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Identification and characterization of a nuclear factor-κ B-p65 proteolytic fragment in nuclei of porcine hepatocytes in monolayer culture

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ABSTRACT

Hepatic responses to proinflammatory signals are controlled by the activation of several transcription factors, including, nuclear factor-κ B (NF-κB). In this study, hepatocytes prepared from suckling pigs and maintained in serum-free monolayer culture were used to define a novel proinflammatory cytokine-specific NF-κB subunit modification. The immunoreactive p65 protein was detected by Western blot analysis at the appropriate molecular weight in the cytosol of control cultures and those incubated with tumor necrosis factor-α (TNF). However, in nuclei, the p65 antisera cross-reacted with a protein of approximately 38 kDa (termed p38) after TNF addition, which was not observed in the cytosol of control or cytokine-treated cells. Specifically, incubation with TNF also resulted in phosphorylation ($P < 0.05$) of the inhibitor complex protein (IκB), whereas incubation with other cytokines, IL-6, IL-17a, or oncostatin M was not associated with either phosphorylation of IκB or nuclear translocation of p65. Intracellular endothelial nitric oxide synthase was deceased ($P < 0.05$) and plasminogen activator inhibitor-1 secretion was increased ($P < 0.05$) after TNF incubation. The TNF-induced p38 protein was purified from hepatocyte nuclei by immunoprecipitation, concentrated by electrophoresis, and subsequently analyzed by mass spectrometry. Ten unique NF-κB p65 peptides were identified after digestion with trypsin and chymotrypsin; however, all were mapped to the N-terminus and within the first 310 amino acid residues of the intact p65 protein. Although low molecular weight immunoreactive p65 molecules were previously observed in various human and rodent systems, this is the first report to positively identify the p38 fragment within hepatocyte nuclei or after specific cytokine (TNF) induction.

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1. Introduction

The liver plays a key role in the regulation of metabolism during an organism's response to inflammatory challenge. The metabolic response is initiated and maintained in part by cytokines that are primarily secreted by cells of the immune system, adipocytes, or produced locally within the liver. The response is orchestrated by hepatocytes through short-term activation of enzyme processes and transcription factors [1–3]. The present work was initiated to investigate the role of and specificity for tumor necrosis factor-α (TNF) in modulating the stress response in neonatal porcine hepatocytes. The complexity of the cellular responses associated with the TNF regulation of liver homeostasis and pathology is well documented. Indeed, TNF paradoxically has both hepatic protective roles and is also associated with induction of apoptosis, carcinogenesis,

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and other inflammatory diseases [4]. Moreover, TNF plays a pivotal role in regulating other hepatic cytokines, including IL-6, and activates nuclear factor-κ B (NF-κB), one of the primary transcription factors involved in inflammatory processes [4]. Across a myriad of potential stresses to cells, evolutionarily conserved levels of cellular stress responses are coordinated via NF-κB processing in regard to the process termed "cross tolerance," wherein the fundamental nature of stress detection and response is distilled down to a few overarching mechanisms [5,6]. Transcription programs regulated by NF-κB also control cell survival, differentiation, and cellular proliferation [7]. Regulation via NF-κB is further complicated by the molecular composition of the transcription factor, which exists as a family of distinct interacting protein components [7,8]. These include members of the Rel family with DNA-binding domains, the inhibitor (IκB) family that forms an inhibitor complex with the Rel proteins, and kinases that are required for activation [7]. In hepatocytes, NF-κB RelA/p65 exists as a heterodimer in the cytosol bound within the IκB inhibitory complex. On the binding of TNF to its receptor, phosphorylation of the IκB inhibitor complex results in degradation of the complex and freeing of the RelA dimer for nuclear translocation [9]. Once inside the nuclear envelope, the Rel proteins interact with DNA to regulate transcriptional activity.

Although the intact RelA molecule may be further activated by phosphorylation [9], lower molecular weight forms of p65/RelA have been previously observed in several experimental systems. Cressman and Taub [10] reported the presence of a 35-kDa (p35/RelA) fragment in nuclei from livers of control and partially hepatectomized rodents which was reactive with antisera to the N-terminal region of p65. In macrophages infected with *Leishmania*, a nuclear p35 fragment was observed, which was a product of proteolytic activity derived from the parasites [11]. Similarly, poliovirus-infected HeLa cells also produce a lower molecular weight p65 fragment that was shown to be the product of viral protease activity [12]; however, this fragment was cytoplasmic and not nuclear. In contrast, a C-terminal p65 fragment (~55 kDa) was observed in T cells infected with human immunodeficiency virus [13].

The objectives of the present study were to determine whether TNF alone was sufficient to activate NF-κB in normal hepatocytes prepared from suckling pigs and whether activation was associated with phosphorylation of IκB, translocation of p65 to the nuclear compartment, and subsequent changes in protein expression. Further, because nuclear translocation of the full-length p65 molecule was not observed, a main focus of this investigation was aimed at identifying the unique protein that was observed in the nucleus of TNF-treated cells.

2. Materials and methods

2.1. Hepatocyte isolation

Care and treatment of all pigs in this study were approved by the Institutional Animal Care and Use Committee of the US Department of Agriculture. Suckling crossbred male piglets (7–24 d of age) were euthanized with a solution that contained ketamine, telazol, xylazine, and butorphanol, and laparotomy was performed. Livers were excised intact, the left lateral lobe was removed, and the portal vein was cannulated and sewed in place. Hepatocytes were isolated by a 2-step collagenase digestion procedure essentially as previously described [14–16], using the entire lateral lobe. Briefly, 500 mL of HEPES-buffered saline (HBS; 10 m*M* HEPES, 142 m*M* NaCl, 6.7 m*M* KCl, 5.5 m*M* glucose) containing 0.5 m*M* ethyleneglycol-bis-(β-amino-ethyl ether) N,N′-tetra acetic acid was pumped through the liver lobe, and pump speed was gradually elevated to 50 mL/min; an additional 500 mL of HBS was perfused. A collagenase solution (150 mL) that contained 100 mL of HBS (with 100 m*M* HEPES and 5 m*M* $CaCl_2$), 50 mL of Medium 199 with Earle's salts, 121 U/mL collagenase (class 2; Worthington, Lakewood, NJ, USA), 0.3 g of glucose, 0.15 g of BSA, and 1.0 mg of bovine insulin was recirculated through the liver for approximately 30 min. The digested tissue mass was teased apart in cell wash (Medium 199 with Hanks' salts and 10 m*M* HEPES) and filtered through cheesecloth. Hepatocytes were isolated with repeated (6 times) washing with cell wash by low speed centrifugation (50 × *g*) in a swinging bucket rotor. Viability of hepatocytes was 81.5% ± 0.8% by trypan blue dye exclusion (n = 9).

2.2. Hepatocyte culture

Hepatocytes (4.5×10^6) were seeded into vented T-25 flasks (Corning Inc, Corning, NY, USA) precoated with bovine skin collagen (A10644-01; Life Technologies, Grand Island, NY, USA) and cultured in William's Media as previously described [15]. Except when noted, all media, cell culture, and assay reagents were purchased from Sigma (St Louis, MO, USA). Recombinant cytokines used were porcine (p)TNFα, pIL-6, human oncostatin M (OSM; R&D Systems, Minneapolis, MN, USA), and pIL-17a (Kingfisher Biotech, St Paul, MN, USA). All basal Williams' E medium was prepared with 100 μ*M* β-mercaptoethanol, 10 m*M* HEPES, 10 n*M* Na_2SeO_3, 2 m*M* glutamine, and antibiotics (gentamicin, penicillin, streptomycin, and amphotericin B). Cells were initially maintained in medium that contained insulin-transferrin-selenium and 10% fetal bovine serum. After a 3-h attachment period, flasks were washed with warm HBS and 5 mL of medium that contained 5% fetal bovine serum, and insulin-transferrin-selenium was added to each flask. On the next day, flasks were washed twice with warm HBS, and medium was replaced with 4.5 mL of serum-free basal medium that contained 1 m*M* carnitine, 0.01% dimethyl sulfoxide, 0.1% BSA (A1595), 10 n*M* dexamethasone, and 1 ng/mL bovine insulin (control medium). In cultures in which media samples were analyzed, the concentration of BSA was lowered to 0.01%.

For all protein determination studies, experimental conditions were established after serum-free conditions were initiated and maintained for an additional 48 h. All media were changed daily, and experiments were terminated approximately 72 h after the initiation of culture. For short-term incubations (5–80 min), monolayers maintained in control medium were washed with warm HBS, fresh control medium was added, and cultures were placed back in the incubator for 2 h before addition of cytokines. At