macrophage

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The production of nitric oxide (NO) and the expression of inducible nitric oxide synthase (iNOS) are known to be modulated by a variety of factors. Recent study showed that endotoxin-induced NO synthesis and iNOS expression were greatly enhanced by elevation of extracellular glucose concentration in murine macrophages. Although this was suggested to be due to the activation of protein kinase C (PKC) via sorbitol pathway, there was lack of evidence for this speculation. This study was performed to delineate the underlying intracellular mechanisms of glucose-enhancing effect on endotoxin-induced NO production in Raw264.7 macrophages. The levels of NO release induced by lipopolysaccharide (LPS) significantly increased by the treatment of glucose in a concentration dependent manner and also, this effect was observed in LPS-preprimed cells. Concurrent incubation of cells with PKC inhibitors, H-7 or chelerythrine, and LPS resulted in the diminution of NO production regardless of glucose concentration but this was not in the case of LPS-prepriming, that is, chelerythrine showed a minimal effect on the glucose- enhancing effect. PMA, a PKC activator, did not show any significant effect on glucose-associated NO production. Modulation of sorbitol pathway with zopolrestat, an aldose reductase inhibitor, did not affect LPS-induced NO production and iNOS expression under high glucose condition. And also, sodium pyruvate, which is expected to normalize cytosolic NADH/NAD^+ ratio, did not show any significant effect at concentrations of up to 10 mM. Glucosamine marginally increased the endotoxin-induced nitrite release in both control and high glucose treated group. 6-diazo-5-oxonorleucine (L-DON) and azaserine, glutamine: fructose-6-phosphate amidotransferase (GFAT) inhibitors, significantly diminished the augmentation effect of high glucose on endotoxin-induced NO production. On the other hand, negative modulation of GFAT inhibitors was not reversed by the treatment of glucosamine, suggesting the minimal involvement, if any, of glucosamine pathway in glucose-enhancing effect. In summary, these results strongly suggest that the hexosamine biosynthesis pathway and the activation of PKC via sorbitol pathway do not contribute to the augmenting effect of high glucose on endotoxin induced NO production in macrophage-like Raw264.7 cells.

Key Words: Nitric oxide, Endotoxin, Glucose, Protein kinase C

INTRODUCTION

Nitric oxide (NO) is a highly reactive molecule with a multitude of biological effects ranging from vasodilation to cytotoxicity (Moncada et al, 1991; Nathan, 1992). It is formed in an oxygen-dependent reaction during which L-arginine is converted into L-citrulline by the enzyme, NO synthase (NOS). 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 one (iNOS) first found in macrophages (Nathan & Xie, 1994). iNOS is a high-output isoform compared with the two constitutive isoforms and its expression in macrophage following exposure to cytokines or endotoxins is

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frequently associated with a generalized or localized inflammatory response resulting from infection or tissue injury (Nathan & Xie, 1994).

The production of NO and the expression of iNOS are known to be modulated by a variety of factors. Recent study showed that endotoxin-induced iNOS expression is enhanced in murine macrophage and mesangial cells by elevation of extracellular glucose concentration (Sharma et al, 1995). Although the activation of protein kinase C (PKC) has been suggested as a possible mechanism for the augmenting effect of high glucose, there was lack of evidence for this speculation. It is quite conceivable that high glucose mediates its adverse effects via multiple mechanisms, since glucose and its metabolites are utilized through diverse pathways. Glucose metabolism via sorbitol pathway is known to increase the $NADH/NAD^+$ ratio in the cytosol, which increases de novo synthesis of diacylglycerol (DAG), and in turn, activates PKC (Lee et al, 1989). And also, recent evidences suggest the intracellular protein glycosylation through hexosamine biosynthesis pathway mediates some of regulatory as well as toxic effects of glucose (Marshall et al, 1991; Roos et al, 1996).

In this study, we examined whether these metabolic schemes contribute to the glucose enhancing effect on endotoxin induced nitrite production and iNOS expression in Raw264.7 macrophages and found that hexosamine biosynthesis pathway and the activation of PKC via sorbitol pathway are not involved in the regulation of iNOS by high glucose.

METHODS

Materials

E.coli lipopolysaccharide (011B : 4), H-7, chelerythrine, staurosporine, phorbol-12-myristic-13-acetate (PMA), sodium pyruvate and 6-diazo-5-oxonorleucine (DON) were from Sigma Chemical (St. Louis, MO, USA). Polyclonal antibody to iNOS was purchased from UBI (Lake Placid, NY, USA). Zopolrestat was a kind gift from Dr. Pagani (Pfizer Inc., Central Research Division, Groton, CT, USA).

Cell culture

Macrophage-like cell line Raw264.7 was obtained from American Type Culture Collection. Cells were grown in Dulbecco's Modified Eagles Medium supplemented with 10% fetal bovine serum (Gibco-BRL), and antibiotics (100 units/ml penicillin-G and 100 units/ml streptomycin). Cultures were maintained in humidified 5% CO_2 atmosphere at 37°C. Confluent cells were trypsinized (0.125% trypsin containng 1.3 mM EDTA), resuspended in fresh media and added to 96 well plates (2×10^5 cells/well). After one day, cultures were treated with various stimuli as indicated.

Assay for Nitrite production

Nitrite was measured spectrophotometrically with Griess reagent. At the end of the incubation, 100 μl of the culture medium was mixed with an equal volume of Griess reagent (1 part of 0.1% naphtylethylenediamine hydrochloride and 1 part of sulfanilamide in 10% phosphoric acid). The reaction was completed after 10 min of incubation, the absorbance at 540 nm was measured and the nitrite concentration was determined using a curve calibrated on sodium nitrite standards.

Western blotting

The cells were lysed with 200 μl of buffer containing 150 mM NaCl, 10 mM Na_2HPO_4 (pH 7.4), 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 2 mM EDTA, 20 μM leupeptin, 1 μM pepstatin A, and 2 mM phenylmethylsulfonyl fluoride (PMSF). The lysates were then incubated on ice for 10 min, and centrifuged at 12,000 g for 10 min at 4°C. And the supernatants were subjected to 7.5% SDS-PAGE using buffer system of Laemmli (1970). The separated proteins were electrophroretically transferred to polyvinylidene difluoride (PVDF) membranes. The blots were soaked for 1 hr in 5% non-fat dried milk and then incubated with the anti-iNOS antibody (1 : 1,500). The iNOS proteins were visualized by incubation with color development reagent (Bio-Rad, Hercules, CA, USA,) containing NBT and BCIP.

Reverse transcriptase-PCR

Total cellular RNA was extracted from cell monolayers using an RNAzol B (Tel-test, Friendswood, TX, USA), and reverse-transcribed using AMV reverse transcripatase (Boeringer Mannheim, Germany). cDNA samples were subjected to 26 cycles of PCR

reaction for iNOS specific primers (sense: 5'-GTG-TTC-CAC-CAG-GAG-ATG-TTG-3', antisense: 5'-CTC-CTG-CCC-ACT-GAG-TTC-GTC-3') and 30 cycles of PCR reaction for GAPDH specific primers (sense: 5'-GTG-AAG-GTC-GGT-GTG-AAC-GGA-TTT-3', antisense: 5'-CAC-AGT-CTT-CTG-AGT-GGC-AGT-GAT-3').

Statistical analysis

All data are presented in the mean±SE. Statistical differences between groups were evaluated by the Student's t-test and differences were considered significant when $p<0.05$.

RESULTS

Enhanced production of NO by high glucose

As shown in Fig. 1, the levels of nitrite release induced by 10 μg/ml of lipopolysaccharide (LPS) were significantly increased by the elevation of glucose concentration in a dose dependent manner. This effect of glucose was maximal at the concentration of 4 mg/ml (22 mM). This was not an osmotic effect as the adjustment of osmolarity same as 4 mg/ml of D-glucose using mannitol did not augment the endotoxin-induced NO production. The enhancing effect of glucose was observed when cells were pre-primed with LPS for 2 hrs and further incubated in fresh media with varying concentration of glucose for 24 hrs (Fig. 2B).

Effects of PKC modulators

Concurrent incubation of cells with PKC inhibitors, H-7 or chelerythrine, and LPS resulted in the diminution of NO production (Fig. 2A), and this was observed in both control and high glucose treated group. But in the case of LPS pre-priming experiment, differential sensitivity to PKC inhibitors was

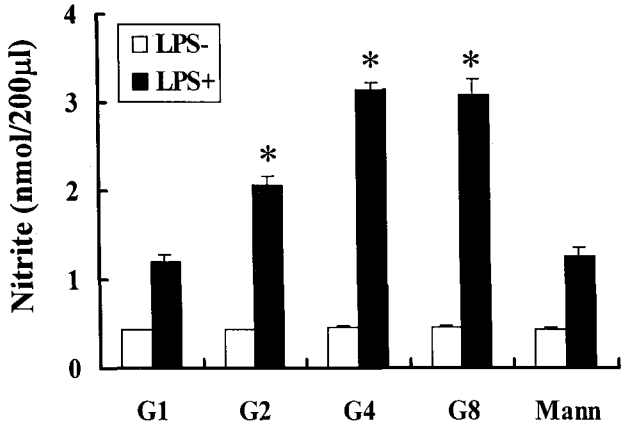

Fig. 1. Effects of glucose on nitrite production. Confluent cells were incubated with varying concentrations of glucose in the presence or absence of LPS (10 μg/ml) for 24 hrs. (G1: 1 mg/ml of glucose, G2: 2 mg/ml of glucose, G4: 4 mg/ml of glucose, G8: 8 mg/ml of glucose) Mann: 3 mg/ml of mannitol in G1 media (Osmolarity was same as that of G4 media) *$p<0.01$ vs G1+LPS

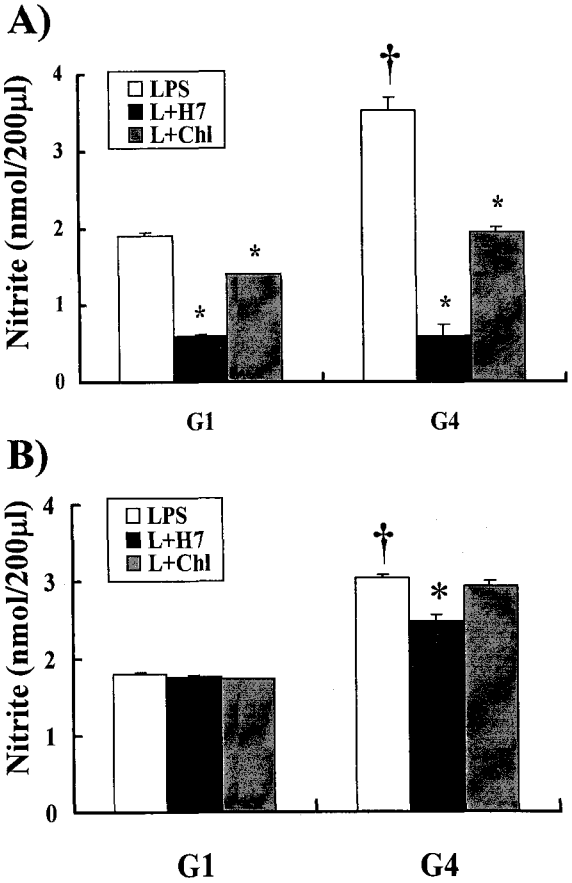

Fig. 2. Effects of PKC inhibitors on nitrite production. *A)* Cells were incubated with LPS (10 μg/ml) in the presence or absence of PKC inhibitors for 24 hrs. *B)* Cells were primed with LPS (10 μg/ml) for 2 hrs. After thorough washing, cells were incubated with different glucose concentrations in the presence or absence of PKC inhibitors for 24 hrs. H-7 (25 μM), Chl (chelerythrine, 5 μM) were used as PKC inhibitors. *$p<0.05$ vs LPS with same glucose concentration. †$p<0.05$ vs G1+LPS.

Table 1. Effects of PMA on nitric oxide production in Raw264.7 cells stimulated with LPS

Glucose	Nitrite accumulation (nmol/200 μl)							
	G1				G4			
	Control	PMA	LPS+PMA	LPS	Control	PMA	LPS+PMA	LPS
I	0.69±0.090	0.66±0.13	1.77±0.23*	1.78±0.13*	0.71±0.17	0.74±0.20	6.91±0.18**	7.29±0.35**
II	0.67±0.078	0.90±0.030	1.62±0.039*	–	0.64±0.003	0.98±0.025	6.59±0.18**	–
III	0.66±0.010	0.68±0.01	0.82±0.017	–	0.64±0.010	0.68±0.003	0.78±0.05	–

Cells were preincubated with PMA (100 nM) for 3 hrs (Group I) or 24 hrs (Group III) and further incubated with LPS (10 μg/ml) for 24 hrs in G1 or G4 media. In another set of experiment, cells were simultaneously treated with PMA and LPS for 24 hrs. (Group II) G1: glucose 1 mg/ml; G4: glucose 4 mg/ml; PMA: PMA (100 nM) only; LPS+PMA: PMA (100 nM)+LPS (10 μg/ml); LPS: LPS (10 μg/ml) only.
*$p<0.05$ vs G1 control, **$p<0.01$ vs G4 control

observed (Fig. 2B). Chelerythrine, a selective PKC inhibitor, showed a minimal effect on NO production. On the contrary, H-7, a less selective inhibitor, significantly diminished the glucose-enhancing effect.

The effects of PMA, a PKC activator, on endotoxin-induced NO synthesis under varying conditions of glucose concentration were summarized in Table 1. Cells were treated with PMA in different time schedule. First, cells were pretreated with PMA (10^{-7} M) for 30 min before stimulating the cells with LPS (Table 1, group I), but in this maneuver, PMA did not show any significant effect on NO production irrespective of glucose concentrations. And the second, PMA was treated with LPS simultaneously for 24 hrs, which had no effect on enhanced-NO production by glucose (Table 1, group II). Prolonged preincubation of the cells with PMA for 24 hrs (Table 1, group III), which is known to down-regulate PKC activity, abolished the endotoxin stimulated NO production to control level in both normal and high glucose concentrations.

Effects of sodium pyruvate and aldose reductase inhibitor, zopolrestat

In order to prevent glucose metabolism through sorbitol pathway, cells were treated with an aldose reductase inhibitor, zopolrestat. Treatment of zopolrestat (100 μM) failed to influence NO production augmented by high D-glucose (Fig. 3A). And also, sodium pyruvate, which is expected to normalize the altered $NADH/NAD^+$ ratio in the cells exposed to high D-glucose, did not affect NO production at concentrations up to 10 mM (Fig. 3B).

Effects of glucose on iNOS expression

Western blot and RT-PCR were performed to determine whether the glucose effect operated at the level of the expression of iNOS. The incubation of Raw 264.7 cells with high glucose (4 mg/ml) and LPS (10 μg/ml) led to increase in the expression of iNOS in parallel with nitrite production (Fig. 4). In the absence of LPS, iNOS was not detected in both control and high glucose group. The addition of zopolrestat resulted in the slight decrease of the iNOS expression, but did not alter the pattern of difference in iNOS expression between control and high glucose treated group.

Effects of 6-diazo-5-oxonorleucine, azaserine and gluocosamine

In order to address the possible involvement of hexosamine pathway in glucose-enhancing effect, 6-diazo-5-oxonorleucine (L-DON) and azaserine, glutamine: fructose-6-phosphate amidotransferase (GFAT) inhibitors, and glucosamine were applied to our experimental system. As shown in Fig. 5, L-DON and azaserine significantly diminished the augmenting-effect of high glucose on endotoxin-induced nitrite production. On the other hand, azaserine did not affect LPS induced NO production. Glucosamine treatment resulted in slight increase in nitrite release

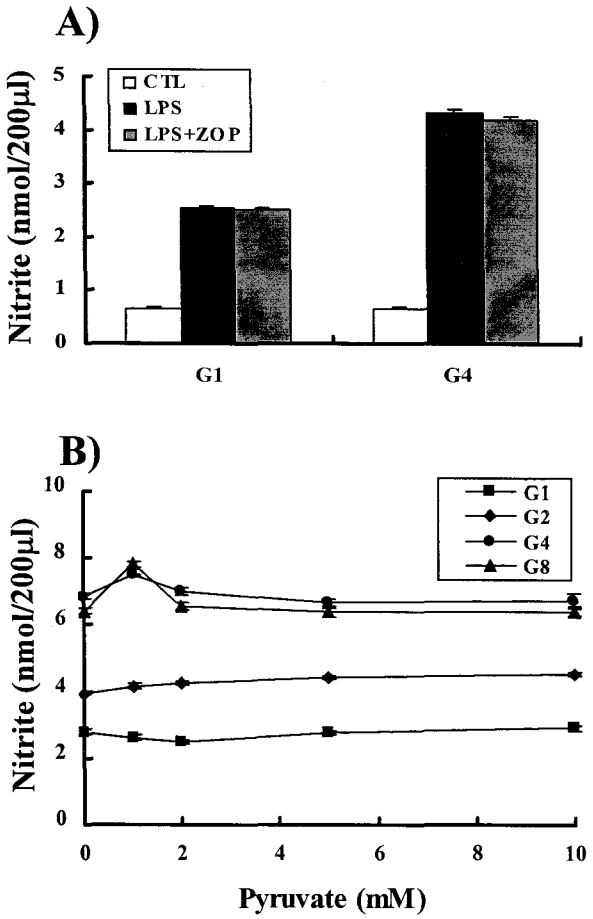

Fig. 3. Effects of zopolrestat and pyruvate on the nitrite production. *A)* Cells were incubated with zopolrestat (ZOP, 100 μM), an aldose reductase inhibitor, in the presence of LPS (10 μg/ml) for 24 hrs. *B:* Cells were incubated with varying concentrations of sodium pyruvate and glucose in the presence of LPS for 24 hrs. (G1: 1 mg/ml of glucose, G2: 2 mg/ml of glucose, G4: 4 mg/ml of glucose, G8: 8 mg/ml of glucose).

(Fig. 5), but did not reverse the effect of L-DON (Data are not shown).

DISCUSSION

Because of its potent vasodilating activity, it is well accepted that excessive release of nitric oxide (NO) contributes to the diminished vascular tone in models of sepsis (Manthous et al, 1993). In this context, it might imply the increase in endotoxin-induced NO production in diabetes that incidence of septic shock is much greater in diabetes compared with non-diabetic patients (Lang et al, 1987). This

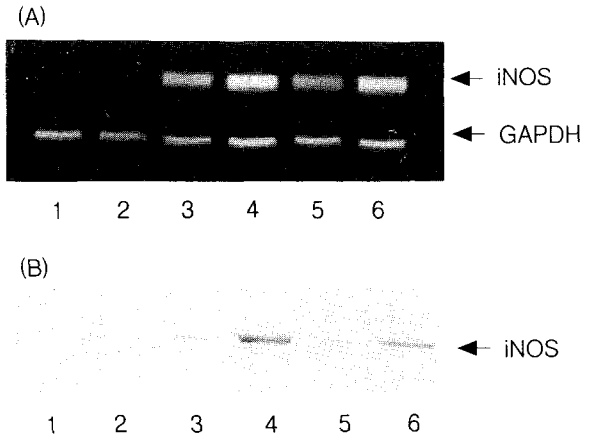

Fig. 4. Effect of glucose on iNOS expression in Raw 264.7 cells stimulated with LPS. *A)* Cells were incubated with or without zopolrestat (100 μM) and LPS (10 μg/ml) for 24 hrs, and harvested for western blot analysis. *B)* Cells were incubated with or without zopolrestat (100 μM) and LPS (10 μg/ml) for 6 hrs, and total RNA was extracted for RT-PCR analysis. lane 1: G1 control; lane 2: G4 control; lane 3: G1+LPS (10 μg/ml); lane 4: G4 +LPS (10 μg/ml); lane 5: G1+LPS (10 μg/ml)+zopolrestat (100 μM); lane 6: G4+LPS (10 μg/ml)+ zopolrestat (100 μM); G1: 1mg/ml of glucose, G4 4 mg/ml of glucose. Results are representative of three separate experiments.

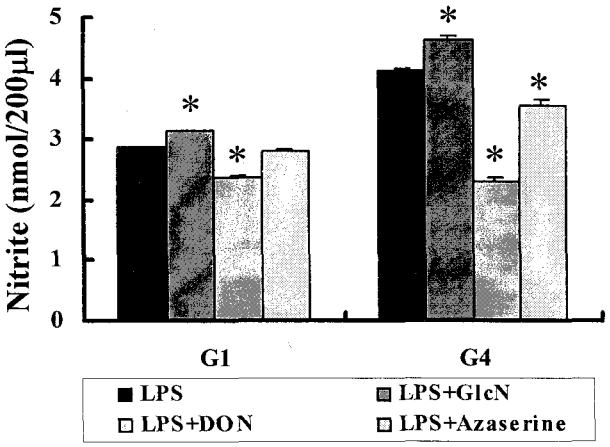

Fig. 5. Effect of D-glucosamine and GFAT inhibitors. Cells were incubated with D-glucosamine (GlcN, 500 μM) or GFAT inhibitors (L-DON 50 μM, Azaserine, 100 μM) in the presence of LPS (10 μg/ml) for 24 hrs. And supernatants were assayed for nitrite. * $p<0.05$ vs LPS with same glucose concentration.

implication could be supported by several reports which described enhanced production of NO metabolites in the urine of diabetic rats (Bank & Aynedjian, 1993; Tolins et al, 1993). And also, various lines of evidences, albeit indirect, suggested that enhanced NO production in early diabetes might be via the iNOS pathway (Corbett et al, 1992; Tolins et al, 1993; Craven et al, 1994). Indeed, recent study showed that the instillation of high concentration of glucose enhanced iNOS expression and thereby increased NO production in macrophage like Raw264.7 cells stimulated with LPS (Sharma et al, 1995). In that study, the activation of PKC was suggested as a possible mechanism of the glucose-enhancing effect although no direct evidence was presented.

High glucose has previously been shown to increase de novo production of DAG and in turn, activate PKC in many cell types including Raw264.7 cells (Craven et al, 1990; Williamson et al, 1993; Hill et al, 1998). Based on these reports, it was hypothesized that the activation of PKC via sorbitol pathway could explain the glucose-enhancing effect on endotoxin-induced NO production. But the addition of PKC inhibitors, H-7 and staurosporine diminished LPS-induced NO production regardless of glucose concentration (Sharma et al, 1995) and similar results were obtained in our experiment (Fig. 2A). And also, down regulation of PKC by prolonged incubation of PMA reduced endotoxin-induced NO production to basal level in both control and high glucose treated cells (Table 1). These results suggest that PKC activation is required for induction of the iNOS in Raw264.7 cells stimulated with LPS. Thus, with concurrent treatment of PKC inhibitors and LPS, it cannot be clarified whether the activation of PKC is responsible for the glucose-enhancing effect. In order to circumvent this limitation, we designed the LPS pre-priming experiments. That is, cells were pretreated with LPS for 2 hrs and further incubated in fresh media with varying concentration of glucose for 24 hrs. Not only glucose-enhancing effect was observed with this experimental maneuver, but the amount of NO produced during incubation was similar to that of concurrently treated group (Fig. 1). But in this case, chelerythrine, a PKC selective inhibitor, did not show any significant effect on NO production during high glucose treatment. These results suggest that the activation of PKC might not be involved in the augmentation of endotoxin-induced NO production by glucose. This speculation is supported by the facts that the activation of PKC by PMA failed to increase the LPS-induced nitrite release (Table 1). And also, glucose-enhancing effect was unaffected when the glucose metabolic scheme leading to the activation of PKC was modulated by zopolrestat, an aldose reductase inhibitor, and sodium pyruvate (Fig. 3 & Fig. 4). Taken together, these results strongly suggest that the activation of PKC via sorbitol pathway does not contribute to the augmenting effect of high glucose on endotoxin induced NO production, at least in macrophage like Raw264.7 cells.

It has been reported that there are differences in tissue and species specificity in relation to PKC activation and iNOS expression. The activation of PKC by phorbol esters promoted the induction of iNOS in rat peritoneal macrophages and hepatocytes. On the other hand, the activation of PKC was reported to inhibit cytokine-induced NO production in mesangial cells and vascular smooth muscle cells (Muhl & Pfeilshifter, 1994; Geng et al, 1994). In addition, a recent study showed differential regulation by protein kinase C isoforms of nitric oxide synthase induction in Raw264.7 macrophages and rat aortic smooth muscle cells (Paul et al, 1997). These reports imply the possibility that different cells, expressing different subtypes of PKC family, employ this enzyme in different regulatory loop. And also, it is well known that individual PKC isozymes have differential regulating activities even in the same cell type (St-Denis et al, 1998). In this regard, it is conceivable that glucose-enhancing effect might be due to multiple mechanisms, including differential regulation of PKC isotypes. In fact, with our experimental protocol, it was unable to discriminate the possible roles of PKC subtypes in iNOS expression in response to LPS and high glucose. Therefore, these unexplored questions should be addressed to get concrete conclusion on the role of PKC in the glucose enhancing effect.

Besides the sorbitol pathway, there might be other mechanisms which could explain the augmenting-effect of high glucose. One of the possible hypotheses is the involvement of hexosamine biosynthesis pathway which has been reported to mediate some of the regulatory as well as the deleterious effects of glucose (Marshall et al, 1991; Crook et al, 1995). Recent study showed that many transcription factors including Sp1 and nuclear proteins, have been shown to be posttranslationally modified by the O-linked

monosaccharide N-acetylglucosamine (GlcNAc), of which the synthesis is regulated by L-glutamine: D-fructose-6-phosphate amido transferase (GFAT) (Roos et al, 1996). Thus increased activity of GFAT or exposure of cells to glucosamine would increase the availability of GlcNAc and this increased substrate availability might promote the glycosylation of transcription factors and other proteins. Based on this hypothesis, the effects of L-DON, azaserine, GFAT inhibitors, and glucosamine on endotoxin-induced nitrite production were investigated. Results showed the diminution in NO production by GFAT inhibitors and negative modulation of L-DON was not reversed by the treatment of glucosamine, suggesting the minimal involvement, if any, of glucosamine pathway in glucose-enhancing effect (Fig. 5).

Recently, it was reported that high D-glucose initiated the formation of superoxide anions in several experimental systems (Hunt et al, 1990; Tesfamariam & Cohen, 1992; Maziere et al, 1995) and this was responsible for the glucose mediated enhancement of nitrite formation to agonists, such as bradykinin in endothelial cells (Graier et al, 1996). But this hypothesis for the involvement of O_2^- in changes in Ca^{+2}/EDRF signaling seemed not work in our experimental conditions. Treatment with O_2^- generating system, xanthine oxidase/hypoxanthine did not mimic the effect of high glucose, and also co-incubation of SOD did not show any significant effect on glucose-mediated enhancement of endotoxin-induced NO formation (Data are not shown).

The murine promtor region of iNOS has proved to be quite complex, containing at least 24 consensus sequences for the binding of transcriptional factors; among them, proteins of NF-kB family appear to be essential components for the transactivation of iNOS (Xie et al, 1994; Kim et al, 1997). In this context, it is noteworthy that leukocyte-endothelial interaction is augmented by high glucose concentrations in a NF-kB dependent fashion (Morigi et al, 1998). Indeed, an intense activation of NF-kB under high glucose condition was observed in our preliminary study (Data are not shown). However, this does not necessarily mean the activation of NF-kB is responsible for the glucose enhancing effect. Together with the precise signaling mechanism leading to the activation of NF-kB under our experimental conditions, its role(s) in the glucose-enhancing effect needs to be elucidated.

In summary, our experimental results strongly suggest that the hexosamine biosynthesis pathway and the activation of PKC via sorbitol pathway do not contribute to the augmentation of endotoxin-induced NO production by high glucose.

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