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Original Research

Role of Janus-associated kinases in somatostatin analog preconditioning of human umbilical-vein endothelial cells

Tzong-Luen Wang [a,b,*], Yu-Hui Yang [c]

[a] Department of Emergency Medicine, Shin-Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan
[b] Medical School, Fu-Jen Catholic University, Taipei, Taiwan
[c] Central Laboratory, Shin-Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan

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Abstract

Background: Somatostatin (SST) has been proven to have cardioprotective effects, but its effects on endothelial cells has not yet well studied.
Aim: To confirm the phenomenon of SST-induced preconditioning (PC) and the cellular mechanisms involved, we designed an *in vitro* study to investigate the effects of SST analogs on tumor necrosis factor (TNF)-α-induced endothelial nuclear factor (NF)-κB activation with subsequent interleukin (IL)-6 and IL-8 release.
Methods: Experiments were performed on primary human umbilical-vein endothelial cells (HUVECs). IL-6 and IL-8 were measured using commercial enzyme-linked immunosorbent assay kits. An electrophoretic mobility shift assay (EMSA) was used to demonstrate NF-κB activation. The effects of pretreatment with octreotide, an SST analog, and/or *N*-acetyl-cysteine (NAC) were tested.
Results: TNF-α stimulated IL-6 and IL-8 production from HUVECs. SST PC using octreotide at concentrations $>10^{-8}$ M attenuated TNF-α-induced IL-6 and IL-8 release, but NAC did not inhibit SST-treated endothelial cells stimulated by TNF-α. EMSA revealed that TNF-α treatment was associated with activation of NF-κB, which could be inhibited by SST PC. By contrast, wortmannin and AG-490 reversed the inhibitory effects of octreotide on TNF-α-induced NF-κB activation, but neither had any definite effects on TNF-α-induced NF-κB activation in the absence of octreotide. Western blots confirmed that octreotide modulated Iκκ at 10^{-8} M.
Conclusion: SST PC modulates Iκκ in a PI3K- and JAK-2-dependent pathway, which in turn attenuates activation of NF-κB induced by TNF-α in HUVECs.

Keywords: HUVECs; Interleukins; Preconditioning; Somatostatin; TNF-α

1. Introduction

We previously demonstrated many modes of preconditioning (PC) in experiments to show cardioprotective effects.[1–3] PC represents a powerful intervention that reduces infarct size after ischemia–reperfusion or other pretreatments such as stimulation or induction of the adrenergic system,[4] adenosine receptors,[5] ATP-sensitive potassium channels,[6] heat shock proteins,[7] and oxidative stress.[8] Some investigators further studied the possible effector cells of such PC. Although we proposed that neutrophils are target cells for PC,[1] endothelial cells are still the main effectors cells used in most research.[2,9–11]

Endothelial cells are associated with many pathophysiological changes that may be related to PC. Theoretically, endothelial PC should be closely related to the mechanisms of PC mentioned above.[6] However, the real link between endothelial PC and myocardial PC deserves further investigation. One of the mechanisms involved in endothelial PC is oxidative stress. It has been suggested that oxidative stress and cellular redox status are significant modulators of intracellular signaling.[12] Zahler et al[9] demonstrated that endothelial cells can be preconditioned by transient intracellular redox stress to reduce

* Corresponding author. Department of Emergency Medicine, Shin-Kong Wu Ho-Su Memorial Hospital, 95 Wen Chang Road, Taipei, Taiwan.
E-mail address: m002183@ms.skh.org.tw (T.-L. Wang).

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responsiveness to an inflammatory mediator. Translocation of the transcription factor nuclear factor (NF)-κB may facilitate novel approaches directed at alleviating reperfusion damage. Because it has been demonstrated that mechanical stretch can activate matrix metalloprotease expression via tumor necrosis factor (TNF)-α and the NF-κB pathway,[13] NF-κB may be the central mediator of endothelial PC, whereas mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channels are believed to be the core mediators of myocardial PC. We previously demonstrated a reduction in infarct size due to SST PC,[3] so it seemed reasonable to investigate if SST PC could also decrease damage due to TNF-α as an additional protective mechanism.

We thus designed an *in vitro* study to investigate the phenomenon of SST-induced PC and the signaling mechanisms involved in human endothelial cells.

2. Methods

2.1. Primary culture of human umbilical-vein endothelial cells

Endothelial cells were cultured from human umbilical veins (<24 hours post partum) using a modification of the method described by Jaffe et al.[14] After cannulation at both ends, cords were flushed with 120 mLof HEPES-buffered saline (10 mM) and incubated with 0.2% collagenase (Sigma, St. Louis, MO, USA) at 37°C for 15 minutes. HUVECs were grown to 100% confluence on gelatin-coated T25 flasks (Becton Dickinson, Franklin Lakes, NJ, USA) in medium 199 supplemented with 20% fetal calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, and 0.2 M L-glutamine (Sigma) at pH 7.4 and 37°C. Purification was determined by flow cytometry (FACS, Becton Dickinson) with a factor VIII-related antigen antibody. Endothelial cells in the first passage were used for experiments.

2.2. STT PC experimental protocol

In PC experiments, medium was removed from confluent HUVECs and replaced with PBS containing octreotide (an SST analog at 10^{-10} M, 10^{-8} M, or 10^{-6} M) for 5 minutes at 37°C. The supernatant was then removed and cells were covered with standard medium again and incubated with TNF-α (2.5 ng/mL) for 4 hours. The supernatant was then sampled for measurement of IL-6 and IL-8. Cells were detached. Time-matched controls were treated with neither octreotide nor TNF-α. Further experimental groups consisted of cells treated with octreotide (5 minutes) or TNF-α (4 hours) alone. For comparison, experiments were repeated in cells pretreated with the intracellular radical scavenger *N*-acetyl-cysteine (NAC, 1 mM) for 30 minutes in comparative groups. After incubation, the cells were washed to remove all extracellular NAC and then subjected to the aforementioned procedures. Some experiments were terminated at 5 minutes or 30 minutes after SST treatment for analysis of subcellular NF-κB localization.

2.3. Interleukin ELISA

Levels of the cytokines IL-6 and IL-8 were determined in cell culture supernatants using commercial enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Woburn, MA, USA). To determine IL-8, samples were diluted 1:100. Sample aliquots of 50 μL per well were used in all cases and measurements were performed according to the manufacturer's instructions. In brief, ELISA plates were incubated with standards or samples for 60 minutes. Unbound cytokine was removed by washing three times, and a second antibody (labeled enzymatically for photometric detection) was added. After further incubation and removal of unbound antibody by washing, the plates were analyzed photometrically using a microplate reader (Dynatech, Guernsey, UK) at 450 nm. As an indicator of cell death, release of lactate dehydrogenase into the supernatant was also measured photometrically at 490 nm.

2.4. NF-κB electrophoretic mobility shift assay

The protein content of nuclear extracts was determined using a BioRad (Hercules, CA, USA) protein assay. Oligonucleotide sequences were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A double-strand NF-κB consensus oligonucleotide probe (5′-AGT TGA GGG GAC TTT CCC AGG-3′) was labeled with ^{32}P and 4 μg of nuclear protein in 2 μL of nuclear extract was mixed with 20 pmol of [γ-^{32}P] dATP-labeled consensus or mutant oligonucleotide in a total volume of 20 μL at room temperature for 30 minutes. The samples were then resolved on a 4% polyacrylamide gel. Gels were dried and imaged by autoradiography at −70°C.

2.5. Signaling pathways for TNF-α and STT PC

To elucidate the underlying signaling mechanisms, HUVECs were subjected to different pretreatment protocols, including AG-490 [100 μM; a Janus-associated kinase (JAK)-2 antagonist], PD98059 [50 μM; a p42/p44 mitogen-activated protein kinase (MAPK) inhibitor], genistein (40 μM; a tyrosine kinase inhibitor) and wortmannin (50 nM; a phosphoinositide-3-kinase (PI3K) inhibitor).

2.6. Iκκ protein expression

HUVECs were lysed in a reducing Triton lysis buffer (Tris 1 mM, NaCl 50 mM, Triton X, sodium vanadate 5 mM, NaF 50 mM, Na pyrophosphate 30 mM, DTT 1 mM, PMSF 1 mM, leupeptin 10 μM, pepstatin 10 μM). Nuclear and cytosolic fractions were separated by centrifugation (10,000 *g* for 5 seconds) and the membrane fraction was removed by centrifugation at 10,000 *g* (4°C) for 15 minutes. The protein concentration in nuclear and cytosolic lysates was determined using a detergent-compatible assay (BCA, Pierce, Rockford, IL, USA). Aliquots (40 μg) of protein were separated on a 12% polyacrylamide gel, transferred to a nitrocellulose membrane (Hybond-enhanced chemiluminescence, Amersham, Braunschweig, Germany), blocked with buffer