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Calcium-mediated signaling and calmodulin-dependent kinase regulate hepatocyte inducible nitric oxide synthase expression

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Abstract

Background—Nitric oxide synthase (iNOS) is induced in hepatocytes by shock and inflammatory stimuli. Excessive NO from iNOS mediates shock-induced hepatic injury and death so understanding the regulation of iNOS will help elucidate the pathophysiology of septic shock. In vitro, cytokines induce iNOS expression through activation of signaling pathways including mitogen-activated protein kinases and Nuclear Factor κ B. Cytokines also induce calcium (Ca^{2+}) mobilization and activate calcium-mediated intracellular signaling pathways, typically through activation of calmodulin-dependent kinases (CaMK). Calcium regulates NO production in macrophages but the role of calcium and calcium-mediated signaling in hepatocyte iNOS expression has not been defined.

Materials and Methods—Primary rat hepatocytes were isolated, cultured, and induced to produce NO with proinflammatory cytokines. Calcium mobilization and Ca^{2+} -mediated signaling were altered with ionophore, Ca^{2+} channel blockers, and inhibitors of CaMK.

Results—The Ca^{2+} ionophore A23187 suppressed cytokine-stimulated NO production while EGTA and nifedipine increased NO production, iNOS mRNA, and iNOS protein expression. Inhibition of CaMK with KN93 and CBD increased NO production but the calcineurin inhibitor FK 506 decreased iNOS expression.

Conclusions—These data demonstrate that calcium-mediated signaling regulates hepatocyte iNOS expression and does so through a mechanism independent of calcineurin. Changes in intracellular calcium levels may regulate iNOS expression during hepatic inflammation induced by pro-inflammatory cytokines.

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Keywords

Hepatocyte; nitric oxide synthase; NOS2; sepsis; cytokines; shock; liver

INTRODUCTION

Hepatic nitric oxide (NO) production is an important component of the host response to inflammatory stimuli. Nitric oxide synthase (NOS) expression is induced in hepatocytes by hemorrhagic shock, sepsis, and ischemia/reperfusion injury (1–3). Excessive NO produces hepatic injury and hepatic inflammation, alters hepatic gene expression, and contributes to death after shock (1). While much has been learned about the mechanisms that govern induced NOS (iNOS) expression (4,5), the intracellular processes that regulate iNOS expression in shock and sepsis continue to be explored.

We have previously demonstrated that hepatocyte iNOS is regulated by cyclic adenosine monophosphate (cAMP) and the cAMP-elevating hormone glucagon (6–8). Cyclic cAMP and glucagon have profound effects on hepatocyte function by regulating glucose metabolism and expression of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme in hepatic gluconeogenesis (9). Cyclic cAMP regulates cell function through several cell signaling pathways including cAMP-dependent protein kinase A (PKA), extracellular signal related kinase (ERK), guanine nucleotide exchange factors and alterations of cellular Ca^{2+} concentrations (10–12). We have shown that the regulation of hepatocyte iNOS by cAMP is mediated by PKA-independent pathways including Akt and guanine nucleotide exchange factors but not ERK (13–15).

Increases in intracellular Ca^{2+} are induced by both glucagon and cAMP (12). Changes in intracellular Ca^{2+} regulate cellular gene expression through either direct effects of Ca^{2+} or through changes in Ca^{2+} -sensitive signal transduction pathways such as Ca^{2+} -dependent protein kinases and Ca^{2+} -dependent transcription factors (16,17). CaMK regulates PEPCK expression in the liver during conditions of increased glucagon secretion such as fasting (18). It is therefore possible that changes in intracellular Ca^{2+} mediate the effect of glucagon and other cAMP-activating agents on hepatocyte iNOS expression. The cytokines that induce hepatocyte iNOS expression also induce changes in intracellular Ca^{2+} (19,20) and Ca^{2+} -dependent mechanisms regulate NO production in macrophages, chondrocytes, neurons, and endothelial cells (21–24). We were therefore interested in determining if Ca^{2+} -mediated signaling pathways regulate iNOS expression and NO production in hepatocytes.

MATERIALS AND METHODS

Reagents

Williams medium E was purchased from Invitrogen Corporation (Carlsbad, CA). Interleukin- β was purchased from Dupont (Boston, MA) and murine recombinant interferon- γ (IFN) was from Invitrogen. The calmodulin-dependent kinase (CaMK) inhibitors CBD and KN93 were purchased from Calbiochem (San Diego, CA). Antibodies to iNOS and I κ B α were from BD Bioscience (Billerica, MA) and antibodies to actin were

from Cell Signaling Technology (Danvers, MA). Nifedepine, A23187, insulin and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Rat hepatocytes were harvested from male Sprague-Dawley rats (Harlan-Sprague-Dawley, Madison, WI) using collagenase perfusion and differential centrifugation as previously described (6,7). The hepatocyte population was > 98% pure and had viability of > 95% (6,7). All experimental protocols were approved by the University of Louisville Animal Care and Use Committee and followed guidelines prescribed by the National Institutes of Health's Guidelines for the Care and Use of Laboratory Animals. Hepatocytes were plated into 12-well or 100 mm gelatin-coated dishes at 2×10^5 cells/well or 5×10^6 cells/plate respectively in Williams medium E containing L-arginine (0.5 mM), insulin (10^{-6} M), HEPES (15 mM), L-glutamine, penicillin, streptomycin, and 10% low endotoxin calf serum (HyClone Laboratories, Logan, VT). After 4 hours, the cells were washed with PBS to remove nonadherent cells, the media replaced with insulin-free media containing 5% calf serum, and hepatocytes cultured overnight at 37°C. After overnight incubation, the cells were washed again with PBS to remove dead and nonadherent cells and the experimental conditions were established. Cultures were performed in duplicate or triplicate and experiments were repeated to ensure reproducibility.

MTT Viability Assay

Cell viability was measured using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) (25). After the indicated incubation, media was replaced with 5 mg MTT/ml in 70% ethanol diluted 1:50 with culture media, incubated for 30 minutes, removed, and 0.5 ml DMSO added. The plates were agitated and 1/10 vol/vol of 2 M Tris buffer (pH 10.5) was added. Absorbance of the sample at 570 nm was measured.

Nitrite Assay

Supernatant nitrite was measured as an index of NO synthesis using the Greiss Reaction as previously described (7).

Western Blot

Cells were rinsed with ice-cold PBS and then lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, and 1 mM PMSF) was added (500 μ l). The cells were lysed at 4°C in 1.5 ml Eppendorf tubes for 30 minutes and then centrifuged at $15,000 \times g$ for 15 minutes to remove cellular debris. Cellular extracts were electrophoresed in 10%–20% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) and 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% non-fat milk for 1 hour. Antibodies were diluted in TBS-T, incubated with membranes at room temperature with agitation for 1–2 hours, washed, and then incubated with secondary antibodies. Detection of immunoreactive protein was performed by chemiluminescence

(Amersham Pharmacia Biotech, Piscataway, NJ). Blots were quantified using Image J software (National Institutes of Health).

Northern Blot

Total cellular RNA was collected by the RNeasy-modified method (7). RNA (20 µg) was electrophoresed in 1% agarose gels, blot-transferred to membranes, and UV autocrosslinked. After hybridization, the membranes were probed with a 2.7kb fragment of the murine macrophage iNOS cDNA (provided by Charles Lowenstein, Johns Hopkins University) and autoradiography performed. The membranes were stripped in boiling EDTA and 0.1% SDS and rehybridized with a probe for GAPDH.

Calcium Measurement

To qualitatively assess hepatocytes for changes in intracellular Ca^{2+} , isolated hepatocytes were cultured on collagen coated dishes and loaded with 5 µM fura-2-AM (Invitrogen) in PBS for 30 minutes. The cells were allowed to recover and then were stimulated with TNFα (500 IU/ml), IL-1β (200 IU/ml), and IFN-γ (100 IU/ml). Immunofluorescence was measured by the ratio of images obtained at 340 and 380 nm with an Olympus IX81 microscope (Olympus America, Inc., Mellville, NY)

Statistics

Data are presented as the mean ± SEM. Data were analyzed by Analysis of Variance followed by Scheffe's post hoc test and $p < 0.05$ was considered statistically significant.

RESULTS

Calcium ionophore decreases hepatocyte NO production

Elevated intracellular Ca^{2+} decreased iNOS expression in macrophages and chondrocytes (21,22). To test whether increased Ca^{2+} regulates hepatocyte iNOS expression, cultured hepatocytes were incubated with the ionophore A23187 and then stimulated to produce NO by IL-1β (200 IU/ml) + IFN-γ (100 IU/ml). The ionophore A23187 decreased cytokine-induced hepatocyte NO_2^- production and iNOS expression (Figure 1A). Altered Ca^{2+} homeostasis produces cell injury and death (26) so we assessed the effect of A23187 on hepatocyte viability using the MTT assay. As shown in Figure 1A, 10^{-7} M A23187 significantly decreased cytokine-stimulated NO_2^- production without decreasing hepatocyte viability while higher doses were toxic to cultured hepatocytes. Concentrations of A23187 greater than 1 µM also decreased viability in hepatocytes cultured without cytokines (data not shown). A combination of TNFα (500 IU/ml), IL-1β (200 IU/ml), and IFNγ (100 IU/ml) is the strongest in vitro stimulus to induce hepatocyte iNOS expression (7). Nitrite production was suppressed by A23187 in a statistically significant manner when this combination of cytokines was used to induce iNOS (Figure 1B). The induction of NOS by combinations of cytokines can be reproduced using high-dose IL-1β as a single stimulus (7) and A23187 suppressed hepatocyte NO production produced by IL-1β alone (500 IU/ml) as well (data not shown).

Decreased Ca^{2+} mobilization increases iNOS

Hepatocytes increase intracellular Ca^{2+} through cell membrane-associated Ca^{2+} channels (27). To establish that proinflammatory cytokines induce Ca^{2+} mobilization in hepatocytes coincident with iNOS expression, isolated hepatocytes were cultured, loaded with fura-2, and stimulated with $\text{TNF}\alpha$ (500 IU/ml), IL-1 β (200 IU/ml), and IFN γ (100 IU/ml). Qualitative immunofluorescence was performed and revealed increased Ca^{2+} -induced fluorescence after cytokine exposure (data not shown). To further evaluate the role of Ca^{2+} mobilization in iNOS regulation, we cultured hepatocytes with the Ca^{2+} -chelating agent EGTA to reduce Ca^{2+} influx. EGTA concentrations of 0.5 and 1.0 mM increased cytokine-induced hepatocyte NO_2^- production (Figure 2A) and iNOS protein expression (Figure 2B) without affecting hepatocyte viability as measured by MTT (data not shown).

We used nifedipine to block plasma membrane Ca^{2+} channels and decrease Ca^{2+} influx. Nifedipine increased cytokine-stimulated NO_2^- production that was statistically significant at 10 μM (Figure 3A). This concentration of nifedipine was associated with increased iNOS protein levels and slightly increased iNOS mRNA levels (Figure 3B & 3C). Hepatocytes cultured with nifedipine alone in the absence of cytokines had no detectable iNOS protein and no change in the low baseline levels of NO_2^- compared to media alone (data not shown).

CaMK regulates iNOS

Calcium-mediated signaling is regulated by several Ca^{2+} -sensitive kinases including the calmodulin-dependent kinases (CaMK)(16,17). Hepatocytes express RNA and protein for CaMK isoforms I, II, and IV (28). We cultured hepatocytes with the CaMK inhibitors CBD and KN93 and then stimulated them to produce iNOS. CBD had no effect on the low baseline levels of NO_2^- in hepatocytes cultured without cytokines (data not shown) but increased supernatant NO_2^- at doses of 0.1–10 μM (Figure 4A). KN93 produced results similar to CBD (Figure 4B).

In Jurkat cells, Ca^{2+} and calcineurin activate NF- κB , NF- κB -dependent DNA binding, and NF- κB -dependent promoter activation (29). In hepatocytes, the calcineurin inhibitor FK506 decreased iNOS expression and NF- κB DNA binding (30) suggesting that Ca^{2+} and calcineurin-mediated effects on NF- κB regulate iNOS. We therefore measured I κB phosphorylation as an index of NF- κB activation in NO-producing hepatocytes cultured with A23187 and nifedipine. IL-1 β +IFN increased phosphorylated I κB and decreased total I κB levels consistent with our prior work (14) and the known mechanism of NF- κB (31). However, A23187 and nifedipine had no significant effect on either phosphorylated I κB or total I κB levels in IL-1 β +IFN-treated hepatocytes (Figure 5) suggesting that the effect of alterations in calcium was not mediated by NF- κB . To compare our hepatocyte cultures to previously published work, we treated cultured hepatocytes with IL-1 β plus the calcineurin inhibitor FK506 and demonstrated a dose-dependent decrease in nitrite (Figure 6) consistent with the work of others (30).

DISCUSSION

The signaling pathways regulating NOS expression in hepatocytes are different from those operative in other cell types (7,23,24). Changes in Ca^{2+} regulate activity of the constitutively present NOS in endothelial cells but the regulation of the expression of iNOS by Ca^{2+} has received comparably less attention. Calcium regulates iNOS expression in selected cells (21,22) and we know that the cytokines that induce hepatocyte iNOS expression alter intracellular Ca^{2+} concentrations (19,20). Therefore, we hypothesized that changes in Ca^{2+} regulate hepatocyte iNOS expression in response to proinflammatory cytokines.

In this study, we demonstrate that the Ca^{2+} mobilizing agent A23187 decreased cytokine-induced iNOS expression but that inhibiting Ca^{2+} influx with nifedipine and EGTA increased hepatocyte iNOS expression. These findings suggest that cytokine-induced Ca^{2+} mobilization activates autoregulatory pathways that reduce or limit iNOS expression and NO production. Our finding that CaMK inhibitors have the same effect as nifedipine and EGTA suggests that the cytokine-induced signaling changes are mediated through CaMK. Ca^{2+} and Ca^{2+} -mediated signaling regulate cellular gene expression through a variety of downstream protein kinases and transcription factors (16,17). Ca^{2+} -mediated alteration of transcription factor activation regulates iNOS expression in neurons, macrophages and colon cells (24,32). CaMK activates the phosphatase calcineurin (CN) which can dephosphorylate NF-AT leading to its import into the nucleus (16,17). Inhibition of CN-induced signaling is associated with a decrease in hepatocyte NF- κ B DNA binding and decreased iNOS expression (30). However, despite regulating iNOS expression, A23187 and nifedipine had no effect on pI κ B or total I κ B levels. This finding suggests that the cytokine-mediated suppression of iNOS through CaMK seen in our study is mediated by a pathway other than NF- κ B.

In hepatocytes, Kaibori showed that the calcineurin inhibitor FK506 decreased IL-1 β -induced iNOS expression through an NF- κ B-dependent mechanism and that calmodulin inhibitors had no effect (30). However, they did not evaluate other Ca^{2+} agents, they used nonspecific calmodulin inhibitors, and cyclosporine A did not produce the same effect as FK506 despite also being a calcineurin inhibitor (16,17,30). Our data demonstrate that the calcineurin inhibitor FK506 decreases iNOS activation, similar to Kaibori (30). This finding suggests that the effects of Ca^{2+} on iNOS are not mediated through calcineurin. We cannot exclude the possibility, though, that FK506 regulates pathways other than calcineurin or inhibits other phosphatases. Our current results are consistent with our previous work demonstrating that glucagon and cAMP, compounds known to increase Ca^{2+} in hepatocytes, suppress iNOS expression (6,8,13). In hepatocytes, adrenergic agents also increase intracellular Ca^{2+} and Collins demonstrated that adrenergic agonists suppress hepatocyte iNOS expression (34). It is therefore possible that any agent that increases hepatocyte Ca^{2+} can affect iNOS expression. CaMK signaling regulates glucagon- and cAMP-mediated PEPCCK expression and our data suggest that it may have a similar role in iNOS expression (18). However, the mechanism mediating the downstream effect of CaMK on hepatocyte iNOS remains to be clarified. CaMK can directly phosphorylate transcription factors such as CREB, Elk, and the coactivator protein CBP as well as histones and other nuclear proteins (35). CaMK also regulates expression of c-Jun (16) and we know that c-Jun suppresses

iNOS expression (13). Determining whether CaMK regulates iNOS through an effect on c-Jun, CREB, or other transcription factors will require further study.

In these studies, we used cytokines as an in vitro model of systemic inflammation and sepsis. Several mediators produced during inflammation can alter cellular Ca^{2+} concentrations including pro-inflammatory cytokines (19, 20), adrenergic agonists such as norepinephrine (34), and counter-regulatory hormones (12). Determining whether these endogenous mediators regulate hepatocyte iNOS during sepsis in vivo will require further study. In addition, Ca^{2+} -channel blockers are commonly used to treat hypertension but whether they affect nitric oxide production in septic patients has not been defined.

In summary, our data demonstrates that Ca^{2+} -mediated signaling decreases cytokine-induced iNOS expression in hepatocytes. Our findings suggest that this effect is mediated through CaMK but not calcineurin. Defining the mechanisms for the CaMK-mediated regulation of iNOS may reveal novel regulatory mechanisms for control of NO production.

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Figure 1.

Calcium ionophore decreases hepatocyte NO production. A) Hepatocytes were cultured with the indicated concentration of A23187 and then stimulated to produce NO with IL-1 β (200 IU/ml) + IFN γ (100 IU/ml) as described in Methods. After 24 hours, supernatants were analyzed for nitrite (open columns) and hepatocyte viability was assessed by the MTT assay (filled columns). Asterisk represents $p < 0.05$ compared to hepatocytes without A23187. B) Hepatocytes were treated in an identical manner as A) but were stimulated to produce NO with TNF α (500 IU/ml), IL-1 β (200 IU/ml) and IFN γ (100 IU/ml). Nitrite was measured at 24 hours. Data represent one of five similar experiments.

Figure 2.

EGTA increases hepatocyte iNOS. Hepatocytes were preincubated with the indicated concentration of EGTA for one hour and then stimulated with IL-1 β +IFN γ to induce iNOS. After 24 hours, supernatants were collected for nitrite (A) and total cellular proteins were collected for Western blot (B). Asterisk represents $p < 0.05$ compared to hepatocytes cultured without EGTA.

Nifedipine (μ M)	0	0	0.1	1.0	10
IL-1 β + IFN γ	--	+	+	+	+

← iNOS

← actin

Figure 3.

Nifedipine increases cytokine-induced iNOS activation. Hepatocytes were preincubated with the indicated concentration of nifedipine for one hour and then stimulated with IL-1 β +IFN γ to produce NO. After 24 hours, supernatants were collected for nitrite (A) and total cellular proteins were collected for Western blot (B). In separate cultures, total RNA was collected after 3 hours (C) and iNOS mRNA measured by Northern analysis. Asterisk represents $p < 0.05$ vs control.

Figure 4.

Inhibition of CaMK increases NO production. Hepatocytes were preincubated for one hour with the indicated concentration of CBD (A) and KN93 (B) and then stimulated with IL-1 β +IFN γ to produce NO. Supernatants were collected after 24 hours and analyzed for nitrite. Open columns represent hepatocytes cultured with KN93 but without IL-1 β +IFN γ . Asterisk represents $p < 0.05$ compared to control hepatocytes without CaMK inhibitors.

Figure 5.

Effect of A23187 and nifedipine on NF-κB. Hepatocytes were cultured with media alone (open columns), A23187 (0.1 μM, filled columns), or nifedipine (10 μM, hatched columns) for 30 minutes and then stimulated with IL-1β+IFNγ or media (control). Cellular proteins were collected at 60 minutes and Western Blot performed for p-IκBα and total-IκBα. Actin was used as a loading control and the protein/actin ratio was normalized to media control. Asterisk represents p<0.05 compared to Media alone.

Figure 6.

FK506 decreases hepatocyte iNOS. Hepatocytes were cultured with the indicated concentration of FK506 and stimulated to produce iNOS with IL-1 β (200 IU/ml). Supernatants were collected at 24 hours and analyzed for nitrite. Asterisk represents $p < 0.05$ vs 0 M FK506.