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The preventive effect of uncarboxylated osteocalcin against free fatty acid-induced endothelial apoptosis through the activation of phosphatidylinositol 3-kinase/Akt signaling pathway

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ABSTRACT

Objective. Increasing evidence suggests that osteocalcin (OC), one of the osteoblast-specific proteins, has been associated with atherosclerosis, but results are conflicting. The aim of this study was to elucidate the independent effect of uncarboxylated osteocalcin (ucOC), an active form of osteocalcin which has been suggested to have an insulin sensitizing effect, on vascular endothelial cells.

Materials and Methods. We used human aortic endothelial cells and treated them with ucOC. Linoleic acid (LA) was used as a representative free fatty acid. Apoptosis was evaluated using various methods including a terminal deoxyribonucleotide transferase-mediated deoxyuridine triphosphate nick-end labeling analysis kit and Western blotting for cleaved caspase 3, cleaved poly (ADP-ribose) polymerase and Bcl-xL. The phosphorylations of Akt and endothelial nitric oxide synthase (eNOS) as well as the level of NO were measured to confirm the effect of ucOC on insulin signaling pathway.

Results. Pretreatment of ucOC (30 ng/ml) prevented LA-induced apoptosis in insulin-stimulated endothelial cells; effects were abolished by pretreatment with the phosphatidylinositol 3-kinase (PI3-kinase) inhibitor, wortmannin. Treatment of ucOC (ranged from 0.3 to 30 ng/ml) significantly increased the phosphorylation of Akt and eNOS and nitric oxide secretion from endothelial cells in a PI3-kinase dependent manner.

Conclusions. Our study is the first to demonstrate the independent effect of ucOC on vascular endothelial cells. Our results further suggest that ucOC could have beneficial effects on atherosclerosis.

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Abbreviations: OC, osteocalcin; cOC, carboxylated osteocalcin; ucOC, uncarboxylated osteocalcin; PI3-kinase, phosphatidylinositol 3-kinase; FFA, free fatty acid; HAEC, human aortic endothelial cell; LA, linoleic aicd; PARP, poly (ADP-ribose) polymerase; eNOS, endothelial nitric oxide synthase; TUNEL, terminal deoxyribonucleotide transferase-mediated deoxyuridine triphosphate nick-end labeling.

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1. Introduction

Bone remodeling is highly costly in terms of energy. This means that there must be cross-talk between bone and the endocrine organs that are involved in energy metabolism. A number of studies seeking to delineate the putative bone-derived signals that are responsible for this cross-talk [1,2] have found that osteocalcin (OC) may play a significant role [1,3]. Although there have been some evidences which suggest that other cell types including adipocyte produce OC, OC is a small peptide that is mainly secreted by osteoblasts and is present in the bone matrix and blood [4]. It is composed of 49 amino acids and is secreted by osteoblasts after undergoing multiple post-translational modifications, including the vitamin K-dependent γ-carboxylation of glutamyl residues into Gla residues [5]. This modification means that two forms of OC exist, namely, carboxylated OC (cOC) and uncarboxylated OC (ucOC) [6]. In vitro experiments on isolated islets and primary adipocytes and a subsequent in vivo experiment revealed that cOC is largely bound to hydroxyapatite, the mineral of bone, and is the inactive form of OC, while ucOC is the active form in terms of energy metabolism [1,3].

Recombinant (i.e. uncarboxylated) OC regulates the gene expression of pancreatic beta cells and adipocytes, thereby improving glucose tolerance [1,3] and insulin resistance of mouse models of hyperphagia and diet-induced obesity [3]. Despite this beneficial effect of ucOC on beta cells and adipocytes, the direct effect of ucOC on vascular cells has not been investigated to date. Given that ucOC stimulates the expression of the anti-atherogenic adipocytokine adiponectin in adipocytes [1,3,7], its effect on vascular cells is expected to be anti-atherogenic. However, the direct and independent effects of ucOC on vascular cells remain to be determined.

A previous study has reported that OC has a hormonal function that links bone to glucose metabolism: intermittent injections of ucOC improved the glucose metabolism of mice fed with a high-fat diet by partially restoring insulin sensitivity [8]. In the present study, it was hypothesised that ucOC may prevent apoptosis in vascular endothelial cells through this insulin-sensitizing effect, specifically by stimulating a representative insulin-signaling pathway, namely, the phosphatidylinositol 3-kinase (PI3-kinase)/Akt signaling pathway [8,9]. Cultured endothelial cells were used to examine whether ucOC affects this insulin-signaling pathway and, if so, whether it protects vascular endothelial cells from free fatty acid (FFA)-induced apoptosis via its insulin-sensitizing activity.

2. Materials and methods

2.1. Cell culture and treatment

Human aortic endothelial cells (HAECs) were obtained from Lonza (Walkersville, MD, USA) and maintained at 37 °C in a humidified incubator supplemented with 5% CO_2 in endothelial basal medium (EBM-2; Lonza) supplemented with 2% fetal bovine serum (FBS) and various growth factors that are required for the growth of endothelial cells. In all experiments, cells were used after six or fewer passages.

Linoleic acid (LA) was obtained from Sigma Chemical (St. Louis, MO, USA) and served as a representative FFA. DMSO was used as a vehicle. The cells were transferred to medium containing 0.5% FBS and incubated for the indicated times in medium containing 100 μmol/L LA. Human ucOC was obtained from US Biological (MA, USA). The amino acid sequence of this purchased human ucOC was as follows: Tyr-Leu-Tyr-Gln-Trp-Leu-Gly-Ala-Pro-Val-Pro-Tyr-Pro-Asp-Pro-Leu-Glu-Pro-Arg-Arg-Glu-Val-Cys-Glu-Leu-Asn-Pro-Asp-Cys-Asp-Glu-Leu-Ala-Asp-His-Ile-Gly-Phe-Gln-Glu-Ala-Tyr-Arg-Arg-Phe-Tyr-Gly-Pro-Val. It has three Glu residues at positions 17, 21, and 24 and no Gla (carboxylated Glu) residues. The anti-apoptotic effect of ucOC was examined by adding OC 1 h before concurrent LA and regular insulin treatment (Lilly, Seoul, Korea). The role of PI3-kinase in the anti-apoptotic effect of ucOC was determined by preincubating the cells for 15 min with 100 nmol/L wortmannin (Calbiochem, Darmstadt, Germany), which is a specific covalent inhibitor of PI3-kinase; thereafter, the cells were treated with ucOC.

2.2. Western blotting analysis

After lysing the cells, the protein samples (20 μg/lane) were resolved by electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were incubated in blocking buffer and then incubated with one or more of the following primary antibodies: anti-Akt (1:1000 Cell Signaling, Danvers, MA, USA), anti-phospho-Akt (Ser473) (1:1000, Cell Signaling), anti-caspase 3 (1:1000, Cell Signaling), anti-cleaved caspase 3 (1:1000, Cell Signaling), anti- poly (ADP-ribose) polymerase (PARP, 1:1000, Cell Signaling), anti-Bcl-xL (1:1000, Cell Signaling), anti-phospho endothelial nitric oxide synthase (eNOS, Ser1177) (1:1000, BD Bioscience, San Jose, CA, USA), anti-eNOS (1:1000, Cell Signaling), anti-β-actin (1:10000, Sigma) and anti-GAPDH antibody (1:1000, Cell Signaling). Thereafter, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA, USA). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Bioscience, UK).

2.3. Analysis of apoptosis

Apoptosis was measured by various methods, namely, a cell death enzyme-linked immunosorbent assay (ELISA) kit that monitors cytoplasmic histone-associated DNA fragmentation (Cell Death Detection ELISAPlus; Roche Diagnostics, Mannheim, Germany), an ApoAlert Caspase 3 Fluorescent Assay Kit (Clontech, CA, USA), a terminal deoxyribonucleotide transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) analysis kit (In Situ Cell Death Detection kit, Fluorescein; Roche Diagnostics), and Western blotting for cleaved caspase 3 [10], cleaved PARP [11], and Bcl-xL expression [12]. The commercial assays were carried out according to the kit supplier's instructions.

2.4. Determination of nitric oxide (NO) concentration

Concentrations of NO in the conditioned media were measured by ELISA kit (Enzo Life Science, NY, USA) according to the kit supplier's instructions.

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