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Cell-specific regulation of iNOS by AMP-activated protein kinase in primary rat hepatocytes

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ABSTRACT

Background: AMP-activated protein kinase (AMPK) regulates several metabolic pathways in hepatocytes that are critical to the hepatic response to sepsis and shock. Induction of nitric oxide synthesis is an important response to sepsis, inflammation and shock and many of the stimuli that upregulate inducible nitric oxide synthase (iNOS) also activate AMPK. AMPK inhibits nitric oxide (NO) production in skeletal and cardiac muscle cells, but the role of AMPK in regulating iNOS expression in hepatocytes has not been determined.

Materials and methods: Primary cultured rat hepatocytes were preincubated with an AMPK inhibitor, AMPK activators, or transfected with AMPK siRNA before being treated with the proinflammatory cytokines interleukin-1β (IL-1β) and interferon-γ (IFNγ). The hepatocyte cell lysate and culture supernatants were collected for Western blot analysis and Griess assay.

Results: IL-1β and IFNγ markedly upregulated iNOS expression and AMPK phosphorylation. IL-1β + IFNγ–induced NO production and iNOS expression were significantly decreased in hepatocytes treated with the AMPK inhibitor compound C and AMPK knockdown by AMPK siRNA. Cytokine-induced iNOS expression was increased by AMPK activators 1-oxo-2-(2H-pyrrolium-1-yl)-1H-inden-3-olate, AMPK signaling activator III and AICA-riboside. Compound C upregulated Akt and c-Jun N-terminal kinase phosphorylation but decreased IκBα phosphorylation. AICA-riboside exerted opposite effects on these signaling pathways in hepatocytes.

Conclusions: In contrast to other cell types, AMPK increased IL-1β + IFNγ–induced NO production and iNOS expression through the Akt, c-Jun N-terminal kinase, and NF-κB signaling pathways in primary hepatocytes. These data suggest that AMPK-altering medications used clinically may have subsequent effects on iNOS expression and proinflammatory signaling pathways.

Introduction

AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine kinase containing α, β, and γ subunits.[1,2] Combinations of these subunits can generate 12 heterotrimeric complexes that show relative tissue specificity.[1,2] Because of its responsiveness to alterations in the AMP/ATP ratio, AMPK is an important regulator of cell metabolism during cellular stress. An increase in the AMP/ATP ratio, which occurs with energy consumption triggers AMP binding to the AMPK γ subunit, phosphorylation of the α subunit and AMPK activation.[3] AMPK can be activated by any cellular stress that depletes cellular ATP including inflammatory cytokines.[4] Once activated, AMPK can phosphorylate many

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downstream proteins to reduce ATP consumption and promote ATP production. This effect of AMPK increases the ATP/ADP ratio and restores energy for cellular metabolism.[3] The restoration of cellular energy metabolism by AMPK is the likely mechanism responsible for the beneficial effect of AMPK activators in reducing alcohol-induced liver injury, decreasing hepatic ischemia/reperfusion (I/R) injury, and improving the results of hepatic transplantation.[5-7]

In the liver, AMPK regulates insulin signaling, contributes to the development of steatohepatitis, and regulates glucose production.[8] AMPK activators are also used clinically to treat diabetes.[8] AMPK stimulates fatty acid oxidation and inhibits cholesterol and triglyceride synthesis by downregulating genes involved in lipogenesis, glucose uptake, and glycolysis in the liver.[4] Infection and inflammation lead to the release of cytokines, induction of cellular stress, and profound alterations in hepatic metabolic pathways[9]; but, the role of AMPK in altering hepatic physiology in sepsis is incompletely defined. Recent studies have demonstrated that AMPK plays an important role in I/R-induced renal injury,[10] the pulmonary response to infection,[11] and facilitates bacterial eradication in sepsis.[12] AMPK exerts antiinflammatory effects by inhibiting nitric oxide (NO)—induced cell death and decreasing endothelial NO synthase.[13,14] AMPK also decreases inducible NO synthase (iNOS) in several cell types[14-18] and promotes recovery from NO-mediated cellular stress.[19,20] While AMPK activators have a beneficial role in reducing hepatic I/R injury,[6] the role of AMPK in the hepatic regulation of iNOS has not been defined. We therefore evaluated the role of AMPK in regulating hepatocyte iNOS expression and demonstrate that, in contrast to other cell types, AMPK activation upregulates hepatocyte iNOS expression.

Materials and methods

Reagents

Williams medium E, human recombinant interleukin-lβ (IL-1β) and interferon-γ (IFNγ) were purchased from Life Technologies (Carlsbad, CA). The AMPK inhibitor compound C and AMPK activators 1-oxo-2-(2H-pyrrolium-1-yl)-1H-inden-3-olate, AMPK signaling activator III (DHPO) and AICA-riboside (AICAr) were purchased from EMD Chemicals, Inc (Gibbstown, NJ). The antibody to iNOS was from BD Bioscience (Billerica, MA) and antibodies to IKBα, AMP kinase, c-Jun N-terminal kinase (JNK), and Akt were from Cell Signaling Technology (Danvers, MA). The siRNA control set A and set B, and AMPKα1 and AMPKα2 siRNA were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). All other reagents were purchased from Sigma Chemical Co (St. Louis, MO).

Primary hepatocyte culture

All experimental protocols were approved by the University of Louisville Animal Care and Use Committee and followed guidelines prescribed by the National Institutes of Healths Guidelines for the Care and Use of Laboratory Animals. Rat hepatocytes were harvested from male Sprague—Dawley rats (Harlan-Sprague-Dawley, Madison, WI) using collagenase perfusion and differential centrifugation as previously described.[21] The hepatocyte population was >98% pure and had a viability of >90%. Hepatocytes were plated into six-well collagen-coated plates at 10^6 cells per well in Williams medium E containing insulin (10^{-6} M), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (15 mM), L-glutamine, penicillin, streptomycin, and 10% fetal bovine serum (HyClone Laboratories Inc, Logan, VT). After 4 h, the cells were washed with phosphate buffered saline to remove nonadherent cells, the media replaced with insulin-free media containing 5% fetal bovine serum, and hepatocytes were cultured overnight at 37°C. After overnight incubation, the cells were washed again with phosphate buffered saline to remove dead and nonadherent cells, and the experimental conditions were established. Cultures were performed in triplicate, and experiments were repeated to ensure reproducibility.

siRNA transfection

Primary hepatocytes were cultured in 12-well plates at a density of 2.5×10^5 cells per well. After 24 h of incubation, a mixture of AMPKα1 and AMPKα2 siRNA (60 pmol each) was transfected into hepatocytes by Xfect siRNA transfection Reagent from Clontech Laboratories (Mountain View, CA) according to the manufacturer's instructions.

Hemorrhagic shock

Sprague—Dawley rats were subjected to hemorrhagic shock (HS) as described.[22] Briefly, the rats were anesthetized with a tracheostomy and vascular cannula placed for monitoring blood pressure and fluid therapy. The mean arterial blood pressure (MAP) and heart rate were continuously monitored. Blood was withdrawn until the MAP decreased to 40% of baseline, and the rats were maintained at this MAP for 60 min. Resuscitation was then performed by returning all the shed blood plus two times the volume of shed blood as saline. The animals were sacrificed 4 h after resuscitation. The liver tissue was snap frozen in liquid nitrogen and maintained at -80°C until protein preparation.

MTT assay

Cell viability was measured using the 3-(4,5 dimethlythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[23] After the indicated incubation time, culture media was replaced with serum-free culture media containing 1:50 diluted MTT stock solution (5 mg MTT/ml in 70% ethanol), incubated for 30 min, removed, and 0.5 mL dimethyl sulfoxide (DMSO) added. The plates were agitated and 1/10 vol/vol of 2 M Tris buffer (pH 10.5) was added. The absorbance of the sample at 570 nm was measured.

Nitrite assay

Supernatant nitrite was measured as an index of NO synthesis using the Griess Reaction as previously described.[24] To measure tissue nitrite, the supernatant from 1 mg of homogenized liver tissue was exposed to nitrate reductase for 60 min to