Antioxidants Inhibit Fatty Acid and Glucose-Mediated Induction of Neutral Endopeptidase Gene Expression in Human Microvascular Endothelial Cells

Pornprom Muangman, MD, Richard N Tamura, PhD, Nicole S Gibran, MD, FACS

BACKGROUND: Neutral endopeptidase (NEP) is a membrane-bound metallopeptidase that degrades tachykinins and may regulate their role in wound repair. NEP enzyme activity is increased in diabetic wounds and skin compared with normal controls. We have shown that unsaturated fatty acids and glucose upregulate NEP activity in human microvascular endothelial cells (HMECs) and that vitamins E and C reduce this effect.

STUDY DESIGN: To determine whether these changes involve NEP gene expression regulation, we analyzed NEP mRNA levels in HMECs cultured with elevated glucose (40 mM) and fatty acids oleate (40 μM) and linoleate (40 μM) for 48 hours or 1 month. Cells were exposed for an additional 48 hours to antioxidants vitamins E or C or *N*-acetylcysteine. Total RNA was extracted and analyzed for NEP mRNA using real-time reverse transcriptase polymerase chain reaction. NEP gene expression was standardized to β-actin mRNA and results were analyzed using ANOVA.

RESULTS: Elevated glucose, oleate, and linoleate upregulated NEP mRNA in short and longterm HMEC cultures, but did not alter rate of NEP mRNA degradation. Vitamins E and C and *N*-acetylcysteine blocked glucose- and fatty acid–induced NEP mRNA ($p \leq 0.05$). The potential role of oxidative stress in NEP activation was confirmed by demonstrating that elevated glucose and fatty acids increase H_2O_2 levels in HMECs.

CONCLUSIONS: Regulation of NEP enzyme activity by glucose and fatty acids appears to include gene expression transcription as well as modulation of enzyme activity. Our results also suggest that oxidative stress may be involved in upregulation of NEP by fatty acids and glucose. (J Am Coll Surg 2005; 200:208–215. © 2005 by the American College of Surgeons)

Peripheral neuropathy is a major complication associated with diabetes mellitus (DM). Loss of sensation, along with microvasculature abnormalities, contributes to the cause of nonhealing cutaneous ulcers that often lead to amputation. The American Diabetes Association predicts 14% to 24% risk of amputation in diabetic patients with nonhealing foot ulcers.[1] Mechanisms for debilitating nonhealing cutaneous ulcers are complicated and multifactorial. Type 2 DM is especially common in older overweight Americans.[2] Epidemiological data strongly implicate hyperglycemia and also hyperlipidemia as dominant influences on development of diabetic complications.[2] Substance P (SP) is a neuropeptide of the tachykinin family produced in sensory neurons and stored at the terminal ends of unmyelinated cutaneous nerve fibers.[3] Sensory nerves release SP to modulate response to injury, including vasodilatation,[4] neutrophil infiltration,[5] enhanced adhesion molecule expression by endothelial cells, macrophage chemotaxis,[6] endothelial cell proliferation,[7] and neovascularization.[8] We have demonstrated that topical SP application improves wound-healing kinetics in a diabetic murine model of delayed wound healing.[9]

SP activity is regulated by neutral endopeptidase (NEP), a cell surface enzyme also known as enkephali-

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From the Department of Surgery, University of Washington, Harborview Medical Center, Seattle, WA.

Correspondence address: Nicole Gibran, MD, FACS, Department of Surgery, Harborview Medical Center, Box 359796, 325 Ninth Ave, Seattle, WA 98104.

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Abbreviations and Acronyms
DM = diabetes mellitus
HMEC = human microvascular endothelial cells
NEP = neutral endopeptidase
PCR = polymerase chain reaction
SP = substance P

nase, neprilysin, common acute lymphoblastic antigen, or CD10 that cleaves vasoactive peptides such as neuropeptides, atrial natriuretic peptide, bradykinin, angiotensin I, and endothelin.[10] Much of the NEP molecule, including the active site, is extracellular where it degrades peptides at the cell surface by hydrolyzing bonds on the amino side of hydrophobic residues. The amino acid sequence of NEP includes a single sequence of 20 hydrophobic residues near the N-terminal that anchor the enzyme to the plasma membrane.[11]

NEP localization and enzyme activity are increased in diabetic wounds and skin compared with normal controls.[12] Topical application of the NEP inhibitor thiorphan onto diabetic mice wounds accelerates wound-closure kinetics compared with normal saline treatment.[1] So upregulation of NEP enzymatic activity may reduce neuropeptide levels and contribute to the delayed response to injury observed in diabetic murine wounds.

Elevated unsaturated fatty acids and glucose levels, as seen in type 2 DM, upregulate NEP enzyme activity in human microvascular endothelial cells (HMECs); the antioxidants vitamin E and vitamin C significantly abrogate this response.[13] In this study, we examine whether elevated glucose and lipids effects on NEP activity are associated with increased NEP gene expression. In addition, we evaluate the potential role of oxidative stress associated with hyperlipidemia and hyperglycemia.

METHODS

Endothelial cells

HMECs were purchased from Cascade Biologicals and were used by passage six for these experiments. Cells were cultured as described previously.[13] Confluent HMEC monolayers were cultured for either 48 hours (short term) or 1 month (longterm) with media containing unsaturated fatty acid, including oleic acid (40 μM, 18:1 ω-9), linoleic acid (40 μM, 18:2 ω-6), and elevated glucose (40 mM). The unsaturated fatty acids oleic acid and linoleic acid were selected basedacid upon previous studies that included unsaturated, saturated, and ω-3 fatty acids, in which only oleic acid and linoleic acid at a maximal concentration of 40 μM significantly upregulated NEP enzyme activity in HMECs ($p < 0.05$).[13]

Short-term and longterm cultures were cultured with and without antioxidants for the last 48 hours of the experiment. As optimized in previous studies, antioxidant concentrations included: 5 mM vitamin C for 48 hours; 100 μM vitamin E for 4 hours followed by 10 μM vitamin E for 48 hours; and 1 mM *N*-acetylcysteine for 48 hours. Two phases of vitamin treatment facilitated early (high concentration for a short time) incorporation of this lipid soluble molecule into the cell surface membrane and subsequent to lower less cytotoxic concentration over the longer (48 hours) exposure period. Each condition was performed in duplicate and the experiments were repeated multiple times. Supernatant was collected to measure hydrogen peroxide release by the cells as described in the following text. Cell monolayers were harvested and processed to measure either NEP enzymatic activity or NEP mRNA expression.

NEP enzymatic assay

The NEP enzymatic assay was performed as described previously.[14] The specific NEP inhibitor, DL-thiorphan, was used to differentiate NEP enzyme activity from nonspecific endopeptidase activity. Nonspecific enzyme activity was used as a control to verify that the effects of lipids and glucose were not generalized to all endopeptidases. Data of enzyme activity are expressed as picomoles 4-methoxy-2-naphthylamine (MNA)/h/μg protein.

Hydrogen peroxide assay

Glucose- and fatty acid–induced oxidative stress was evaluated by measuring the amount of hydrogen peroxide present in the tissue culture media after use of commercially available Colorimetric Hydrogen Peroxide Kit (Assay Designs, Inc) according to manufacturer's protocol. The reaction product was read using a spectrophotometric microplate reader (Spectra MAX Plus; Molecular Devices) at 550 nm. Data were exported to data analysis software Microsoft Excel 2003 to calculate sample concentrations.

Quantitative reverse transcriptase polymerase chain reaction

HMEC monolayers were lysed with TRIzol Reagent (Life Technologies). Total RNA was isolated according