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High glucose enhances inducible nitric oxide synthase expression. Role of protein kinase C-βII

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Abstract

The aim was to determine whether high glucose levels interfere with nitric oxide (NO) production and inducible NO synthase (iNOS) protein expression in interleukin-1β-stimulated vascular smooth muscle cells from normotensive Wistar Kyoto and spontaneously hypertensive rats. Cells were incubated with either normal (5.5 mM) or high (22 mM) D-glucose for 72 h and with interleukin-1β (10 ng/ml) for the last 24 h. High glucose increased nitrite levels, iNOS expression and protein kinase C activity in cells from normotensive rats and had no effect in cells from hypertensive rats. High glucose effects on nitrite production and iNOS expression was abolished by the selective inhibitor for the protein kinase C-βII, 5,21:12,17-dimetheno-18*H*-dibenzo[*i*,*o*]pyrrolo[3,4-1] [1,8]diacyclohexadecine-18,20 (19*H*)-dione, 8-[(dimethylamino) methyl]-6,7,8,9,10,11-hexahydro-monomethanesulfonate (LY379196, 30 nM). Calphostin C (1 μM) and LY379196 (10 μM) reduced nitrite levels and iNOS expression only in cells from normotensive rats treated with both media. These results suggest that high glucose increases inducible nitric oxide synthase induction and subsequent NO production by activating the protein kinase C-βII; this mechanism seems to be altered in hypertension.

Keywords: High glucose; Hypertension; Nitric oxide; Protein kinase C-βII; Vascular smooth muscle cell

1. Introduction

Cardiovascular complications are the leading cause of morbidity and mortality in patients with diabetes mellitus (Kannel and McGee, 1979; Ruderman and Haudenschild, 1984). The results of the Diabetes Control and Complications Trial clearly established hyperglycemia as a major causal factor for the development of diabetic complications (The Diabetes Control and Complications Trial Research Group, 1993). Multiple biochemical mechanisms have been proposed to explain the manner in which hyperglycemia could cause vascular dysfunctions, but they are still unclear. Attention has been focused on a potential role for protein kinase C (PKC) as a mediator of diabetes-induced vascular injury (Way et al., 2001), since it plays a pivotal role in the regulation of many biological functions within various cell types. In vascular smooth muscle, PKC has been implicated in the regulation of permeability, contractility, extracellular matrix, cell growth, angiogenesis and cytokine actions, all of which are abnormal in diabetes (Meier and King, 2000). Moreover, it has been suggested that activation of the PKC-β isoform is involved in the development of some early abnormalities in the vascular complications of diabetes (Joy et al., 2005). Kunisaki et al. (1996) demonstrated that elevated levels of glucose in smooth muscle cells from aorta preferentially increased PKC-βII.

Induction of inducible nitric oxide synthase (iNOS) capable of sustained nitric oxide (NO) production is a response to a variety of agents and cytokines, in several types of cells. Thus, interleukin-1β, a macrophage-derived cytokine, stimulates the release of large amounts of NO from vascular smooth muscle cells (VSMC) in vitro (Beasley et al., 1991; Pacheco et al., 2000). NO is not only a potent vasodilator, but it also inhibits platelet adherence and aggregation, reduces adherence of

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leukocytes to the endothelium, and suppresses proliferation of VSMC. Activation of PKC has been reported to up-regulate the cytokine-induced increase in iNOS expression in murine macrophages (Sharma et al., 1995) and glomerular mesangial cells (Noh et al., 2002; Sharma et al., 1995). Recently, Salonen et al. (2006) have suggested that the PKC isoenzyme β mediates the lipopolysaccharide-induced upregulation of iNOS expression and NO production in macrophages. Moreover, it has been reported that cytokines, such as interleukin-1β, are released during both atherogenesis and arterial injury (Libby and Hansson, 1991; Muniyappa et al., 1998). Therefore, alterations in NO release by VSMC or its regulation might have an important role in the pathogenesis of diabetic complications. However, data on NO production from iNOS in response to high glucose are contradictory. Several studies have found evidence for increased NO production in mesangial cells (Noh et al., 2002; Sharma et al., 1995), macrophages (Noh et al., 2002; Sharma et al., 1995), islets of Langerhans (Andersen et al., 1996; Sprinkel et al., 2001), retinal cells (Du et al., 2004), human keratinocyte cell lines (Nakai et al., 2003) as well as in rat aortic endothelial cells (Xu et al., 1999). However, others have found no effect (Hishikawa and Lüscher, 1998) or decreased iNOS expression in VSMC (Muniyappa et al., 1998; Nishio and Watanabe, 1996). In addition, it has been reported that high glucose exposition abolished the upregulation of iNOS induction elicited by insulin in VSMC (Begum and Ragolia, 2000).

It is widely accepted that the development of vascular disease in patients with diabetes is greatly accelerated by the coexistence of hypertension. Patients with diabetes are more commonly hypertensive than those without diabetes (Williams, 1995). Evidence has been found that an altered immune response may exist in spontaneously hypertensive rats (SHR) and that hypertension may be related to an abnormal regulation of iNOS activity and expression (Briones et al., 2000; Chou et al., 1998; Hong et al., 2000; Pacheco et al., 2000; Wu et al., 1996).

The aim of this study was to determine the effect of high glucose on interleukin-1β-stimulated NO production and iNOS expression in vascular smooth muscle cells from Wistar Kyoto (WKY) and SHR rats and to examine the involvement of the PKC-βII isoform in this effect.

2. Materials and methods

2.1. Experimental animals

Male WKY and SHR rats, 6 month old, were obtained from the Animal Quarters of Facultad de Medicina of the Universidad Autónoma de Madrid. Rats were euthanized by decapitation. Thoracic aortas were dissected for primary cultures. All experiments comply with the current Spanish and European laws (R.D. 223/88 MAPA and 609/86).

2.2. Cell culture

Primary cultures of VSMC were obtained according to the method described elsewhere (Pacheco et al., 2000). Briefly, thoracic aortas from WKY and SHR were cut into small pieces, placed in Dulbecco's modified Eagle medium (DMEM, Reactiva) with 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri, USA) and 4 mg/ml of collagenase (type II, Worthington), and incubated for 90 min at 37 °C in a humidified atmosphere of CO_2 (5%) and air (95%). The resulting cell suspension was washed three times by centrifugation and then resuspended in DMEM supplemented with 10% fetal calf serum (Reactiva), 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2.5 μg/ml of amphotericin B (Sigma). Cells were characterized as smooth muscle by immunocytochemical staining with smooth muscle-specific monoclonal antibody to α-actin. Three or more different VSMC cultures were used for each type of experiment; each culture was obtained using three rat aortas from one of the two strains. Cultures from identically treated lines for both WKY and SHR between passages 3 and 8 were used.

2.3. Experimental protocol

VSMC from both strains were plated into either 24-well plates at a density of approximately 5×10^4 cells/well or 6-well plates at a density of approximately 2×10^5 cells/well, for nitrite assay and Western blotting of iNOS, respectively, and cultured until confluence. The medium was then replaced with fresh DMEM plus 1% fetal calf serum and 0.1% bovine serum albumin with either normal (5.5 mM) or high (22 mM) D-glucose, which contained the drugs of interest, during 72 h. Interleukin-1β (10 ng/ml) was added to both normal and high glucose medium for the last 24 h. The cellular viability, measured by the lactate dehydrogenase activity, was not decreased by high glucose incubation in VSMC from both WKY and SHR (results not shown). To study the role of the NOS isoform in the interleukin-1β-induced nitrite release, a specific iNOS inhibitor, 1400W, was used. To analyse the role of PKC in the effect of high glucose on interleukin-1β-induced nitrite release and on iNOS expression, we used an activator, phorbol-12-myristate-13-acetate (PMA), and two different PKC inhibitors, the non-selective inhibitor calphostin C, and the selective inhibitor for the PKC-βII isoform, LY379196. The last drug is selective for this isoform when used at 30 nM and non-selective at 10 μM. Medium with 5.5 mM D-glucose + 16.5 mM L-glucose was also used as osmotic control.

2.4. Nitrite assay

After a 72-h incubation, NO production was determined by measuring the nitrite contents of a 75-μl aliquot from each well with 225 μl of Griess reagent (Green et al., 1982). The absorbance at 540 nm was measured and the nitrite concentration was determined by using a calibration curve of standard sodium nitrite concentrations versus absorbance. Cells were washed twice with phosphate-buffered saline (PBS, Reactiva), dissolved with 0.2 M NaOH overnight at 4 °C and used for protein assay with Bio-Rad DC Protein Assay Reagents Package (Bio-Rad Laboratories, Spain) and BSA as a standard. Nitrite levels were corrected by protein measurement and data were shown per mg protein.

2.5. VSMC lysate preparation and Western blotting of iNOS

After appropriate treatments, VSMC were washed twice in ice-cold buffer (25 mM Tris–HCl, 1 mM EDTA, pH 7.4). The cells were scraped and lysed in the following homogenization buffer: 1 mM sodium vanadate, 1% sodium dodecyl sulfate (SDS), 10 mM Tris–HCl, pH 7.4. The protein content of each sample was determined by Bio-Rad DC Protein Assay Reagents Package.

Proteins from the whole cell lysates (16 μg) were separated on a 7.5% SDS-polyacrylamide gel and electrophoretically transferred to polyvinyl difluoride membranes in Tris–Glycine transfer buffer with 20% methanol in a Bio-Rad Trans-Blot Cell (Bio-Rad Laboratories, Hercules, California, USA). Membranes were blocked for 90 min at room temperature with 5% non-fat dry milk in Tris-buffered saline (in mM): 10 Tris, 100 NaCl, pH 7.5 containing 0.1% Tween 20, and incubated with a monoclonal primary mouse antibody against iNOS, 1:10000 (Transduction Laboratories, Lexington, UK) for 75 min at room temperature. The membranes were washed thoroughly and incubated with horseradish peroxidase-coupled anti-mouse IgG antibody (1:2000; Transduction Laboratories) for 1 h. After thorough washing, the bound antibodies were visualized by enhanced chemiluminescence (ECL) (Amersham International plc, Little Chalfton, Germany) and exposure to Kodak X-OMAT film. Signals on the inmunoblot were quantified using a computer program (NIH Image V1.56). Multiple exposure of each blot was performed to ensure that signals were within the linear range of the film.

2.6. PKC activity assay

Relative protein kinase C activity was determined using a non-radioactive PKC assay kit (StressGen Bioreagents, Ann Arbor, MI, USA), according to the manufacturer's instructions. The activity was measured in the cytosolic fraction of smooth muscle cells from WKY and SHR rats incubated for 72 h in normal or high glucose media and during the last 24 h with interleukin-1β (10 ng/ml). A decreased in that activity indicates an increase in the PKC translocation to the membrane and then an activation of this enzyme. The results were expressed as arbitrary units using the following equation: Relative kinase activity = (average absorbance$_{sample}$ − average absorbance$_{blank}$)/μg protein.

2.7. Drugs and statistical evaluation

Interleukin-1β (mouse; recombinant), phorbol-12-myristate-13-acetate (PMA) and calphostin C were purchased from Sigma (Spain) and *N*-[3-(Aminomethyl)benzyl]acetamidine, Dihydrochloride (1400W) from Calbiochem (Spain). 5,21:12,17-Dimetheno-18*H*-dibenzo[*i*,*o*] pyrrolo[3,4-1] [1,8] diacyclohexadecine-18,20 (19*H*)-dione, 8-[(dimethylamino) methyl]-6,7,8,9,10,11-hexahydro-monomethanesulfonate (LY379196) was a generous gift from Eli Lilly and Company (Indianapolis, USA). Data reported are mean values ± S.E.M. of the different cultures used for the experiments. Treatments were assayed in triplicates (replicates) for each VSMC culture and their mean was considered a single observation in the statistical analysis. Mean values from replicate wells of the different VSMC cultures used were averaged for representation in the figures. Student's *t*-test for unpaired observations and two-way analysis of variance (ANOVA) were used for statistical analysis, as indicated in the corresponding figure legend. A *P* value below 0.05 was considered significant. The Kolmogorov–Smirnov test was used to confirm the Gaussian distribution of our population in both WKY and SHR.

3. Results

3.1. Effect of high glucose on interleukin-1β induced nitric oxide production

The effect of high glucose on nitrite release from VSMC treated with different concentrations of interleukin-1β is shown in Fig. 1. Cells from normotensive and hypertensive rats incubated with interleukin-1β (1–20 ng/ml) for 24 h, in normal glucose conditions caused a concentration-dependent increase in nitrite accumulation. The treatment of VSMC from normotensive rats with 22 mM glucose medium significantly potentiated the interleukin-1β-induced nitrite release when compared to the control (normal glucose medium, Fig. 1A). However, in VSMC from hypertensive rats, incubation with high glucose medium did not cause a significant difference in the amount of interleukin-1β-induced nitrite release with respect to the release in the normal glucose medium (Fig. 1B). In cells from normotensive rats, the maximal effect of high glucose medium was reached at interleukin-1β concentrations between 5 and 20 ng/ml. This was not an osmotic effect since the addition of L-glucose to the normal glucose medium to produce the same osmolarity as in the high glucose medium did not reproduce the high glucose effect (results not shown).

Since iNOS is regulated mainly at the level of expression (Forstermann et al., 1995), we sought to determine whether high glucose modified interleukin-1β-induced iNOS protein expression. No iNOS protein expression was detected in cell lysates from VSMC of normotensive rats in the absence of interleukin-1β (data not shown). Exposure to interleukin-1β (1, 5 and 10 ng/ml) induced an iNOS protein expression that was higher in the high glucose than the normal glucose medium (Fig. 1C). This difference between cells treated with normal and high glucose was clearly observed at 10 ng/ml of interleukin-1β, the concentration at which the maximum effect of high glucose medium on nitrite release was observed. For that reason, we chose this concentration of interleukin-1β for all further experiments.

The specific iNOS inhibitor, 1400W (10 μM), abolished the nitrite release induced by 10 ng/ml interleukin-1β in VSMC from both normotensive and hypertensive rats treated with normal or high glucose medium (Fig. 2).

3.2. Role of PKC on high glucose effect on nitric oxide production

To assay the involvement of PKC in the potentiatory effect of high glucose medium on interleukin-1β-stimulated nitrate

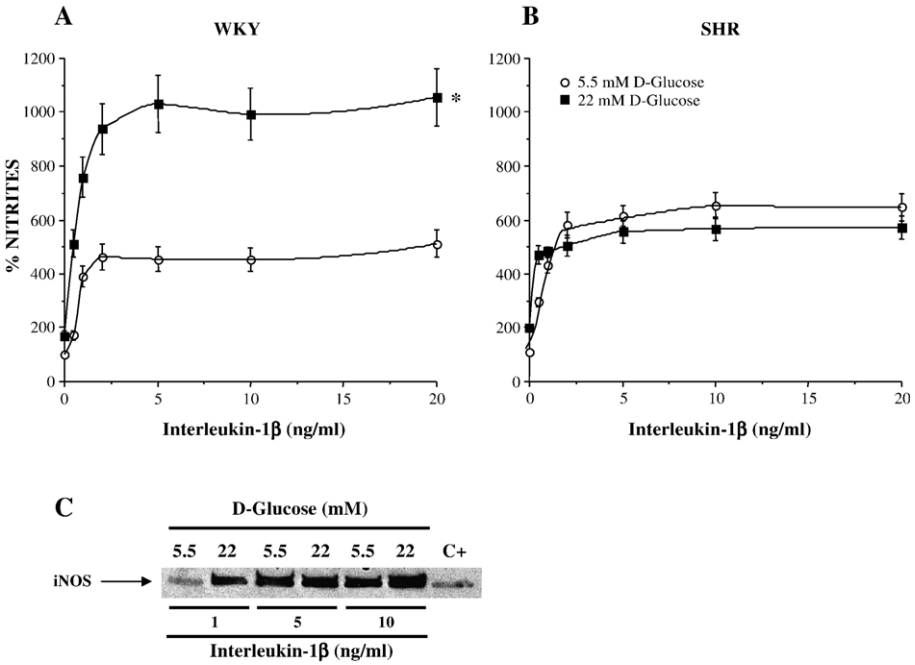

Fig. 1. Effect of interleukin-1β on nitrite production in cultured vascular smooth muscle cells from normotensive (WKY) and hypertensive (SHR) rats (A, B). Cells were exposed for 72 h to normal glucose (5.5 mM D-glucose) or high glucose (22 mM D-glucose) medium; interleukin-1β was added for the last 24 h. Results are expressed as a percentage of the nitrite release observed in the absence of interleukin-1β. Data are means±S.E.M. of 5 different VSMC cultures and each treatment was analyzed in triplicate. *$P<0.05$ between normal and high glucose medium, determined by two-way analysis of variance (ANOVA). Representative Western blot for iNOS protein expression in vascular smooth muscle cells from normotensive cells (C). C+: positive control (macrophages).

release, we determined the effect of a PKC activator, PMA and of two inhibitors, LY379196 and calphostin C, on this effect.

As shown in Fig. 3, incubation with PMA (10 nM) had no effect on interleukin-1β-stimulated nitrite levels in VSMC from either rat strain when compared to their controls (normal and high glucose media).

Calphostin C (1 μM), a potent and widely used inhibitor of PKC, reduced the interleukin-1β-stimulated nitrite levels, in VSMC from normotensive rats treated with normal and high glucose medium (Fig. 3). However, calphostin C did not modify interleukin-1β-stimulated nitrite levels in VSMC from hypertensive rats exposed to normal or high glucose medium (Fig. 3).

To determine the possible participation of the PKC isoform βII in the altered nitrite release and iNOS expression observed in cells treated with high glucose medium, we used LY379196 at 30 nM, a concentration that is selective for the PKC-βII isoform. As observed in Fig. 4, this inhibitor significantly reduced the interleukin-1β-stimulated nitrate release in VSMC from normotensive rats in both incubation media. This reduction was greater in cells incubated with high glucose; so, in the presence of this antagonist, no differences in interleukin-1β-stimulated nitrite release between normal and high glucose media were observed. A higher concentration of LY379196 (10 μM) further decreased interleukin-1β-stimulated nitrite release in VSMC

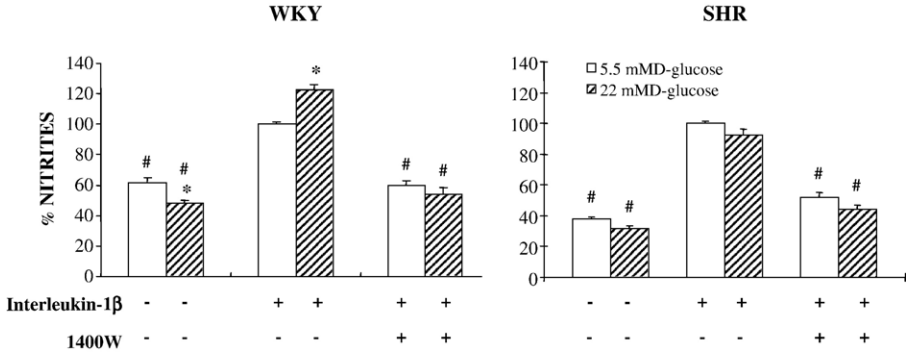

Fig. 2. Effect of 1400W (10 μM) on interleukin-1β (10 ng/ml)-stimulated nitrite production in cultured vascular smooth muscle cells from normotensive (WKY) and hypertensive (SHR) rats. Cells were exposed for 72 h to normal glucose (5.5 mM D-glucose) or high glucose (22 mM D-glucose) medium; interleukin-1β was added for the last 24 h. Results are expressed as a percentage of the nitrite release induced by interleukin-1β in cells treated with 5.5 mM glucose in each strain. Data are means±S.E.M. of 5 to 8 different VSMC cultures and each treatment was analyzed in triplicate. *$P<0.05$ versus normal glucose medium; $^{\#}P<0.05$ versus interleukin-1β, determined by Student's *t*-test analysis for unpaired observations.

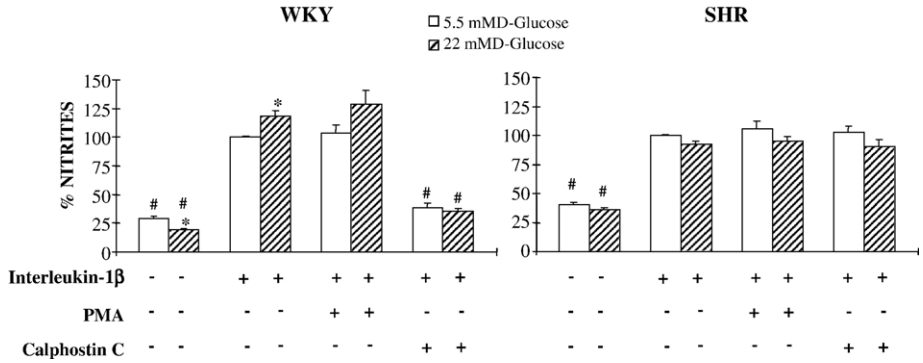

Fig. 3. Effects of phorbol 12-myristate 13-acetate (PMA; 10 nM) and calphostin C (1 μM) on interleukin-1β (10 ng/ml)-stimulated nitrite production in cultured vascular smooth muscle cells from normotensive (WKY) and hypertensive (SHR) rats. Cells were exposed by 72 h to normal glucose (5.5 mM D-glucose) or high glucose (22 mM D-glucose) medium; interleukin-1β was added for the last 24 h. Results are expressed as a percentage of the nitrite release induced by interleukin-1β in cells treated with 5.5 mM D-glucose. Data are means±S.E.M. of 6 to 12 different VSMC cultures and each treatment was analyzed in triplicate. *$P<0.05$ versus normal glucose medium; #$P<0.05$ versus interleukin-1β treatment, determined by Student's *t*-test analysis for unpaired observations.

from normotensive rats treated with both normal or high glucose media also abolishing the differences observed in nitrite release. Nevertheless, in VSMC from hypertensive rats treated with normal or high glucose media neither 30 nM nor 10 μM induced any effect on the nitrite release induced by 10 ng/ml interleukin-1β (Fig. 4).

LY379196 at 30 nM and 10 μM induced a concentration-dependent inhibition of iNOS expression in VSMC from normotensive rats (Fig. 5); in the presence of both concentrations of LY379196, no difference in iNOS expression in VSMC treated with normal and high glucose medium was observed. Nevertheless, neither 30 nM nor 10 μM LY379196 affected iNOS expression in VSMC from hypertensive rats treated with normal and high glucose media (Fig. 5).

3.3. Effect of high glucose effect on PKC activity

The cytosolic PKC activity of VSMC from SHR incubated in normal glucose medium was lower than that from WKY (0.243±0.02 vs. 0.146±0.02 arbitrary units for WKY and SHR, respectively, $P<0.05$). After incubation in high glucose media, this activity was decreased only in VSMC from WKY (Fig. 6).

4. Discussion

Diabetes and hypertension are major risk factors associated with increased cardiovascular mortality and morbidity. Hypertension aggravates the cardiovascular complications associated with diabetes and vice-versa. Thus, the concurrence of the two disorders results in more devastating structural and functional impairments than those caused by either disease alone (Alderman et al., 1999; Sowers et al., 2001). Hyperglycemia is thought to be a major factor in the pathogenesis of vascular complications of diabetes. The results of the present study show that high glucose levels increase the induction of iNOS and the subsequent NO production, through the activation of the PKC-βII isoform in VSMC from normotensive but not from hypertensive rats. To our knowledge, this is the first study to describe the loss of the high glucose effect on iNOS induction in VSMC from hypertensive rats.

Apart from the classical role of NO in causing vascular dilatation, local production of large amounts of NO may play a role in cell toxicity or growth inhibition. Different studies provide evidences of enhanced NO production via the iNOS pathway after high glucose exposure (Andersen et al., 1996; Du

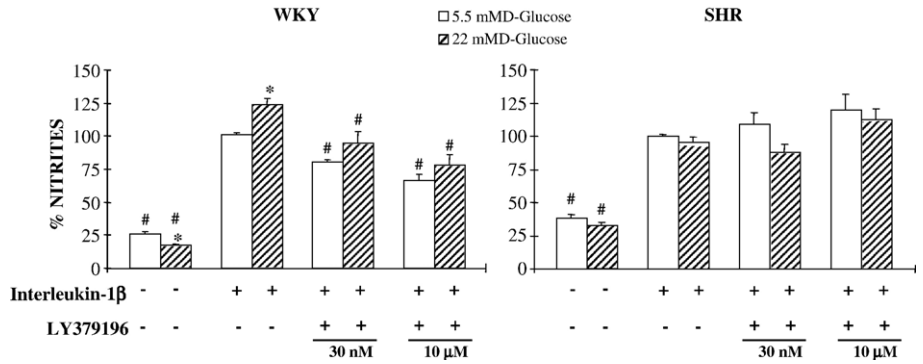

Fig. 4. Effects of LY379196 (10 μM and 30 nM) on interleukin-1β (10 ng/ml)-stimulated nitrite production in cultured vascular smooth muscle cells from normotensive (WKY) and hypertensive (SHR) rats. Cells were exposed for 72 h to normal glucose (5.5 mM D-glucose) or high glucose (22 mM D-glucose) medium; interleukin-1β was added for the last 24 h. Results are expressed as a percentage of the nitrite release induced by interleukin-1β in cells treated with 5.5 mM D-glucose in each strain. Data are means±S.E.M. of 8 to 12 different VSMC cultures and each treatment was analyzed in triplicate. *$P<0.05$ versus normal glucose medium; #$P<0.05$ versus interleukin-1β, determined by Student's *t*-test analysis for unpaired observations.

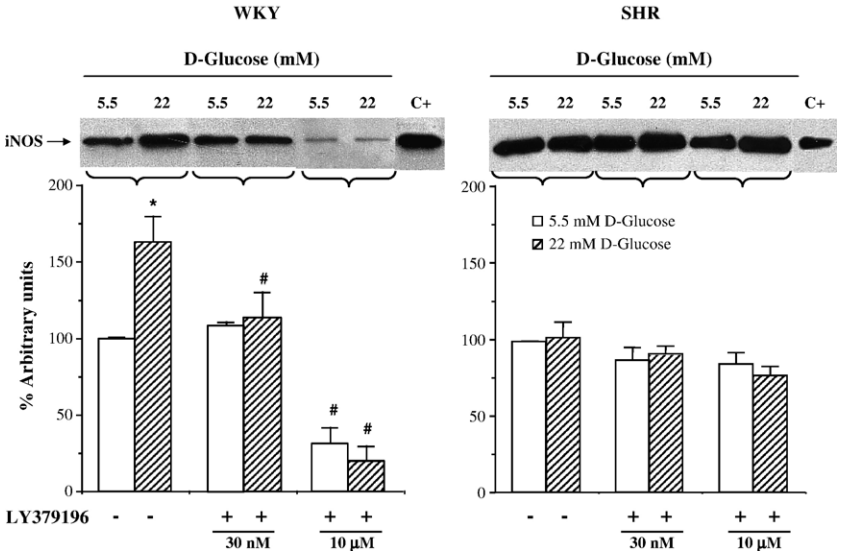

Fig. 5. Effects of LY379196 (10 μM and 30 nM) on interleukin-1β (10 ng/ml) stimulated inducible nitric oxide synthase (iNOS) protein expression in vascular smooth muscle cells from normotensive (WKY) and hypertensive (SHR) rats. Representative Western blot of four to five independent experiments is represented above. Cells were exposed for 72 h to normal glucose (5.5 mM D-glucose) or high glucose (22 mM D-glucose) medium; interleukin-1β was added for the last 24 h. Cell lysates were subjected to SDS-PAGE followed by inmunoblot analysis using the anti-iNOS antibody. Results are expressed as a percentage of the iNOS expression induced by interleukin-1β in cells treated with 5.5 mM D-glucose. Bars represent means±S.E.M. of 4 to 5 different VSMC cultures. *$P<0.05$ versus normal glucose medium; #$P<0.05$ versus interleukin-1β, determined by Student's *t*-test analysis for unpaired observations. C+: positive control (macrophages).

et al., 2004; Nakai et al., 2003; Noh et al., 2002; Sharma et al., 1995; Sprinkel et al., 2001). In the present study, treating VSMC from normotensive rats with high glucose medium significantly potentiated the interleukin-1β-induced nitrite release. The selective inhibitor of iNOS, 1400W, abolished the effect produced by interleukin-1β on nitrite release from VSMC treated with normal or high glucose medium. Hence, the observed effects are due to iNOS-mediated NO production.

Since the observed effect on nitrite production might be the result of an alteration in iNOS expression, we sought to determine whether the normal or high glucose medium modified iNOS expression in VSMC. In agreement with the effect on nitrite release, we found that different concentrations of interleukin-1β induced greater iNOS protein expression in the VSMC exposed to the high glucose than to the normal glucose medium. All these data suggest that the treatment with high glucose potentiates interleukin-1β-induced nitrite release at the level of the expression and activity of the iNOS protein. Changes in osmolarity of the medium are not responsible for the observed effect of high glucose, since neither nitrite release nor iNOS protein expression were altered in VSMC exposed to a similar concentration of L-glucose. Accelerated atherogenesis is a key abnormality in diabetes-associated vascular disease (Sowers and Epstein, 1995). Induction of iNOS in VSMC by cytokines may have a role in the vascular response to injury. Thus, cytokines such as interleukin-1β, which are released during atherogenesis (Libby and Hansson, 1991), and iNOS activity have been demonstrated in the VSMC of atherosclerotic plaques (Buttery et al., 1996). Thus, it can be postulated that increased iNOS induction in VSMC through cytokines could have an important role in the vascular alteration that occurs in diabetes.

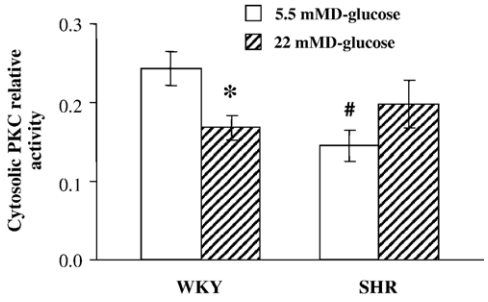

Fig. 6. Cytosolic PKC activity of cultured vascular smooth muscle cells from normotensive (WKY) and hypertensive (SHR) rats. Cells were exposed for 72 h to normal glucose (5.5 mM D-glucose) or high glucose (22 mM D-glucose) medium; interleukin-1β was added for the last 24 h. Results (arbitrary units) are expressed as relative kinase activity [(average $absorbance_{sample}$ − average $absorbance_{blank}$)/μg protein]. Data are means±S.E.M. of 5 different VSMC cultures. *$P<0.05$ between normal and high glucose medium, #$P<0.05$ versus WKY, determined by Student's *t*-test analysis for unpaired observations.

In agreement with our data, other authors have found that endothelial (Xu et al., 1999) or mesangial cells (Noh et al., 2002; Sharma et al., 1995) exposed to high glucose show stimulated iNOS mRNA, protein expression and NO production. However, these results differ from the findings of Nishio and Watanabe (1996) and Muniyappa et al. (1998), who reported that in VSMC exposed to LPS or interleukin-1β, high glucose inhibits NO production by inhibiting iNOS expression and iNOS activity. The reasons for the discrepancy between our results and those of these investigators are unclear, but may have to do with differences in the VSMC, their origin and obtention of culture cells and in the experimental protocols. In our study, VSMC were isolated from WKY rat thoracic aorta by enzymatic dissociation. In contrast, Muniyappa et al. (1998) used Sprague–Dawley rats and Nishio and Watanabe (1996) did not mention the strain of the rat and the cells they used were

obtained by explants. In addition, in our study, VSMC were incubated with either normal or high glucose medium plus 1% fetal calf serum for 72 h and stimulated with interleukin-1β (10 ng/ml) for the last 24 h, whereas, in the study of Muniyappa et al. (1998), cells were incubated in a serum-free medium. Serum contains various growth factors that can influence on iNOS activity or expression (Trachtman et al., 1998). Other reason to explain these discrepancies would be the duration of high glucose exposition. Thus, Nakai et al. (2003) have reported enhancement of cytokine-induced NO production in human keratinocytes after 24 h high glucose whereas treatment for 10 days reduced both mRNA and nitrite production.

Hypertension is associated with elevated proinflammatory cytokine blood levels (Chou et al., 1998; Virdis and Schiffrin, 2003; Wu et al., 1996). Some authors have described increased iNOS protein expression and activity in vascular tissues from SHR (Briones et al., 2000; Chou et al., 1998; Hong et al., 2000; Pacheco et al., 2000; Wu et al., 1996) while others have reported that iNOS activity is depressed in VSMC from stroke-prone SHR rats (Mizutani et al., 2000). However, in contrast to the observations in cells from WKY rats, in VSMC from SHR rats, the incubation with high glucose medium did not modify the amount of interleukin-1β-induced nitrite release or iNOS protein expression when compared to levels in the normal glucose medium. These results indicate that the increased iNOS expression after high glucose treatment observed in VSMC from normotensive rats could be an adaptive mechanism that would be lost in hypertension. Although more experiments are necessary to explain the loss of the high glucose effect on the iNOS pathway in VSMC from SHR, two hypotheses may be proposed: (1) Since iNOS expression is increased in VSMC from SHR (Pacheco et al., 2000), it would be more difficult to detect some additional enlargement. (2) The mechanisms involved in the intracellular effects caused by high glucose in VSMC from SHR would be altered or coincide with the mechanisms involved in the increased iNOS expression observed in these cells. Thus, an increase in the intracellular calcium concentration by the activation of voltage-operated calcium channels would be responsible for the increased iNOS expression observed in VSMC from SHR (Pacheco et al., 2000) and in some of the effects induced by high glucose (Muniyappa et al., 1998; Paul et al., 1997; Scivittaro et al., 2000).

High glucose has previously been shown to increase de novo synthesis of diacylglicerol, which is a potent activator of PKC in many cellular types (Wolf et al., 1991). In addition, diabetic tissues such as aorta, heart and glomeruli are all in a state of chronic PKC activation (Inoguchi et al., 1992). On the other hand, PKC activation (Paul et al., 1997), especially of PKC β isoforms (Salonen et al., 2006), is required to induce iNOS expression in VSMC and macrophages, respectively, although negative modulation has also been described (Geng et al., 1994). In the present study, we found that the incubation in high glucose medium induced a decrease of PKC activity of the cytosolic fraction. This indicates an increase in the PKC translocation to the membrane and then an activation of this enzyme, as reported by Muniyappa et al. (1998). Thus, the enhancing effect of high glucose on the iNOS pathway in VSMC from WKY may be due to PKC activation, as has been suggested in macrophages and mesangial cells (Noh et al., 2002; Sharma et al., 1995).

Immunoblotting studies have identified, at least twelve PKC isoforms (Idris et al., 2001). The pattern of PKC isoform expression varies in the different tissues. In rat aortic smooth muscle cells exposed to high glucose, increases were observed in the membranous fraction for the activated pool of PKC-βII, but not PKC-α (Kunisaki et al., 1994). Distinguishing a specific isoform can be difficult, since few selective agents exist. Fortunately, LY379196 is selective for PKC-βII when administered at 30 nM (Braiman et al., 1999). At this concentration, LY379196 reversed the potentiator effect of high glucose treatment on interleukin-1β-stimulated nitrate release and iNOS expression, in VSMC from normotensive rats. These results confirm the participation of the PKC-βII isoform in the observed effect of high glucose in VSMC from normotensive rats. Using different inhibitors it has been suggested that PKC-βII isoform is implicated in the effect of high glucose on H_2O_2 production (Shaw et al., 2003) as well as in vascular cell adhesion molecule 1 upregulation (Kouroedov et al., 2004). Other authors have reported that the oral treatment of diabetic rats with the inhibitor of PKC-β, ruboxistaurin, ameliorated the glomerular filtration rate, albumin excretion rate and retinal circulation (Ishii et al., 1996). In addition, the treatment with ruboxistaurin prevented the reduction in endothelium-dependent vasodilation induced by acute hyperglycemia in healthy humans (Beckman et al., 2002). Hence, abnormal activation of PKC, in particular its β isoenzymes, may underlie some of the vascular complications of diabetes.

The non-selective PKC inhibitor, calphostin C, and a high concentration of LY379196 not only abolished the potentiator effect of high glucose but also reduced interleukin-1β-stimulated nitrite release and iNOS expression after both normal or high glucose treatment. These results confirm that PKC activation is required to induce iNOS protein expression in VSMC, as suggested (Noh et al., 2002; Paul et al., 1997; Sharma et al., 1995). Yan et al. (1999) reported that the augmented iNOS expression in VSMC from Wistar rats was associated with enhanced nuclear transcription factor κB (NFκB) activation. Moreover, it has been demonstrated that the PKC inhibitor calphostin C can completely attenuate NFκB activation by high glucose (Yerneni et al., 1999). Thus, it is possible that NFκB contributes to abnormal vascular function in diabetes and under hyperglycemic conditions. However, incubation with a PKC activator, PMA, had no effect on interleukin-1β-stimulated nitrite levels in our VSMC. It is difficult to interpret these results but they could be related with its rapid degradation by non-specific esterases (Asaoka et al., 1991) or by other additional inhibitory mechanisms induced in the high glucose-treated cells (e.g. increased calcium), as suggested by Muniyappa et al. (1998).

In contrast to the observations in VSMC from normotensive rats, the two LY379196 concentrations used and calphostin C did modify neither nitrite release nor iNOS expression in VSMC from SHR treated with normal or high glucose medium. PKC activity was greater in VSMC from SHR than WKY, as

previously reported (Yasunari et al., 1995); however high glucose medium did not alter PKC activity in VSMC from hypertensive rats. These results indicate the non-participation of PKC activation in interleukin-1β-induced iNOS expression in this strain and suggest the existence of hypertension-related alterations in the signals that mediate the expression of this protein. The alteration in iNOS activity and expression previously described in vascular tissues of hypertensive animals (Briones et al., 2000; Chou et al., 1998; Hong et al., 2000; Pacheco et al., 2000; Wu et al., 1996) would be related with these results. However, further studies are necessary to prove this hypothesis.

In conclusion, high glucose levels seem to increase iNOS induction and, subsequently, NO production, by activating the PKC-βII isoform in VSMC from normotensive animals but not from hypertensive animals. The above findings indicate that the induction of this enzyme by different stimuli might be altered in hypertension, thereby reflecting a loss of the adaptive role assigned to iNOS in vascular response to injury in this condition. The results obtained with the selective inhibitors of PKC-βII isoform should assist in defining their potential therapeutic value and future clinical use in the treatment of diabetic vascular disease in normotensive patients. Further studies are needed to elucidate the signal transduction pathway involved in the pathogenesis of diabetes and hypertension.

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