The roles of hyperglycaemia and oxidative stress in the rise and collapse of the natural protective mechanism against vascular endothelial cell dysfunction in diabetes

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Abstract

Vascular endothelial cell (VEC) dysfunction in diabetes has been associated with hyperglycaemia-induced intra- and extracellular glycation of proteins and to overproduction of glucose-derived free radicals. VEC protect their intracellular environment against an increased influx of glucose in face of hyperglycaemia by reducing the expression and plasma membrane abundance of their glucose transporter-1 (GLUT-1). We investigated the hypothesis that glucose-derived free radicals induce this down-regulatory mechanism in VEC, but proved the contrary. In fact, pro-oxidants significantly increased the expression and plasma membrane abundance of GLUT-1 and the rate of glucose transport in VEC while abolishing high-glucose-induced down-regulation of the hexose transport system. The resulting uncontrolled influx of glucose followed by overproduction of glucose-derived ROS further up-regulates the rate of glucose transport, and vice versa. This perpetuating glycoxidative stress finally leads to the collapse of the auto-regulatory protective mechanism and accelerates the development of dysfunctional endothelium in blood vessels.

Key words: Anti-oxidants, bilirubin, free radicals, glucose, glucose transport, glucose transporter, GLUT-1, hexose, 4-hydroxy tempol, hyperglycaemia, oxidative stress, pro-oxidants, reactive oxygen species, substrate auto-regulation, vascular endothelial cells.

Abbreviations: dGlc, 2-deoxy-D-glucose; GLUT, glucose transporter; H$_2$DCFDA, 5, (and 6)-carboxy-2-7'-dichlorofluoresceine diacetate; ROS, reactive oxygen species; TPL, 4-hydroxy tempol; VEC, vascular endothelial cells.

Introduction

Micro- and macrovascular diseases are serious complications of Type-1 (insulin-dependent) and Type-2 (non-insulin dependent) diabetes. Microvascular dysfunction often results in severe pathologies, such as retinopathy or nephropathy, whereas macrovascular dysfunction leads to an accelerated development of endothelial cell dysfunction and atherosclerosis (Schalkwijk and Stehouwer, 2005). Chronic hyperglycaemia is considered a major risk factor for the development of both types of vascular diseases (Stratton et al., 2000; Study, 1995; Wei et al., 1998; Yamagishi et al., 2007; Yu and Lyons, 2005). Recent studies suggest that post-prandial hyperglycaemic excursions also adversely affect the vascular wall (Heine et al., 2004; Monnier et al., 2006; Yamagishi et al., 2007). Therefore, an optimal short- and long-term glycaemic control is required to decrease the risk of vascular complications in diabetic patients. Other independent risk factors such as, hyperlipidaemia, obesity or hypertension, also participate in the aetiology of cardiovascular disease (Rosenberg et al., 2005). Recent findings on a cross-talk between signalling events induced by glucose and by lipids point to an adverse synergism between hyperglycaemia and such factors in the development of cardiovascular disease in diabetic patients (Kanter et al., 2007).

Normally, the endothelial cell monolayer in blood vessels forms a barrier that contains the blood within vessel lumen. This monolayer also forms an antithrombotic surface due to surface expression of molecules, such as heparan sulphate, and through the synthesis and release of anti-thrombogenic substances (i.e., prostacyclin). Endothelium-dependent dilation of blood vessels follows a receptor-mediated activation of nitric oxide synthase (eNOS)
and synthesis of NO that relaxes the neighbouring smooth muscle cell layer. It has been suggested that the combination of hyperglycaemia-induced impeded proliferation and increased apoptosis of VEC is of the earliest events in lesion formation in blood vessels (Lorenzi et al., 1985). When the VEC monolayer is injured its anti-thrombotic and dilatory properties are compromised along with its effectiveness to form an impermeable barrier to blood cells. Moreover, injured endothelium secretes increased amounts of chemotactic factors that attract monocytes and smooth muscle cells to the intima layer to form early atherosclerotic plaques (Morales, 1993). Hyperglycaemia also alters mRNA expression of integrin receptor subunits, adhesion molecules, thrombospondin-1 and VEGF, which are important for normal interactions of VEC with the extracellular matrix substratum in blood vessels (Stenina, 2005, and references therein).

Evidence that diabetes creates an oxidative environment in vivo came from the observations on elevated levels of oxidative stress markers, on one hand, and of subnormal levels of low-molecular weight anti-oxidants, on the other, in the blood and tissues of diabetic patients or animal models of diabetes (Cowell and Russell, 2004; Dandona et al., 1996; Davi et al., 2004; Elangovan et al., 2000; Thornalley et al., 1996; Vincent et al., 2004). It has been suggested that the preservation of the anti-oxidant defence in cells and/or anti-oxidants therapies may ameliorate high glucose-induced complications (Tsuneki et al., 2007). Similarly, endothelial cell dysfunction at large, and in diabetes in particular, has been attributed to deleterious effects of free radicals (Ceriello, 2003; Nishikawa et al., 2000). For instance, superoxide radicals uncouple the interaction between eNOS and its cofactor tetrahydrobiopterine in VEC and avert the physiological production of NO to an abnormal generation of superoxide radicals (Guzik et al., 2002; Milstien and Katusic, 1999). Hyperglycaemia-induced oxidative stress in VEC is produced by two independent mechanisms: first, metabolic production of reactive oxygen species (ROS) due to disruption of mitochondrial function and electron leakage, NADH overload, increased polyol and glucose-amine pathways flux, methylglyoxal and glyoxal production, activation of NADPH oxidase, diacylglycerol-dependent activation of conventional and novel PKC isoenzymes or inhibition of the thioredoxin system. Second, glycoxidation and non-enzymatic glycation of proteins lead to an excessive production of free radicals (Halliwell and Gutteridge, 2007a; Hudson et al., 2005; Wautier and Schmidt, 2004).

The process of hyperglycaemia-induced VEC dysfunction may be blocked or delayed by lowering the rate of glucose influx. We have discovered a natural defence mechanism in VEC that down-regulates the glucose transport capacity by nearly 50% in face of hyperglycaemia and protects the cells against deleterious effects of an uncontrolled influx of glucose and a glycoxidative stress (Alpert et al., 2005; Alpert et al., 2002; Sasson et al., 1996). Figure 1 describes this auto-regulatory mechanism: VEC that had been maintained at 5.5 or 23 mM glucose up- or down-regulated their rates of hexose transport, respectively. Cells that were initially exposed to normal glucose level and then received high glucose medium down-regulated the rate of transport, while the opposite medium change unregulated it. This auto-regulatory response entails a 24-h lag period and is complete within 48 h. We found that the underlying molecular mechanism is glucose-induced destabilization of GLUT-1 mRNA and a subsequent reduction in GLUT-1 protein content and plasma membrane abundance (Alpert et al., 2005; Alpert et al., 2002; Totary-Jain et al., 2005). The glucose-derived metabolites and/or other intracellular signals that operate this auto-regulatory mechanism in VEC have not been identified yet. Interestingly, it has been shown that ROS significantly reduced the expression of the insulin sensitive transporter, GLUT-4, in skeletal muscle cells and adipocytes (Pessler et al., 2001). These findings led to a working hypothesis that similarly linked the reduced expression of GLUT-1 in VEC to an elevated production of glucose-derived free radicals. Our studies, however,
have proven the opposite: the mechanism of high glucose-induced auto-regulatory protective collapses in the presence of other potent pro-oxidants. These findings explain the critical role of the combination of hyperglycaemia and an intense oxidative stress in the development of endothelial cell dysfunction.

**Pro-oxidants augment the rate of hexose transport in cultured VEC**

The indication that a direct oxidative stress increases the rate of hexose transport in VEC came from a simple experiment in which primary cultures of bovine aortic endothelial cells were exposed to increasing concentrations of hydrogen peroxide. It has been shown that hydrogen peroxide increases intracellular levels of superoxide radicals in VEC due to aberrant interactions with eNOS and NADPH oxidase (Coyle et al., 2006). Figure 2 shows that 25 μM hydrogen peroxide increased significantly the rate of uptake of the glucose analogue [3H]-2-deoxy-D-glucose ([3H]-dGlc) 1.42 ± 0.03-fold over the basal rate within 3 h. Higher concentrations of hydrogen peroxide were less effective and even cytotoxic.

Similar evidence for the hexose transport up-regulatory role of pro-oxidants came from our study on the interactions of unconjugated bilirubin with VEC (Cohen et al., 2006). Unconjugated bilirubin exhibits cytoprotective effects at normal levels but becomes cytotoxic at higher concentrations. This duality in bilirubin action results from its respective dose-dependent anti- and pro-oxidative properties (Kapitulnik, 2004). The free contact between the endothelial cell monolayer in blood vessels and circulating bilirubin allows such protective or damaging effects to occur, depending on the concentration of this haem metabolite in the blood. For instance, at concentration as low as 10 μM, bilirubin induced apoptosis in cultured bovine brain endothelial cells within 24 h (Akin et al., 2002). At higher concentrations it inhibited (Ki = 137 μM) the rate of hexose uptake into isolated brain capillaries of rats (Katoh-Semba and Kashiwamata, 1980). A link between an increased haem oxygenase activity and subsequent biliverdin and bilirubin production to the pathogenesis of diabetes-induced endothelial cell damage has also been proposed (Chen et al., 2004; Dulak et al., 2002). On the other hand, favourable anti-oxidant properties in VEC have also been documented (Stockert et al., 1987), such as suppression of MHC class II expression, prevention of cell sloughing, attenuation of pro-inflammatory responses and some protection against oxidative damage (Kawamura et al., 2005; Oberle et al., 2003; Rodella et al., 2006; Wu et al., 2005).

We performed dose-response and time-course analyses of the effect of bilirubin on the rate of hexose transport in primary cultures of bovine aortic endothelial cells, which had been pre-conditioned at 5.5 or 23 mM glucose for 48 h. Figure 3 shows that the basal rate of hexose uptake was reduced (−44 ± 3%, p < 0.05, n = 3) in cells exposed to the high glucose concentration in comparison with the normal glucose incubation. These findings agree with our previous reports on high glucose-induced down-regulation of hexose transport in these cells (Alpert et al., 2005). Low concentrations of bilirubin had no significant effects on the rate of hexose transport, which remained similar to that of the control cultures. High levels of bilirubin, however, augmented the rate of transport 1.7–2.6-fold in comparison with the respective control values.

![Figure 2](image-url) **Figure 2.** Hydrogen peroxide augments the rate of hexose transport in bovine aortic endothelial cell primary cultures. Cell cultures were grown and maintained at 5 mM glucose. Hydrogen peroxide was added at the indicated concentrations and incubated for 3 h. The cells were then washed and taken to a standard [3H]-dGlc uptake assay (Alpert et al., 2002). Mean ± SEM, n = 3, p < 0.05 in comparison with untreated cells, Student’s t-test. Reproduced from Altman et al. (2004) with kind permission of the Romanian Academy Publishing House.

![Figure 3](image-url) **Figure 3.** Unconjugated bilirubin stimulates hexose transport in bovine aortic endothelial cell primary cultures. Cell cultures were maintained at 5.5 (close circles) or 23 mM glucose (open circles) in the absence or presence of the indicated concentrations of bilirubin for 48 h. The cells were then washed and taken to a standard [3H]-dGlc uptake assay (Alpert et al., 2002). Mean ± SEM, n = 3, p < 0.05 in comparison with untreated cells, Student’s t-test. Reproduced from Cohen et al. (2006) with kind permission of the Society for Biomedical Diabetes Research.
measured at 5.5 and 23 mM glucose, respectively. Maximal effects were obtained with 20 to 40 μM bilirubin. The hexose transport stimulating effect of bilirubin entailed a 24-lag period and became maximal within 36–48 h (data not shown). The bilirubin-induced stimulation of hexose transport resulted from a ~2-fold increased expression of GLUT-1 in the cells (Cohen et al., 2006). Of importance is the observation that at these effective concentrations bilirubin exhibited potent pro-oxidant properties in VEC cultures: we measured cell-associated ROS production with the fluorescent dye H₂DCFDA (5, (and 6)-carboxy-2’7’-dichlorofluoresceine diacetate) (Kehrer and Paradathathu, 1992) in naïve- and bilirubin-treated cells and found a substantial increase in cell-associated fluorescence in the latter (Figure 4). This study indicates that a bilirubin-induced oxidative stress is associated with stimulation of the glucose transport system in VEC regardless of the ambient glucose levels.

While studying anti- and pro-oxidant functions of 4-hydroxy tempol (TPL) in VEC (Alpert et al., 2004) we obtained further support to the findings on oxidative stress-induced stimulation of the glucose transport system. TPL dramatically increased the production of ROS in primary cultures of bovine aortic endothelial cells: cultures that had been maintained at 5 or 23 mM glucose for 48 h were exposed to TPL (1 or 5 mM) during the last 12 h of incubation and the level of cell-associated ROS was determined using the H₂DCFDA assay. The results were striking: the relative fluorescence measured in VEC exposed to 1 and 5 mM TPL was ~4- and 6-fold higher than that measured in untreated cells, either at 5 or 23 mM glucose. Dose-response analyses revealed that half-maximal and maximal ROS production were obtained with 0.25–0.5 and 5 mM TPL, respectively (Alpert et al., 2004).

Oxidation of proteins causes irreversible carbonylation that renders them dysfunctional and resistant to proteasomal degradation (Dalle-Donne et al., 2006). Figure 5 shows profiles of ROS-induced protein carbonylation in lysates prepared from VEC cultures following exposure to 5.5 or 33 mM glucose for 72 h without or with 5 mM TPL. This high concentration of glucose was chosen in order to visualize better the glucose-dependent protein carbonylation during the relative short incubation period. High glucose induced a moderate increase in protein carbonylation in comparison with the 5.5 mM glucose incubation. However, this effect seems minute in view of the marked TPL-induced carbonylation, which was 3–4 fold higher than that measured in its absence.

In parallel, TPL augmented the rate hexose transport system in VEC. The incubation with 5 mM TPL increased the rate of uptake [³H]-dGlc uptake 1.70 ± 0.12 and 2.01 ± 0.25-fold higher than the rates measured in the untreated cultures at 5 and 23 mM glucose, respectively. Figure 6 shows that

Figure 4. Pro-oxidant properties of bilirubin in bovine aortic endothelial cell primary cultures. The cells were seeded and grown on fibronectin-coated glass cover-slips and treated with bilirubin at the indicated concentrations for 4 h. The cells were loaded with H₂DCFDF, washed, fixed and taken for fluorescent microscopic determinations as described (Cohen et al., 2006). A summary of 20 field measurements in 3 different slides of each treatment is depicted. Mean ± SEM, p < 0.05 in comparison with control, Student’s t-test. Reproduced with modifications from Cohen et al. (2006) with kind permission of the Society for Biomedical Diabetes Research.

Figure 5. High glucose and TPL induce protein carbonylation in bovine aortic endothelial cell primary cultures. Cell cultures were incubated with 5.5 or 33 mM glucose without or with 5 mM TPL for 72 h. Another culture was incubated with 5 mM glucose and 28 mM L-glucose (osmolarity control). The cells were then washed and lysed, and protein-bound carbonyl moieties were derivatized with 2,4-dinitrophenylhydrazine to produce 2,4-dinitrophenylhydrazone, which were detected using a specific antibody following separation by SDS-PAGE and Western blot analysis, using the OxyBlot™ oxidation detection kit (Chemicon International). Reproduced from Alpert et al., (2004) with kind permission of Elsevier, Inc.
alterations in VEC (Bishara et al., 2006) and found to ameliorate oxidative stress-induced down-regulation of glucose transport due to destabilization of GLUT-1 mRNA. Aminoguanidine is a hydrazine scavenging compound that prevents the interaction of dicarbonyl precursors (i.e., methylglyoxal, glyoxal, 3-deoxyglucosone) with arginine or lysine residues in proteins and abolishes the formation of advanced glycation end products (Degenhardt et al., 1998; Thormaehl, 2003; Thormaehl et al., 2000). N-acetylcysteine scavenges ROS and replenishes glutathione stores in cells (Cotgreave, 1997). Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a cell-permeable and water soluble analogue of vitamin E that scavenges peroxy radicals and inhibits lipid peroxidation (Mickle et al., 1989; Wu et al., 1990). In vitro anti-oxidant effects of vitamin C (ascorbic acid) include scavenging of hydrogen peroxide and peroxyl, hydroperoxyl, superoxide, hypochlorous acid or peroxynitrous acid radicals (Halliwell and Gutteridge, 2007b). We studied the effects of all four compounds on high glucose-induced protein carbonylation in VEC. Figure 7 shows that each of these anti-oxidants significantly inhibited high-glucose-induced protein carbonylation in VEC cultures.

In parallel, we measured the rate of hexose transport in these anti-oxidant-treated VEC cultures (Altman et al., 2004). Despite their obvious anti-oxidant effects (Figure 7) none of these compounds modified significantly the rate of hexose transport in VEC: the rate of [3H]-dGlc uptake in control cultures exposed to 23 mM glucose was 45 ± 2 pmol/10^6 cells/min. The corresponding rates in aminoguanidine-, N-acetylcysteine-, trolox- and vitamin C-treated cultures were 38 ± 3, 38 ± 6, 47 ± 2 and 41 ± 2 pmol/10^6 cells min (mean ± SEM, n = 3), respectively. These results further support the conclusion on a functional dissociation between high glucose-induced oxidative stress and high glucose-induced down-regulation of the glucose transport system in VEC.

**Discussion**

Two processes occur simultaneously in VEC cultures exposed to high glucose level: first, an augmented production of ROS due to metabolic effects and non-enzymatic glycation of glucose, and second, the down-regulation of glucose transport due to destabilization of GLUT-1 mRNA. Pro-oxidants also induce oxidative stress in VEC, but the effect is different. Hyperglycaemia down-regulates while other pro-oxidants up-regulate the glucose transport system in VEC. This disparity in the mode of regulation of the glucose transport system by two oxidative stressful conditions is intriguing. A theory

**Anti-oxidants ameliorate high glucose-induced oxidative stress in VEC**

Another line of evidence that dissociates the effects of glucose-derived free radicals from the regulation of hexose transport in VEC came from our findings on the failure of the anti-oxidants aminoguanidine, N-acetylcysteine, trolox, and vitamin C to alter this auto-regulatory mechanism (Altman et al., 2004). Each of these compounds was tested in other studies and found to ameliorate oxidative stress-induced alterations in VEC (Bishara et al., 2002; Chen et al., 2006; Chen et al., 2007; Forstermann and Munzel, 2006; Hornig, 2002; Huwiler et al., 2001; Ji et al., 2003; Kowluru and Koppolu, 2002; Laurent et al., 2006; Lehr et al., 2006; Navarro-Antolin et al., 2007; Price et al., 2001; Ram and Hiebert, 2004; Rodriguez-Manas et al., 2003; Siow et al., 1999; Sun and McCrae, 2006; Wautier and Guillausseau, 2001).
that glucose-derived free radicals belong to a different class of ROS, which is unique and specifically elicit down-regulatory interactions, is unlikely since the low molecular weight anti-oxidants tested effectively abolished high glucose-induced protein carbonylation without altering high glucose-induced down-regulation of the glucose transport. Moreover, applying an intense oxidative stress (in the presence of hydrogen peroxide, bilirubin or 4-hydroxy tempol) not only blocked high glucose-induced down-regulation of the hexose transport system but in fact led to a substantial up-regulation of the hexose transport system in cells that were maintained at either normal or high glucose levels (Alpert et al., 2004; Cohen et al., 2006).

Glucose-derived advanced glycation-end products (AGEs) interact with the AGE-receptor in endothelial cells to generate ROS and induce overexpression of vascular endothelial growth factor (VEGF) (Soro-Paavonen and Forbes, 2006). Whether AGE- and possibly pro-oxidants-induced expression of VEGF is involved in the regulation of GLUT-1 expression in VEC remains to be investigated.

In summary, VEC significantly limit the rate uptake of glucose when exposed to hyperglycaemic-like conditions. Subsequently, the production of glucose-derived free radicals remains low and below a threshold level, above which up-regulation of the glucose transport is ensued. When pro-oxidants are added to these high-glucose cultures they impose an intense oxidative stress that exceeds this threshold level and operates the up-regulatory reaction. The model in Figure 8 summarized these interactions: hyperglycaemia-induced down-regulation of the hexose transport system is the principal mechanism by
which VEC protects their intracellular environment against an excessive high glucose-induced oxidative damage. Abnormal and pathological conditions, such as potent pro-oxidants, counteract this natural protective mechanism and establish a vicious cycle that allows for an uncontrolled influx of glucose, which leads to an over-production of ROS. The resulting strong oxidative stress further augments the expression of GLUT-1 and the influx of glucose, which in turn aggravates the oxidative stress, and vice versa, until endothelial cell dysfunction becomes inevitable.

We suggest that when hyperglycaemia in diabetic patients is complicated with other pro-oxidative challenges, the endothelial cell monolayer in blood vessels loses its first line of defence, namely down-regulation of glucose transport, and becomes vulnerable to detrimental oxidative interactions. Among pathological conditions known to create an oxidative stress are conditions associated with the metabolic syndrome, such as hyperlipidaemia or hypertriglyceridaemia (Galle et al., 2006; Nitenberg et al., 2006; Rebollo and Actis Dato, 2005) and prooxidative function of various or micro- and macronutrients (Bienvenu et al., 1992; Dandonet al., 2005), dietary supplements, environmental pollutants and certain pharmaceuticals (Gonzalez-Flecha, 2004; Parke and Sapota, 1996; Tao et al., 2003). Hence, the prevention of such harmful conditions and exposures in hyperglycaemic individuals may better preserve the autoregulatory potential of VEC and delay or prevent endothelial cell dysfunction.

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