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Original Contribution

Heme oxygenase-1-derived bilirubin protects endothelial cells against high glucose-induced damage

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ABSTRACT

Hyperglycemia and diabetes are associated with endothelial cell dysfunction arising from enhanced oxidative injury, leading to the progression of diabetic vascular pathologies. The redox-sensitive transcription factor nuclear factor-E2-related factor 2 (Nrf2) is a master regulator of antioxidant genes, such as heme oxygenase-1 (HO-1), involved in cellular defenses against oxidative stress. We have investigated the pathways involved in high glucose-induced activation of HO-1 in endothelial cells and examined the molecular mechanisms underlying cytoprotection. Elevated D-glucose increased intracellular generation of reactive oxygen species (ROS), leading to nuclear translocation of Nrf2 and HO-1 expression in bovine aortic endothelial cells, with no changes in cell viability. Superoxide scavenging and inhibition of endothelial nitric oxide synthase (eNOS) abrogated upregulation of HO-1 expression by elevated glucose. Inhibition of HO-1 increased the sensitivity of endothelial cells to high glucose-mediated damage, while addition of bilirubin restored cell viability. Our findings establish that exposure of endothelial cells to high glucose leads to activation of endogenous antioxidant defense genes via the Nrf2/ARE pathway. Upregulation of HO-1 provides cytoprotection against high glucose-induced oxidative stress through the antioxidant properties of bilirubin. Modulation of the Nrf2 pathway in the early stages of diabetes may thus protect against sustained damage by hyperglycemia during progression of the disease.

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1. Introduction

Hyperglycemia plays a major role in the pathogenesis of diabetic vascular complications [1,2]. Upregulation of GLUT1 results in an increase in intracellular glucose levels in endothelial cells [3], favoring flux through the polyol and hexosamine pathways, and activation of PKC isoforms [4]. In addition, glucose and glucose-derived dicarbonyl compounds react nonenzymatically with the basic amino acids lysine and arginine in proteins to form AGEs both extra- and intracellularly [4,5]. These changes in turn increase the generation of reactive oxygen species (ROS), leading to oxidative and endothelial cell proinflammatory activation or damage and ultimately diabetic vascular pathology [6, 7]. However, ROS are produced continuously as natural by-products of normal metabolism of oxygen and play important roles in redox signaling [2,8–10].

Cells have evolved highly regulated endogenous antioxidant defense systems to counteract an overproduction of reactive oxygen species, and previous studies have investigated the ability of endothelial cells to react to diabetic-like conditions by upregulating antioxidant responses [11–13]. The transcription factor nuclear factor erythroid 2-related factor (Nrf2) plays a key role as redox sensor. Under physiological conditions, Nrf2 is retained in the cytosol via the actin binding protein Kelch-like ECH-associated protein (Keap1), which negatively regulates Nrf2 by targeting it for ubiquitination and proteasomal degradation [14–16]. GSK-3β phosphorylation may also lead to Nrf2 degradation through an adaptor protein β-TrCP independent of Keap1 [17]. Oxidative and

Abbreviations: BAEC, bovine aortic endothelial cells; Bil, bilirubin; DCF-DA, 2,7-dichlorofluorescin diacetate; HO-1, heme oxygenase-1; L-NAME, N^G-nitro-L-arginine methyl ester; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; Nrf2, nuclear factor erythroid 2-related factor; SnPPIX, tin protoporphyrin IX; ZnPPIX, zinc (II) protoporphyrin IX

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electrophilic stress induce alterations in the Nrf2–Keap1 complex, preventing proteasomal degradation and promoting nuclear accumulation of Nrf2 via *de novo* synthesis [18] and potentially favoring a shuttling process between cytosol and nuclei [19]. In the nucleus, Nrf2 forms heterodimers with small Maf proteins and binds to the antioxidant/electrophile response element (ARE/EpRE) in the promoter region of phase II detoxifying (e.g., NAD(P)H:quinone oxidoreductase 1) and antioxidant (e.g. heme oxygenase-1) enzymes to upregulate their expression [18].

Heme oxygenase is the first and rate-limiting enzyme in the degradation of heme into biliverdin, carbon monoxide (CO), and free iron [20]. Heme oxygenase-1 (HO-1) is a 32 kDa stress protein present at low levels in most mammalian tissues [21], and its expression is induced by a wide variety of stress stimuli, including ROS, nitric oxide, and inflammatory cytokines [22–24]. HO-1 has been described as a key regulator of adaptive cellular responses to oxidative stress and plays a role in cellular homeostasis through the strong antioxidant activity of bilirubin, derived from biliverdin, ferritin, induced by free iron, and the antiapoptotic effect of carbon monoxide [25–27].

The upregulation of Nrf2 in the induction of antioxidant enzymes in response to hyperglycemia has been reported [28,29]; however, the molecular mechanisms underlying endothelial cytoprotection remain to be established. This study in bovine aortic endothelial cells (BAEC) demonstrates that short-term exposure to a high glucose concentration, via the generation of reactive oxygen species and Nrf2 activation, upregulates HO-1, which we propose exerts cytoprotection through the generation of bilirubin.

2. Methods

2.1. Endothelial cell culture

Bovine aortic endothelial cells (BAEC) were isolated from fresh aortae using 0.5 $mgml^{-1}$ collagenase (Boehringer) and cultured in DMEM containing 10% fetal calf serum (FCS), 5.5 mM D-glucose, 5 mML-glutamine, penicillin/streptomycin (100 IU ml^{-1}) and maintained at 37 °C under 5% CO_2 humidified atmosphere [30].

2.2. Cell culture reagents and inhibitors

All cell culture reagents, Tiron, N^G-nitro-L-arginine methyl ester (L-NAME), zinc (II) protoporphyrin IX (ZnPPIX), tin protoporphyrin IX (SnPPIX), 2,7-dichlorofluorescin diacetate (DCF-DA), and bilirubin (Bil) were obtained from Sigma (Gillingham, UK and Milan, Italy) and the chemiluminescence probe L-012 from Wako Pure Chemical Industries Ltd (Osaka, Japan).

2.3. Cell viability assays

Cell viability was evaluated using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) and AlamarBlue (Invitrogen) assays. Confluent cells were equilibrated in DMEM (1% FCS) for 24 h and then treated for 24 h with DMEM (control, 5 mM D-glucose) or DMEM containing 25 mM D-glucose (25 G) or an iso-osmotic control (5 mM glucose + 20 mM D-mannitol, 25 M), in the absence or presence of the HO-1 inhibitors ZnPPIX (30 μM) and SnPPIX (50 μM). For the MTT assay, cells were incubated with 5 μgml^{-1} MTT for 3 h at 37 °C. Insoluble formazan salts were dissolved in DMSO with absorbance at 570 nm measured in a spectrophotometric plate reader (EL-808 BIO-TEK Instruments Inc.). The AlamarBlue viability test was performed following the manufacturer's instructions. Mean values from each treatment were calculated as a percentage relative to untreated control cells.

2.4. Chemiluminescence detection of reactive oxygen species in intact bovine aortic endothelial cells

Generation of reactive oxygen species (ROS) was measured in intact BAEC monolayers using the luminol analogue L-012 (8-amino-5-chloro-7-phenylpyridol[3,4–d] pyridazine-1,4-(2*H*,3*H*) dione sodium salt) [30,31]. In a cell-free system neither D-mannitol nor D-glucose affected L-012 chemiluminescence (data not shown). Confluent cells were equilibrated in DMEM (1% FCS) for 24 h and then treated for 0–24 h with DMEM (control, 5 mM D-glucose) or DMEM containing 20 mM D-mannitol plus 5 mM D-glucose (25 M) or 25 mM D-glucose (25 G) in the absence or presence of the superoxide chelator 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron, 10 μM) or inhibitor of eNOS (L-NAME, 100 μM). Cells were incubated at 37 °C in Krebs buffer containing 25 M or 25 G (in the continued absence or presence of 25 M, 25 G, and/or inhibitors) and L-012 luminescence was monitored immediately after addition of L-012. Mean light units were recorded in a microplate luminometer (Chameleon V, Hidex), and maximal values obtained over a 20–40 min interval in 3–4 independent endothelial cell cultures averaged and expressed as mean light units per milligram protein.

2.5. Detection of 2,7-dichlorofluorescin diacetate-positive cells

Generation of peroxides and reactive oxygen species (ROS) was also measured in intact BAEC by means of fluorimetric detection of 2,7-dichlorofluorescin diacetate (DCF-DA)-positive cells. Confluent cells were equilibrated in DMEM (1% FCS) for 24 h and then treated for 0–24 h with DMEM (control, 5 mM D-glucose) or DMEM containing 25 M or 25 G in the absence or presence of SnPPIX (50 μM) and bilirubin (0.5 μM). At the end of incubation, cells were treated with DCF-DA (20 μM) for 30 min at 37 °C and washed and fluorescence was recorded using an Attune acoustic focusing cytometer (Life Technologies). Mean values from each treatment were calculated as a percentage relative to untreated control cells.

2.6. ELISA assay

Generation of 4-hydroxynonenal (4-HNE) was measured in cell lysates using a competitive enzyme immunoassay, following the manufacturer's instructions (bovine 4-hydroxynonenal Elisa Kit, BlueGene Biotech, Shangai, China). Briefly, cells were equilibrated in DMEM (1% FCS) for 24 h and then treated for 6–24 h with DMEM (control, 5 mM D-glucose) or DMEM containing 25 M or 25 G in the absence or presence of SnPPIX (50 μM) and bilirubin (0.5 μM). At the end of incubation, cells were detached, washed, and subjected to ultrasonication. After centrifugation, to remove cell debris, samples were assayed. The optical density of samples was recorded at 450 nm using a microplate reader (EL-808 BIO-TEK Instruments Inc.). 4-HNE concentrations following each treatment were normalized relative to protein content and expressed as nanogram 4-HNE per microgram protein.

2.7. Quantitative RT-PCR

Cells were treated for specified times with DMEM (control, 5 mM glucose) or DMEM containing 25 M or 25 G. Total RNA was isolated using the Nucleospin 96-well plate format RNA isolation kit (Machereye-Nagel, Düren, Germany). Cells were lysed, and RNA was purified using a Machereye-Nagel RNA extraction kit, quantified, and reverse-transcribed using a QuantiTect RT kit (Qiagen, Manchester, UK). Expression of HO-1 was analyzed using a quantitative RT-PCR system (Corbett Rotor-gene, Corbett Research UK, Cambridge, UK), and mRNA levels were normalized to the geometric

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