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Superoxide dismutase 1 and glutathione peroxidase 1 are involved in the protective effect of sulodexide on vascular endothelial cells exposed to oxygen–glucose deprivation

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ABSTRACT

Sulodexide (SDX) is widely used in the treatment of both arterial and venous thrombotic disorders. In addition to its recognized antithrombotic action, SDX has endothelial protective potential, which is independent of the coagulation/fibrinolysis system. However, the detailed molecular mechanisms of the endothelioprotective action of the drug are still unresolved.

The aim of the present study was to determine whether treatment with SDX at concentrations of 0.125–0.5 lipase releasing unit (LRU)/ml have on the expression and activity of antioxidant enzymes in ischemic endothelial cells and how these effects might be related to the antiapoptotic properties of SDX.

In the present study, human umbilical vein endothelial cells (HUVECs) were subjected to ischemia-simulating conditions (combined oxygen and glucose deprivation, OGD) for 6 h to determine the protective effects of SDX. SDX (0.25 and 0.5 LRU/ml) in OGD significantly increased the cell viability and prevented mitochondrial depolarization in the HUVECs. Moreover, SDX protected the HUVECs against OGD-induced apoptosis. At concentrations of 0.25 and 0.5 LRU/ml, the drug increased both superoxide dismutase 1 (SOD1) and glutathione peroxidase 1 (GPx1) mRNA/protein expression together with a significant attenuation of oxidative stress in ischemic HUVECs. Our findings also demonstrate that an increase in both SOD and GPx activity is involved in the protective effect of SDX on ischemic endothelial cells. Altogether, these results suggest that SDX has a positive effect on ischemia-induced endothelial damage because of its antioxidant and antiapoptotic properties.

Introduction

Sulodexide (SDX) is a highly purified mixture of glycosaminoglycans (GAGs) containing 80% fast-moving heparin (FMH) fraction and 20% dermatan sulfate (DS) fraction with well documented antithrombotic and profibrinolytic activity. FMH and DS show an additive effect on the inhibition of thrombin because thrombin inhibition is caused by their specific interactions with antithrombin and heparin cofactor II (HCII), respectively (Cosmi et al., 2003). SDX is effective in the treatment of both arterial and venous thrombotic disorders (Broekhuizen et al., 2010; Andreozzi, 2012). In addition to its recognized antithrombotic action, SDX has a protective potential for endothelial cells that is independent of the coagulation/fibrinolysis system (Lauver and Lucchesi, 2006). The drug has been shown to maintain or restore damaged endothelial glycocalyx as well as to exert antiproliferative, anti-inflammatory, anti-proteolytic and anti-ischemic effects (Broekhuizen et al., 2010).

Interestingly, despite the relatively large amount of clinical data on the anti-ischemic properties of SDX, so far, the direct protective effect of the drug on vascular endothelial cells has only been reported in one experimental model of ischemia-induced endothelial damage (Lauver et al., 2005). The beneficial effect of SDX on ischemic-injury may involve multiple mechanisms, such as decreasing the deposition of C-reactive protein without any change in activated thromboplastin time (Lauver et al., 2005), inhibiting the inflammatory response (Ciszewicz et al.,

Abbreviations: CAT, catalase; DCF-DA, 2′,7′-dichlorofluorescein diacetate; DS, dermatan sulfate; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; FMH, fast-moving heparin fraction; GAGs, glycosaminoglycans; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPx, glutathione peroxidase; GSH, glutathione; HBSS, Hank's buffered salt solution; HRP, horseradish peroxidase; HUVEC, human umbilical vein endothelial cells; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl-carbocyanine iodide; LRU, lipase releasing unit; MDA, malonyldialdehyde; MPTP, mitochondrial permeability transition pore; MTP, mitochondrial transmembrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor kappa B; OGD, oxygen and glucose deprivation; PKC, protein kinase C; RIPA, radioimmunoprecipitation assay buffer; ROS, reactive oxygen species; SDX, sulodexide; SOD, superoxide dismutase.

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2009), reducing endothelial apoptosis (Young, 2008) and decreasing oxidative damage (Suminska-Jasinska et al., 2011). However, the detailed molecular mechanisms of the endothelioprotective action of the drug are still unresolved.

Tissue ischemia is a condition shared by different pathologic conditions associated with endothelial dysfunction, including myocardial infarction, peripheral vascular disease and cerebrovascular disorders (Cai and Harrison, 2000). Oxidative stress-induced vascular endothelial dysfunction is the first key step in the pathogenesis of ischemic injury. An imbalance between reactive oxygen species (ROS) formation and the capacity of the endothelium to destroy them leads to remodeling, platelet aggregation, loss of vasodilation and inflammation (Higashi et al., 2014). The direct effects of ROS on the cell include damage to DNA, enzymes, and structural proteins; lipid peroxidation; and mitochondria dysfunction, and ROS also disturb intracellular redox processes, diminish antioxidant levels (particularly glutathione, GSH) and oxidize other intracellular thiols (Halliwell, 1992).

The most common antioxidant enzymes that have been used to evaluate ischemia-induced oxidative damage are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Li and Shah, 2004).

In humans, there are three isoforms of SOD: copper/zinc superoxide dismutase (Cu/Zn SOD/SOD1) situated mostly in the cytoplasm, manganese superoxide dismutase (MnSOD/SOD2) located predominantly in the mitochondria and extracellular SOD (EC-SOD/SOD3) (Matés et al., 1999). SOD1 is believed to play a major role in the first line of defense against the superoxide radical, and the enzyme catalyzes its dismutation into hydrogen peroxide (H_2O_2), which is utilized by CAT or GPx. Furthermore, increasing evidence suggests that SOD1 blocks the release of mitochondrial cytochrome c and could thereby reduce apoptosis after ischemia (Fujimura et al., 2000). At high steady state concentrations of H_2O_2, CAT, which is predominantly located in cellular peroxisomes, converts H_2O_2 to water and molecular oxygen. In contrast, under lower steady-state levels of H_2O_2, GPx, which is a selenoperoxidase, removes not only H_2O_2 but also lipidic and non-lipidic hydroperoxides by catalyzing their conversion to hydroxyl acids in the presence of GSH. For this reason, GPx is considered the major source of protection against low levels of oxidant stress (Matés et al., 1999). GPx exists in several isoforms, and the most abundant intracellular isoform in human endothelial cells is cytosolic/mitochondrial GPx1 (cGPx) (Lei et al., 2007).

The aim of the present study was to determine if treatment with SDX at concentrations of 0.125–0.5 lipase releasing unit (LRU)/ml with proven endothelioprotective potential (Ciszewicz et al., 2009) might have an effect on the expression and activity of the ROS-inactivating enzymes, SOD1, CAT and GPx1, in ischemic endothelial cells and whether this effect might be related to its potentially positive influence on cell viability. These effects seem to be very important because endothelial cells are involved in numerous processes that affect vascular tone, leukocyte function, and smooth muscle responsiveness during and after ischemic events (Deanfield et al., 2007). The degenerated endothelium might not be effective enough to remove and inactivate excessive amounts of ROS, leading to loss of the glycocalyx, alterations of endothelial cell-cell contacts, an increase in vascular permeability and, thus, damage to nearby tissues (Szocs, 2004).

To determine the mechanism of the cytoprotective action of SDX, we subjected human umbilical vein endothelial cells (HUVECs) to ischemia-simulating conditions (combined oxygen and glucose deprivation, OGD).

Materials and methods

Reagents

Antibodies against SOD1, CAT and GPx1, 40% solution acrylamide/bis-acrylamide, Bradford reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33,342, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1), 2′,7′-dichlorofluorescein diacetate (DCF-DA), Hank's buffered salt solution (HBSS), a lipid peroxidation (MDA) assay kit, a protease inhibitor cocktail, radioimmunoprecipitation assay buffer (RIPA) and TRI Reagent were purchased from Sigma-Aldrich (St. Louise, MO, USA). M199 medium and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA, USA). Antibodies against cytochrome c, p-eNOS (Thr^{495}), e-NOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Amersham ECL Prime Western blotting Detection Reagent was obtained from GE Healthcare (Little Chalfont, UK). Nuclease-free water, a reverse transcriptase Kit, and Taq PCR Master Mix 2× Kit were obtained from the Promega Corporation (Madison, WI, USA). Reagent kits for measuring the total SOD, CAT and GPx activity were obtained from Abcam (Cambridge, CA, USA). A Calbiochem nitric oxide synthase assay kit was purchased from EMD Chemicals (Darmstadt, Germany).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Walkersville MD, USA) and maintained in M199 medium supplemented with 10% heat-inactivated FBS and 2 ng/ml basic fibroblast growth factor (FGF-2) at 37 °C in 95% air and 5% CO_2 at 95% relative humidity (CO_2 incubator, Kebo-Assab, Sweden). Experiments were performed with cells from passages 3 through 5.

Treatment of HUVEC cultures

Prior to the experiment, the cells were incubated overnight with fresh medium. HUVEC cultures were placed in medium deprived of glucose and serum. The osmolarity of the medium was measured and adjusted to 319 mOsm with 20% mannitol (0.9 ml/100 ml). The cultures were incubated for 6 h under ischemia-simulating conditions (OGD): 92% N_2, 5% CO_2 and 3% O_2 at 37 °C (gassed incubator BB 6060, Heraeus, Hanau, Germany) (Urbanek et al., 2014). The cells were treated with SDX (Vessel Due F, Alfa Wasserman, Alanno Scalo, Italy) at concentrations of 0.125, 0.25 and 0.5 LRU/ml. SDX was added directly to the medium at the start of OGD. Samples were processed immediately after 6 h of OGD without any reoxygenation. To investigate the effect of SDX on the cell viability, the HUVECs were treated with SDX for 6 and 24 h under OGD. Control cultures (normoxia) were placed in standard M199 containing 10% FBS (glucose concentration of 5.6 mM) and exposed to humidified 95% air/5% CO_2 at 37 °C for the same duration.

Cell viability and morphology

The cell viability of the HUVECs treated with SDX was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion method (Mosmann, 1983). The ability of the cells to convert MTT indicates mitochondrial integrity and activity, which in turn indicates cell viability. The cleavage of the tetrazoline ring of MTT takes place mainly with the participation of mitochondrial succinate dehydrogenase and depends on the activity of the respiratory chain and the redox state of the mitochondria (Shearman et al., 1995). MTT (final concentration — 0.25 mg/ml) was added to the medium 3 h before the scheduled end of the experiment, and then the cultures were incubated at 37 °C under proper conditions. At the end of the experiment, after being washed twice with PBS, the cells were lysed in 100 μl dimethyl sulfoxide, which enabled the release of the blue reaction product formazan. The absorbance was read on a microplate reader at 570 nm, and the results were expressed as a percentage of the absorbance measured in the control cells (normoxia).