BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 159, 928–938

0007-1188/10
www.brjpharmacol.org

RESEARCH PAPER

Beneficial effect of the oligomerized polyphenol oligonol on high glucose-induced changes in eNOS phosphorylation and dephosphorylation in endothelial cells

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Background and purpose: Hyperglycaemia is known to reduce nitric oxide (NO) bioavailability by modulating endothelial NO synthase (eNOS) activity, and polyphenols are believed to have cardiovascular benefit. One possible mechanism could be through interaction with eNOS.
Experimental approach: The effects of the oligomerized polyphenol oligonol on eNOS phosphorylation status and activity were examined in porcine aortic endothelial cells cultured in high glucose concentrations.
Key results: Exposure to high glucose concentrations strongly inhibited eNOS phosphorylation at Ser-1177 and dephosphorylation at Thr-495 in bradykinin (BK)-stimulated cells. These inhibitory effects of high glucose were significantly prevented by treatment with oligonol. Akt and p38 mitogen-activated protein kinase (MAPK) were activated in BK-stimulated cells. High glucose inhibited Akt activation but enhanced p38 MAPK activation, both of which were reversed by oligonol treatment. The phosphatidylinositol 3-kinase inhibitor wortmannin blocked the reversal by oligonol of phosphorylation at Ser-1177, but not dephosphorylation at Thr-495, in BK-stimulated cells exposed to high glucose. The effect of oligonol on BK dephosphorylation under high glucose was mimicked by protein kinase C (PKC) ε-neutralizing peptides. These data suggest that the effects of oligonol on high glucose-induced attenuation of eNOS Ser-1177 phosphorylation and Thr-495 dephosphorylation may be regulated by Akt activation and PKCε inhibition respectively. Oligonol also prevented high glucose-induced attenuation of BK-stimulated NO production.
Conclusions and implications: Oligonol prevented the impairment of eNOS activity induced by high glucose through reversing altered eNOS phosphorylation status. This mechanism may underlie the beneficial cardiovascular health effects of this oligomerized polyphenol.
British Journal of Pharmacology (2010) **159,** 928–938; doi:10.1111/j.1476-5381.2009.00594.x; published online 29 January 2010

Keywords: Akt; endothelial cells; endothelial nitric oxide synthase (eNOS); hyperglycaemia; polyphenol; protein kinase C (PKC)

Abbreviations: BH4, tetrahydrobiopterin; BK, bradykinin; BSA, bovine serum albumin; DAF-2 DA, 4,5-diaminofluorescein diacetate; DCFA, 2′7′-dichlorofluorescein diacetate; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; KRP, Krebs-Ringer's phosphate; LDL, low-density lipoprotein; NAME, N^G-nitro-arginine methyl ester; NO, nitric oxide; PBS, phosphate-buffered saline; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase

Introduction

It is widely held that cardiovascular disease is the leading cause of mortality in patients suffering from diabetes mellitus (Stamler *et al.*, 1993). Hyperglycaemia is one of the important pathogenic factors that have been put forward to explain the development of cardiovascular disease during diabetes (Bartnik *et al.*, 2007), and impaired nitric oxide (NO) bioavailability is said to result from high glucose levels (Cai and Harrison, 2000). NO is a pivotal component of cardiovascular homeostasis through regulation of vascular tone, arterial blood pressure, platelet activity, leukocyte adhesion to the endothelium, vascular smooth muscle cell proliferation and

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Received 11 June 2009; accepted 10 October 2009

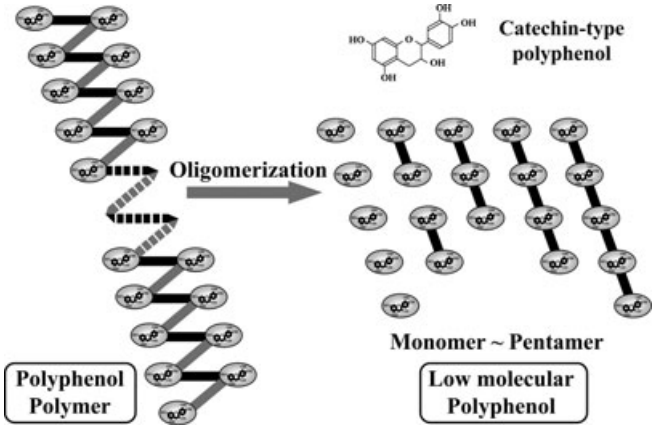

Figure 1 The schema of catechin-type polyphenol structure and the principles of polyphenol oligomerization. When polyphenol polymers are oligomerized, low molecular polyphenols, which are usually no more than pentamers, are formed. Monomer and oligomers are more readily absorbed from the intestine than the polymer.

angiogenesis (Albrecht *et al.*, 2003). Endothelial NO synthase (eNOS) is the major source of NO production in vascular endothelial cells, and this enzyme is subject to various forms of regulation including availability of cofactors and substrates, sub-cellular localization, protein–protein interaction, and phosphorylation (Govers and Rabelink, 2001).

Several lines of evidence have suggested that eNOS is regulated, in part, through the coordinated phosphorylation and dephosphorylation of the amino acid residues Ser-1177 and Thr-495 (Fulton *et al.*, 1999; Fleming *et al.*, 2001; Harris *et al.*, 2001; Michell *et al.*, 2001). Multiple stimuli, including growth factors and fluid shear stress, signal through the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway to activate eNOS by Ser-1177 phosphorylation (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999; Gallis *et al.*, 1999; Michell *et al.*, 1999; Hisamoto *et al.*, 2001; Montagnani *et al.*, 2001). On the other hand, phosphorylation of Thr-495 by AMP-activated protein kinase *in vitro* reduces eNOS activity (Chen *et al.*, 1999). Conversely, bradykinin (BK) can promote Thr-495 dephosphorylation, leading to an increase in eNOS activity (Fleming *et al.*, 2001; Harris *et al.*, 2001).

Polyphenols are believed to be the active principles of a wide range of medicinal plants used to provide cardiovascular protection. Red wine and green tea polyphenols have been shown to induce eNOS-mediated endothelium-dependent relaxation in rat aortic rings (Andriambeloson *et al.*, 1997; Lorenz *et al.*, 2004). Furthermore, red wine polyphenols can enhance eNOS expression in human umbilical vein endothelial cells (Leikert *et al.*, 2002), suggesting that polyphenols may not only activate the enzyme but also increase its levels. Interestingly, chronic oral treatment with green tea polyphenols has been demonstrated to improve insulin sensitivity and to raise plasma adiponectin levels in spontaneously hypertensive rats (Potenza *et al.*, 2007). Therefore, polyphenols may provide safe and effective adjunctive treatment of diabetes and its cardiovascular complications because of the improvement of endothelial dysfunction and insulin resistance.

Oligonol (Figure 1) is a novel biotechnology product derived from the oligomerization of polyphenols, typically proanthocyanidins from a variety of fruits including lychees, grapes, apples and persimmons. Although fruits and plants originally have only low levels of oligomeric proanthocyanidins, the process of oligomerization enables the production of oligonol which contains mainly catechin-type monomers and oligomeric proanthocyanidins (Aruoma *et al.*, 2006). It has been shown that oligonol exhibits significant protection against β-amyloid- and high glucose-induced cytotoxicity in rat pheochromocytoma PC12 cells and the porcine proximal tubule cell line LLC-PK_1 respectively (Li *et al.*, 2004; Fujii *et al.*, 2006).

The purpose of the present study was to examine the effect of oligonol on high glucose-induced modulation of eNOS phosphorylation status and activity in endothelial cells and to delineate the signalling mechanism involved. The present study used BK as a stimulant, which is a well-known endogenous vasoactive peptide that promotes vasodilation by stimulating the release of NO from endothelial cells via eNOS activation. Here we have demonstrated that the oligomerized polyphenol oligonol can restore NO release from porcine aortic endothelial cells in response to BK, impaired under high-glucose conditions, through improvement of the coordinated regulation of the phosphorylation status of the enzyme.

Methods

Cell culture

Porcine aortas were obtained from a slaughterhouse and preserved in phosphate-buffered saline (PBS). Porcine aortic endothelial cells were isolated as previously described (Tomioka *et al.*, 2001) by gently scraping the intima of the descending part of porcine aortas. After centrifugation at 250 *g* for 10 min in M199 solution, the pellet of endothelial cells was purified from this suspension, resuspended in M199 solution supplemented with 100 U mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin, and 10% fetal bovine serum (FBS), then aliquoted into polybiphenyl dishes, fixed on 10 × 10-mm glass coverslips, and incubated at 37°C in 5% CO_2. The medium was renewed every day. Cells were identified as endothelial cells, as described in our previous report (Tomioka *et al.*, 2001). Thus, cultured cells were virtually free of contaminating cells as indicated by staining with diiodoacetyl-low-density lipoprotein (LDL); ~99% of the cells took up diiodoacetyl-LDL. The percentage of cells taking up diiodoacetyl-LDL was determined when the nuclei became visible under bisbenzimide staining. Cells were seeded into 6- or 24-well plates and used at two to five passages. Before the experimental procedure was started, the medium was removed and replaced with phenol red-free M199 supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were growth-arrested by removal of FBS for 24 h. After washing twice in PBS, they were incubated with two different glucose concentrations (5.6 and 22.4 mM) for 5 h. We defined the control as the status under 5.6 mM glucose concentration, which is the same concentration as normal human plasma glucose. Mannitol was used to rule out the effect of osmotic pressure and was confirmed to produce substantially the same results as the control. To perform robust statistical analyses,

three independent replicate experiments were performed on each of the aortic cells from three or four animals using a separate stock of cells for each experiment.

Imaging of intracellular NO and reactive oxygen species (ROS) by fluorescence microscopy

To detect NO production, we loaded endothelial cells with 4,5-diaminofluorescein diacetate (DAF-2 DA; Sekisui Chemical CO., Osaka, Japan). This membrane-permeable dye is hydrolysed intracellularly by cytosolic esterases releasing DAF-2, which is converted in the presence of NO into a fluorescent product, DAF-2 triazole (Kojima *et al.*, 1998; Nakatsubo *et al.*, 1998). The DAF-2 DA was loaded at a concentration of 10 μM in Krebs-Ringer's phosphate (KRP) buffer [composition (mM) 154 NaCl, 5.6 KCl, 1.1 $MgSO_4$, 2.2 $CaCl_2$, 0.85 NaH_2PO_4, 2.15 Na_2HPO_4, 5.6 glucose and 0.5% bovine serum albumin (BSA); pH 7.4] in the dark at 37°C for 30 min. The KRP buffer contained either 5.6 or 22.4 mM glucose as well as 100 μM L-arginine. When N^G-nitro-L-arginine methyl ester (L-NAME) or N^G-nitro-D-arginine methyl ester (D-NAME) was used, at a concentration of 100 μM, it was added with DAF-2 DA. To detect intracellular ROS, we loaded endothelial cells with 2′7′-dichlorofluorescein diacetate (DCFA; final concentration 10 μM) for 30 min in PBS at 37°C in the dark. At the end of an experiment, cells were rinsed with PBS and then harvested by trypsinization followed by resuspension in 1 mL of PBS. This cell suspension was mixed with 0.1 M Tris (pH 8.0) and analysed with an F-2000 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan). Background fluorescence was subtracted and values were normalized to cell number.

Superoxide dismutase (SOD) activity assay

Endothelial cells were harvested by adding lysis buffer to the well and were pelleted by centrifugation at 12 000 *g* for 10 min at 4°C. The protein concentrations of the supernatants were determined by the method of Lowry *et al.* (1951) using BSA standard. SOD activity was measured from 50 μL of supernatant with a commercially available SOD assay kit (Wako Pure Chemical, Osaka, Japan), as described in the manufacturer's manual.

Western blot analysis

Immunoblotting was performed basically according to the method described previously (Matsuda *et al.*, 2003; 2006). Briefly, each sample of cell extraction (10–20 μg of protein) was run on 8–10% SDS-polyacrylamide gel and transferred to a polyvinylidine difluoride filter membrane. The membrane was blocked with 1% BSA in PBS containing 0.1% Tween 20 for 1 h at room temperature, followed by overnight incubation with primary antibody at 4°C. The membrane was washed three times with PBS-Tween buffer and incubated with horseradish-conjugated secondary antibody, which was an anti-rabbit or anti-mouse IgG (1:5000 dilution with PBS-Tween buffer; Bio-Rad Laboratories, Hercules, CA, USA), for 1 h at room temperature. After being washed three times in PBS-Tween buffer, the blots were visualized using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA), exposed to X-ray film and analysed by Image J (National Institutes of Health, Bethesda, MD, USA).

The following antibodies, which are commercially available, were used: anti-human eNOS mouse monoclonal antibody (BD Biosciences, San Jose, CA, USA), anti-human phospho-eNOS (Ser-1177) rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human phospho-eNOS (Thr-495) rabbit monoclonal antibody (Upstate Technology, Lake Placid, NY, USA), anti-human Akt rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA), anti-mouse phospho-Akt (Ser-473) rabbit polyclonal antibody (Cell Signaling Technology), anti-human p38 mitogen-activated protein kinase (MAPK) rabbit polyclonal antibody (Cell Signaling Technology), anti-human phospho-p38 MAPK (Thr-180/Tyr-182) rabbit polyclonal antibody (Cell Signaling Technology) and anti-mouse β-actin mouse monoclonal antibody (Abcam, Cambridge, UK).

Statistical analysis

Data are expressed as means ± s.e.mean. Statistical assessment of the data was performed using Student's *t*-test or a repeated-measure one-way ANOVA followed by Neeman's multiple comparison test when appropriate. A P value of <0.05 was considered to be statistically significant.

Materials

Oligonol (Figure 1) used in this study is a catechin-type polyphenolic compound that was extracted from lychee fruits and purified, as described previously (Fujii *et al.*, 2007). Oligonol was of highest purity (>95%) and contained ~30–50% oligomeric polyphenols. L-NAME was from Cayman Chemical, Ann Arbor, MI, USA.; D-NAME was from BIOMOL, Plymouth Meeting, PA, USA; and bradykinin was from Sigma-Aldrich, St. Louis, MO, USA.

Results

Changes in the phosphorylation status of eNOS

In unstimulated porcine aortic endothelial cells, eNOS was strongly phosphorylated at Thr-495 but only weakly at Ser-1177 (Figure 2). Stimulation of endothelial cells with BK (30 nM) elicited a transient increase in phosphorylation of Ser-1177, peaking at 5 min and returning to the control level within 15 min. On the other hand, BK stimulation of endothelial cells resulted in gradual dephosphorylation of eNOS at Thr-495. A significant decrease in Thr-495 phosphorylation occurred at 5 min after BK stimulation. A maximum effect was observed at 15 min, after which it appeared to be re-phosphorylated. Differences observed were not attributed to differences in the total amount of eNOS protein because equal amounts of eNOS protein were detected for all time points when every blot was reprobed with a non-phospho-specific anti-eNOS-antibody recognizing total eNOS.

Under high-glucose conditions (22.4 mM glucose), BK induced a significantly smaller increase in eNOS phosphory-

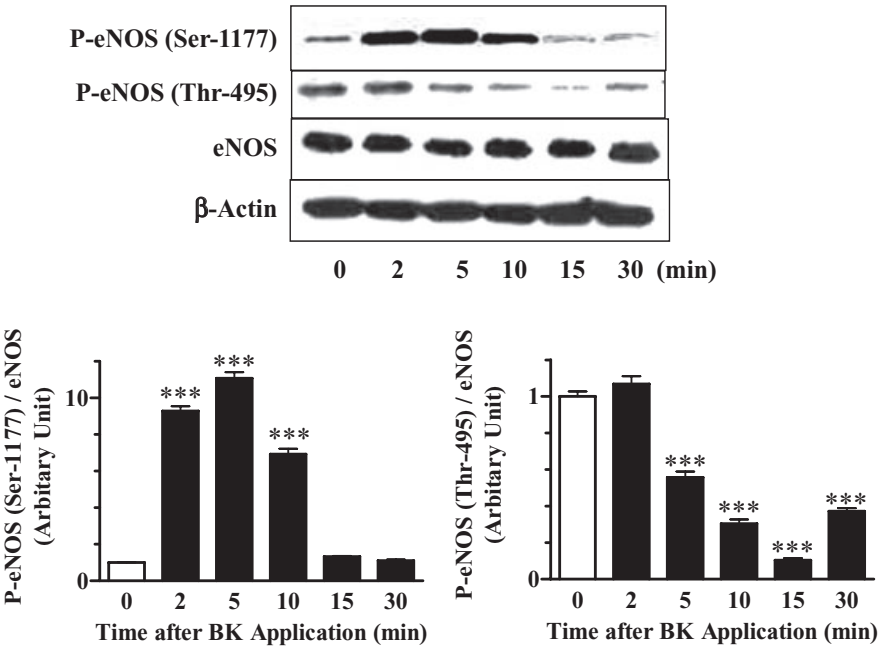

Figure 2 Time course of BK-induced eNOS phosphorylation at Ser-1177 and dephosphorylation at Thr-495 in porcine aortic endothelial cells under normal glucose concentrations. Representative Western blots and statistical analysis showing the effect of BK (30 nM) on the phosphorylation status of eNOS are presented. No apparent change in total eNOS protein after BK stimulation was noted. To ensure equal loading of each lane, β-actin served as loading control, and relative phosphorylation was quantified densitometrically based on the ratio of phospho-eNOS/β-actin. Values are means ± s.e.mean of three separate experiments. ***$P < 0.001$ versus zero time. BK, bradykinin; eNOS, endothelial nitric oxide synthase.

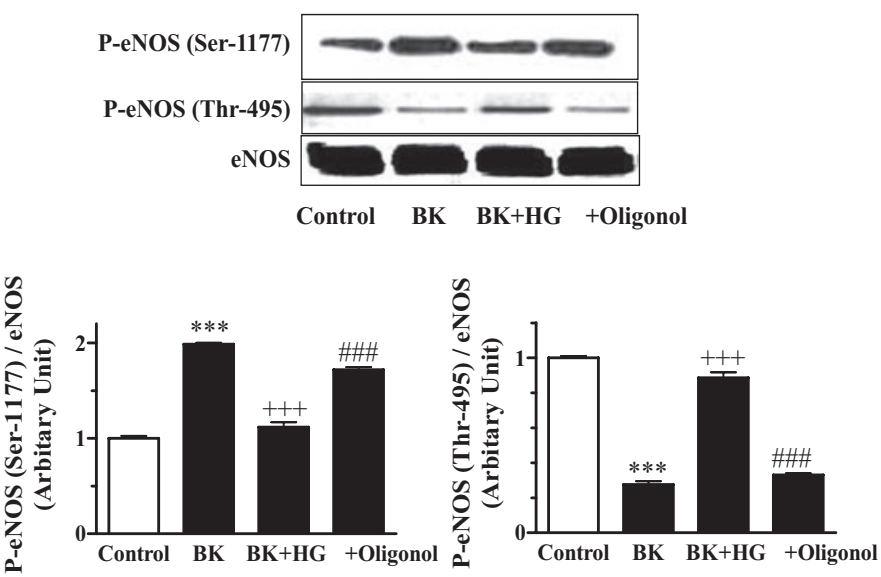

Figure 3 Effect of oligonol treatment on alterations in BK-induced eNOS phosphorylation at Ser-1177 and dephosphorylation at Thr-495 in porcine aortic endothelial cells exposed to high glucose. Representative Western blots and statistical analysis showing the changes in the effect of BK (30 nM) on Ser-1177 phosphorylation at 10 min after its application when cells were untreated and treated with 100 μg·mL^{-1} oligonol from 5 h before BK under high-glucose (HG) conditions. Values are means ± s.e.mean of three separate experiments and are expressed as percentage of the respective control (normal glucose) result without any treatment. ***$P < 0.001$ versus control. +++$P < 0.001$ versus BK stimulation under normal glucose. ###$P < 0.001$ versus BK stimulation under HG. BK, bradykinin.

lation at Ser-1177 than that observed in normal glucose medium (Figure 3). When endothelial cells were treated with the oligomerized polyphenol oligonol at a dose of 100 μg mL^{-1}, high glucose-induced attenuation of BK-stimulated phosphorylation at Ser-1177 was significantly prevented.

Exposure of endothelial cells to high glucose strikingly blocked BK-stimulated dephosphorylation of eNOS at Thr-495 (Figure 3). Treatment with oligonol resulted in a complete preservation of the ability of BK to induce eNOS dephosphorylation at Thr-495.

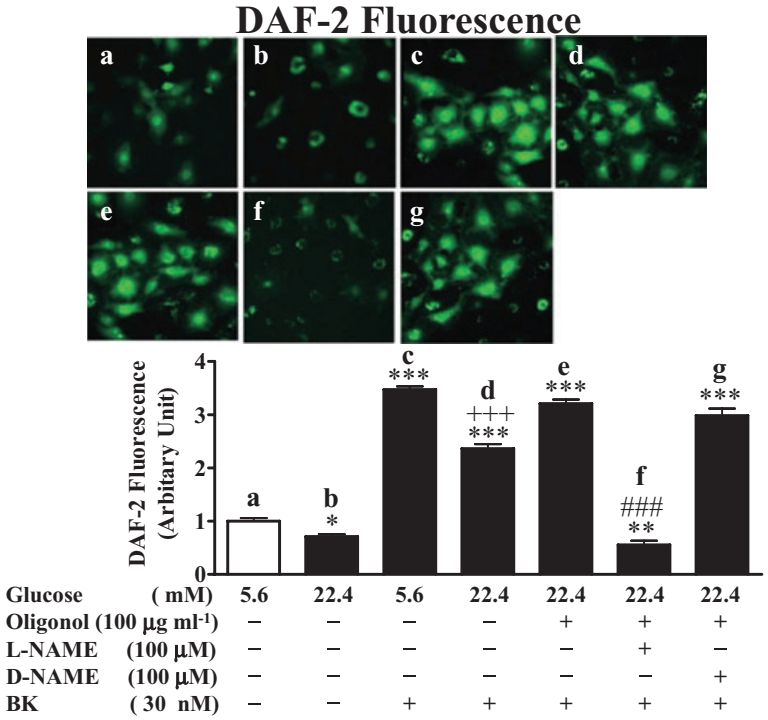

Figure 4 Effect of oligonol treatment on BK-stimulated NO production from porcine aortic endothelial cells under high-glucose conditions. Cells were incubated for 5 h with 5.6 or 22.4 mM glucose. Where indicated, oligonol (100 μg·mL^{-1}), L-NAME (100 μM), or D-NAME (100 μM) was present in high-glucose medium. We loaded endothelial cells with the NO-sensitive dye DAF-2 DA and examined them 10 min after BK stimulation by fluorescence microscopy. Actual micrographs showing the fluorescence signal are presented on the upper panels. Values are means ± s.e.mean of four separate experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 versus non-stimulated cells under normal glucose. +++P < 0.001 versus BK stimulation under normal glucose. ###P < 0.001 versus BK stimulation in the presence of oligonol under high glucose. BK, bradykinin; DAF-2 DA, 4,5-diaminofluorescein diacetate; NO, nitric oxide.

NO production from endothelial cells

To assess whether oligonol treatment can counteract changes in BK stimulation of eNOS activity under high-glucose conditions, we examined the levels of NO, which was produced from endothelial cells 10 min after stimulation with BK, using the fluorescent dye DAF-2 DA (Figure 4). In normal glucose medium, the NO level increased, approximately 3.5-fold from unstimulated control, in response to BK. However, BK-stimulated NO production from endothelial cells significantly declined under high-glucose conditions. Treatment with oligonol at a dose of 100 μg mL^{-1} showed a significant recovery of the decline in NO production from endothelial cells stimulated with BK due to high levels of glucose. This response of NO production was sensitive to L-NAME but unaffected by D-NAME.

Changes in SOD activity and ROS production

Hyperglycaemia may lead to glycosylation of the radical-scavenging enzyme SOD, thereby rendering it inactive and consequently elevating superoxide anion levels (Arai *et al.*, 1987). Whether oligonol treatment has a significant effect on the high glucose-induced changes in the activity of this endogenous antioxidant enzyme was investigated. Endothelial cells exposed to high glucose exhibited a marked decrease in SOD activity (Figure 5). Treatment with oligonol restored the activity of this antioxidant enzyme in a dose-dependent manner.

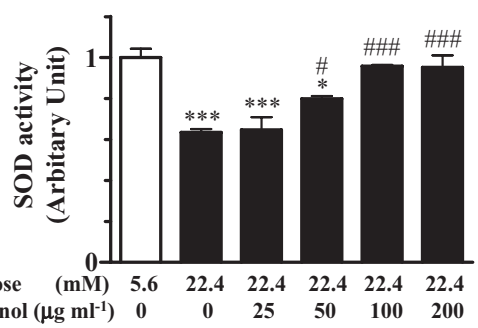

Figure 5 Effect of oligonol treatment on SOD activity in porcine aortic endothelial cells under high-glucose conditions. Cells were incubated for 5 h with 5.6 or 22.4 mM glucose. Where indicated, oligonol was present at doses of 25, 50, 100, and 200 μg·mL^{-1} in high-glucose medium. Values are means ± s.e.mean of three separate experiments. *P < 0.05, ***P < 0.001 versus normal glucose. #P < 0.05, ###P < 0.001 versus high glucose. SOD, superoxide dismutase.

Intracellular ROS were visualized using the fluorescent dye DCFA. Cells exposed to high glucose had a 1.2-fold increase in intracellular fluorescence (Figure 6). Oligonol, in a dose-dependent manner, prevented this increase in ROS-induced intracellular fluorescence under high-glucose conditions.

Signalling pathways involved in the effects of oligonol on altered eNOS phosphorylation under high glucose

Phosphorylation of eNOS at Ser-1177 is characteristic of the serine/threonine kinase Akt (Dimmeler *et al.*, 1999; Fulton

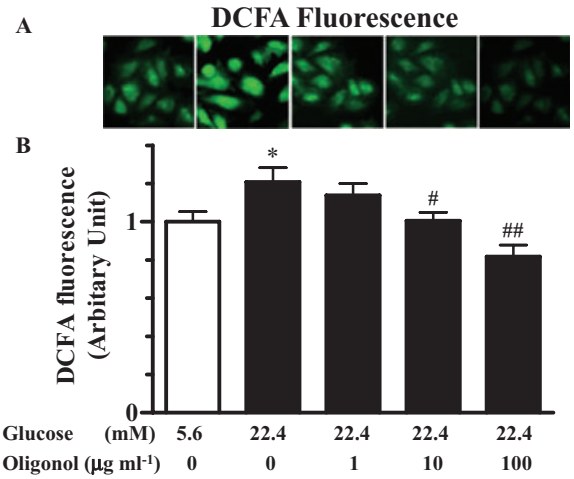

Figure 6 Effect of oligonol treatment on DCFA fluorescence in porcine aortic endothelial cells under high-glucose conditions. (A) Representative images indicating intracellular reactive oxygen species visualization with the use of DCFA. (B) Averaged values shown in bar graphs. Cells were incubated for 5 h with 5.6 or 22.4 mM glucose. Where indicated, oligonol was present at doses of 1, 10 and 100 μg·mL^{-1} in high-glucose medium. Values are means ± s.e.mean of three separate experiments. *$P < 0.05$ versus normal glucose. #$P < 0.05$, ##$P < 0.01$ versus high glucose. DCFA, 2′7′-dichlorofluorescein diacetate.

et al., 1999; Gallis *et al.*, 1999; Michell *et al.*, 1999). When Akt activation was assessed by immunoblotting endothelial cell lysates with an antibody specific for activated Akt (i.e. phosphorylated at Ser-473), we observed that stimulation of 30 nM BK resulted in a time-dependent increase in activation of Akt (Figure 7A). BK-induced Akt activation was significantly reduced under high-glucose conditions, as indicated by a significant decrease in Ser-473 phosphorylation of Akt (Figure 7B). This reduction in BK-stimulated activation of Akt was prevented by treatment with oligonol. The total expression levels of Akt in endothelial cells were unchanged by BK regardless of whether the glucose level was normal or high.

Previous reports have implicated MAPKs in eNOS activation by high-density lipoprotein (Mineo *et al.*, 2003) and by hydrogen peroxide (Cai *et al.*, 2003). Furthermore, activation of p38 MAPK has been demonstrated to lead to an Akt-dependent increase in eNOS activity (Anter *et al.*, 2004). When activation of p38 MAPK was assessed by immunoblotting endothelial cell lysates with an antibody specific for the phosphorylated form of p38 MAPK (Thr-180/Tyr-182), we found that BK stimulated a transient increase in p38 MAPK activation with maximum activity observed at 5 min (Figure 8A). Exposure to high glucose significantly enhanced BK-induced phosphorylation of p38 MAPK, which was reversed to the phosphorylation levels observed in normal glucose medium by treatment with oligonol (Figure 8B). None of BK, high glucose or oligonol treatments had any influence on total expression levels of p38 MAPK.

Because Akt functions as a downstream effector of PI3-K (Fruman *et al.*, 1998), we examined the effect of the PI3-K inhibitor wortmannin and inhibition of p38 MAPK with SB202190 in our experimental system. Effective but relatively low concentrations of wortmannin (5 nM) and SB202190 (5 μM) were used by themselves to assess any direct effects on BK-induced eNOS phosphorylation and dephosphorylation. We found that treatment with neither wortmannin nor SB202190 had a significant effect on BK-mediated Ser-1177 phosphorylation and Thr-495 dephosphorylation of eNOS at the concentrations used in this study (data not shown). However, as shown in Figure 9, treatment with wortmannin significantly blocked the ability of oligonol to prevent high glucose-induced attenuation of BK-stimulated eNOS Ser-1177 phosphorylation. In contrast, the ability of oligonol to prevent high glucose-induced attenuation of BK-stimulated eNOS Thr-475 dephosphorylation was maintained in the presence of wortmannin. SB202190 marginally prevented both of the effects of oligonol on phosphorylation of Ser-1177 and on dephosphorylation of Thr-495.

As protein kinase C (PKC) may be one of the important factors for eNOS regulation in hyperglycaemia (Fleming *et al.*, 2001; Michell *et al.*, 2001), we investigated the possible role of PKC in the effect of oligonol on the BK regulation of eNOS phosphorylation under high glucose (Figure 10). The pan-PKC isoform inhibitor B3806 (5 μM) blocked high glucose-induced attenuation of BK-stimulated eNOS Thr-475 dephosphorylation, as did oligonol. B3806 was without effect on the high glucose-induced decrease in BK-stimulated eNOS Ser-1177 phosphorylation (data not shown). The same effect was obtained with PKCε-neutralizing peptides (5 μM). Neither PKCζ-neutralizing peptides (10 μM) nor the conventional isoform inhibitor Gö6976 (100 nM) mimicked the effect of oligonol on BK-stimulated eNOS de-phosphorylation in high-glucose media.

Discussion

Hyperglycaemic impairment of endothelial function and consequent NO production contributes to cardiovascular disorders in diabetic patients. It has been well documented that experimental hyperglycaemia diminishes basal and stimulated NO production in cultured endothelial cells (Ding *et al.*, 2000; 2004; Du *et al.*, 2001). In this study, we observed that BK-stimulated NO production was significantly attenuated in porcine aortic endothelial cells exposed to high glucose (22.4 mM) for 5 h. It has been proposed that phosphorylation of Ser-1177 and dephosphorylation of Thr-495 determine the activity of eNOS in BK-stimulated endothelial cells, leading to increased NO production (Fleming *et al.*, 2001). We found that BK-induced Ser-1177 phosphorylation of eNOS was markedly decreased by high glucose. This is in good agreement with the results of other investigators using bovine aortic and human umbilical vein endothelial cells (Du *et al.*, 2001; Schnyder *et al.*, 2002). On the other hand, we showed that exposure of endothelial cells to high glucose also had a strongly inhibitory effect on BK-stimulated eNOS dephosphorylation at Thr-495. To the best of our knowledge, this is the first report demonstrating that the regulation of eNOS activity by agonist-induced dephosphorylation at Thr-495 is disturbed under hyperglycaemic conditions.

Several laboratories have indicated that eNOS serine phosphorylation is catalysed by the Akt protein kinase.

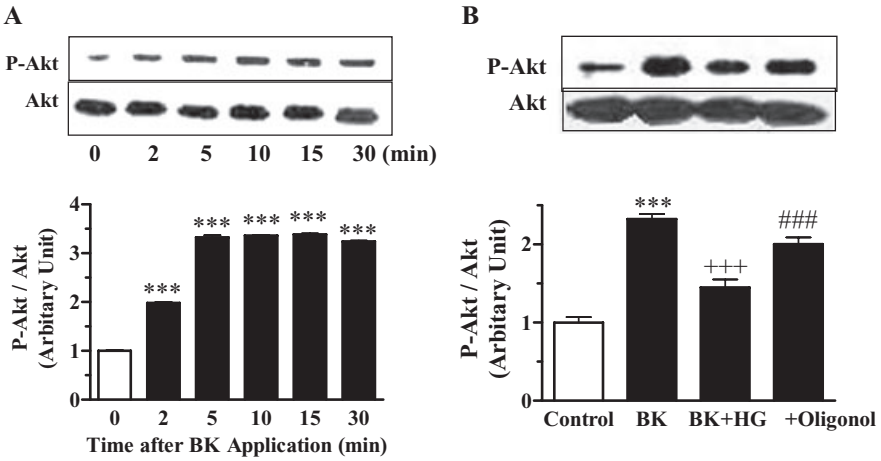

Figure 7 Effect of oligonol treatment on alternations in BK-induced activation of Akt in porcine aortic endothelial cells exposed to high glucose (HG). (A) Representative Western blots and statistical analysis showing the time course of changes in Akt phosphorylation at Ser-473 and total Akt after application of 30 nM BK under normal glucose. No apparent change in total Akt protein after BK stimulation was noted. Values are means ± s.e.mean of three separate experiments and are expressed as percentage of the respective result before BK application. ***P < 0.001 versus zero time. (B) Representative Western blots and statistical analysis showing the changes in the effect of BK (30 nM) on Ser-473 phosphorylation of Akt at 5 min after its application when cells were untreated and treated with 100 μg·mL^{-1} oligonol from 5 h before BK under HG conditions. Total Akt remained unchanged. Values are means ± s.e.mean of three separate experiments and the data represent phospho-Akt/Akt expressed relative to the respective control (normal glucose) result without any treatment. ***P < 0.001 versus control. +++P < 0.001 versus BK under normal glucose. ###P < 0.001 versus BK under HG. BK, bradykinin.

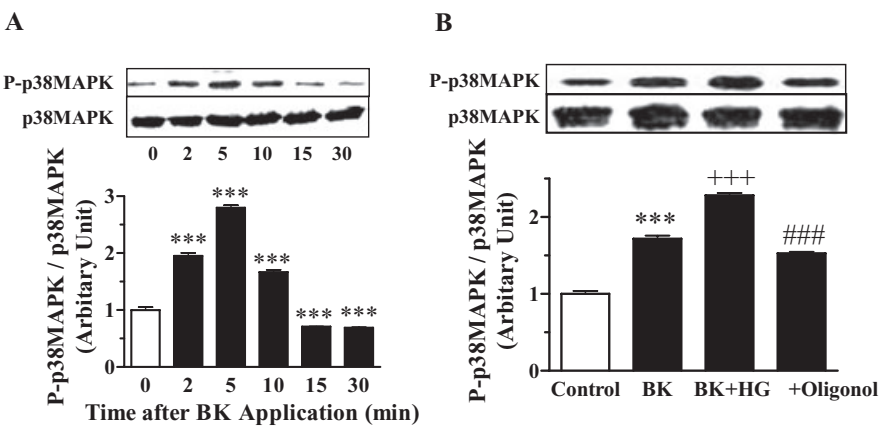

Figure 8 Effect of oligonol treatment on alterations in BK-induced activation of p38 MAPK in porcine aortic endothelial cells exposed to high glucose (HG). (A) Representative Western blots and statistical analysis showing the time course of changes in p38 MAPK phosphorylation at Thr-180/Tyr-182 after application of 30 nM BK. No apparent change in total p38 MAPK protein after BK stimulation was noted. Values are means ± s.e.mean of three separate experiments and the data represent phospho-p38 MAPK/p38 MAPK expressed relative to the respective result before BK application. ***P < 0.001 versus zero time. (B) Representative Western blots and statistical analysis showing the changes in the effect of BK (30 nM) on Thr-180/Tyr-182 phosphorylation of p38 MAPK at 5 min after its application when cells were untreated and treated with 100 μg·mL^{-1} oligonol from 5 h before BK under HG conditions. Total p38 MAPK remained unchanged. Values are means ± s.e.mean of three separate experiments and the data represent phospho-p38 MAPK/p38 MAPK expressed relative to the respective control (normal glucose) result without any treatment. ***P < 0.001 versus control. +++P < 0.001 versus BK under normal glucose. ###P < 0.001 versus BK under HG. BK, bradykinin; MAPK, mitogen-activated protein kinase.

Akt-mediated serine phosphorylation of eNOS in endothelial cells is thus stimulated by vascular endothelial growth factor, insulin-like growth factor-1 and insulin (Fulton *et al.*, 1999; Michell *et al.*, 1999; Kim *et al.*, 2001). Signal transduction pathway of growth factor receptors, however, can largely differ from those of G-protein-coupled receptors such as BK receptors. Indeed, although BK activated Akt in HeLa cells (Xie *et al.*, 2000), whether eNOS is phosphorylated by Akt at Ser-1177 in BK-stimulated endothelial cells was quite doubtful (Fleming *et al.*, 2001). And yet, strong evidence has been clearly provided that BK-stimulated Akt activation results in serine phosphorylation of eNOS in bovine aortic endothelial cells (Harris *et al.*, 2001). Consistent with this, the present study showed that BK significantly stimulated a time-dependent increase in Akt activity in porcine aortic endothelial cells. Importantly, exposure to high glucose strongly inhibited BK-stimulated Akt activation, suggesting that the impairment of BK stimulation of eNOS Ser-1177 phosphorylation under high-glucose conditions may be the result of the inhibition of BK-induced Akt activation.

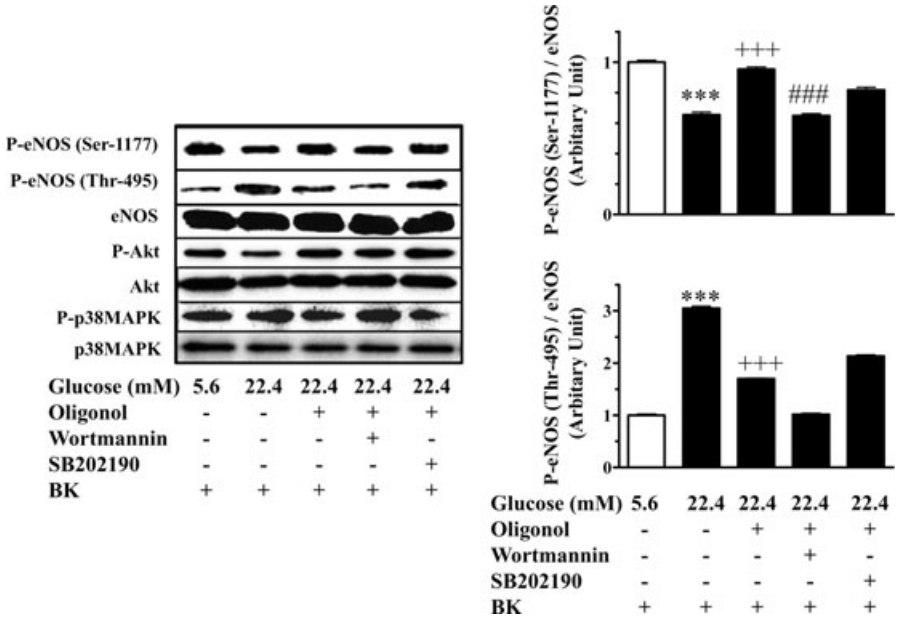

Figure 9 Effects of wortmannin and SB202190 on the oligonol effect on alterations in BK-induced Ser-1177 and Thr-495 phosphorylation of eNOS in porcine aortic endothelial cells exposed to high glucose. Representative Western blots and statistical analysis showing the changes in the effect of BK (30 nM) on Ser-1177 and Thr-495 phosphorylation at 5 min after its application when cells were untreated and treated with 100 μg·mL^{-1} oligonol from 5 h before BK under high-glucose (22.4 mM) conditions in the absence and presence of 5 nM wortmannin or 5 μM SB202190. Wortmannin and SB202190 were added 30 min before BK challenge. Typical Western blots include the data showing that wortmannin and SB202190 effectively inhibited phosphorylation of Akt and of p38 MAPK respectively. Values are means ± s.e.mean of three separate experiments and the data represent phospho-eNOS/eNOS expressed relative to the respective result with BK stimulation under normal-glucose (5.6 mM) conditions. ***$P < 0.001$ versus BK under normal glucose. +++$P < 0.001$ versus BK under high glucose. ###$P < 0.001$ versus BK under high glucose and oligonol treatment. BK, bradykinin; eNOS, endothelial nitric oxide synthase.

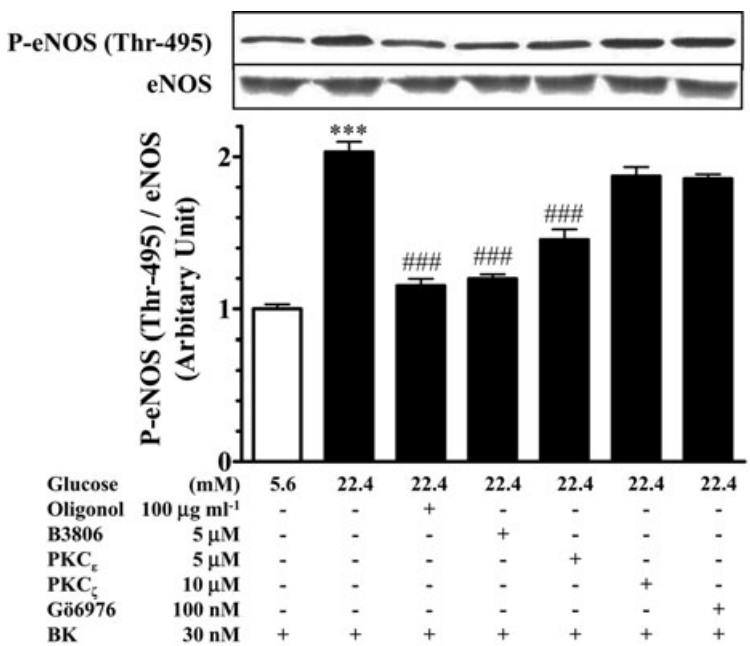

Figure 10 Effects of the pan-PKC isoform inhibitor B3806, the conventional isoform inhibitor Gö6976, PKCε-neutralizing peptides and PKCζ-neutralizing peptides on BK-induced Thr-495 dephosphorylation of eNOS in porcine aortic endothelial cells exposed to high glucose. Representative Western blots and statistical analysis showing the changes in the effect of BK (30 nM) on Thr-495 dephosphorylation at 5 min after its application under high-glucose (22.4 mM) conditions in the absence and presence of 100 μg·mL^{-1} oligonol, 5 μM B3806, 5 μM PKCε-neutralizing peptides, 10 μM PKCζ-neutralizing peptides or 100 nM Gö6976. PKC inhibitors and neutralizing peptides were added 30 min before BK challenge. Values are means ± s.e.mean of three separate experiments and the data represent phospho-eNOS/eNOS expressed relative to the respective result with BK stimulation under normal-glucose (5.6 mM) conditions. ***$P < 0.001$ versus BK under normal glucose. ###$P < 0.001$ versus BK under high glucose. BK, bradykinin; eNOS, endothelial nitric oxide synthase; PKC, protein kinase C; SOD, superoxide dismutase.

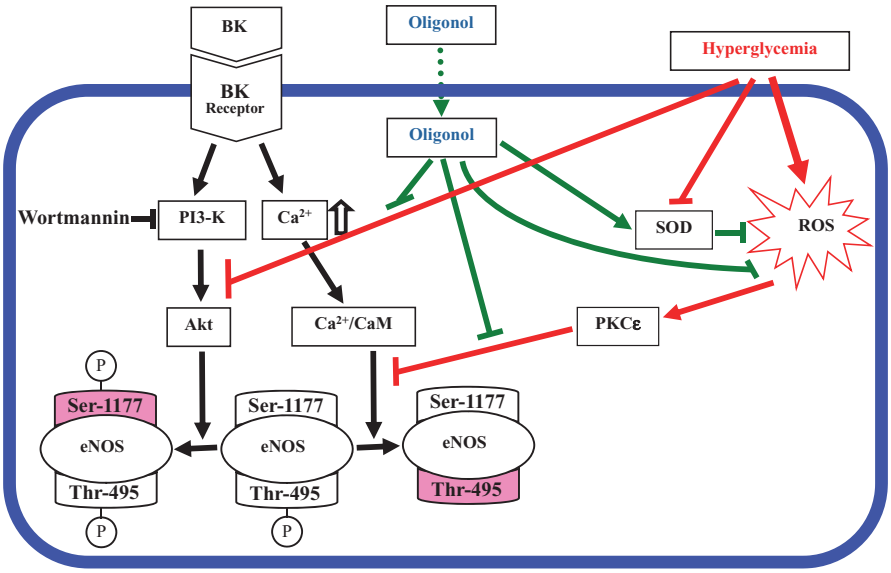

Figure 11 Schematic diagram of the signalling pathways in vascular endothelial cells that contribute to the beneficial effects of oligonol on hyperglycaemia-induced eNOS dysfunction. BK activates eNOS by phosphorylation of Ser-1177 via the PI3-K/Akt pathway and by dephosphorylation of Thr-495 possibly due to the Ca^{2+}/calmodulin (CaM)-dependent pathway. Hyperglycaemia reduces BK-stimulated phosphorylation of eNOS at Ser-1177 by inhibiting the PI3-K/Akt pathway. Oligonol reverses activation of the PI3-K/Akt pathway leading to the improved phosphorylation level of eNOS at Ser-1177. Hyperglycaemia also generates ROS with inactivation of SOD. ROS are upstream signalling molecules to PKCε which is involved in the hyperglycaemia-induced blockade of dephosphorylation of eNOS at Thr-495. Oligonol upregulates SOD and counteracts the action of PKCε. BK, bradykinin; eNOS, endothelial nitric oxide synthase; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase.

The recent work has identified p38 MAPK as an upstream mediator of Akt-dependent eNOS activation in endothelial cells (Anter *et al.*, 2004). We found that BK exerted transient phosphorylation of p38 MAPK, which was temporally similar to eNOS phosphorylation at Ser-1177. However, hyperglycaemic conditions led to a further increase in the activated forms of p38 MAPK despite the marked inhibition of eNOS Ser-1177 phosphorylation in BK-stimulated endothelial cells. This can be supported by a great deal of evidence showing high glucose-mediated activation of p38 MAPK in endothelial cells (Liu *et al.*, 2000; McGinn *et al.*, 2003; Takaishi *et al.*, 2003). In this regard, it is interesting to note an earlier report that Akt can down-regulate p38 MAPK in endothelial cells (Gratton *et al.*, 2001). Therefore, it would be logical to conclude that activation of the p38 MAPK pathway does not play a crucial role in the regulation of Ser-1177 phosphorylation of eNOS in BK-stimulated endothelial cells.

The regulation of the constitutive phosphorylation of a second residue, that is, eNOS Thr-495, remains to be definitely established. Because growth factors do not stimulate dephosphorylation of eNOS at Thr-495 (Harris *et al.*, 2001), signalling through the PI3-K/Akt pathway does not appear to be relevant for the regulation of eNOS Thr-495 phosphorylation. Instead, signalling through PKC has been proposed to be involved in Thr-495 phosphorylation of eNOS. Indeed, the PKC inhibitor Ro-318220 could attenuate the basal level of phosphorylation of eNOS Thr-495, as well as rephosphorylation of this site after completion of BK stimulation in endothelial cells (Fleming *et al.*, 2001; Michell *et al.*, 2001). PKC may be one of the important factors for eNOS regulation in hyperglycaemia. High glucose is widely held to cause a variety of effects on endothelial cells via activation of PKC (Hempel *et al.*, 1997; Consentino *et al.*, 2003; Chu and Bohlen, 2004). Our results with PKC inhibitors and specific PKC isoform neutralizing peptides indicate that the novel isoform PKCε may be involved in the high glucose-induced blockade of BK-stimulated dephosphorylation of eNOS at Thr-495.

Interestingly, oligonol treatment resulted in a significant improvement of diminished Akt activity in BK-stimulated cells exposed to high glucose. Moreover, the PI3-K inhibitor wortmannin significantly blocked the reversal by oligonol of the high glucose-induced inhibition of BK stimulation of phosphorylation of eNOS at Ser-1177. These data support the view that this reversal by oligonol may be, in part, regulated by Akt activation. A recent report has shown that black tea polyphenols lead to p38 MAPK-mediated activation of the PI3-K/Akt pathway which appears to involve coordinated eNOS phosphorylation at Ser-1177 and dephosphorylation at Thr-495 (Anter *et al.*, 2004). This does not appear to be the case in our system, as pharmacological inhibition of p38 MAPK with SB202190 displayed only a marginal inhibition of the reversal by oligonol on the two eNOS phosphorylation events under high-glucose conditions. Wortmannin enhanced rather than prevented the inhibition by oligonol of increased phosphorylation at Thr-495 seen under hyperglycaemic conditions, implying no involvement of Akt activation in this oligonol effect. While PKC is known to generate ROS, high glucose-induced PKC activation in glomerular mesangial cells (Studer *et al.*, 1997; Hua *et al.*, 2003) and aortic endothelial cells (Nishikawa *et al.*, 2000) is mediated by ROS. Oligonol treatment protected SOD activity which was markedly decreased by high glucose, indicating that the effects of elevated ROS levels under high glucose may be mitigated by oligonol. Moreover, we showed that oligonol

dose-dependently reduced ROS-induced intracellular fluorescence under high glucose. In this regard, eNOS uncoupling due to oxidation of its cofactor tetrahydrobiopterin (BH4) and subsequent production of ROS instead of NO may be an important component of a mechanism by which oligonol rescues NO production. Our recent work has demonstrated that the beneficial effect of 17β-oestradiol on eNOS in bovine aortic endothelial cells under high glucose was associated with the reversal of down-regulation of GTP cyclohydrolase I, a rate-limiting enzyme for BH4 synthesis (Miyazaki-Akita *et al*., 2007). We speculate that the consequent reduction in ROS-mediated activation of PKCε may contribute to the reversal by oligonol on diminished BK-stimulated Thr-495 dephosphorylation of eNOS under hyperglycaemic conditions. The present observations and their consequences for the beneficial effects of oligonol in endothelial cells under hyperglycaemic conditions are summarized schematically in Figure 11.

In conclusion, the data presented here indicate that the oligomerized polyphenol oligonol had a clearly beneficial effect on high glucose-induced impairment of eNOS phosphorylation at Ser-1177 and dephosphorylation at Thr-495 with diminished eNOS activity in BK-stimulated endothelial cells. The mechanism of oligonol in leading to the recovery of eNOS phosphorylation at Ser-1177 and dephosphorylation at Thr-495 appeared to involve activation of the PI3-K/Akt pathway and inhibition of PKC respectively. Given the importance of endothelial dysfunction in the pathophysiology of diabetes and its cardiovascular complications, the present results may be relevant to understanding the molecular mechanisms underlying the benefit of oligonol as an effective adjunctive treatment for metabolic and cardiovascular health, as well as for overall mortality, in diabetes.

Acknowledgement

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflicts of Interest

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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