HIGH GLUCOSE MEDIATES PROOXIDANT AND ANTIOXIDANT ENZYME ACTIVITIES IN CORONARY ENDOTHELIAL CELLS

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Short title: Hyperglycaemia regulates antioxidant enzyme activity

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Abstract

Objective: Excess levels of free radicals such as nitric oxide (NO) and superoxide anion (O_2^-) are associated with the pathogenesis of endothelial cell dysfunction in diabetes mellitus. This study was designed to investigate the underlying causes of oxidative stress in coronary microvascular endothelial cells (CMEC) exposed to hyperglycaemia.

Methods: CMEC were cultured under normal (5.5 mmol/L) or high glucose (22 mmol/L) concentrations for 7 days. The activity and expression (protein level) of eNOS, iNOS, NAD(P)H oxidase and antioxidant enzymes, namely, superoxide dismutase (SOD), catalase and glutahione peroxidase (GPx) were investigated by specific activity assays and Western analyses, respectively while the effects of hyperglycaemia on nitrite and O_2^- generation were investigated by Griess reaction and cytochrome *C* reduction assay, respectively.

Results: Hyperglycaemia did not alter eNOS or iNOS protein expressions and overall nitrite generation, an index of NO production. However, it significantly reduced the levels of intracellular antioxidant glutathione by 50% ($p < 0.05$) and increased the protein expressions and/or activities of p22-phox, a membrane-bound component of pro-oxidant NAD(P)H oxidase and antioxidant enzymes ($p < 0.05$). Free radical-scavengers, namely, Tiron and MPG (0.1-1 µmol/L) reduced hyperglycaemia-induced antioxidant enzyme activity and increased glutathione and nitrite generation to the levels observed in CMEC cultured in normoglycaemic medium ($p < 0.01$). The differences in enzyme activity and expressions were independent of the increased osmolarity generated by high glucose levels as investigated by using equimolar concentrations of mannitol in parallel experiments.

Conclusions: These results suggest that hyperglycaemia-induced oxidative stress may arise in CMEC as a result of enhanced prooxidant enzyme activity and diminished generation of

antioxidant glutathione. By increasing the antioxidant enzyme capacity CMEC may protect

themselves against free radical-induced cell damage in diabetic conditions.

Introduction

Nitric oxide (NO) is generated from amino acid L-arginine by a class of enzymes termed as NO synthases (NOSs) which include two constitutively expressed isoforms, namely, endothelial NOS (eNOS) and neuronal NOS (nNOS) and an inducible isoform (iNOS) [1, 2]. NO, generated within healthy endothelium by eNOS, plays pivotal roles in the maintenance of vascular tone [3] and in the inhibition of leukocyte adhesion [4] and vascular smooth muscle cell (VSMC) proliferation [5]. The functions and/or integrity of endothelium change in several pathological conditions including diabetes mellitus leading to a phenomenon called "endothelial dysfunction", a hallmark of diabetic vascular complications which is characterised by impaired endothelial cell function and enhanced anti-haemostatic properties [6]. Current data as to the pathogenesis of diabetic endothelial cell dysfunction are extremely diverse in that several mechanisms have so far been proposed to account for this phenomenon. These include inefficient utilisation of substrate L-arginine by NOS for NO synthesis [7], an abnormal NOS activity due to inadequate presence of co-factor tetrahydrobiopterin (H_4B) [8], concurrent release of endothelium-derived constricting factor arising from the cyclooxygenase pathway [9] and NO quenching by advanced glycation end-products (AGEs) [10].

Despite a lack of consensus on the expression and activity of eNOS and therefore generation of NO in diabetic conditions, an overwhelming body of evidence has in recent years indicated that reactive oxygen species (ROS) are released at greater levels in diabetic animals and cells cultured under high glucose conditions [11, 12]. ROS are, in part, also generated as a direct consequence of constant and prolonged exposure of cells to hyperglycaemia that leads to non-enzymatic glycation of plasma proteins [13]. The glycated products undergo further spontaneous reactions to produce free radicals including superoxide anion (O_2^-), the foundation radical [14].

O_2^- is also formed through the reduction of molecular oxygen by several enzymes such as cyclooxygenase, xanthine oxidase, mitochondrial NADH dehydrogenase, NOSs and NAD(P)H oxidase [15, 16]. Amongst these enzymes NAD(P)H oxidase has attracted much of the attention in recent years and has been characterised in coronary microvascular endothelial cells (CMEC) as the main source of ROS [17]. It has also been associated with oxidative stress-mediated endothelial dysfunction in the central retinas of type II diabetic rats [18]. NAD(P)H oxidase, consists of a membrane-bound cytochrome b_{558} that in turn consists of p22-phox and gp91-phox, and several cytosolic subunits, namely, p47-phox, p67-phox, p40-phox and small GTP-binding protein *rac* [19]. The importance of p22-phox subunit for the activity and stability of the enzyme as a whole has been further emphasised by a recent study in that polymorphism of the p22-phox gene has been linked to initiation and progression of atherosclerotic disease [20].

Under physiological conditions O_2^- is converted to hydrogen peroxide (H_2O_2), another ROS, by superoxide dismutases (SODs), namely CuZn-containing (CuZn-SOD) or Mn-containing isoform (Mn-SOD) (21). Once generated, H_2O_2 is metabolised to H_2O by enzymatic activities of catalase and glutathione peroxidase (GPx) [21]. However, in the absence of adequate levels of SODs or intracellular antioxidants such as glutathione [22], O_2^- rapidly scavenges NO within the vascular wall to reduce its biological half-life and causes contraction of vascular smooth muscle [23, 24]. Indeed, diminished levels of glutathione have been reported in aortic endothelial cells from diabetic rabbits [25] but not in human umbilical vein endothelial cells cultured in 20 mmol/L glucose for 14 days [26]. It is noteworthy in this context that hyperglycaemia may lead to glycosylation of SOD enzymes thereby rendering them inactive and consequently elevating O_2^- levels [27]. In support of this hypothesis, a recent study has shown that pretreatment with SOD enhances basal and agonist-stimulated endothelium-dependent vascular relaxant responses

in diabetic rat aorta therefore suggesting an ample presence of O_2^- in diabetic tissues [28]. It is possible that pathologies in the regulation of catalase and GPx, H_2O_2-scavenging enzymes, may further complicate the oxidative stress status in diabetes and indeed account for its aetiology.

Hence, the current study was designed to investigate the underlying causes of coronary endothelial cell oxidative stress in hyperglycaemic conditions with reference to: 1- availability of intracellular antioxidant glutathione and nitrite, a stable end-product of NO; 2- expression and/or activity of enzymes that may be involved in the regulation of cellular oxidative stress i.e. eNOS, iNOS, SODs, GPx and catalase, NAD(P)H oxidase.

Materials and Methods

Isolation and Characterisation of CMEC

The hearts were mounted and perfused retrogradely on a constant-flow Langendorff system with 0.04% collagenase. The ventricles were then chopped and collagenase digestion was quenched by the addition of bovine serum albumin to the perfusate. CMEC were obtained by sedimentation of myocytes and incubated in 0.01% trypsin at 37°C for the prevention of non-endothelial cell attachment. Cells were then activated by washing in calcium and suspended in Medium 199 (Gibco-BRL) supplemented with 10% foetal calf serum, 10% newborn calf serum, benzylpenicillin 250 U/ml, streptomycin 250 μg/ml, amphotericin B 12.5 μg/ml and gentamycin 50 μg/ml. Cell suspensions were plated and incubated at 37°C under 5% CO_2. After 1 h incubation, unattached cells were washed off with saline and remaining cells were cultured to confluence.

For the different experiments, CMEC were cultured for 7 days in the growth medium containing either 5.5 mmol/L (normal) or 22 mmol/L (high) D-glucose. When there was a statistically significant difference between 5.5 mmol/L and 22 mmol/L D-glucose, CMEC were

also cultured in the presence of 5.5 mmol/L D-glucose + 16.5 mmol/L L-glucose and 5.5 mmol/L D-glucose + 16.6 mmol/L mannitol to investigate the involvement of extracellular glycation and increased osmolarity, respectively in the differences. Growth medium was changed on a daily basis and all experiments were performed on CMEC up to and including passage number 5. Cultured CMEC were characterised by their typical "cobblestone" morphology and and their ability to form capillary-like tubes on the Matrigel [29].

Western Blotting

Total cellular proteins were isolated by lysis of CMEC with a boiling solution containing 1% SDS, 10 mmol/L Tris pH 7.4. Equal amounts of protein were run on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were then incubated overnight with either monoclonal (for eNOS, iNOS and p22-phox antibodies from Transduction Labs.) or polyclonal primary antibodies raised against rat Cu/Zn-SOD, rat Mn-SOD (Calbiochem) or bovine catalase (Chemicon). Horseradish peroxidase-linked secondary antibodies were then used and the bands were detected by use of a chemiluminescence agent (Amersham Pharmacia). Blots were assessed by densitometry of bands with subtraction of the background counts measured outside loaded lanes. Since, a species-specific antibody for GPx protein could not be obtained, Western analyses for this protein were not performed.

Nitrite Detection

Nitrite levels were measured by Griess reaction as an index of NO generation following conversion of nitrate to nitrite by nitrate dehydrogenase [30]. An aliquot of the cellular homogenate was mixed with an equal volume of Griess reagent (sulfanilamide 1% w/v, naphthylethylenediamine dihydrochloride 0.1% w/v and orthophosphoric acid 2.5% v/v) and incubated at room temperature for 10 min prior to measurement of absorbances at 540 nm. The

amount of nitrite formed was compared to those of known concentrations of sodium nitrite and normalised to the protein content of the respective flask.

Superoxide anion (O_2^-) Production

O_2^- was measured by cytochrome *C* reduction assay. Briefly, CMEC grown under normoglycaemic or hyperglycaemic conditions at the density of 5×10^6 cells/ml were incubated with 50 µmol/L cytochrome *C* for 30 min at 37 °C in Hepes buffer. Absorbances were recorded using a Cobas-Fara centrifugal analyser at 550 nm with 90 seconds intervals for 12 min. Production of O_2^- was calculated as previously described and results were expressed as pmoles O_2^- per 10^6 cells [31].

SOD Assay

SOD activity was also measured by a reaction dependent upon the inhibition of cytochrome *C* by endogenous SOD in cellular homogenates using a Cobas Fara centrifugal analyser. The O_2^-, required for reduction, was generated by a reaction of xanthine-xanthine oxidase (XO). One unit of XO activity was defined by the amount of homogenate required to inhibit, by 50%, the rate of cytochrome *C* reduction. For assay of total SOD activity, 0.1 mmol/L xanthine was dissolved in 50 mmol/L $NaCO_3$ buffer. A dilute stock solution was added to a 10 µmol/L solution of cytochrome *C*, 50 µmol/L xanthine, 0.1 mmol/L EDTA and 50 mmol/L sodium carbonate to produce a change in absorbance of 0.0250/min at 550 nm at pH 10. This was followed by the measurement of Mn-SOD activity, which is resistant to incubation at room temperature with 3 mmol/L potassium cyanide (BDH Chemicals Ltd) for 45 minutes. For Mn-SOD activity the protocol was identical except that carbonate buffer was replaced with 50 mmol/L potassium phosphate and pH adjusted to 7.8. CuZn-SOD activity was subsequently calculated by the subtraction of Mn-SOD activity from total SOD activity.

Catalase Assay

The activity of catalase was determined by a photometric method where the activity was determined by monitoring the decomposition of H_2O_2 at 240 nm in the presence of methanol which produces formaldehyde which in turn reacts with Purpald (4-amino-3-hydazino-5-mercapto-1,2,4-triazole) and potassium periodate to produce a chromophore. Quantification was performed in comparison to the results obtained with catalase and formaldehyde standards.

GPx Assay

The activity of GPx was determined in cellular homogenates using a method developed by McMaster et al [32]. Briefly, a fresh solution containing 0.3 U/ml glutathione reductase, 1.25 mmol/L reduced glutathione and 0.19 mmol/L NADPH in 50 mmol/L potassium buffer (pH 7.4) was prepared. Homogenates of 100 µg total protein were added to this solution and incubated for 3 minutes prior to addition of 12 mmol/L t-butylhydroperoxide to commence the reaction. Absorbances were read at 340 nm for 4 min. Activities were calculated as nmole glutathione/mg protein/min.

Glutathione Assay

A modified technique of Griffith was used to measure both reduced (GSH) and oxidized (GSSG) glutathione levels using a Cobas Fara centrifugal analyser [33]. To detect GSSG, samples were treated with 2-vinylpyridine that effectively inhibited GSH. Due to low abundance of GSSG, CMEC were pooled from at least eight T75 flasks prior to analysis.

Cell Viability Assay

A small aliquot of CMEC grown under different conditions was incubated in 0.1% trypan blue for 3-4 min and viewed under a light microscope. Dead cells were permeable to trypan blue and thus become coloured. By counting 100 cells, the percentage of viable cells was calculated.

Statistical Analysis

Results were presented as mean ± SEM. Statistical analysis was performed by a Student's t-test and P <0.05 was considered statistically significant.

Results

Effects of hyperglycaemia on eNOS, iNOS, p22-phox and antioxidant enzyme protein expressions

The treatment of CMEC with high glucose concentrations (22 mmol/L) failed to alter eNOS and iNOS protein expressions but significantly increased p22-phox, CuZn-SOD, Mn-SOD and catalase protein levels ($P<0.05$ for each protein) as assessed by Western analyses (Fig. 1). The differences in protein expression were independent of both extracellular glycation and the increase in osmolarity as equimolar concentrations of L-glucose and mannitol did not alter the level of protein expressions, respectively (data not shown).

Effects of hyperglycaemia on nitrite and O_2^- generation and antioxidant enzyme activities

In accordance with the observed increases in protein expression, hyperglycaemia enhanced the O_2^- generation ($P<0.05$) and activities of CuZn-SOD, Mn-SOD, catalase and GPx ($P<0.05$ for each enzyme). However, it failed to change the levels of nitrite production compared to CMEC cultured under normoglycaemic conditions ($P>0.05$). Equimolar concentrations of L-glucose or mannitol did not have any impact on enzyme activities or nitrite generation as compared to cells grown under normoglycaemic conditions ($P>0.05$) (Table 1).

Effects of free radical scavengers on nitrite and O_2^- generation and antioxidant enzyme activities

Concurrent incubation of CMEC with 22 mmol/L glucose plus one of the free radical scavengers, namely, mercaptopropionylglycine (MPG, 0.1 -1 μmol/L) or Tiron (0.1 -1 μmol/L)

reduced antioxidant enzyme activity to the levels observed in CMEC cultured with 5.5 mmol/L

glucose ($P<0.05$). These treatments significantly increased nitrite generation and diminished O_2^-

availability in a dose-dependent manner ($P<0.05$). These alterations were not observed in CMEC

cultured in equimolar mannitol (Table 2).

Effects of hyperglycaemia on intracellular glutathione levels

Exposure of CMEC to high glucose levels (22 mmol/L) resulted in a significant decrease in

both GSH and GSSG levels compared to CMEC grown in equimolar mannitol or

normoglycaemic conditions ($P<0.01$). However, treatment of CMEC with 1 µmol/L

concentration of either Tiron or MPG significantly increased glutathione levels ($P<0.01$) (Table

3).

Effects of hyperglycaemia on CMEC viability

There were no significant differences in CMEC viability between cells cultured in different

concentrations of glucose as assessed by trypan blue exclusion assay. Approximately 85±8% vs

79±5% of normoglycaemic and hyperglycaemic cells were viable, respectively ($P>0.05$).

Discussion

The endothelium synthesises and/or releases a wide variety of substances including

angiotensin II, prostacyclin and NO in response several chemical, physical and humoral stimuli

[6]. Because of their ability to release several vasoactive agents and their strategic localisation, as

they constitute the first port of call for any exogenous or endogenous insult, it was critical to

study the response of endothelial cells in relation to pathogenesis of hyperglycaemia-induced

oxidative stress. NO, the most potent endothelium-derived vasodilator, plays pivotal roles in the

maintenance of normal vascular tone, in the modulation of inflammatory and immune responses

and in the inhibition of low-density lipoprotein oxidation, VSMC proliferation and leukocyte

migration and adhesion [3 – 5]. Three isoforms of NOSs have been characterised to date, all of which are the products of separate genes and are divided into two classes with respect to their requirement of Ca^{2+} for their enzymatic activity [34]. The isoforms originally found in endothelial cells (eNOS) and in neuronal cells (nNOS) are constitutively expressed and require Ca^{2+} for their activity while an inducible isoform (iNOS) is solely expressed in response to several stimuli including cytokines and does not require Ca^{2+} for its enzymatic activity [1, 2]. Although eNOS is constitutively expressed, many pathological stimuli like genetic hypertension [35] regulate its expression. However, the activity and expression of eNOS therefore eNOS-mediated generation of NO remain rather controversial under many other pathological states such as diabetes mellitus, a metabolic disorder characterised by hyperglycaemia. Indeed, both enhanced and diminished expression of eNOS and production of NO has so far been reported in patients with diabetes mellitus, in animal models of diabetes and in cells cultured under high glucose conditions [7, 11, 12, 36, 37].

In contrast to eNOS, iNOS produces greater amounts of NO that may lead to cell damage, inflammation, atherosclerosis and myocardial dysfunction in diabetic conditions [38, 39]. Indeed, the inhibitors of iNOS namely, aminoguanidine and NOX-101 have been shown to be beneficial in preventing diabetic vascular permeability and endothelial dysfunction thereby indicating the significance of iNOS-mediated generation of NO in disease settings [40, 41]. In the current study nitrite production, a stable end-product of NO, has been shown to be similar in CMEC cultured with normal (5.5 mmol/L) and high (22 mmol/L) glucose concentrations for 7 days thus suggesting similar rate of expression for both potential NO-generating enzymes. In order to substantiate this hypothesis, the levels of eNOS and iNOS protein expressions were investigated. Our findings have shown a similar degree of protein expressions for both enzymes and similar

rates of cell viability in CMEC grown under aforementioned conditions. Taken together these data suggest that alteration in NO levels cannot account, at least during the duration of our experiments, for the vascular changes and endothelial cell dysfunction.

The amount of functional NO is also determined by the availability of free radicals in particular O_2^- which rapidly scavenges NO to reduce its biological half-life, causes contraction of VSMC and diminishes Ca^{2+} signals [23, 24]. At lower concentrations, free radicals modulate an endothelium adaptation to ensure endothelial vasomotion control while at higher concentrations they affect several intracellular pathways such as kinases, phosphatases and transcription factors such as NFκB and AP-1. The activation of these pathways may subsequently lead to endothelial barrier disruption, cell lysis potentially by producing other potent free radicals like the hydroxyl radical catalysed by iron during the Fenton reaction [42].

NAD(P)H oxidase has recently been shown as the main source of free radicals in rat CMEC where the contributions of other O_2^--generating enzymes including cyclooxygenase, xanthine oxidase and NOSs to oxidative stress were found to be negligible using the selective inhibitors of these enzymes [17]. Hence, in the present study the levels of O_2^- and the expression of p22-phox protein, a membrane-bound component of NAD(P)H oxidase that plays a central role in the activity and stability of the whole enzyme complex [19], were investigated in CMEC cultured in normoglycaemic and hyperglycaemic medium. The results have revealed a marked increase in both O_2^- production and expression of p22-phox protein in CMEC cultured under high (22 mmol/L) glucose concentrations. In support of the current data the previous studies have shown NAD(P)H oxidase-mediated increases in O_2^- levels in both diabetic rat aorta and endothelial cells grown under hyperglycaemic conditions [11, 12]. It is noteworthy that ROS may also be generated as a direct consequence of constant and prolonged in vivo or in vitro exposure of cells

to hyperglycaemia [13, 14]. This process leads to protein kinase C activation and non-enzymatic glycation of plasma proteins which then undergo further spontaneous reactions resulting in the excess production of ROS like O_2^-. Many other biochemical pathways associated with hyperglycaemia e.g. polyol pathway and eicosanoid synthesis may also increase the production of O_2^- [13, 14].

In the present study, the relationship between free radicals and coronary endothelial cell dysfunction was further investigated by use of one of the two structurally distinct O_2^--scavengers namely, Tiron and MPG. Both agents have been shown to be beneficial in ameliorating endothelial cell oxidative stress in that they significantly reduced the elevated levels of O_2^- and increased the levels of nitrite in CMEC cultured in high (22 mmol/L) glucose concentrations in a dose-dependent manner. This finding was in good agreement with a previous study showing that free radical scavengers and antioxidant agents like probucol alleviate endothelial cell dysfunction in the abdominal aorta of diabetic rats [43]. These results may suggest a potential role for ROS-scavengers in eNOS gene regulation in addition to their well-known free radical-scavenging effects.

The rise in glucose concentration has been associated with a concurrent marked decrease in both oxidised (GSSG) and reduced (GSH) glutathione levels in CMEC that was reversed by the addition of either Tiron (1 μmol/L) or MPG (1 μmol/L). Glutathione is a key aqueous phase antioxidant in cells and its depletion has been previously reported in retinal pericytes [22] and endothelial cells [25] cultured in high glucose concentrations where increases in malondialdehyde as well as CuZn-SOD and Mn-SOD mRNA levels were also reported. Although the underlying mechanisms of glutathione depletion remain to be determined in full, decreases have been reported in the gene expression and activity of γ-glutamyl cysteine

synthetase (γ-GCS), the rate-limiting enzyme in glutathione synthesis, with high glucose

concentrations [44]. Moreover, glucose-derived modification of this enzyme has been implicated

in the predisposition of erythrocytes to oxidative stress thereby implying the possibility that a

similar mechanism could also be responsible for decreased glutathione in CMEC which would

predispose them to oxidant-mediated damage [45]. However, it is also possible that free radicals

rather than glucose *per se* may account for inactivation of γ-GCS.

Hyperglycaemic-oxidative stress may be further exacerbated by the inactivation or deficiency

of SODs that metabolise O_2^- to H_2O_2 (21), another ROS that causes irreversible endothelial

damage linked with diminished NO and is detoxified to H_2O by either catalase or GPx [21]. If

H_2O_2 is not dismutated sufficiently, then excess H_2O_2 is converted to toxic hydroxyl radicals

through the Fenton reaction [42]. Thus, investigating the high glucose-induced changes in

antioxidant enzyme expression and activities was an appropriate way to elucidate

hyperglycaemia-related oxidative injuries in vitro. We have found in the current study that

hyperglycaemia elicits significant increases in Mn-SOD, CuZn-SOD, GPx and catalase protein

expression and/or activity in rat CMEC. The increases in antioxidant enzyme activities were in

keeping with their well-known induction in response to oxidative stress and have also been

previously reported in human endothelial cells [46] and in patients with type II diabetes mellitus

[47]. In contrast to these reports, a recent study has shown that gene transfer of eNOS but not

CuZn-SOD failed to improve the endothelium-dependent vascular relaxation in carotid arteries

from diabetic rabbits [48]. This may be explained by either inability of CuZn-SOD to penetrate

to vascular smooth muscle layer or by hyperglycaemia-induced glycosylation and therefore

inactivation of SOD enzymes [27]. In support of these hypothesis, a recent study has shown that

pretreatment with cell-permeable SOD enhances basal and agonist-stimulated endothelium-

dependent vascular relaxant responses in diabetic rat aorta [28]. It therefore appears reasonable to hypothesise that elevated expression and/or activity of antioxidant enzymes is an adaptive response of CMEC to meet the biological demand exerted by hyperglycaemic oxidative stress. Although the current study did not investigate the underlying causes of the increases in antioxidant gene expression, a recent study has revealed that transcriptional, translational and post-translational regulations as major determinants of local antioxidant enzyme levels in the renal cortex of diabetic rats [49].

In conclusion, the current study has shown that the aetiology of hyperglycaemia-induced oxidative stress in CMEC is multi-factorial. Endothelial cell dysfunction is associated with increased O_2^- production as opposed to inadequate synthesis of NO thereby suggesting the idea that decreasing O_2^- levels may have beneficial therapeutic implications. By increasing the antioxidant enzyme capacity, CMEC may equip themselves better protected against free radicals-induced cell damage in diabetic conditions.

Acknowledgements

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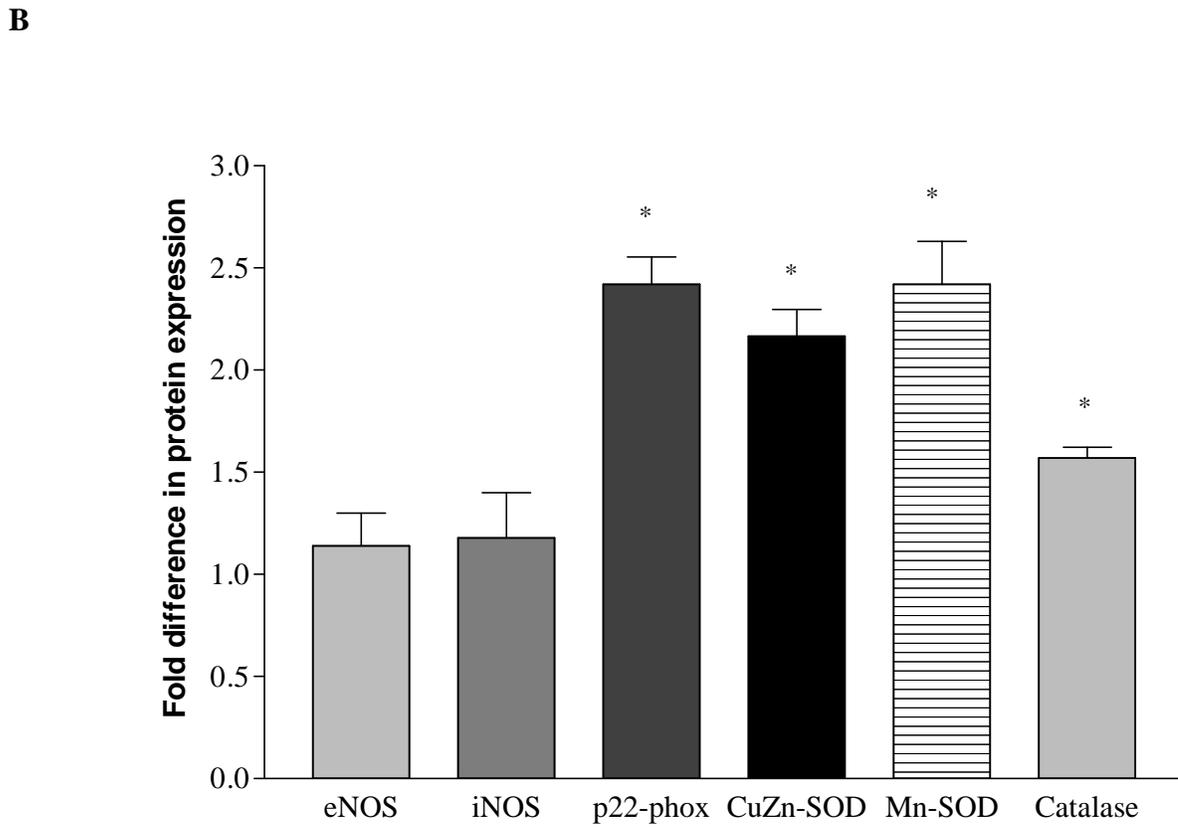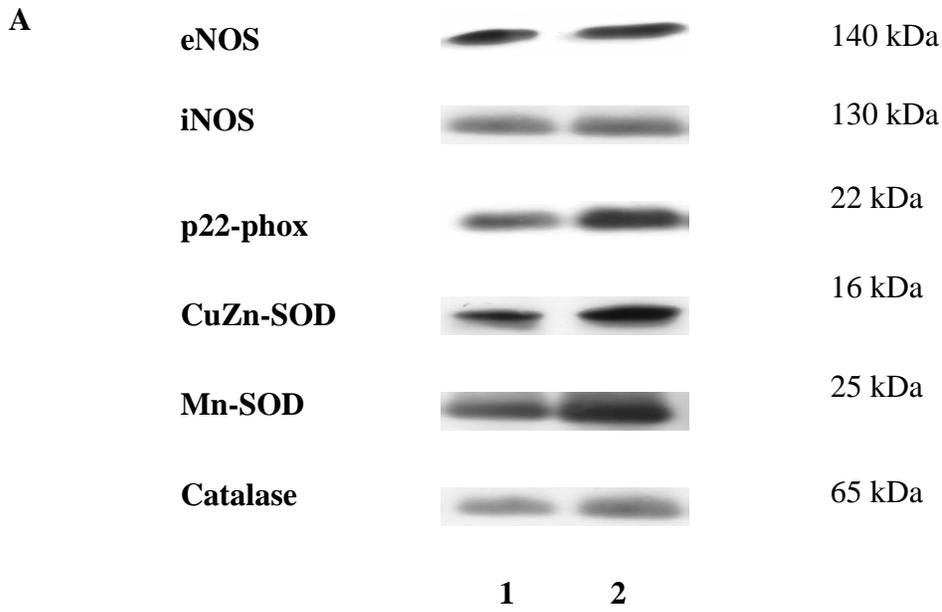
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Figure Legends

Fig. 1. (A) Representative Western blots showing the protein expression of endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), p22-phox, CuZn-SOD, Mn-SOD and catalase in CMEC cultured under normoglycaemic (lanes 1; 5.5 mmol/L) and hyperglycaemic (lanes 2; 22 mmol/L glucose) conditions for 7 days. (B) Histogram showing the fold differences in protein expression observed in CMEC cultured under hyperglycaemic versus normoglycaemic conditions. Data are expressed as mean ± SEM, n=3, * $P < 0.05$, normoglycaemic vs hyperglycaemic conditions.

A

eNOS			140 kDa
iNOS			130 kDa
p22-phox			22 kDa
CuZn-SOD			16 kDa
Mn-SOD			25 kDa
Catalase			65 kDa

1　　**2**

B

Table 1 Effect of glucose on nitrite and O_2^- production and antioxidant enzyme activities.

	Nitrite (nmol/mg protein)	O_2^- levels (pmoles/10^6 cells)	CuZn-SOD (mU/mg protein)	Mn-SOD (mU/mg protein)	Catalase (U/mg protein)	GPx (mU/mg protein)
NG	12.13 ± 0.61	45 ± 5	630 ± 110	80 ± 20	4.12 ± 0.86	236 ± 47
HG	13.31 ± 0.66	$98\pm11^*$	$960 \pm 130^*$	$170 \pm 40^*$	$7.12 \pm 0.93^*$	$555 \pm 59^*$
Control	12.39 ± 0.66	42 ± 6	670 ± 110	70 ± 23	3.69 ± 0.73	246 ± 41

NG, 5.5 mmol/L glucose; HG, 22 mmol/L glucose; Control, NG + 16.5 mmol/L mannitol. The levels of nitrite, O_2^- and antioxidant enzyme activities in CMEC cultured in NG, HG or Control medium for 7 days were measured as described in the Materials and Methods. Results are expressed as means means $\pm$ s.e.m. from 4 separate experiments. $^*P<0.05$ difference compared to NG group.

Table 2 Effects of free-radical scavengers, Tiron and MPG, on nitrite and O_2^- levels and antioxidant enzyme activities.

	Nitrite (nmol/mg protein)	O_2^- levels (pmoles/10^6 cells)	CuZnSOD (mU/mg protein)	MnSOD (mU/mg protein)	Catalase (U/mg protein)	GPx (mU/mg protein)
NG	12.13 ± 0.61	45 ± 5	630 ± 110	80 ± 20	4.12 ± 0.86	236 ± 47
HG	13.31 ± 0.66	$98\pm11^*$	$960 \pm 130^*$	$170 \pm 40^*$	$7.12 \pm 0.93^*$	$555 \pm 59^*$
NG + 1 µM MPG	13.42 ± 0.76	38 ± 7	580 ± 60	60 ± 10	4.74 ± 0.81	216 ± 31
NG + 1 µM Tiron	13.77 ± 0.81	41 ± 4	595 ± 55	60 ± 12	4.38 ± 0.60	222 ± 33
HG + 0.1 µM MPG	$18.81 \pm 1.01^{*\dagger}$	$63\pm7^{*\dagger}$	660 ± 110	100 ± 21	4.96 ± 0.81	261 ± 42
HG + 1 µM MPG	$20.61 \pm 1.21^{*\dagger}$	$41\pm6^{\dagger}$	490 ± 70	60 ± 9	3.78 ± 0.69	201 ± 17
HG + 0.1 µM Tiron	$18.23 \pm 0.96^{*\dagger}$	$57\pm5^{*\dagger}$	715 ± 105	110 ± 30	4.77 ± 0.88	312 ± 52
HG + 1 µM Tiron	$20.99 \pm 1.09^{*\dagger}$	$39\pm5^{\dagger}$	520 ± 100	65 ± 10	4.11 ± 0.66	223 ± 38

NG, 5.5 mmol/L glucose; HG, 22 mmol/L glucose. The levels of nitrite, O_2^- and antioxidant enzyme activities were measured in CMEC cultured in NG or HG medium for 7 days in the absence and presence of free-radical scavengers, Tiron and MPG. Results were expressed as means ± s.e.m. from 4 separate experiments. $^*P<0.05$ difference compared to NG group, $^{\dagger}P<0.05$ difference compared to HG group.

Table 3 Effect of glucose and free radical-scavengers, Tiron and MPG on

reduced (GSH) and oxidised glutathione (GSSG) levels.

	GSSG (nmol/mg protein)	GSH (nmol/mg protein)
NG	0.064 ± 0.009	2.26 ± 0.28
HG	$0.029 \pm 0.004^{*}$	$1.23 \pm 0.13^{*}$
Control	0.068 ± 0.008	2.17 ± 0.23
HG + 1 µM Tiron	$0.071 \pm 0.007^{\dagger}$	$2.47 \pm 0.32^{\dagger}$
HG + 1 µM MPG	$0.073 \pm 0.010^{\dagger}$	$2.26 \pm 0.21^{\dagger}$

NG, 5.5 mmol/L glucose; HG, 22 mmol/L glucose. Results were expressed as

means $\pm$ s.e.m. from 4 separate experiments. $^{*}P<0.05$ difference compared

to NG group, $^{\dagger}P<0.05$ difference compared to HG group.