Published in final edited form as:

Shock. 2011 August ; 36(2): 196–202. doi:10.1097/SHK.0b013e3182205e07.

HYPOXIA-INDUCED OVEREXPRESSION OF BNIP3 IS NOT DEPENDENT ON HIF-1 α IN MOUSE HEPATOCYTES

Rajaie A. Namas¹, Mallikarjuna R. Metukuri², Rajeev Dhupar¹, Claudia Velosa¹, Bahiyyah S. Jefferson¹, Evan Myer¹, Greg M. Constantine^{3,4}, Timothy R. Billiar^{1,4}, Yoram Vodovotz^{1,4}, and Ruben Zamora^{1,*}

¹Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213

²Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213

³Department of Mathematics, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213f

⁴Center for Inflammation and Regenerative Modeling, McGowan Institute for Regenerative Medicine, Pittsburgh, PA 15219, USA

Abstract

We sought to investigate the expression of the cell death protein BNIP3 in hypoxic hepatocytes, as well as the role that HIF-1 α plays in the upregulation of BNIP3 in hypoxic primary mouse hepatocytes and in the livers of mice subjected to ischemia/reperfusion. Freshly isolated mouse hepatocytes were exposed to 1% hypoxia for 1, 3, 6, 24 and 48 h, and the RNA and protein were isolated for RT-PCR and Western blot analysis. Similarly, livers from mice subjected to segmental (70%) hepatic warm ischemia for 30 min or 1 h, or to 1 h ischemia followed by 0.5–4 h reperfusion, were collected and subjected to Western blot analysis for HIF-1 α protein. We showed that hypoxic stress increases the formation of the BNIP3 homodimer while decreasing the amount of the monomeric form of BNIP3 in primary mouse hepatocytes. In contrast to RAW264.7 macrophages, there is a basal expression of HIF- α protein in normoxic primary mouse hepatocytes that does not change significantly upon exposure to hypoxia. Using siRNA technology, we demonstrated that reduced HIF-1 α protein levels did not block the hypoxia-induced overexpression of BNIP3. In contrast to the effect on BNIP3 expression reported previously, livers from ischemic animals demonstrated only a modest increase in HIF-1 α protein as compared to resting livers from control animals; and this expression was not statistically different from sham controls. These results suggest that HIF-1 α does not mediate the hypoxia-induced upregulation of BNIP3 in mouse hepatocytes *in vitro*, and possibly in the liver *in vivo*.

Keywords

Liver; Ischemia; Reperfusion; Transcription factor; Cell culture

***Contact Information:** Ruben Zamora, *PhD*, Research Associate Professor of Surgery, W943 Biomedical Sciences Tower, 200 Lothrop St., Pittsburgh, PA 15213, Tel.: 412-383-6998, Fax: 412-383-5946, zamorar@pitt.edu.

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INTRODUCTION

Hypoxia-inducible factor-1 (HIF-1) is a short lived, heterodimeric protein that is composed of a constitutively expressed HIF-1 β subunit and an O₂-regulated HIF-1 α subunit. Oxygen regulates the rate at which HIF-1 α protein is degraded and in normoxia HIF-1 α protein is rapidly degraded, resulting in essentially no detectable HIF-1 α protein. During hypoxia, HIF-1 α becomes stabilized and translocates from the cytoplasm to the nucleus, where it dimerizes with HIF-1 β , and the HIF complex formed becomes transcriptionally active (1). HIF-1 has been shown to activate the transcription of many genes that code for proteins involved in a growing number of cellular processes such as angiogenesis, glucose metabolism, cell proliferation/survival, and invasion/metastasis (2).

The cell death protein Bcl-2/adenovirus E1B 19kD-interacting protein 3 (BNIP3) is a membrane-associated protein localized to mitochondria and other cytoplasmic membrane structures (3) that was initially described as a pro-apoptotic protein, but that has also been implicated in necrotic cell death in a number of human and animal cell lines (4). BNIP3 and Nix (a BNIP3 homologue also known as BNIP3L) are the only members of the Bcl-2 family of apoptotic factors induced in response to hypoxia (4). We have focused our attention on the expression and regulation of BNIP3 in hepatocytes *in vitro* and in the liver *in vivo*. We have previously demonstrated that BNIP3 is upregulated *in vitro* and *in vivo* in both experimental and clinical settings of redox stress (5–7). *In vitro*, BNIP3 was expressed constitutively and localized to the nucleus of normoxic hepatocytes, while being upregulated and localized to the cytoplasm under hypoxic conditions (7). BNIP3 contributes to hypoxic injury in hepatocytes, since this injury can be attenuated by knockdown of BNIP3 mRNA. We also showed that hepatic BNIP3 was upregulated in two different models of liver stress *in vivo*, namely hemorrhagic shock and ischemia/reperfusion, suggesting that the upregulation of BNIP3 is one mechanism of hepatocyte cell death and liver damage in these settings (7).

In many cell types, increased HIF-1 α leads to transactivation of BNIP3. The HIF-1 α -mediated pathway of BNIP3 induction has been clearly established in cardiac myocytes and various non-tumor and tumor cell lines (8,9). One might assume therefore that HIF-1 α plays a significant role on the hypoxia-induced BNIP3 expression in hepatocytes. Surprisingly, in a recent study, the absence of nuclear induction of HIF-1 α protein in hypoxic primary rat hepatocytes suggested that although hepatocytes do respond to hypoxia, the contribution of HIF-1 α to this adaptation may be minor or transient at best, probably due to the translocation of HIF-1 α to peroxisomes rather than to the nucleus in hypoxia (10). HIF-1 α is a transcription factor known to regulate BNIP3 expression in various cell types and biological settings. Accordingly, we decided to further investigate the expression of BNIP3 in hypoxic hepatocytes and the role that HIF-1 α plays in the upregulation of BNIP3 in primary mouse hepatocytes cultured in hypoxic conditions as well as in livers of mice subjected to ischemia/reperfusion.

Our results demonstrate that hypoxia induces BNIP3 upregulation by increasing the formation of BNIP3 homodimers while decreasing BNIP3 monomers in primary mouse hepatocytes. More significantly and contrary to prior literature in other cell types, we demonstrated that the HIF-1 α pathway is not responsible for the hypoxia-induced overexpression of BNIP3 in mouse hepatocytes, despite the fact that this transcription factor is generally involved in the hypoxia-induced regulation of BNIP3 and numerous other genes *in vitro* and *in vivo*. Our results suggest that the regulation of hepatocyte BNIP3 under hypoxic conditions should be investigated more closely, and that hepatocyte BNIP3 may be a target for relatively selective therapeutic modulation under disease settings that involve liver hypoxia.

MATERIALS AND METHODS

Reagents

Williams Medium E, penicillin, streptomycin, L-glutamine, and HEPES were purchased from Invitrogen (Carlsbad, CA). Insulin (Humulin®) was purchased from Eli Lilly (Indianapolis, IN), and calf serum was obtained from HyClone Laboratories (Logan, UT). Tissue culture dishes were from Corning Glass Works (Corning, NY). Unless indicated otherwise, all other chemicals and proteins were purchased from Sigma-Aldrich (St. Louis, MO).

Hepatocyte Isolation and Culture

All procedures involving animals were approved by the Animal Care and Use Committee of the University of Pittsburgh. Primary hepatocytes were harvested from C57BL/6 mice (Charles River Laboratories, Wilmington, MA). Hepatocytes were isolated by collagenase perfusion using the method of Seglen (11) and purified to >98% purity by repeated centrifugation at 50g, followed by further purification over 30% Percoll. Viability at time of plating was checked by trypan blue exclusion. Highly purified hepatocytes (>98% purity and >95% viability by trypan blue exclusion) were suspended in Williams' E medium supplemented with 10% heat-inactivated calf serum, 15 mM HEPES (pH 7.4), 16 units insulin, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. The cells were plated on collagen-coated cell culture dishes (3×10⁶ cells/6-cm dish or 5×10⁶ cells/10-cm dish) and cultured overnight at 37°C under normoxic conditions (5% CO₂). The old medium was then removed and cells were further incubated with fresh media containing 5% heat-inactivated calf serum. Hypoxic conditions were obtained by placing the cells into a modular incubator chamber (Billups-Rothenburg, Del Mar, CA) flushed with a hypoxic gas mixture containing 1% O₂, 5% CO₂ and 94% N₂. Hepatocytes incubated under normoxic conditions (21% O₂) served as controls. For the data analysis and unless otherwise indicated, the number of independent experiments (*n*) refers to the number of separate mice from which hepatocytes were harvested.

siRNA Treatment and Knockdown of HIF-1α and E2F1

For transient knockdown experiments, hepatocytes were transfected with mouse-specific siRNAs to HIF-1α or E2F1 (ON-TARGET plus SMART pool) or control, non-silencing siRNA (SMARTpool® siRNA reagents, Dharmacon, Chicago, IL). Briefly, primary mouse hepatocytes were plated onto 6-cm or 10-cm cell culture dishes. On the next day, both siRNAs (silencing and non-silencing) were diluted to a final concentration of 5 nM (5–50 nM for E2F1) in Opti-Mem (Invitrogen life Technologies, San Diego, CA). DharmaFECT 1 (HIF-1α) or DharmaFECT 4 (E2F1) siRNA Transfection Reagent (Dharmacon) was added and incubated at room temperature for 20 min. The cells were then incubated with the siRNAs in serum-free conditions for 6 h at 37°C. After removing the transfection mixture, the cells were incubated in William's E media supplemented with 5% calf serum for 24 h followed by exposure to hypoxia for another 6 h. Additional technical details, reagent preparation, storage, and incubation times were as per manufacturer's recommendation.

Protein Isolation and Western Blot Analysis

Cultured hepatocytes (3×10⁶ cells/60 mm Petri dish) were washed twice with ice-cold PBS and resuspended in 200 µl of ice-cold 1X lysis buffer (Cell Lysis Buffer 10X from Cell Signaling Technology, Danvers, MA) containing 2 mM Tris-HCl buffer (pH 7.5), 15 mM NaCl, 0.1 mM EDTA and EGTA, 0.1 % Triton X-100, 250 µM sodium pyrophosphate, 100 µM β-glycerolphosphate, 100 µM Na₃VO₄, and the protease inhibitors leupeptin (0.1 µg/mL) and phenylmethylsulfonyl fluoride (PMSF, 1 mM). After 5 min incubation on ice, the

cells were scraped off the dish and transferred to microcentrifuge tubes. The cells were sonicated two times for 10–15 s each time with 1 min on ice between each sonication. Cell debris was then removed by centrifugation at 10,000 rpm for 10 min and the supernatant was used as cell lysate and stored at -80°C when necessary. An aliquot was used to determine protein concentration using the BCA Protein Assay Kit from Pierce (Rockford, IL) with bovine serum albumin (BSA) as standard. Protein samples (75 μg) were separated on 12% (for BNIP3) or 7.5% (for HIF-1 α) SDS-polyacrylamide gels and the gels were electroblotted onto PVDF nitrocellulose membranes. Immunodetection of BNIP3 protein was done using a mouse monoclonal antibody against human BNIP3 (1:1000 dilution) purchased from Sigma-Aldrich (St. Louis, MO). Lysates from MCF-7 cells and RAW267.4 macrophages exposed to hypoxia for 36 or 6 h respectively, were used as positive control. For immunodetection of HIF-1 α and in order to increase sensitivity (10), three mouse anti-HIF-1 α monoclonal antibodies (NB100–131, NB100–123 and NB100–105, all from Novus Biologicals, Littleton, CO) were combined and used at 1:500 dilution. For normalization, the membranes were stripped and reprobed with an anti- β -actin antibody from Abcam (Cambridge, MA). Immunoreactive bands were visualized after incubation with the SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL). Instructions for the kit were provided by the supplier. Where indicated, protein expression (band density relative to β -actin loading control) was represented as fold change vs. respective non-treated control (normoxia).

Viability Assay

Cell viability was assessed by measuring the release of the cytoplasmic enzyme Lactate dehydrogenase (LDH) from damaged cells into the supernatant using a colorimetric assay (Cytotoxicity Detection Kit-LDH assay; Roche Applied Science, Indianapolis, IN).

RT-PCR

After harvesting, cell and liver samples were immediately submerged in RNeasy lysis reagent (Qiagen, Crawfordsville, IN), as per manufacturer's instructions and processes for RNA isolation or stored at -80°C until further analysis. Semi-quantitative RT-PCR was performed using standard methods. Briefly, two microliters of reverse transcribed cDNA was used as template for PCR amplification in an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA.). Master mix and cDNA template were sealed into PCR capillary tubes. The amplification mixture contained the following reactants: sense and antisense primers (10 μM) and 1 $\times$ Sybr Green ROX mix (Biorad). Each gene product was amplified using the appropriate specific mouse primers from Integrated DNA Technologies (IDT) (Coralville, IA): **HIF-1 α** , forward http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www_results_help.cgi -PRIMER_THREE (5'-CGC CTC TGG ACT TGT CTC TT-3'), reverse (5'-GAA GTG GCA ACT GAT GAG CA-3'); **HIF-2 α** , forward http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www_results_help.cgi -PRIMER_THREE (5'-GAG GGT TTC ATT GCT GTG GT-3'), reverse 5'-CTC ACG GAT CTC CTC ATG GT-3'); **HIF-3 α** forward (5'-ACC TGT CGG AAA ATG TCA GC-3'), reverse (5'-GGG CTT CCA GCT TCT TCT TT-3'). The relative abundance of each gene product (relative to the GAPDH internal control) was determined by comparative cycle threshold analysis and was calculated using the equation $2^{-(\text{Ct}_{\text{gene}} - \text{Ct}_{\text{GAPDH}})}$. The efficiency of the PCR reactions was consistently above 95%.

Mouse model of Ischemia/Reperfusion

The experimental protocol was approved by the University of Pittsburgh Institutional Animal Care and Use Committee and conformed to the NIH guidelines for the care and use of laboratory animals. We used a non-lethal model of segmental (70%) hepatic warm

ischemia (12). In this model, all structures in the portal triad (hepatic artery, portal vein, bile duct) to the left and median liver lobes were occluded with a microvascular clamp for 30 or 60 min (ischemia) followed by removal of the clamp (reperfusion). Sham-operated animals underwent anesthesia, laparotomy and exposure of the portal triad without hepatic ischemia. Following euthanasia, liver were isolated and prepared for protein isolation and Western blot analysis as described above.

Liver damage assessment

Hepatic injury following liver ischemia and ischemia/reperfusion (I/R) was assessed by measuring serum alanine aminotransferase (sALT) levels using the Opera Clinical Chemistry System (Bayer Co., Tarrytown, NY, USA).

Statistical Analysis

Experiments were performed at least three times independently and data are presented as mean $\pm$ SE. Statistical differences were assessed by one-way analysis of variance (ANOVA) followed by a *post-hoc* test, where appropriate, using SigmaPlot 11 software (Systat Software, San Jose, CA). Differences were considered significant at the 95% confidence interval ($P < 0.05$). Two-way ANOVA was carried out using the Analysis of Variance (aov) program in the Statistical package S-plus from TIBCO Spotfire (Somerville, MA) as indicated.

RESULTS

Effect of hypoxia on BNIP3 expression in mouse hepatocytes in vitro

We have previously reported the effect of low oxygen tension, which is relevant to conditions where hypoxia plays an important role (e.g. ischemia-reperfusion injury), on BNIP3 expression (7). However, our previous studies did not show the changes in BNIP3 expression over time. Herein we measured the hypoxia-induced BNIP3 protein levels at 30 min, 1, 3, 6, 24 and 48 h. Freshly isolated mouse hepatocytes were plated and allowed to adhere and equilibrate overnight (18 h). The following day, we cultured them under hypoxic conditions (1% O₂) for the times indicated. Hepatocytes cultured under normoxic conditions served as control. We found that exposure to hypoxia (1% O₂) led to a time-dependent increase in BNIP3 protein expression (30 kDa band) in mouse hepatocytes that peaked at 6 h (Fig. 1A and 1B), while longer exposure resulted in lower levels of BNIP3 protein probably due to cell death and protein degradation (Fig. 1A and 1B). BNIP3 migrates on SDS-PAGE as a major band of 60 kDa and a minor band of 30 kDa when over-expressed transiently (3). We analyzed and quantified both bands in those experiments where the two bands were present. The results confirm that in fact the two bands are not expressed to the same extent. In our studies, the ~30 kDa band (monomer) decreases over time, while the ~60 kDa (dimer) increases and peaks at 3–6 h following hypoxia, and then decreases but to a lesser extent than the ~30 kDa band. Statistically significant changes were observed over time in both the lower ($P < 0.0001$) and upper bands ($P < 0.004$) (Fig. 1C and 1D). Furthermore, analysis by two-way ANOVA revealed a statistically significant interaction ($P = 0.0206$) between the two bands (30 and 60 kDa) over time, suggesting that these two BNIP3 variants do not vary independently. Confirming our previous observations (7), while exposure to 6 h hypoxia did not result in a significant loss of viability, incubation under hypoxic conditions for 24 or 48 h inevitably led to cell death as measured by LDH release (Fig. 1E).

HIF-1 α , HIF-2 α and HIF-3 α mRNA expression in mouse hepatocytes in vitro: Effect of hypoxia vs. normoxia

We incubated freshly isolated mouse hepatocytes under hypoxic (1% O₂) and normoxic conditions (control) for 30 min, 1, 3, 6, 24 and 48 h. At the end of the experiments, total RNA was isolated and expression of the HIF-1 α , HIF-2 α , and HIF-3 α target genes was determined by semi-quantitative RT-PCR. Exposure to hypoxia did not result in significant changes in the HIF-1 α mRNA levels up to 6 h as compared to normoxic controls but decreased significantly at 24 and 48 h probably due to cell death and protein degradation (Fig. 2A). In a similar fashion, the mRNA levels of HIF-2 α did not change in hypoxic cells up to 6 h as compared to normoxic controls but decreased significantly at 24 and 48 h (Fig. 2B). Surprisingly, at those later time points we found a marked increase in both HIF-1 α and HIF-2 α mRNA levels in control normoxic cells (Fig. 2A and 2B). In contrast to HIF-1 α and HIF-2 α , no (values under detection limit) or very low levels of HIF-3 α were detected by RT-PCR (not shown, n=3).

Effect of hypoxia on HIF-1 α protein expression in mouse hepatocytes in vitro

We next performed Western blotting analysis to determine the protein levels of HIF-1 α under the same experimental conditions as above. Freshly isolated mouse hepatocytes were exposed to hypoxia (1% O₂) for 3, 6, 24, 48 and 72 h. Untreated hepatocytes served as controls. Because cell density has been shown to affect the stabilization of HIF-1 α (13), we performed all the experiments using the same plating density (3 $\times$ 10⁶ cells/60 mm dish). At the end of the incubation, total protein was isolated and expression of the HIF-1 α was determined using a cocktail of monoclonal antibodies as described in *Materials and Methods*. As shown in Fig. 3, in contrast to resting RAW macrophages (used as a negative control for BNIP3) we observed a basal expression of HIF-1 α protein in normoxic primary mouse hepatocytes that did not change significantly upon exposure to hypoxia up to 72 h.

Silencing of HIF-1 α or E2F1 does not affect the hypoxia-induced overexpression of BNIP3 in mouse hepatocytes

Although the involvement of the HIF-1 α pathway in BNIP3 induction has been clearly established in various normal and tumor cell lines (8,9), the exact role of BNIP3 in hepatocytes has not yet been fully elucidated. In order to study whether or not the expression of BNIP3 is mediated by HIF-1 α in hepatocytes, we employed siRNA technology to reduce HIF-1 α mRNA expression. Primary mouse hepatocytes were exposed to 6 h of hypoxia with or without transient transfection with HIF-1 α (5 nM) or control non-silencing siRNA. We confirmed our previous results showing that incubation under hypoxia for 6 h leads to overexpression of BNIP3 protein in primary mouse hepatocytes (7). Under the same experimental conditions, transient transfection with HIF-1 α siRNA had no effect on the hypoxia-induced overexpression of BNIP3 as shown in Fig. 4A for the dimeric form of the protein, while it significantly reduced the expression of HIF-1 α (Fig. 4B) as measured by Western blot analysis. Transfection with the non-silencing RNA (negative control) or transfection reagent (Dharm.) alone had no significant effect on either BNIP3 (Fig. 4A) or HIF-1 α (Fig. 4b) expression induced by hypoxia. This suggests that the hypoxia-induced upregulation of BNIP3 in hepatocytes is independent of HIF-1 α .

Recently, the hypoxic expression of BNIP3 was associated with E2F1, a member of the E2F family of transcriptional regulators that is upregulated by hypoxia (14). Using siRNA technology, we attempted to explore the role of E2F1 under the same experimental conditions as for the experiments with HIF-1 α siRNA. However, we found that in 3 out of 5 independent experiments (data not shown) transient transfection with E2F1 siRNA, while it reduced the expression of E2F1 protein, had no effect on the hypoxia-induced overexpression of BNIP3 as measured by Western blot analysis. This result suggests that,

like HIF-1 α , E2F1 does not play a significant role in the hypoxia-induced of BNIP3 in mouse hepatocytes.

Hepatic expression of HIF-1 α in a mouse model of ischemia/reperfusion (I/R) in vivo

We determined the hepatic expression of HIF-1 α protein in a mouse model of ischemia/reperfusion (I/R) injury (7,12). As shown in Fig. 5A, livers from ischemic animals demonstrated only a modest increase in HIF-1 α protein expression as compared to resting livers from control animals. Furthermore, expression of HIF-1 α in livers from mice subjected to ischemia (1 h) followed by reperfusion (0.5 and 1 h) was not different from sham and only slightly elevated at later (2, 3 and 4 h) periods of reperfusion (Fig. 5A and 5B). In contrast to the effect on BNIP3 expression (7), statistical analysis revealed no significant differences in HIF-1 α protein expression between the different experimental groups. As expected, reperfusion, but not ischemia alone, led to significant hepatic injury as measured by serum ALT levels (Fig. 5C).

DISCUSSION

BNIP3 and Nix (a BNIP3 homologue also known as BNIP3L) are the only members of the Bcl-2 family of apoptotic factors induced in response to hypoxia (4). Although hypoxia is regarded as the major physiological inducer of BNIP3 expression, much of the data on BNIP3 have been derived from cellular over-expression experiments, performed in the absence of any hypoxic stress (15). BNIP3 was initially described as a cytoplasmic, membrane-associated protein localized to mitochondria and other cytoplasmic membrane structures (3). However, it was also found to be primarily localized to the nucleus of glial cells of the normal human brain, as well as in the malignant glioma cell line U251 where it localized with the mitochondria upon exposure to hypoxia (16). Similarly, we recently found that under normoxic conditions BNIP3 is mostly localized to the nucleus with some cytoplasmic staining in primary mouse hepatocytes, an effect reversed upon exposure to hypoxia (7). The present study is an attempt to further elucidate the regulation of BNIP3 in the liver by hypoxia.

We hypothesized that the hypoxia-induced upregulation of BNIP3 would be mediated by HIF-1 α in a fashion similar to the regulation of BNIP3 in other cells (15). The BNIP3 promoter contains a functional HIF-1-responsive element (HRE) and is potently activated by both hypoxia and forced expression of HIF-1 α (4). Many genes that are regulated by hypoxia, including BNIP3, are regulated by HIF-1 α (8,9,15). The transcription and synthesis of HIF-1 α are constitutive and seem not to be affected by oxygen. However, under normoxic conditions, the HIF-1 α protein is rapidly degraded, resulting in essentially no detectable HIF-1 α protein. When cells are subjected acutely to hypoxia, stabilization of HIF-1 α is followed by translocation from the cytoplasm to the nucleus, where HIF-1 α dimerizes with HIF-1 β . This HIF complex then binds to its cognate sequence in numerous target genes, resulting in increased transcription of these genes (17,18). At oxygen tensions used in standard cell culture experiments such as the normoxic conditions described herein (which arguably may or may not reflect the conditions under which these cells would exist *in vivo*), most tissues have undetectable or very low levels of BNIP3. However, BNIP3 mRNA is induced under hypoxic conditions through a 5' promoter containing a HIF-1 binding site (19). Others have suggested that under low oxygen tensions, HIF-1 is the major mediator of increased BNIP3 expression in a number of human and animal cell lines (15).

However, a recent study reported a lack of nuclear induction of HIF-1 α protein in hypoxic primary rat hepatocytes (10). These results suggest that although hepatocytes do respond to hypoxia, the contribution of HIF-1 α to this adaptation may be minor or transient at best probably due to its translocation to peroxisomes rather than to the nucleus in hypoxia (10).

Although the involvement of the HIF-1 α pathway in BNIP3 induction has been clearly established in both normal and tumor cell lines (8,9), the exact role of HIF-1 α in hepatocytes is not known. Our present results clearly show that in contrast to resting RAW macrophages, which do not express HIF-1 α , there is a basal expression of HIF- α protein in normoxic primary mouse hepatocytes that does not change significantly upon exposure to hypoxia. These results suggest that the upregulation of BNIP3 is not preceded by changes in the expression of HIF-1 α protein. One possibility is that the HIF-1 complex might move to the nucleus without increasing total HIF-1 α . However, we found that knocking down HIF-1 α mRNA resulted in a significant reduction in HIF-1 α protein levels, yet did not affect the hypoxia-induced upregulation of BNIP3. These studies support the notion that HIF-1 α does not play a role in hypoxia-induced overexpression of BNIP3 in mouse hepatocytes.

Furthermore, MAPK activity has been shown to regulate the induction and/or degradation of certain transcription factors, including HIF-1 α (2). Treatment with PD98059, an upstream inhibitor of the ERK1/2 MAPK pathway, inhibited HIF-1 α protein phosphorylation and target gene activation (20). However, we have demonstrated previously that this inhibitor had no significant effect on BNIP3 expression in primary mouse hepatocytes (7), lending further support to our conclusion that hypoxia-induced overexpression of BNIP3 is not dependent on HIF-1 α in this cell type.

Liver hypoxia is associated with inflammatory conditions such as ischemia/reperfusion (I/R) and hemorrhagic shock (HS) (21). We have previously shown that ischemia leads to upregulation of BNIP3 protein expression as compared to sham-operated animals in a mouse model of ischemia/reperfusion (I/R) injury (7). In the same animal model, livers from ischemic animals demonstrated only a modest increase in HIF-1 α protein expression as compared to resting livers from control animals. Furthermore, the expression of HIF-1 α in livers from mice subjected to ischemia and followed by shorter periods of reperfusion was not different from sham controls, and was only slightly elevated at later periods of reperfusion. The lack of statistically significant differences in HIF-1 α protein expression between the different experimental groups stands in contrast to the effect on BNIP3 expression previously reported (7), and suggest that the ischemia-induced hepatic upregulation of BNIP3 *in vivo* is, like in the hypoxic hepatocytes *in vitro*, not preceded by changes in the expression of the HIF-1 α protein. Thus, although hypoxia is partly responsible for the upregulation of BNIP3 in the liver *in vivo*, the contribution of HIF-1 α appears to be minor.

In addition to hypoxic signals, the HIF-1 pathway is also activated by a wide variety of oxygen-independent signals, including growth factors and cell density (22). In contrast to HIF-1 α , the HIF-2 α isoforms have been reported to suppress BNIP3 expression (15). Interestingly, we found that primary mouse hepatocytes cultured under normoxia, but not under hypoxia, upregulated not only HIF-1 α but HIF-2 α mRNA. Accumulation of HIF-2 α during normoxic conditions was first reported in HeLa cells, where cells seeded at low density contained less stabilized HIF-1 α and HIF-2 α protein as compared to confluent monolayers. The authors proposed that the high levels observed for both HIF proteins at high cell density might explain why certain HIF-1 target genes were seen to be up-regulated in their expressions (23). The accumulation of HIF-1 α and HIF-2 α protein during normoxic conditions has been previously demonstrated (13), but to our knowledge this phenomenon has not been reported in primary hepatocytes. Since a high rate of oxygen consumption due to increased cell density creates an oxygen gradient within the cellular microenvironment that is similar, but not identical, to hypoxic conditions, and because cell density has been shown to affect the stabilization of HIF-1 α (13), it is worth emphasizing that we performed all the experiments using the same plating density. Furthermore, studies in HepG2 cells have shown that while hyperbaric oxygen increases HIF-1 α protein levels and DNA binding,

there is no corresponding increase in transcriptional activity (24). Whether or not the observed upregulation of HIF-1 α and HIF-2 α mRNA in normoxic hepatocytes translates into *de novo* protein synthesis with relevant biological activity remains to be investigated. A previous study has shown basal levels of HIF-3 α in the nuclei of normoxic hepatocytes that shifted out of the nucleus in hypoxia, and unlike HIF-2 α , did co-localize with the peroxisomal enzyme catalase, suggesting a similar targeting mechanism as HIF-1 α (10). In contrast to HIF-1 α and HIF-2 α , however, in the present study very low or no levels of HIF-3 α were detected by RT-PCR using specific mouse primers. Thus, the question still remains whether or not HIF-3 α is involved in the regulation of BNIP3 in hepatocytes or in any other cell type.

If HIF-1 α does not mediate the hypoxia-induced elevation of BNIP3, what other mechanisms might be at work? The response to hypoxia involves the activation of several transcription factors and regulators associated with inflammation, tumor invasion, angiogenesis, cell-cycle arrest, and apoptosis. These include the NF- κ B family, activating protein-1 (AP-1), p53, stimulatory proteins 1 and 3 (SP1/SP3), Myc family members, nuclear factor for Interleukin 6 (NF-IL6), and early growth response-1 (Egr-1) (reviewed in (25,26)). Apart from HIF-1 α , the hypoxic expression of BNIP3 has been recently associated with E2F1, a member of the E2F family of transcriptional regulators that is upregulated by hypoxia (14). In rat ventricular myocytes, the BNIP3 promoter is subject to strong negative repression under normoxic conditions but is highly induced in cells during hypoxia in a manner dependent on the binding to E2F1 (27). Using siRNA technology, we attempted to explore the role of E2F1 under the same experimental conditions as for the experiments with HIF-1 α siRNA. However, we found that transient transfection with E2F1 siRNA had no effect on the hypoxia-induced overexpression of BNIP3 protein as measured by Western blot analysis, suggesting that E2F1 does not play a significant role in the hypoxia-induced of BNIP3 in mouse hepatocytes.

One reported anomaly regarding the detection of BNIP3 protein by Western blotting is that, despite its calculated molecular mass of 21.54 kDa, when transiently expressed it migrates on SDS-PAGE as a major band of 60 kDa and a minor band of 30 kDa (3). It has been shown in certain epithelial tumors (28), intestinal mucosa (6), and in cardiac myocytes (29), that the two bands are differentially expressed and that the magnitude of upregulation of the 60 kDa band does not parallel that of the 30 kDa protein. Similarly, in hepatocytes we routinely find that the two bands are not always expressed to the same extent (7). Therefore, we decided to analyze and quantify both bands in those experiments where the two bands were detected and the results confirmed the differential expression of the protein: while the ~30 kDa band (monomer) decreases over time, the ~60 kDa (dimer) increases following hypoxia. This suggests that cellular stresses such as hypoxia increase the formation of the BNIP3 homodimer while it decreases the amount of the monomeric form of BNIP3 in primary mouse hepatocytes. This phenomenon does not appear to be cell-specific since it has recently been shown that the BNIP3 homodimer increases in rat hearts subjected to I/R and in isolated cardiac myocytes exposed to hypoxia/reoxygenation or hydrogen peroxide treatment (29). Furthermore, BNIP3 is regulated post-transcriptionally under hypoxic conditions, existing as multiple monomeric and dimeric phosphorylated forms. Moreover, phosphorylation appears to increase the stability of BNIP3 in human tumor cells (30).

In summary, we have expanded our understanding of the regulation of BNIP3 in hepatocytes under hypoxic stress. Herein, we have confirmed that hypoxic stress increases the formation of the BNIP3 homodimer while it decreases the amount of the monomeric form of BNIP3 in primary mouse hepatocytes. Contrary to our initial hypothesis, we found that the hypoxia-induced overexpression of BNIP3 is not dependent on HIF-1 α in mouse hepatocytes *in vitro*, and possibly in the liver *in vivo*. Expression of several HIF-1 target genes, such as

BNIP3, is induced by hypoxia in many cell types. However, gene expression is induced by hypoxia in a cell-typespecific manner for the majority of HIF-1 target genes (2). Since HIF-1 activity is induced by hypoxia in almost all cell types, HIF-1 alone cannot account for this cell type-specific gene expression. We suggest that the functional interaction of HIF-1 with other transcription factors may determine the subgroup of HIF-1 target genes that is activated in any particular hypoxic cell, as has been proposed by others (2). We are currently investigating which transcription factors and pathway(s), alone or in conjunction with HIF-1 α , are involved in the hypoxia-induced upregulation of BNIP3 in hepatocytes and liver.

Acknowledgments

The authors thank Ms. Jinling Yin for technical assistance with RNA isolation, and Ms. Carol Meiers and Ms. Danielle Reiser for isolation and preparation of primary hepatocyte cultures.

This study was supported by P50-GM-53789-09 NIH/NIGMS grant (TRB, YV, RZ, GC) AND UO1 DK072146-05 (YV, GC)

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FIG. 1. Effect of hypoxia on BNIP3 protein expression in mouse hepatocytes

Freshly isolated mouse hepatocytes were exposed to hypoxia (1% O₂) for different time intervals as indicated and the total protein was isolated and analyzed as described in *Materials and Methods*. Control samples (0 h) were hepatocytes cultured under normoxic conditions. Western blot analysis for BNIP3 protein in mouse hepatocytes, where (A) and (C) are representative Western blots; and (B) and (D) are the densitometric quantification expressed as the mean $\pm$ SE of n independent experiments as follows: (B) $n = 6-15$ mice, $*P = 0.001$ vs. Ctrl, analyzed by one-way ANOVA followed by Fisher LSD method. (D) $n = 3-6$ mice, analyzed by two-way ANOVA as described in *Materials and Methods*. (E) Viability of hepatocytes exposed to hypoxia (1% O₂) for 1–48 h measured by LDH release as described in *Materials and Methods*. Data are mean $\pm$ SE ($n = 6$ mice, time 0 h represents Normoxia Ctrl). $*P < 0.001$ vs. Ctrl and 1 h hypoxia; $**P < 0.001$ vs. Ctrl, 1, 3 and 6 h hypoxia, analyzed by one-way ANOVA followed by Holm-Sidak method.

FIG. 2. Effect of normoxia and hypoxia on HIF-1α and HIF-2α transcripts in primary mouse hepatocytes

Total RNA was collected from primary mouse hepatocytes cultured under normoxic (21% O₂) and hypoxic (1% O₂) conditions at different time intervals, reverse-transcribed into cDNA, and quantitatively examined for HIF-1α (A) and HIF-2α (B) as described in *Materials and Methods*. Bars represent the relative amount of each gene product relative to the GAPDH internal control and are the mean ± SE from 5 independent PCR amplification experiments (n=5 mice). Data were analyzed by one-way ANOVA followed by Fisher LSD method: (A) **P*<0.05 vs. 0.5, 1 and 3 h; ***P*<0.05 vs. 0.5, 1, 3 and 6 h; ##*P*<0.001 vs. 0.5, 1, 3, 6 and 24 h. (B) **P*<0.05 vs. 0.5, 1 and 3 h; \$*P*<0.01 vs. 0.5 h; #*P*<0.01 vs. 0.5, 1, 3 and 6 h; ##*P*<0.001 vs. 0.5, 1, 3, 6 and 24 h.

FIG. 3. Effect of hypoxia on HIF-1 α protein expression in mouse hepatocytes

Freshly isolated mouse hepatocytes from 5–7 mice were exposed to hypoxia (1% O₂) for different time intervals as indicated and the total protein was isolated and analyzed as described in *Materials and Methods*. Control samples (0 h) were hepatocytes cultured under normoxic conditions and RAW267.4 macrophages exposed to hypoxia for 6 h were used as positive control. A representative Western blot (**A**) and the densitometric quantification for HIF-1 α protein (**B**) of 5–7 independent experiments are shown ($P=0.607$, analyzed by one-way ANOVA).

FIG. 4. Effect of HIF-1α siRNA on BNIP3 and HIF-1α protein expression in mouse hepatocytes
Following transient transfection with Dharmacon (transfection reagent), non-silencing (scrambled) RNA and HIF-1α siRNA (5 nM), primary mouse hepatocytes from 3 mice were exposed to 6 h of hypoxia and the total protein was isolated and analyzed for BNIP3 and HIF-1α as described in *Materials and Methods*. Representative Western blots showing BNIP3 (**A**) and HIF-1α (**B**) protein expression under the same experimental conditions are shown on top of densitometric quantification of bands normalized to β-actin (n=3 independent experiments, * $P < 0.005$ vs. scr. siRNA and * $P < 0.01$ vs. Dharm., analyzed by one-way ANOVA followed by Fisher LSD method).

FIG. 5. Hepatic expression of HIF-1 α in an *in vivo* model of ischemia/reperfusion

Livers from 5 mice subjected to segmental (70%) hepatic warm ischemia for 30 min or 1 h, or 1 h ischemia followed by 0.5–4 h reperfusion were collected and Western blot analysis for HIF-1 α protein was performed as described in *Materials and Methods*. Livers from sham animals served as control. (A) A representative Western blot and (B) the densitometric quantification (mean $\pm$ SE) of 5 independent experiments are shown. (C) Serum ALT levels were analyzed as a measure of hepatocellular injury. Data represent the mean $\pm$ SE of 4 independent experiments (* P <0.05 vs. Sham and Ischemia only, ** P =0.028 vs. Sham, analyzed by one-way ANOVA followed by Fisher LSD method).