Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.JournalofSurgicalResearch.com

# Hepatocyte autophagy is linked to C/EBP-homologous protein, Bcl2-interacting mediator of cell death, and BH3-interacting domain death agonist gene expression

*Junlin Zhang, PhD, Nitesh Singh, MS, Kendra S. Robinson-Taylor, MS, Wanda A. Dorsett-Martin, DVM, Michael W. Morris Jr., MD, Truman M. Earl, MD, and Christopher D. Anderson, MD**

*Department of Surgery, University of Mississippi Medical Center, Jackson, Mississippi*

ARTICLE INFO

*Article history:*
Received 30 September 2014
Received in revised form
6 January 2015
Accepted 22 January 2015
Available online 28 January 2015

*Keywords:*
Autophagy
Endoplasmic reticulum stress
BIM
BID
CHOP
Hepatocyte

ABSTRACT

*Background:* Endoplasmic reticulum (ER) stress and autophagy each play important roles in hepatocyte cell injury. We hypothesized that gene expression of C/EBP-homologous protein (CHOP) and the BH3 proteins Bcl2-interacting mediator of cell death (BIM) and BH3-interacting domain death agonist (BID) are involved in a complex interplay that regulates ER stress-induced autophagy and cell death.

*Materials and methods:* Hepatocytes were cultured from lean Zucker rats. Confluent hepatocytes were incubated with single or combined small interfering RNA for CHOP, BIM, and/or BID for 24 h providing gene inhibition. Incubation with tunicamycin (TM) for another 24 h stimulated ER stress. Quantitative real-time polymerase chain reaction determined the expression levels of CHOP, BIM, and BID. Immunostaining with microtubule-associated protein 1 light chain 3 measured autophagy activity. Trypan blue exclusion determined the cell viability.

*Results:* TM treatment increased the messenger RNA levels of CHOP and BIM but decreased the messenger RNA levels of BID. TM increased autophagy and decreased cell viability. Individual inhibition of CHOP, BIM, or BID protected against autophagy and cell death. However, simultaneous treatment with any combination of CHOP, BIM, and BID small interfering RNAs reduced autophagy activity but increased cell death independent of ER stress induction.

*Conclusions:* Autophagy in hepatocytes results from acute ER stress and involves interplay, at the gene expression level, of CHOP, BIM, and BID. Inhibition of any one of these individual genes during acute ER stress is protective against cell death. Conversely, inhibition of any two of the three genes results in increased nonautophagic cell death independent of ER stress induction. This study suggests interplay between CHOP, BIM, and BID expression that can be leveraged for protection against ER stress-related cell death. However, disruption of the CHOP/BH3 gene expression homeostasis is detrimental to cell survival independent of other cellular stress.



* *Corresponding author*. Division of Transplant and Hepatobiliary Surgery, Department of Surgery, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216. Tel.: +1 601 815 3135; fax: 601-984-2962.
E-mail address: cdanderson@umc.edu (C.D. Anderson).

0022-4804/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jss.2015.01.039

## 1. Introduction

Endoplasmic reticulum (ER) stress is important to many pathophysiologic conditions in the liver. ER stress is induced under a number of situations such as calcium depletion with thapsigargin, glycosylation inhibition with tunicamycin (TM), alterations in redox protein folding, and lipid loading of the ER such as with palmitate [1]. ER stress has a dual role. It, in general, is a protective mechanism for cells during times of stress. However, acute or exacerbated ER stress will induce cell death [2,3]. After a cellular stressor, the chaperone protein glucose related/binding immunoglobulin protein 78 recognizes misfolded proteins within the ER lumen and activates three major ER stress mediators and their downstream pathways as follows: pancreatic eukaryotic translation initiation factor kinase, inositol-requiring enzyme-1α, and activating transcription factor-6 [2]. As the downstream target of pancreatic eukaryotic translation initiation factor kinase, activating transcription factor-4 activated C/EBP-homologous protein (CHOP) is the most implicated pathway resulting in ER stress-related cell death [4].

We have previously demonstrated the importance of CHOP in cold ischemia and reperfusion injury of the liver [5], and we have demonstrated autophagy as a mediator of ER stress-induced cell death in hepatocytes [6]. The main function of autophagy is to clear unwanted components in the cytoplasm, and it is believed to be beneficial to cell survival. Autophagy has many roles in the liver. It contributes to normal homeostasis but it also is a major contributor to numerous liver diseases [7]. In many of its disease state roles, autophagy is linked to ER stress [8,9]. Autophagy contributes to cell death under certain circumstances [10]. Therefore, similar to ER stress, autophagy also acts as a double-edged sword in mediating cell fate.

The link among ER stress, autophagy, and cell death in hepatocytes is not well understood. It is known that CHOP is an important mediator of ER stress-induced autophagy and cell death in rat cardiac cells [11] and human glioma cells [12]. CHOP is also reported to be closely related to proapoptotic BH3 proteins, such as Bcl2-interacting mediator of cell death (BIM) and BH3-interacting domain death agonist (BID) [13,14]. BID has also been implicated in ER stress-related cell death [15]. In the present study, we hypothesized that in addition to CHOP, BIM and BID gene regulations are also involved in ER stress-induced autophagy and cell death in rat hepatocytes.

## 2. Methods and materials

Experimental procedures were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee and complied with the Association for Assessment and Accreditation of Laboratory Animal Care International and National Institutes of Health guidelines.

### 2.1. Cell culture of primary hepatocytes

After 1 wk acclimatization, 300-g male lean Zucker rats ordered from Charles River (Wilmington, MA) received standard hepatectomy using isoflurane. The liver was placed on a filter membrane with a 20G angiocath cannulated in the portal vein. A Masterflex L/S microprocessor pump (Cole Parmer, Vernon Hills, IL) was connected to the angiocath. The liver was flushed with 100-mL liver perfusion medium (Gibco, Grand Island, NY), followed by 100-mL liver digest medium (Gibco) for 45 min, both mediums contain penicillin streptomycin (1:100; Gibco) and were kept at 37°C. The liver was then disrupted, filtered through a 230-μm mesh, and followed by a layer of sterile cotton gauze. The mixed liver suspension was washed twice with hepatocyte wash medium (Gibco) at 4°C. Nonviable hepatocytes were removed using Percoll solution (Sigma, St Louis, MO). The hepatocyte cell pellet was washed and then suspended in complete hepatocyte culture medium (Lonza, Allendale, NJ). After calculating the cell viability using Trypan blue exclusion, the hepatocytes were plated in different sizes of petri dishes at the concentration of $1 \times 10^5$ cells/mL and incubated at 37°C with 5% carbon dioxide for normal growth. The culture medium was changed 6 h after plating, and the cells were allowed to grow until about 70%–80% confluent and then used for small interfering RNA (siRNA) and drug treatments.

### 2.2. Delivery of siRNA and induction of ER stress

N-TER Nanoparticle siRNA transfection system was used to delivery siRNA to cultured hepatocytes according to manufacturer instructions. The sequence of CHOP siRNA (sense 5′-GGAAGAACUAGGAAACGGTT-3′ and anti-sense 5′-UCCGUUUCCUAGUUCUUCCTT-3′) was created based on a previous report [16]. TM, CHOP siRNA, BIM siRNA (SASI_Rn01 00083125), and BID siRNA (SASI_Rn01 00043867) were purchased from Sigma.

For the first cohort study, hepatocyte culture medium was removed and replaced with fresh culture medium containing 50 nM of negative siRNA, CHOP siRNA, BIM siRNA, BID siRNA, CHOP/BIM siRNA, CHOP/BID siRNA, BIM/BID siRNA, or CHOP/BIM/BID siRNA. After siRNA treatment, cells were allowed to grow for 24 h before harvest. In the second cohort study, the hepatocytes were treated with the previously mentioned individual siRNA and combined siRNAs for 24 h first followed by another 24 h of incubation in hepatocyte culture medium containing 1 μg/mL TM to induce ER stress. Controls were treated with negative siRNA or negative siRNA + TM. Data from three separate hepatocyte isolations were included in each treatment.

### 2.3. Quantitative real-time polymerase chain reaction

The hepatocytes were harvested using 0.05% Trypsin-EDTA (Gibco). Hepatocyte RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the instructions, and its concentration was measured by Synergy HT microplate reader (BioTek, Winooski, VT). Complementary DNA was acquired through the thermal cycler (Bio-Rad, Hercules, CA) using complementary DNA reverse transcription kit (Applied Biosystems, Foster City, CA). The messenger RNA (mRNA) levels were quantified by TaqMan probe-based quantitative real-