Insulin inhibits hepatocyte iNOS expression induced by cytokines by an Akt-dependent mechanism

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Harbrecht BG, Nweze I, Smith JW, Zhang B. Insulin inhibits hepatocyte iNOS expression induced by cytokines by an Akt-dependent mechanism. Am J Physiol Gastrointest Liver Physiol 302: G116–G122, 2012. First published October 28, 2011; doi:10.1152/ajpgi.00114.2011.—Hepatocyte inducible nitric oxide synthase (iNOS) expression is a tightly controlled pathway that mediates hepatic inflammation and hepatocyte injury in a variety of disease states. We have shown that cyclic adenosine monophosphate (cAMP) regulates cytokine-induced hepatocyte iNOS expression through mechanisms that involve protein kinase B/Akt. We hypothesized that insulin, which activates Akt signaling in hepatocytes, as well as signaling through p38 and MAPK p42/p44, would regulate iNOS expression during inflammation. In primary rat hepatocytes, insulin inhibited cytokine-stimulated nitrite accumulation and iNOS expression in a dose-dependent manner. Inhibition of MAPK p42/p44 with PD98059 had no effect on iNOS activation, whereas SB203580 blocked p38 reversed insulin’s inhibitory effect. However, insulin did not increase p38 activation and inhibition of p38 signaling with a dominant negative p38 plasmid had no effect on cytokine- or insulin-mediated effects on iNOS. We found that SB203580 blocked insulin-induced Akt activation. Inhibition of Akt signaling with LY294002 or a dominant negative Akt plasmid increased cytokine-stimulated nitrite production and iNOS protein expression and blocked the inhibitory effects of insulin. NF-κB induces iNOS expression and can be regulated by Akt, but insulin had no effect on cytokine-mediated IkBα levels or NF-κB p65 translocation. Our data demonstrate that insulin inhibits cytokine-stimulated hepatocyte iNOS expression and does so through effects on Akt-mediated signaling.

necrotic injury; necrotic acid synthase; sepsis; inflammation; liver

HEPATIC DYSFUNCTION WAS IDENTIFIED decades ago as a component of the multiple organ failure syndrome (10). Liver dysfunction can be manifested in a variety of ways and is associated with increased morbidity and mortality in critically ill patients (13, 18, 25). Nitric oxide synthesis is an important pathway in the host’s response to inflammation and mediates hepatic dysfunction and hepatic injury in models of shock and organ failure (19, 28). Proinflammatory cytokines stimulate hepatic nitric oxide production, and we have shown that glucagon and cyclic AMP inhibit hepatocyte inducible nitric oxide synthase (iNOS) expression (14–16). The role of other counterregulatory mediators produced in shock and sepsis on iNOS regulation remain incompletely studied (8). Glucagon and cAMP regulate hepatocyte iNOS expression through intracellular signaling pathways involving JNK and NF-κB (15, 20, 44). Our laboratory and others have demonstrated that cAMP activates protein kinase B/Akt in hepatocytes (9, 27, 43, 45). Akt-mediated signaling changes have profound effects on hepatocyte function and physiology (9, 27, 43), and we have shown that the cAMP-induced changes in iNOS expression are mediated in part through Akt-induced signaling (45).

Insulin is a potent modulator of hepatocyte metabolism and increases hepatocyte gluconeogenesis by regulating hepatocyte gene expression (6, 31, 35). Signaling mechanisms activated by insulin include PI3K, protein kinase B/Akt, and mitogen-activated protein kinases (MAPK) such as p38, p42/p44, and JNK (2, 3, 6, 21, 24, 33). Several of these signaling pathways, such as p38 and MAPK p42/p44, regulate iNOS expression in other tissues and cell types (7, 23, 26). In the liver, the effect of insulin on different signal transduction pathways varies depending on whether primary hepatocytes or hepatic cell lines are studied (2, 3, 6, 21). Although insulin can regulate the expression and activity of several hepatic transcription factors, and therefore the expression of numerous hepatocyte genes (6, 21, 33, 35), insulin’s regulation of hepatic function and physiology in sepsis, other than glucose metabolism, has largely been unexplored. Given that insulin activates Akt and the MAPK signaling pathways that have been implicated in iNOS regulation, we hypothesized that insulin would regulate hepatocyte iNOS expression and activity in hepatocytes.

MATERIALS AND METHODS

Reagents. Williams Medium E, penicillin, streptomycin, L-glutamine, HEPES, human recombinant interleukin 1β (IL-1β) and murine recombinant interferon (IFN-γ) were from Invitrogen Life Science (Carlsbad, CA). Insulin was from Lilly (Indianapolis, IN). Polyclonal antibodies to iNOS were purchased from BD Bioscience (Billerica, MA). Antibodies to Akt, p38, p65, and actin were purchased from Cell Signaling Technology (Danvers, MA), and the antibody to PCNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The plasmid expressing the dominant negative Akt was kindly provided by Drs. Burgering and Triest (Utrecht University). The plasmid expressing a dominant negative p38α MAPK (AdDNp38) was purchased from Cell Biolabs (San Diego, CA). All other chemicals were reagent grade and were purchased from Sigma (St. Louis, MO).

Hepatocyte isolation and culture. Primary hepatocytes were isolated from male Sprague-Dawley rats (200–250 g) using the modified collagenase perfusion technique as previously described (14–16). Hepatocytes were >98% pure and >95% viable as measured by trypan blue exclusion. Hepatocytes were cultured onto collagen-coated 100-mm dishes at 1 × 10^6 cells/ml (5 ml) or collagen-coated 6-well plates (10^6 cells/well) in Williams Medium E with L-arginine (0.5 mM), L-glutamine (2 mM), HEPES (15 mM), insulin (1 μM), penicillin, streptomycin, and 10% low endotoxin calf serum (HyClone Laboratories, Logan, UT). After 4 h to allow adherence, the hepatocytes were washed with PBS and cultured overnight in insulin-free media containing 5% calf serum. The cells were then washed with
PBS, and the experimental conditions established in insulin-free media except as otherwise described. Experimental conditions were performed in duplicate or triplicate cultures, and experiments were repeated to ensure reproducibility.

**Plasmid transfection.** Hepatocytes were plated in 6-well plates and transfected using LipofectAMINE as previously described (44). For experiments investigating p38, recombinant adenovirus expressing the dominant negative p38 \( \frac{1}{2} \) MAPK or adenovirus expressing GFP were used. For all other transfection experiments, hepatocytes were transfected with an empty vector (0.2 mg/well pIETLacZ) or the indicated plasmids for 6 h, washed, and allowed to recover for 16 h before experimental conditions were established.

**Western blot.** Hepatocytes were washed twice with ice-cold PBS and lysed with 500 μl of lysis buffer (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1 mM Na2-EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM PMSF). They were scraped into Eppendorf tubes, lysed for 30 min at 4° C, centrifuged (15, 000 g for 15 min), and stored at −80°C until use.

Proteins were separated on SDS-PAGE and blot-transferred to nitrocellulose membranes. Nonspecific binding was blocked with TBS-T (50 mM Tris·HCl, ph 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk for 1 h. Primary antibodies were diluted and incubated with membranes for 1–2 h at room temperature or overnight at 4°C with agitation. After washing three times with TBS-T, secondary antibodies were incubated at 1:10,000 dilution for 1 h. After five additional washes with TBS-T, the bands were visualized with chemiluminescence according to the manufacturer’s instructions. The membranes were stripped and reprobed for total unphosphorylated proteins or actin where indicated as loading control. Blots were quantified using Image J software (National Institutes of Health).

**MTT viability assay.** Cell viability was assessed by the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described (32). Briefly, 5 mg/ml of MTT in 70% ethanol was diluted 1:50 with culture media immediately before use. Hepatocytes were cultured overnight in 6-well plates and then stimulated with cytokines and insulin as indicated. After 24 h, the media was aspirated and replaced with the MTT solution. The cells were then incubated for 30 min, the MTT solution was aspirated, and 0.5 ml of DMSO was added. After agitation of plate for ~5 min, 1/10 vol/vol of 2 M Tris buffer (pH 10.5) was added, the wells were mixed thoroughly, and then a sample was taken to measure absorbance at 570 nm.

**NO measurement.** Supernatant NO\(_2\^-\) was measured as an index of NO production by the Griess reaction as described (10). Data are presented as means ± SD, and ANOVA was used to determine statistical significance. A P value of <0.05 was used to determine statistical significance.

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**Fig. 1.** Insulin decreases cytokine-stimulated inducible nitric oxide synthase (iNOS). Hepatocytes were cultured overnight and then stimulated with cytokines [IL-1β 200 U/ml; interferon (IFN) 100 U/ml] in the presence of the indicated concentration of insulin. A: samples were collected 24 h later and analyzed for nitrite (top) and iNOS protein (bottom). Data represent one of three similar experiments. *Significant difference vs. media control (P < 0.01; n = 6). B: densitometry analysis. C: hepatocyte viability was measured after 24-h cultures with the indicated concentration of insulin by the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

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RESULTS

To test the hypothesis that insulin regulates hepatocyte iNOS expression, we cultured hepatocytes with increasing concentrations of insulin in the presence and absence of proinflammatory cytokines to stimulate iNOS. In culture supernatants and cellular proteins collected at 24 h, insulin decreased IL-1β/IFN-stimulated NO2 production and iNOS protein expression in a dose-dependent manner (Fig. 1). Similar findings were evident when hepatocytes were stimulated to produce iNOS with a combination of multiple cytokines (14, 15) (Fig. 2) or IL-1β alone (data not shown). The MTT assay was performed to assess hepatocyte viability and demonstrated no decrease in hepatocyte viability at the insulin concentrations that were effective at inhibiting iNOS expression (Fig. 1C). Decreased viability was seen if the insulin concentration was increased to 50 μM.

Insulin regulates MAP kinase signaling in hepatic cells (2, 3, 6, 21). To evaluate the role of MAP kinase in mediating the effect of insulin on iNOS activation, we cultured hepatocytes in the presence of SB203580 to inhibit p38 and PD98059 to inhibit MEK/MAPK p42/p44. When measured after 24 h of culture, PD98059 had no effect on the suppression of NO2 production produced by insulin, whereas SB203580 blocked the insulin-induced inhibition of NO (Fig. 3). PD98059 reliably decreased cytokine-stimulated p42/p44 signaling in our hepatocyte cultures at these concentrations (45). However, when we measured p38 activation by Western blot, insulin did not increase p38 phosphorylation either alone (Fig. 3A) or in the presence of cytokines (not shown). Transfection of hepatocytes with a dominant negative p38 plasmid did not alter NO2 production in response to cytokines or cytokines plus insulin (Fig. 3B). Since several protein kinase inhibitors are not completely specific (4, 5), we evaluated the specificity of SB203580. We found that SB203580 slightly decreased p38 phosphorylation at 20 μM (data not shown) but markedly decreased Akt phosphorylation at 10 μM concentration or greater (Fig. 3C).

Insulin activates Akt signaling in both hepatic cell lines (6, 35, 41) and primary rat hepatocytes (24). In our cultures,
insulin increased Akt phosphorylation in a dose-dependent manner and increased Akt phosphorylation in hepatocytes stimulated with IL-1β + IFN to induce iNOS (Fig. 4). IL-1β + IFN alone induced Akt phosphorylation, consistent with our previous work (45), but not to the magnitude of insulin.

To evaluate the role of Akt in mediating the effect of insulin on iNOS, we blocked Akt signaling by inhibiting upstream PI3K with LY294002. Phosphorylation of Akt by insulin was effectively decreased when hepatocytes were cultured with LY294002 (Fig. 5A). The inhibitory effect of insulin on IL-1β + IFN-induced iNOS activation was partially reversed with LY294002 (Fig. 5B). We also inhibited Akt signaling using a dominant negative Akt plasmid (Akt-KD) that decreases Akt phosphorylation (45). Hepatocytes were transfected with either Akt-KD or a control plasmid, allowed to recover, and then stimulated with IL-1β + IFN to produce NO. Insulin decreased NO production and iNOS protein expression in hepatocytes transfected with the control plasmid. Akt-KD partially prevented the inhibition in NO production and iNOS expression by insulin (Fig. 5C). Akt-KD and LY294002 also increased NO production in IL-1β + IFN-stimulated hepatocytes without insulin, similar to our previous findings (45).

NF-κB is an important regulator of iNOS expression in cytokine-stimulated hepatocytes (15, 20, 38). Hepatocyte NF-κB activation has been shown to be regulated by Akt through Akt-mediated effects on IKKα (17, 29). To evaluate whether the effect of insulin on iNOS was mediated through Akt-induced changes in NF-κB, we stimulated hepatocytes with IL-1β + IFN in the presence and absence of insulin and measured IκBα and nuclear p65 by Western blot. Consistent with our previous work and that of others (20, 38), IL-1β + IFN decreased IκBα, which corresponded to increased levels of p65 in the nucleus (Fig. 6). Insulin had no effect on IκBα levels up to 120 min of culture and did not change nuclear p65, suggesting that insulin did not mediate its effects on iNOS through changes in IκBα or p65 translocation to the nucleus.

**DISCUSSION**

The administration of insulin to control glucose levels in critically ill patients has been a topic of considerable interest...
We have shown that limiting excessive NO from iNOS expression during inflammation.

Endogenous mediators that promote Akt activation may therefore downregulate hepatocyte iNOS expression in vitro and in vivo (16, 21) and limit iNOS-induced tissue injury in times of stress. Insulin had little effect on p38 in our experiments, and SB203580 had more potent effects on Akt than p38 in these cultures. We cannot exclude the possibility that insulin or SB203580 altered p38 at a time point we did not measure or that the SB203580-induced inhibition of Akt was mediated by upstream p38 signaling. Our results suggest, however, that Akt signaling is more important in regulating cytokine-induced hepatocyte iNOS in response to insulin than p38.

Akt signaling can regulate downstream NF-κB activity and MAPK p42/p44 activation (17, 34, 48). Our results, however, demonstrate that insulin did not alter IκBα or p65 nuclear translocation. Inhibition of MAPK p42/p44 with PD98059 had no effect on the insulin-mediated suppression of iNOS. Our results suggest that these signaling pathways do not contribute to insulin’s effects on iNOS expression. The identity of the molecular targets of Akt-mediated insulin signaling that inhibit hepatocyte iNOS expression have not been identified. We have previously shown that JNK signaling mediates the cAMP-induced suppression of hepatocyte NO synthesis (44). Akt can regulate JNK in other cell types (1, 11, 12, 46) and activates JNK in hepatocytes (24). It is unknown whether insulin regulates JNK, either directly or through Akt, in hepatocytes stimulated to produce NO during inflammation, and this pathway will require further investigation. We also cannot completely exclude a role for NF-κB in regulating the effect of insulin on iNOS. Although p65 nuclear translocation and IκBα levels were not changed by insulin, NF-κB can potentially mediate the effects of insulin on iNOS through other mechanisms. The p65 subunit of NF-κB can be directly phosphorylated and, once phosphorylated, interacts with the coactivator CBP to interfere with transcription independent of DNA binding (42). The p65 subunit can also bind to other coactivator proteins and regulate transcription (36, 47). Whether insulin regulates other functions of NF-κB in hepatocytes such as competition for coactivators to mediate the expression of iNOS or other genes will require further study.

In conclusion, our data demonstrate that insulin inhibits cytokine-stimulated iNOS expression in primary rat hepatocytes. The suppressive effect of insulin on iNOS was mediated through the upregulation of Akt signaling and was not mediated through effects on MAPK p42/p44 or p38. In addition to regulating glucose levels, insulin may downregulate iNOS expression and limit iNOS-mediated tissue injury and organ dysfunction following shock and inflammation.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
B.G.H. and B.Z. conception and design of research; B.G.H., I.N., and B.Z. analyzed data; B.G.H., I.N., J.W.S., and B.Z. interpreted results of experi-
ments: B.G.H. and B.Z. drafted the manuscript; B.G.H., I.N., J.W.S., and B.Z. edited and revised the manuscript; B.G.H., I.N., and B.Z. approved the final version of the manuscript; I.N. and B.Z. performed experiments; B.Z. prepared figures.

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