Ulker, Sibel and McMaster, Dorothy and McKeown, Pascal and Bayraktutan, Ulvi (2004) Antioxidant vitamins C and E ameliorate hyperglycaemia induced oxidative stress in coronary endothelial cells. Diabetes Obesity and Metabolism, 6 . pp. 442-451.

ANTIOXIDANT VITAMINS C AND E AMELIORATE HYPERGLYCAEMIA-INDUCED OXIDATIVE STRESS IN CORONARY ENDOTHELIAL CELLS

Sibel Ülker, MD; Dorothy McMaster, PhD; Pascal P. McKeown, MD; Ulvi Bayraktutan* DVM, PhD

Department of Medicine, Institute of Clinical Science Block B, Queen`s University Belfast, Belfast, United Kingdom

***Address for correspondence**

Dr. Ulvi Bayraktutan

Department of Medicine,

Institute of Clinical Science Block B

Queen`s University Belfast

Grosvenor Road

Belfast BT12 6BJ

United Kingdom

Tel: +44 28 90632742

Fax: +44 28 90329899

E-mail: u.bayraktutan@qub.ac.uk

Abstract

Objective: Vitamins C and E have protective features in many disease states associated with enhanced oxidative stress. The aim of this study was to investigate whether vitamins C and/or E modulate hyperglycaemia-induced oxidative stress by regulating enzymatic activities of prooxidant i.e. NAD(P)H oxidase and/or antioxidant enzymes, namely endothelial nitric oxide synthase (eNOS), superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), using coronary microvascular endothelial cells (CMEC).

Methods: CMEC were cultured under normal (5.5 mM) or high glucose (22 mM) concentrations for 7 days. The enzyme activities were determined by specific assays. The levels of O_2^- and nitrite were measured by cytochrome *C* reduction and Griess assays, respectively.

Results: Hyperglycaemia did not alter eNOS activity or overall nitrite generation, an index of NO production. However, it increased NAD(P)H oxidase and antioxidant enzyme activities ($p<0.05$). Specific inhibitors of NAD(P)H oxidase i.e. phenylarsine oxide (PAO, 0.1-3 μM) and 4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF, 5-100 μM) and vitamins C and E (0.1-1 μM) significantly reduced prooxidant and antioxidant enzyme activities in CMEC exposed to hyperglycaemia ($p<0.01$). The differences in enzyme activities were independent of increases in osmolarity generated by high glucose levels as investigated by using equimolar concentrations of mannitol in parallel experiments.

Conclusions: Vitamins C and E may protect CMEC against hyperglycaemia-induced oxidative stress by concomitantly regulating prooxidant and antioxidant enzyme activities.

Introduction

Nitric oxide (NO), generated from amino acid L-arginine within healthy endothelium by endothelial type of nitric oxide synthase (eNOS), plays a pivotal role in the regulation of normal vascular tone [1]. The characteristics of endothelium change in several pathological conditions including diabetes mellitus leading to a phenomenon called "endothelial dysfunction" which is characterised by impaired endothelial cell function. Although the actual causes of this pathology are unknown, several mechanisms have thus far been proposed. These include inefficient utilisation of substrate L-arginine by NOS [2], an abnormal NOS activity due to inadequate availability of co-factor tetrahydrobiopterin (BH_4) [3], concurrent release of endothelium-derived vasoconstrictors by cyclooxygenase (COX) pathway [4] and scavenging of NO by advanced glycation end-products (AGEs) [5].

In recent years, enhanced oxidative stress status arising from excess release of reactive oxygen species (ROS) has been documented in diabetic animals and cells cultured under high glucose conditions [6,7] and associated with the pathogenesis of diabetic endothelial dysfunction. ROS may be generated as a result of prolonged exposure of cells to hyperglycaemia that results in non-enzymatic glycation of plasma proteins [8] which then undergo further spontaneous reactions to produce free radicals such as superoxide anion (O_2^-), the foundation molecule of other ROS [9]. O_2^- is also formed by enzymatic activities of COX, xanthine oxidase (XO), uncoupled eNOS and NAD(P)H oxidase [3,10,11]. Amongst these enzymes NAD(P)H oxidase enzyme system has attracted much of the attention. It has been characterised as the main source of ROS in coronary microvascular endothelial cells (CMEC) [12] and has later been coupled to oxidative stress-mediated endothelial dysfunction in the central retinas of type II diabetic rats [13].

Under physiological conditions, O_2^- is converted to hydrogen peroxide (H_2O_2) by superoxide dismutases (SODs) [14] and upon generation H_2O_2 itself is further metabolised to H_2O by catalase and glutathione peroxidase (GPx) [14]. However, under hyperglycaemic conditions the levels of O_2^- may be elevated as a consequence of glycosylation and hence inactivation of SODs [15]. O_2^- readily scavenges NO to diminish its vasoprotective effects and produces vascular smooth muscle (VSM) contractions in the absence of intracellular antioxidants such as glutathione and cysteine [16,17]. Depletion of these antioxidants in diabetic conditions is a common occurrence as demonstrated in aortic endothelial cells isolated from diabetic rabbits [18] and in CMEC cultured with 22 mM D-glucose-containing media for 7 days [19]. Taken together the currently available data imply that the elevation of antioxidant levels in diabetic states may be critical in suppressing hyperglycaemia-induced oxidative stress generation and thus initiation and/or progression of endothelial dysfunction. Several recent studies including our own have supported this hypothesis in that treatments of diabetic patients with antioxidants such as allopurinol and incubation of CMEC grown under hyperglycaemic conditions with free radical scavengers e.g. Tiron displayed beneficial *in vivo* and *in vitro* effects, respectively [19,20]. A growing body of evidence has revealed that vitamins C and E, in addition to other intracellular antioxidants such as glutathione, also improve endothelial function in human subjects and in animal models of diabetes mellitus [21-23]. Moreover, they are associated with increases in NO generation and total antioxidant status and also with reduction in blood pressure [24,25]. Although both antioxidant vitamins are known to stimulate NO generation and have prominent ROS-scavenging effects, the underlying mechanisms of their putative beneficial actions remain to be determined. The present study was therefore set out to investigate whether vitamins C and/or E modulate redox state in CMEC exposed to hyperglycaemic conditions via regulation of

the enzymatic activities of prooxidant i.e. NAD(P)H oxidase and antioxidant enzymes i.e. eNOS, total SOD, catalase and GPx.

Materials and Methods

Isolation and Characterisation of CMEC

CMEC were isolated from 12- to 14-week-old Wistar rat hearts as previously described [26]. Briefly, two 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 (Life Technologies) 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 gentamicin 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 different experiments, CMEC were cultured for 7 days in the growth medium containing either 5.5 mM (normal) or 22 mM (high) D-glucose. CMEC were also cultured with 5.5 mM D-glucose + 16.5 mM L-glucose and 5.5 mM D-glucose + 16.5 mM mannitol in order to investigate the effects of extracellular glycation and increased osmolarity. 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 their ability to form capillary-like tubes on the Matrigel [27].

Measurement of NOS activity

NOS activity was measured by the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline. Briefly, CMEC were homogenised, on ice, in TRIS buffer (50 mM, pH 7.4) containing leupeptin (0.2 μM), pepstatin A (1.5 mM) and phenylmethylsulfonyl fluoride (PMSF, 1 mM). Samples were incubated at 37^{o}C for 30 min in the presence of calmodulin (30 nM), NADPH (1 mM), H_4B (5 μM), Ca^{2+} (2 mM), L-valine (50 mM) and a mixture of unlabelled (0-5 μM) and L-[^{3}H]-arginine (10 mM) (Amersham Pharmacia). To assess the contribution of iNOS (calcium-independent isoform) to overall NOS activity Ca^{2+} was replaced with EGTA (1 mM). Reactions were terminated by the addition of 1 ml HEPES (20 mM, pH 5.5) containing EDTA (1 mM) and EGTA (1 mM). Newly formed L-[^{3}H]citrulline, neutral at pH 5.5, was separated from the incubation mixture by cation exchange resin (Dowex AG 50 W-X8, Bio-Rad) and quantified using a liquid scintillation counter. Results were expressed as pmol L-citrulline/mg protein/min.

Nitrite Detection

Nitrite levels were measured by Griess reaction as an index of NO generation following conversion of nitrate to nitrite by nitrate reductase [28]. 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.

Measurement of NAD(P)H oxidase activity and detection of O_2^- levels

O_2^- levels were measured by cytochrome *C* reduction assays. Briefly, CMEC were collected in Hanks' balanced salt solution (HBSS) at a density of 20 x 10^6 cells/ml. Aliquots (250 μl)

containing 50 μM cytochrome *C* were then incubated for 60 min at 37°C. O_2^- generation was measured as the superoxide dismutase (10 μg/ml)-inhibitable reduction of cytochrome *C* and monitored as the change in absorbance at 550 nm using a Cobas-Fara centrifugal analyser. Absorbances were recorded for 12 min with 90 seconds intervals and production of O_2^- was calculated as pmoles O_2^- per 10^6 cells after subtracting background values measured at 550 nm. NAD(P)H oxidase activity was measured in similar experiments where the aforementioned specific inhibitors of other ROS-generating enzymes i.e. L-NAME (0.1 mM), rotenone (50 μM), allopurinol (100 μM) or indomethacin (50 μM) were added to aliquots during 60 min incubation period prior to determining O_2^- generation.

SOD Assay

SOD activity was 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-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 mM xanthine was dissolved in 50 mM $NaCO_3$ buffer. A dilute stock solution was added to a 10 μM solution of cytochrome *C*, 50 μM xanthine, 0.1 mM EDTA and 50 mM sodium carbonate to produce a change in absorbance of 0.0250/min at 550 nm at pH 10.

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 solutions of known activities (31.2, 15.6, 7.8 and 3.9 U/ml) and formaldehyde standards (25, 50, 100, 200 μM).

GPx Assay

The activity of GPx was determined in cellular homogenates using a method developed by McMaster et al [29]. Briefly, a fresh solution containing 0.3 U/ml glutathione reductase, 1.25 mM reduced glutathione and 0.19 mM NADPH in 50 mM 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 mM t-butylhydroperoxide to commence the reaction. Absorbances were read at 340 nm for 4 min. Activities were calculated as nmole glutathione/mg.

Evaluation of Cell Viability

A small aliquot of cells cultured under different conditions was incubated with 0.1% trypan blue for a few minutes 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. Numbers (n) indicated throughout the article denote the number of separate CMEC isolations and individual experiments. Statistical analyses were performed by both Student's *t*-test and ANOVA where appropriate. $P < 0.05$ was considered statistically significant.

Results

Effects of vitamins C and E on prooxidant and antioxidant enzyme activities

The current study revealed greater prooxidant i.e. NAD(P)H oxidase and antioxidant i.e. total SOD, catalase and GPx enzyme activities in CMEC cultured with high (22 mM) compared to

normal (5.5 mM) glucose concentrations for 7 days (P<0.05 for each enzyme). However, the treatment of CMEC with antioxidant vitamins C (0.1-1 μM) and E (0.1-1 μM) alone or in combinations (0.1/0.1 μM and 1/1 μM vitamin C and vitamin E, respectively) significantly reduced these enzyme activities (Table 1). Equimolar concentrations of L-glucose or mannitol did not have any impact on enzyme activities as compared to cells grown under normoglycaemic conditions (P>0.05) (Table 1).

Hyperglycaemia failed to alter eNOS and iNOS activities in CMEC as assessed by L-[^{3}H]-arginine to L-[^{3}H]-citrulline conversion assay (P>0.05). However, the addition of vitamins C and E alone or in combination to the culture medium significantly enhanced eNOS activity without altering iNOS activity in both normoglycaemic and hyperglycaemic CMEC (P<0.05) (Fig. 1A-B). Equimolar concentrations of L-glucose or mannitol did not have any impact on eNOS or iNOS activities compared to cells cultured under normoglycaemic conditions (P>0.05) (data not shown).

Effects of vitamins C and E on nitrite and O_2^- generation

In accordance with the observed increases in enzyme activities, hyperglycaemia enhanced O_2^- generation (P<0.05) but did not alter nitrite production as compared with CMEC cultured under normoglycaemic conditions (P>0.05) (Table 2). However, the use of vitamins C (0.1-1 μM) and E (0.1-1 μM) alone or in combination (0.1/0.1 μM and 1/1 μM) significantly enhanced nitrite generation and reduced O_2^- production in hyperglycaemic CMEC (P<0.05) (Fig. 2A-B). Equimolar concentrations of L-glucose or mannitol did not have any impact on nitrite generation compared to cells grown under normoglycaemic conditions (P>0.05) (data not shown).

Effects of NAD(P)H oxidase inhibitors on nitrite and O_2^- generation and antioxidant enzyme activities

Incubations of CMEC with one of the two structurally-unrelated inhibitors of NAD(P)H oxidase i.e. either PAO (0.1-3 μM) or AEBSF (5-100 μM) reduced antioxidant enzyme activities in hyperglycaemic CMEC (P<0.05). While decreases in enzyme activities were dose-dependent in case of PAO, they were independent of the dose of AEBSF used in the experiments. Moreover, treatments of hyperglycaemic CMEC with PAO and AEBSF diminished O_2^- availability but increased nitrite generation (P<0.05) (Table 2). These alterations were not observed in CMEC cultured in equimolar mannitol or L-glucose (Table 2).

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 86±11% vs 78±9% of normoglycaemic and hyperglycaemic cells were viable, respectively (P>0.05).

Discussion

The endothelium releases a large number of vasoactive substances including NO to maintain normal vascular tone [1]. Endothelial NOS (eNOS) is associated with production of moderate levels of NO in healthy endothelium while inducible NOS (iNOS) is coupled to excess generation of NO, endothelial cell damage and atherosclerosis in a number of disease settings including diabetes mellitus [30,31]. The inhibitors of iNOS such as aminoguanidine have therefore proven to be critical in preventing diabetic endothelial dysfunction [32]. Despite being a constitutively expressed isoform, the expression and activity of eNOS are affected by many pathological conditions associated with enhanced oxidative stress status such as genetic hypertension [33]. However, the regulation of eNOS in diabetes mellitus remains ambiguous as both enhanced and diminished expression of eNOS have thus far been reported [2,6,7,31]. Recent data have shown that the bioavailability of NO under diabetic conditions is also

determined by ROS, in particular O_2^- which readily scavenges NO to reduce its biological half-life [16]. Although lower concentrations of free radicals may be beneficial in endothelial adaptation to ensure vasomotion control, their higher concentrations may induce several intracellular pathways such as phosphatases and transcription factors e.g. NFκB to disrupt endothelial integrity by producing other potent ROS like the hydroxyl radical via Fenton reaction [34]. NAD(P)H oxidase has recently been characterised and shown as the main source of free radicals in CMEC [12]. Hyperglycaemia-mediated oxidative stress generation may be further exacerbated by the inadequacy of antioxidant enzymes SODs that dimutate O_2^- to H_2O_2 and catalase and/or GPx that metabolise H_2O_2 to H_2O [14].

In light of the currently available data, the present study aimed to investigate whether vitamins C and E alone or in combination maintain a well-balanced oxidative status, a prerequisite for normal endothelial cell function, by regulating prooxidant and antioxidant enzyme activities in CMEC. To study the combinations of vitamin C and E were important in relation to findings indicating that vitamin C, apart from being a free radical-scavenger, is required for regeneration of vitamin E to its active form [35]. Putative beneficial effects of antioxidant vitamins may be attributed to their ability to (i) scavenge free radicals; (ii) regulate NO synthesis or release; (iii) regulate ROS generation; and (iv) regulate antioxidant enzyme activities that metabolise ROS.

It is known that low molecular weight antioxidants such as urate and thiols along with vitamins C and E constitute the first line of defence against oxidative stress in the extracellular environment by scavenging free oxygen radicals and hence preventing oxidation of proteins and lipids [36]. Although consistent beneficial effects of vitamin C have been reported in diabetic patients, animal models of diabetes mellitus and cell culture models, similar studies with vitamin E have produced contradictory findings [21-23]. For instance, the Cambridge Heart Antioxidant

Study (CHAOS) revealed a marked reduction in non-fatal myocardial infarction in patients received 400-800 IU of vitamin E/day compared to patients receiving placebo [21]. However, the subsequent Heart Outcomes Prevention Evaluation (HOPE) study failed to confirm the beneficial effects of vitamin E [37].

The present study has demonstrated similar rates of cellular viability, eNOS and iNOS activities as well as nitrite (the stable end-product of NO) production between CMEC cultured with high (22 mM) and normal (5.5 mM) glucose concentrations for 7 days. These findings are in support of a recent study demonstrating similar levels of eNOS and iNOS protein expressions and nitrite generation in CMEC cultured under identical conditions that were used in the current study [19]. In the present study, the incubation of CMEC with combinations of vitamins C and E or solely with vitamin C or E significantly enhanced nitrite generation and eNOS activity but failed to alter iNOS activity in both sets of cells without dramatically altering cellular viability rates. Similar results with vitamin C have also been reported under *in vivo* conditions in that long-term dietary intake of vitamin C by apolipoprotein E (apoE)-deficient mice have been associated with significant increases in eNOS but not iNOS activities and nitrite generation in apoE-deficient mice aortas [38]. These increases may in part be assigned to the ability of vitamin C to spare intracellular thiols to stabilise NO through controlling the formation of biologically active *S*-nitrosothiols and also to its ability to increase the intracellular levels of eNOS co-factor i.e. BH_4 [39,40]. Vitamin E-mediated significant elevations in endothelial NO release and endothelial function have been documented in diabetic rat aorta [23]. The increases in these parameters were more likely to be a consequence of inhibitory effects of vitamin E on LDL oxidation via suppression of protein kinase C-mediated phosphorylation of muscarinic receptors on endothelial cells rather than its regulatory action on eNOS [41].

In the present study, marked increases in basal levels of O_2^- and NAD(P)H oxidase activity have been determined in CMEC cultured in hyperglycaemic versus normoglycaemic medium for 7 days. Since ROS, under hyperglycaemic conditions, may also be generated by the polyol pathway, eicosanoid synthesis, protein kinase C activation and non-enzymatic glycation of plasma proteins [8,9], it was critical to investigate the extent of NAD(P)H oxidase activity to overall ROS generation in CMEC grown under high glucose concentrations. Hence, two structurally distinct and specific NAD(P)H oxidase inhibitors, namely PAO (0.1-3 μM) and AEBSF (5-100 μM), were used in this study. Both agents reduced antioxidant enzyme activities and O_2^- levels but increased nitrite production in CMEC cultured with high glucose concentrations thereby indicating NAD(P)H oxidase as the main source of ROS in CMEC exposed to hyperglycaemic conditions. These data are in good agreement with those of several previous studies showing that antioxidants and free radical scavengers such as probucol and Tiron improve endothelial function in hyperglycaemic CMEC and in the thoracic aortic rings from SHR [19,25].

The impacts of vitamins C and E on free radical generation were also investigated in this study. Treatments with both vitamins C and E reduced both O_2^- production and NAD(P)H oxidase activity selectively in CMEC cultured with high glucose concentrations. These data confirm the previous findings pertaining to regulatory effects of these vitamins on NAD(P)H oxidase activity in SHR aortas and also their well-known free radical-scavenging effects [25]. Although the mechanisms by which vitamins C and E may modulate NAD(P)H oxidase activity have not been investigated in the current study, both the transcriptional and post-translational modification of NAD(P)H oxidase by vitamins have previously been associated with its regulation [42]. In addition to these, vitamin E may mediate the interactions between membrane-

bound and cytosolic components of NAD(P)H oxidase to form fully active enzyme upon induction [42].

The current study has shown that hyperglycaemia elicits significant increases in total SOD, GPx and catalase activities in rat CMEC. The increases in these antioxidant enzyme activities are in keeping with their well-known induction in response to oxidative stress and have also been previously reported in human endothelial cells [43] and in patients with type II diabetes mellitus [44]. The results of the current study are also supportive of our recent study demonstrating enhanced expression of CuZn-SOD, Mn-SOD and catalase protein levels in rat CMEC cultured with high compared to normal concentrations of D-glucose for 7 days [19]. The increases in antioxidant enzyme protein expressions in the former study and in activities in the current study are independent of changes in osmolarity and extracellular glycation as assessed in parallel experiments where equimolar concentration of mannitol or L-glucose were substituted with D-glucose. Although a recent study has shown that gene transfer of CuZn-SOD failed to improve the endothelium-dependent vascular relaxation in carotid arteries from diabetic rabbits [45], another study using a cell-permeable SOD has shown to enhance basal and agonist-stimulated endothelium-dependent vascular relaxant responses in diabetic rat aorta [6]. These data indicate that the former findings may be due to inability of CuZn-SOD to penetrate VSM layer or due to glycosylation and therefore inactivation of SODs by high glucose levels [46]. Although putative mechanisms by which vitamins C and E may modulate antioxidant enzyme activities remain largely unknown, 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 [47].

In conclusion, the present study indicates that hyperglycaemia-mediated oxidative stress in CMEC does not appear to arise from alterations in eNOS activity or NO availability. However, exaggerated synthesis and release of ROS in particular O_2^- may contribute to pathogenesis of this phenomenon and increases in activity of antioxidant enzymes may be an adaptive response of CMEC to meet the biological demand exerted by hyperglycaemic oxidative stress. Our data demonstrate that elevation of intracellular levels of antioxidant vitamins C and E to the levels that can effectively scavenge O_2^- levels [48] may provide beneficial cellular effects by regulating pro- and antioxidant enzyme activities in diabetic states.

Acknowledgements

This work was kindly supported by the Heart Trust Fund (Royal Victoria Hospital), Belfast, UK.

References

1 Lerman A, Burnett JC Jr. Intact and altered endothelium in regulation of vasomotion. *Circulation* 1992; **86:** III12-III19

2 Pieper GM, Peltier BA. Amelioration by L-arginine of a dysfunctional arginine/nitric oxide pathway in diabetic endothelium. *J Cardiovasc Pharmacol* 1995; **25:** 397-403

3 Pieper GM. Acute amelioration of diabetic endothelial dysfunction with a derivative of the nitric oxide synthase cofactor, tetrahydrobiopterin. *J Cardiovasc Pharmacol* 1997; **29:** 8-15

4 Tesfamariam B, Jakubowski JA, Cohen RA. Contraction of diabetic rabbit aorta caused by endothelium-derived PGH_2-TxA_2. *Am J Physiol* 1989; **57:** H1327-H1333

5 Bucala R, Tracey KJ, Cerami A. Advanced glycosylation products quench NO and mediate defective endothelium-dependent vasodilatation in experimental diabetes. *J Clin Invest* 1991; **87:** 432-438

6 Langenstroer P, Pieper GM. Regulation of spontaneous EDRF release in diabetic rat aorta by oxygen free radicals. *Am J Physiol* 1992; **263:** H257-H262

7 Graier WF, Pusch K, Wascher T. Increased superoxide formation in endothelial cells during hyperglycemia. *Diabet Res Clin Prac* 1999; **45:** 153-160

8 Tames FJ, Mackness MI, Arrol S, Laing I, Durrington PN. Non-enzymatic glycation of apolipoprotein B in the sera of diabetic and non-diabetic subjects. *Atheroscler* 1992; **93:** 237-244

9 Sakari T, Sugioka K, Nakano M. O_2^- generation and lipid peroxidation during oxidation of a glycated polypeptide, glycated polylysine, in the presence of iron-ADP. *Biochim Biophys Acta* 1997; 1043: 27-33

10 Xia Y, Dawson VL, Dawson TM, Snyder SH, Zweier JL. NOS generates superoxide and NO in arginine depleted cells leading to peroxynitrite-mediated cellular injury. *Proc Natl Aca Sci USA* 1994; **93:** 6770-6774

11 Matsumoto S, Koshiishi I, Inoguchi T, Nawata H, Utsumi H. Confirmation of superoxide generation via xanthine oxidase in streptozotocin-induced diabetic mice. *Free Rad Res* 2003; **37:** 767-772

12 Bayraktutan U, Draper N, Lang D, Shah AM. Expression of a functional neutrophil-type NADPH oxidase in cultured rat coronary microvascular endothelial cells. *Cardiovasc Res* 1998; **38:** 256-262.

13 Ellis EA, Grant MB, Murray FT, Wachowski MB. Increased NADH oxidase activity in the retina of the BBZ/WOR diabetic rat. *Free Rad Biol Med* 1998; **24:** 111-120

14 Yu BP. Cellular defenses against damage from reactive oxygen species. *Physiol Rev* 1994; **74:** 139-162

15 Yokoyama I, Ohtake T, Monomura SI et al., Hyperglycemia rather than insulin resistance is related to reduced coronary flow reserve in NIDDM. Diabetes 1998; **47:** 119-124

16 Gryglewski RJ, Palmer RM, Moncada S. O_2^- is involved in the breakdown of NO. *Nature* 1986; **320:** 454-456

17 Kashiwagi A, Asahina T, Ikebuchi M *et al*. Abnormal glutathione metabolism and increased cytotoxicity caused by H_2O_2 in human umbilical vein endothelial cells cultured in high glucose medium. *Diabetologia* 1994; **37:** 264-269

18 Tagami S, Kondo T, Yoshida K, Hirokawa J, Ohtsuka Y, Kawakami Y. Effect of insulin on impaired antioxidant activities in aortic endothelial cells from diabetic rabbits. *Metabolism* 1992; **41:** 1053-1058

19 Weidig P, McMaster D, Bayraktutan U. High glucose mediates pro-oxidant and antioxidant enzyme activities in coronary endothelial cells. *Diabet Obes Metabol* 2004 (in press).

20 Butler R, Morris AD, Belch JJF, Hill A, Struthers AD. Allopurinol normalizes endothelial dysfunction in type 2 diabetics with mild hypertension. *Hypertension* 2000; **35:** 746-751.

21 Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet* 1996; **347:** 781-786

22 Williams SB, Cusco JA, Roddy MA, Johnstone MT, Creager MA. Impaired NO-mediated vasodilation in patients with non-insulin-dependent diabetes mellitus. J Am Coll Cardiol 1996; **27:** 567-574

23 Keegan A, Walbank H, Cotter MA, Cameron NE. Chronic vitamin E treatment prevents defective endothelium-dependent relaxation in diabetic rat aorta. Diabetologia 1995; **38:** 1475-1478

24 Chen X, Touyz RM, Park JB, Schiffrin EL. Antioxidant effects of vitamins C and E are associated with altered activation of vascular NADPH oxidase and superoxide dismutase in stroke-prone SHR. *Hypertension* 2001; **38:** 606-611

25 Ulker S, McKeown PP, Bayraktutan U. Vitamins reverse endothelial dysfunction through regulation of eNOS and NAD(P)H oxidase activities. *Hypertension* 2003; **41:** 534-539

26 Piper HM, Spahr R, Mertens S, Krytzfeldt A, Watanabe H. Microvascular endothelial cells from the heart: In; Piper HM, editor, Cell culture techniques in heart and vessel research, Berlin:Springer, 1990, pp. 158-177

27 Nishida M, Carley WW, Gerritsen ME, Ellingsen O, Kelly RA. Isolation and characterisation of human and rat cardiac microvascular endothelial cells. *Am J Physiol* 1993; **264:** H639-H652

28 Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. *Anal Biochem* 1982; **126:** 131-138

29 McMaster D, Bell N, Anderson P et al., Automated measurement of two indicators of human selenium status and applicability to population studies. *Clin Chem* 1990; **36:** 211-216

30 Iyengar R, Stuehr DJ, Marletta MA. Macrophage synthesis of nitrite, nitrate and N-nitrosoamines: precursors and role of the respiratory burst. *Proc Natl Aca Sci USA* 1987; **84:** 6369-6373

31 Cosentino F, Hishikawa K, Katusic ZS, Luscher TF. High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. *Circulation* 1997; **96:** 25-28

32 Corbett JA, Tilton RG, Chang K et al. Aminoguanidine, a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. *Diabetes* 1992; **41:** 552-556

33 Bayraktutan U, Yang ZK, Shah AM. Selective dysregulation of nitric oxide synthase type 3 in cardiac myocytes but not coronary microvascular endothelial cells of spontaneously hypertensive rat. *Cardiovasc Res* 1998; **38:** 719-726

34 Kvietys PR, Inauen W, Bacon BR, Grisham MB. Xanthine oxidase-induced injury to endothelium: role of intracellular iron and hydroxyl radical. *Am J Physiol* 1989; **257:** H1640-H1646

35 Packer JE, Slater TF, Wilson RL. Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* 1979; **278:** 738-739

36 Stocker R, Frei B. Endogenous antioxidant defenses in human blood plasma. In: Sies H, ed. Oxidative stress, oxidants and antioxidants. London: Academic Press, 1991.

37 Kleinert S. HOPE for cardiovascular disease prevention with ACE inhibitor ramipril. *Lancet* 1999; **354:** 841

38 d'Uscio LV, Milstein S, Richardson D, Smith L, Katusic ZS. Long-term vitamin C treatment increases vascular tetrahydrobiopetrin levels and nitric oxide synthase activity. *Circ Res* 2003; **92:** 88-95

39 Meister A. Glutathione-ascorbic acid antioxidant system in animals. *J Biol Chem* 1994; **269:** 9397-9400

40 Huang A, Vita JA, Venema RC, Keaney JF. Ascorbic acid enhances endothelial nitric oxide synthase activity by increasing intracellular tetrahydrobiopterin. *J Biol Chem* 2000; **275:** 17399-17406

41 Keaney JF, Guo Y, Cunningham D, Shwaery GT, Xu A, Vita JA. Vascular incorporation of α-tocopherol prevents endothelial dysfunction due to oxidised LDL by inhibiting protein kinase C stimulation. *J Clin Invest* 1996; **98:** 386-394

42 Chaudiere J, Ferrari-Iliou R. Intracellular antioxidants: from chemical to biochemical mechanisms. *Food Chem Toxicol* 1999; **37:** 949-962

43 Ceriello A, dello Russo P, Amstad P, Cerutti P. High glucose induces antioxidant enzymes in human endothelial cells in culture. *Diabetes* 1996; **45:** 471-477

44 Aydin A, Orhan H, Sayal A, Ozata M Sahin G, Isimer A. Oxidative stress and nitric oxide related parameters in type II diabetes mellitus: effect of glycemic control. Clin Biochem 2001; **34:** 65-70

45 Lund DD, Faraci FM, Miller FJ, Heistad DD. Gene transfer of eNOS improves relaxation of carotid arteries from diabetic rabbits. *Circulation* 2000; **101:** 1027-1033

46 Arai K, Izuka S, Tada Y, Oikawa K, Taniguchi N. Increase in the glycosylated form of erythrocyte CuZn superoxide dismutase in diabetes and close association of the nonenzymatic glycosylation with the enzyme activity. *Biochim Biophys Acta* 1987; **924:** 292-296

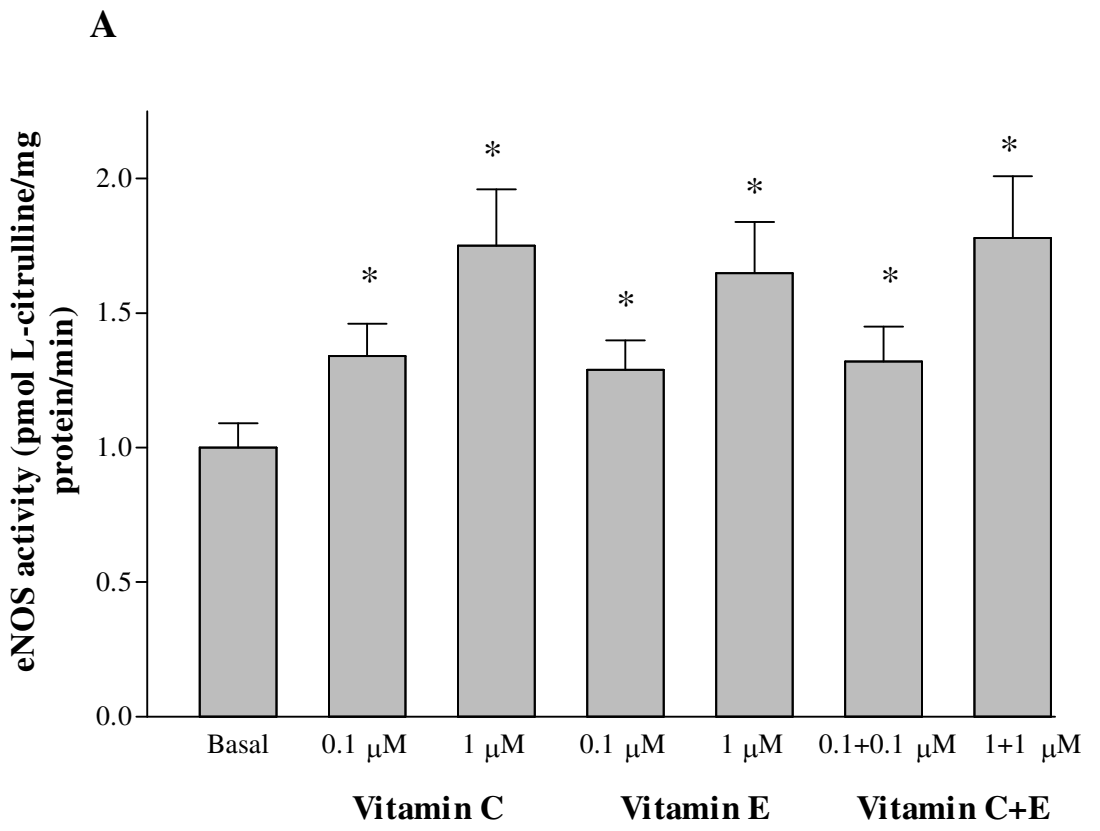
47 Limaye PV, Raghuram N, Sivakami S. Oxidative stress and gene expression of antioxidant enzymes in the renal cortex of streptozotocin-induced diabetic rats. *Mol Cell Biochem* 2003; **243:** 147-152

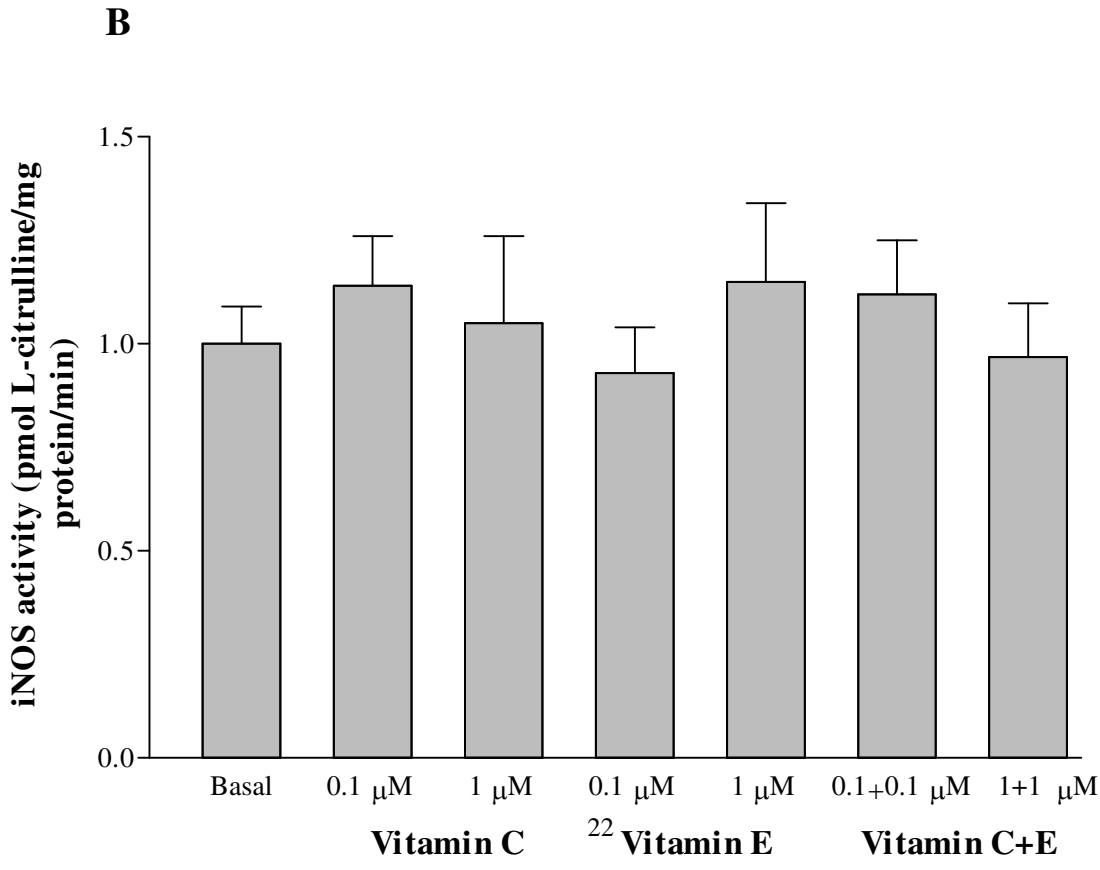
48 Ek A, Strom K, Cotgreave IA. The uptake of ascorbic acid into human umbilical vein endothelial cells and its effect on oxidant insult. *Biochem Pharmacol* 1995; **50:** 1339-1346

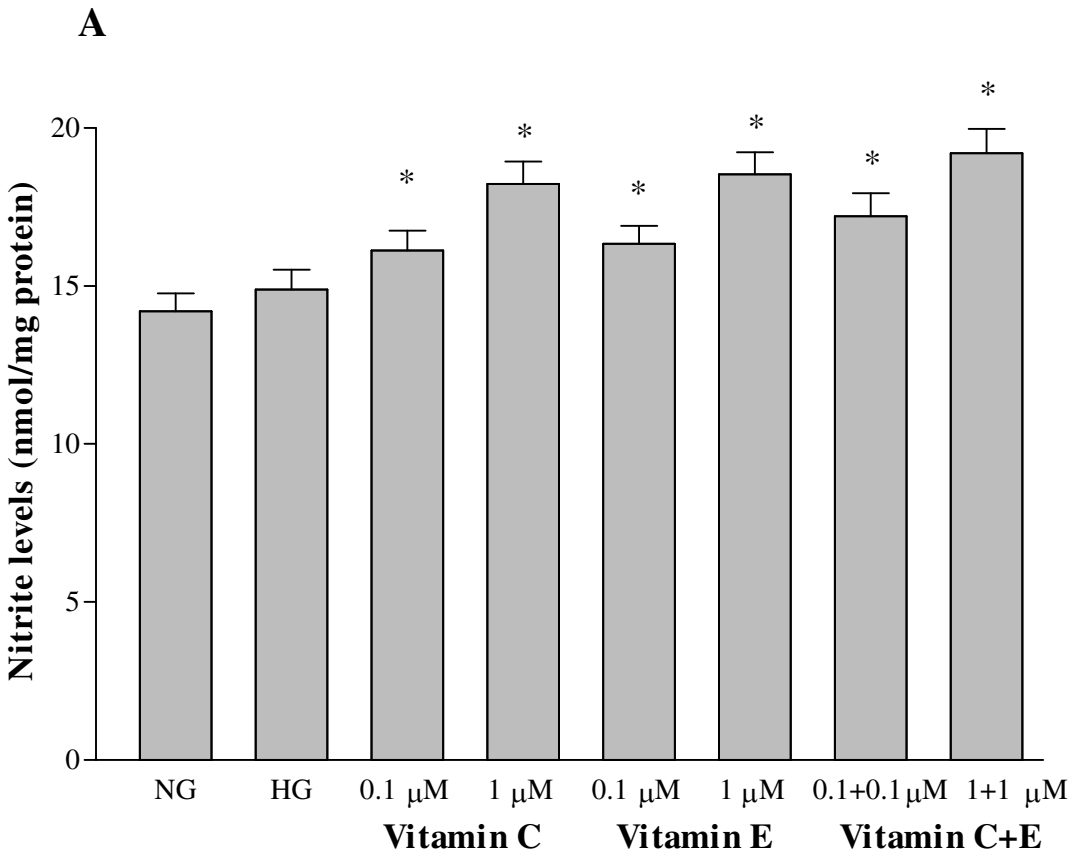
Figure Legends

Fig. 1. (A) The levels of endothelial Nitric Oxide Synthase (eNOS) activity in coronary microvascular endothelial cells (CMEC) grown under normoglycaemic (NG, 5.5 mM D-glucose) conditions and hyperglycaemic (22 mM D-glucose) conditions in the absence and presence of vitamins C and E alone or in combination. (B) The levels of inducible Nitric Oxide Synthase (iNOS) activity in coronary microvascular endothelial cells (CMEC) grown under normoglycaemic (NG, 5.5 mM D-glucose) conditions and hyperglycaemic (22 mM D-glucose) conditions in the absence and presence of vitamins C and E alone or in combination. Data from 4 separate experiments are expressed as mean ± SEM. *P<0.05 difference compared to NG group.

Fig. 2. (A) The levels of nitrite in coronary microvascular endothelial cells (CMEC) grown under normoglycaemic (NG, 5.5 mM D-glucose) conditions and hyperglycaemic (22 mM D-glucose) conditions in the absence and presence of vitamins C and E alone or in combination. (B) The levels of superoxide anion (O_2^-) in coronary microvascular endothelial cells (CMEC) grown under normoglycaemic (NG, 5.5 mM D-glucose) conditions and hyperglycaemic (22 mM D-glucose) conditions in the absence and presence of vitamins C and E alone or in combination. Data from 4 separate experiments are expressed as mean ± SEM. *P<0.05 difference compared to NG group and $^{\#}$ P<0.05 difference compared to HG group.


A
eNOS activity (pmol L-citrulline/mg protein/min)
2.0
1.5
1.0
0.5
0.0
*
Basal
0.1 μM
1 μM
0.1 μM
1 μM
0.1+0.1 μM
1+1 μM
Vitamin C
Vitamin E
Vitamin C+E


B
iNOS activity (pmol L-citrulline/mg protein/min)
1.5
1.0
0.5
0.0
Basal
0.1 μM
1 μM
0.1 μM
1 μM
0.1+0.1 μM
1+1 μM
Vitamin C
Vitamin E
Vitamin C+E


A
Nitrite levels (nmol/mg protein)
20
15
10
5
0
NG
HG
0.1 μM
1 μM
0.1 μM
1 μM
0.1+0.1 μM
1+1 μM
Vitamin C
Vitamin E
Vitamin C+E

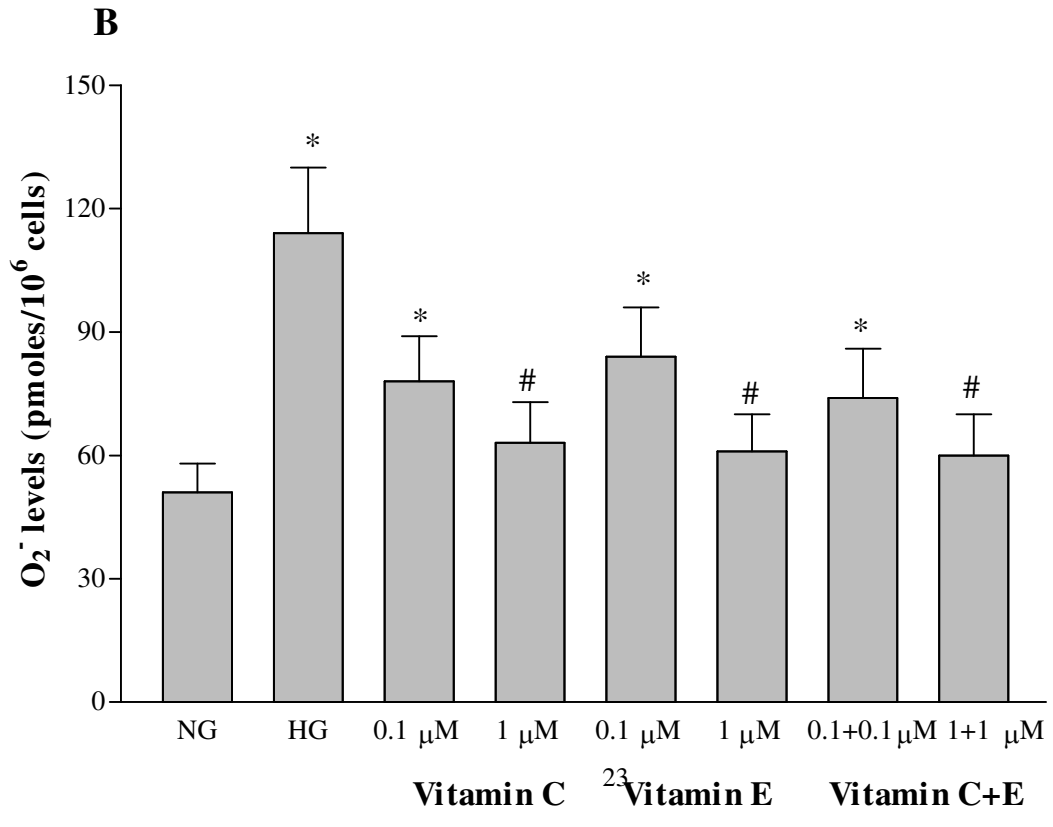

B
O2- levels (pmoles/10^6 cells)
150
120
90
60
30
0
NG
HG
0.1 μM
1 μM
0.1 μM
1 μM
0.1+0.1 μM
1+1 μM
Vitamin C
Vitamin E
Vitamin C+E

Table 1 Effects of antioxidant vitamins C and E on prooxidant (NAD(P)H oxidase) and antioxidant enzyme activities

	NAD(P)H Oxidase (pmoles/10^6 cells)	Total SOD (mU/mg protein)	Catalase (U/mg protein)	GPx (mU/mg protein)
5.5 mM Glucose (NG)	28 ± 3	862 ± 105	4.16 ± 0.63	239 ± 16
22 mM Glucose (HG)	59 ± 7*	1230 ± 145*	7.76 ± 0.91*	585 ± 24*
NG + 16.5 mM L-Glucose	26 ± 3	802 ± 80	4.65 ± 0.67	243 ±17
NG + 16.5 mM Mannitol	26 ± 4	821 ± 95	4.71 ± 0.60	256 ± 21
Vitamin C (0.1 μM)	33 ± 5†	395 ± 50*†	1.93 ±0.26*†	367 ± 18*†
Vitamin C (1 μM)	29 ± 4†	365 ± 43*†	1.94 ±0.20*†	350 ± 24*†
Vitamin E (0.1 μM)	35 ± 6†	425 ± 55*†	1.98 ± 0.28*†	351 ± 21*†
Vitamin E (1 μM)	26 ± 3†	413 ± 50*†	2.05 ±0.30*†	337 ± 26*†
Vitamin C +E (0.1 + 0.1 μM)	31 ± 5†	380 ± 55*†	2.00 ± 0.19*†	345 ± 11*†
Vitamin C + E (1 + 1 μM)	26 ± 4†	345 ± 40*†	1.89 ± 0.21*†	350 ± 15*†

Results are expressed as means ± S.E.M. (n=4). $^{*}P<0.05$ difference compared to NG group, $^{\dagger}P<0.05$ difference compared to HG group.

Table 2 Effect of NAD(P)H oxidase inhibitors on nitrite and O_2^- production and pro- (NAD(P)H oxidase) and anti-oxidant enzyme activities

	Nitrite (nmol/mg protein)	O_2^- levels (pmoles/10^6 cells)	NAD(P)H Oxidase (pmoles/10^6 cells)	Total SOD (mU/mg protein)	Catalase (U/mg protein)	GPx (mU/mg protein)
NG	14.21 ± 0.56	51 ± 7	28 ± 3	862 ± 105	6.10 ± 0.94	289 ± 53
HG	14.89 ± 0.63	114 ± 16*	61 ± 7*	1230 ± 145*	9.12 ± 1.11*	685 ± 73*
L-Glucose	14.09 ± 0.48	47 ± 6	26 ± 3	813 ± 80	6.27 ± 0.92	301 ± 55
Mannitol	13.91 ± 0.46	50 ± 6	26 ± 4	821 ± 95	5.95 ± 0.90	280 ± 48
PAO (0.1 μM)	16.36 ± 0.67*†	65 ± 8†	35 ± 5†	934 ± 95†	7.01 ± 0.83†	354 ± 59†
PAO (3 μM)	18.37 ± 0.72*†	56 ± 7†	32 ± 5†	878 ± 83†	6. 65 ± 0.76†	331 ± 50†
AEBSF (5 μM)	15.78 ± 0.61*†	71 ± 9†	37 ± 5†	975 ± 98†	7.01 ± 0.79†	365 ± 58†
AEBSF (100 μM)	16.03 ± 0.69*†	69 ± 8†	35 ± 5†	943 ± 86†	6.96 ± 0.77†	350 ± 45†

NG, 5.5 mM D-glucose; HG, 22 mM D-glucose; L-Glucose and Mannitol, 5.5 mM D-Glucose and 16.5 mM L-glucose or 16.5 mM mannitol, respectively; PAO, phenylarsine oxide; AEBSF, 4-(2-Aminoethyl)bebzenesulfonyl fluoride. Results are expressed as means ± S.E.M. (n=4). $^{*}P<0.05$ difference compared to NG group, $^{\dagger}P<0.05$ difference compared to HG group.