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Inhibition of endoplasmic reticulum stress and oxidative stress by vitamin D in endothelial cells

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ABSTRACT

Endoplasmic reticulum (ER) stress and oxidative stress promote endothelial dysfunction and atherosclerosis. Since vitamin D has been shown in several studies to lower the risk of cardiovascular disease, we examined the effects of vitamin D on ER stress and oxidative stress in endothelial cells. ER stress was measured using the placental secreted alkaline phosphatase assay and oxidative stress was measured by hydroethidine fluorescence. Expression of ER stress markers, including glucose-regulated protein 78, c-*jun* N-terminal kinase 1 phosphorylation, and eukaryotic initiation factor 2α phosphorylation, as well as X-box binding protein-1 splicing were measured in tunicamycin (TM)-treated human umbilical endothelial cells (HUVEC) treated with 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and other vitamin D analogs. When TM and 1,25-$(OH)_2D_3$ were added simultaneously, 1,25-$(OH)_2D_3$ prevented ER stress. However, the effect was much stronger when cells were pre-treated with 1,25-$(OH)_2D_3$ for 24-h. However, ER stress was not inhibited by 25-OH vitamin D_3 (25-OHD_3) or the vitamin D analog EB1089. Both ZK191784 and the vitamin D metabolite 24,25-dihydroxyvitamin D_3 were as effective as 1,25-$(OH)_2D_3$ in preventing ER stress. Similar effects were observed dextrose-induced stress. All of the compounds tested, except for 25-OHD_3, inhibited dextrose-induced (27.5 mM) oxidative stress and ER stress. Although TM with and without 1,25-$(OH)_2D_3$ had no effect on VDR expression, inhibition of VDR expression via siRNA prevented 1,25-$(OH)_2D_3$, ZK191784, EB1089, and 24,25-dihydroxyvitamin D_3 from inhibiting dextrose-mediated SO generation. Furthermore, each vitamin D analog, with the exception of 25-OHD_3, prevented dextrose-induced toxicity. These results suggest that vitamin D has a protective effect on vascular endothelial cells.

1. Introduction

Coronary artery disease is the leading cause of mortality in the United States [1]. Several risk factors predict the development of coronary artery disease, including dyslipidemia and the presence of diabetes, obesity, and the metabolic syndrome [2–4]. Recently, several studies have shown that plasma vitamin D levels are inversely correlated with atherosclerosis [5–7]. While it is not clear how vitamin D impacts the development of coronary artery disease, several studies have indicated that part of the mechanism may involve vitamin D's effects on immune cells, especially macrophages [8,9]. In some trials however, treatment of vitamin D-deficient patients with vitamin D had little or no effect on lowering triglyceride, low-density lipoprotein (LDL) and total cholesterol levels, and raising high-density lipoprotein levels [10,11].

Endothelial cells form an essential barrier function and are involved in regulating the passage of plasma constituents and immune cells into the extravascular space. Pro-inflammatory cytokines, free-fatty acids, and hyperglycemia have been shown to induce intracellular stress responses in endothelial cells, promoting oxidative stress and endoplasmic reticulum (ER) stress, as well as the production of chemo-attractant molecules such as monocyte chemo-attractant protein-1 (MCP-1), intercellular adhesion molecule 1 (ICAM 1), and vascular cell adhesion molecule 1 (VCAM

Abbreviations: CSPD, 3-(diethylamino)-1-(2,2-dimethyl-3-H-1-benzofuran-7-yl)-propan-1-one; eIF2α, eukaryotic initiation factor 2α; DMSO, dimethylsulfoxide; EB, EB1089; ER, endoplasmic reticulum; ES-TRAP, placental secreted alkaline phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRP 78, glucose-regulated protein 78; HBSS, Hank's balanced salt solution; HDL, high-density lipoprotein; HUVEC, human umbilical vein endothelial cell; ICAM 1, intercellular adhesion molecule 1; JNK1, c-*jun* N-terminal kinase 1; LDL, low-density lipoprotein; MCP-1, monocyte chemo-attractant protein-1; N.S., not significant; PCR, polymerase chain reaction; PI, propidium iodide; R.L.U., relative light units; SEAP, secreted alkaline phosphatase; SO, superoxide; TM, tunicamycin; Tris-Cl, tris(hydroxymethyl)aminomethane hydrochloride; VCAM 1, vascular adhesion molecule 1; VDR, vitamin D receptor; XBP-1, X-box binding protein-1; ZK, ZK 1911784

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1) [12–15]. Extravasation of monocytes and passage of oxidized LDL through a damaged endothelium allows for the formation of lipid-laden foam cells. Access to the sub-endothelial space also allows for activation of clotting and complement-based injury.

Several circulating factors have been shown to have favorable effects on endothelial barrier function. Antioxidants, estradiol, testosterone, and high-density lipoprotein (HDL) have been shown to inhibit oxidative stress, ER stress, chemo-attractant molecule expression [16–18], while promoting NO release and relaxation of vascular tone [19,20]. Since elevated plasma vitamin D levels are inversely correlated with atherosclerosis and cardiovascular disease, we assessed the ability of vitamin D to inhibit ER stress and hyperglycemia-induced oxidative in human primary endothelial cells.

2. Materials and methods

2.1. Materials

1,25-dihydroxy vitamin D_3 (1,25-$(OH)_2D_3$), 25-hydroxyvitamin D_3 (25-OHD_3), and 24,25-dihydroxyvitamin D_3 (24,25-$(OH)_2D_3$) were obtained from Sigma-Aldrich (St. Louis, MO.) The chemiluminescent alkaline phosphatase substrate 3-(diethylamino)-1-(2,2-dimethyl-3-H-1-benzofuran-7-yl)-propan-1-one (CSPD) was purchased from Clontech Laboratories, Inc. (Mountain View, CA). Lipofectamine and hydroethidine were obtained from Invitrogen (Carlsbad, CA.) Tunicamycin (TM) was purchased from Cayman Chemical (Ann Arbor, MI). EB1089 (EB) was a gift from LEO Pharma (A/S, Ballerup, Denmark) and ZK-191784 (ZK) was a gift from (Schering A.G., Berlin, Germany). EB1089 (seocalcitol) is a low-calcemic analog of 1,25-$(OH)_2D_3$ that has been examined extensively for its growth inhibitor effects on various cancer cells both *in vitro* and in clinical trials. ZK-191784 was developed as a vitamin D receptor antagonist [21]. All other chemicals were obtained from Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA).

2.2. Cell culture

Primary human umbilical endothelial cells (HUVEC) were obtained from Invitrogen and maintained in complete, phenol red-free endothelial cell growth medium (Invitrogen). For each experiment, cells were released with trypsin and plated in six-well plates until 70–80% confluent. Cells were maintained in a humidified incubator at 37 °C and 5% CO_2. Cells between passages 2–8 were used in each experiment.

2.3. ER stress measurement

ER stress was measured using the placental secreted alkaline phosphatase (ES-TRAP) assay [22] (Great EscAPe™, Clontech Laboratories, Inc.). Briefly, HUVEC at 70–80% confluence in six-well plates were transfected with the plasmid pSEAP2-Control, containing a truncated placental alkaline phosphatase gene adjacent to the simian virus 40 early gene promoter, and 24-h later treated with 5.5 or 27.5 mM dextrose with and without the indicated amounts of each compound or the solvent ethanol. After 24-h, secreted alkaline phosphatase (SEAP) activity was measured by mixing 25-μl of conditioned medium with 75-μl of dilution buffer, which was incubated at 65 °C for 30-min in 1.5 ml microcentrifuge tubes. After cooling on ice for three minutes, 100 μl of CSPD was added to each tube. The samples were incubated at room temperature for 30-min and SEAP activity was measured with a Modulus luminometer (Promega, Madison, WI). The solvent dimethylsulfoxide (DMSO) (diluted 1:1000) had no effect on SEAP activity. As a negative control, SEAP activity was measured in cells transfected with pSEAP2-Basic, which contains the placental SEAP gene but lacks a eukaryotic promoter to drive expression.

2.4. Superoxide generation

Superoxide (SO) generation was measured using hydroethidine fluorescence [23]. HUVEC were trypsinized and transferred to 96-well black, clear bottom fluorescence assay microtiter plates at a density of 50,000 cells per well. Twenty four-hours after plating, the cells were treated as described in the text, then washed with sterile Hank's balanced salt solution (HBSS) containing 1.26 mM $CaCl_2$, 5.37 mM KCl, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2 \cdot 6H_2O$, 0.41 mM $MgSO_4 \cdot 7H_2O$, 136.7 mM NaCl, 4.2 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, and 5.5 mM D-glucose. Hydroethidine (in HBSS) was added to a final concentration of 10 μmol/L and fluorescence was measured every 10-min for a total of 60-min, using a microplate fluorescence spectrophotometer (Wallac 1420 Multilabel Counter, Turku, Finland) with 488 nm excitation and 610 nm emission. The rate of SO generation was calculated from the rate of appearance of the oxidized hydroethidine within the first hour of adding the indicator. Each experiment was repeated six times.

2.5. Western blotting

HUVEC were treated with solvent (DMSO) and 0.1 μM TM with or without 50 nM 1,25-$(OH)_2D_3$ and after 24-h protein extracts were prepared by suspending the cells in 200 μl of lysis buffer (12.5% glycerol, 40 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-Cl) (pH8.0), and 0.1% sodium dodecylsulfate). Protein content was measured as described [24] and 50 μg of protein extract was fractionated by electrophoresis on a 10%-sodium dodecylsulfate polyacrylamide gel [25]. After transfer to nitrocellulose [26], the membrane was blocked with 10% newborn calf serum (NCS) in Tris-buffered saline/Tween 20 (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% Tween 20) and incubated overnight with antibodies to vitamin D receptor (VDR) (EMD Millipore, Billerica, MA), GRP 78, JNK1, phospho-JNK1, eIF2α, or phospho-eIF2α (all from Cell Signaling Technology, Danvers, MA) (all diluted 1:750) followed by a goat-anti-rabbit secondary antibody conjugated to horseradish peroxidase (Sigma) (diluted 1:5000) and binding was detected by enhanced chemiluminescence with reagents from Pierce (Rockford, IL). The membranes were then stripped and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were measured as a loading control with an antibody from Santa Cruz Biotechnology (Dallas, TX).

2.6. XBP1 mRNA splicing

Total RNA was isolated from endothelial cells treated with ER stress inducers with and without 50 nM 1,25-$(OH)_2D_3$ as described [27]. One μg of RNA was reverse transcribed with avian myeloblastosis virus reverse transcriptase and 100 ng of cDNA was used in each polymerase chain reaction (PCR) with the following primers: XBP1 forward, 5′-TTA CGA GAG AAA ACT CAT GGC C-3′; XBP1 reverse, 5′-GGG TCC AAG TTG TCC AGA ATG C-3′. After PCR, the samples were fractionated by electrophoresis on a 5% polyacrylamide gel in 1 × Tris-borate-ethylene diaminetetraacetic acid. The gel was stained with ethidium bromide and photographed.

2.7. Inhibition of VDR expression via siRNA silencing

To knockout VDR expression, HUVEC were transfected with 10 nM of control siRNA or 5 and 10 nM of VDR-specific siRNA (both from Santa Cruz Biotechnology) using Lipofectamine for 72-h and VDR levels were measured by Western blotting.

To examine the effect of VDR on SO generation, HUVEC were