Semmelweis University
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Acetaminophen-induced redox stress in the endoplasmic reticulum

Ph. D. thesis

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INTRODUCTION

In the eukaryotic cell, the endoplasmic reticulum (ER) presents a major stage in the secretory pathway of proteins. The co- and posttranslational covalent modification and folding of secretory and membrane proteins – a delicately regulated process that results in proteins adopting their native conformation – takes place in the ER lumen. Covalent modification involves formation of intra- and intermolecular disulfide bonds. Correct protein folding is dependent on luminal homeostasis and strictly monitored by chaperones. Upon entering the ER lumen a nascent protein – i.e. a client to the folding machinery - is non-covalently bound by chaperones and will remain bound through the folding process until it leaves the ER for the Golgi.

The ER lumen differs significantly from the cytoplasm in its metabolite composition, redox and calcium homeostasis and protein content. An altered luminal homeostasis will inevitably result in the appearance and accumulation of unfolded and misfolded client proteins. The disturbance of protein folding is readily sensed and interpreted by chaperones as ER stress, which activates a bunch of signal transduction pathways jointly termed the *unfolded protein response* (UPR). The UPR aims to reduce the burden of client proteins by a general inhibition of translation, also, it increases chaperone capacity by specific up-regulation of transcription and translation of ER chaperones, as well as by facilitating clearance of misfolded proteins from the lumen. Taken together, the UPR helps restoring proper ER functions and homeostasis; also, it coordinates luminal with cellular homeostasis. Besides its pro-survival features, the UPR also has other components leading to programmed cell death (apoptosis). These pro-apoptotic components dominate the response if the stress is too severe to cope with.

Three UPR proteins have been shown until now to initiate ER-dependent apoptosis: GADD153, JNK and caspase-12. GADD153 *(growth arrest- and DNA damage-inducible gene 153)* is a pro-apoptotic transcription factor which is specifically induced upon ER stress. Members of the JNK (c-Jun N-terminal kinase) family of protein kinases are activated upon several types of stress stimuli. Caspase-12 is the only caspase known to reside in the ER membrane; its pro-form (procaspase-12) is bound to the cytosolic side and is activated by cleavage in ER stress. The final outcome of the UPR will be determined by the balance between pro-survival and pro-apoptotic features.

ER stress is now known to play a causative role in several acute and chronic diseases. Thus, the study of ER stress and stress response is winning on importance; *in vivo* and *in vitro* models exist for the better understanding of the underlying molecular mechanisms. My thesis is concerned with modeling redox imbalance-based ER stress.

Proper ER redox homeostasis is a prerequisite of oxidative protein folding. Formation of disulfide bonds in client proteins is dependent on an oxidative environment, a condition aptly supplied by the ER lumen. Compared to the cytosol, the ER lumen is more oxidative, which is reflected by the glutathione system, the major luminal redox puffer. Whereas the cytosol is characterized by a reduced-to-oxidized glutathione ratio of 30-100:1, the ER lumen has a ratio of 1-3:1. Oxidative protein folding is indirectly dependent on the cytosolic redox status, as reduced glutathione (GSH) has been shown to be transported into the ER lumen. It is proposed that glutathione promotes the efficient rearrangement of disulfide bonds in client proteins until native conformation is reached, a theory well supported by the observation that more than half of the ER glutathione is present as mixed glutathione-protein disulfides.

The ER plays an essential role in drug metabolism due to its wide array of biotransformation enzymes. Acetaminophen (AAP) is a widely used analgesic and antipyretic agent. However added in overdose it may cause severe glutathione depletion, oxidative stress, mitochondrial permeability transition, nuclear damage and consequent death of hepatocytes. AAP-induced hepatocellular damage is a well-known example of drug-induced oxidative stress. In spite of decades of intensive research centered on AAP-toxicity, many questions concerning the exact mechanism still remain to be elucidated.

The redox sensitivity of luminal protein folding and the central role of the ER in AAP metabolism led us to assume that AAP overdose leads to ER stress, which may play a role in AAP-induced hepatocellular damage. Our assumption was supported by recent advances in the understanding of the ER; namely, the organelle acts not only as an executioner but also as an orchestrator of cellular functions. AAP-induced ER-stress and stress response are the subjects of my PhD thesis.

SPECIFIC AIMS

In my PhD work, I set out to answer the following questions:

1. Does AAP overdose lead to depletion of the ER glutathione pool and to an altered redox status of the ER lumen?
2. Is there an ER stress response after AAP overdose? Which branches will dominate the response if there is one? Are there any changes in the expression of ER chaperones or an up-regulation of pro-apoptotic factors?
3. Is depletion of ER glutathione *per se* enough to promote ER stress? For depletion of glutathione BSO (buthio….) was used.
4. Which role does the used AAP model plays in AAP-induced hepatocellular injury?
5. Is there any difference between AAP-induced *in vivo* and *in vitro* stress and stress response mechanisms?

AAP-induced hepatocellular injury may be viewed as an *in vivo* model for redox imbalance-based ER stress, thus our *in vivo* experiments may present a relevant tool for the study of acute liver injury in humans.

METHODS

Animal experiments, cell cultures

4-5-weeks-old CD-1 male mice were used for animal studies. Mice were kept under standard housing conditions. Mice were treated after 24 hours of fasting. For cell culture studies two human cell lines produced by the permanent transfection of the HepG2 hepatocellular line were used. C34 cells carry an empty vector, whereas E47 cells carry a plasmid coding for CYP2E1. Hepa1c1c7, a mouse hepatoma line, was also used.

Serum enzyme activities

Blood samples collected from the *vena cava inferior* of sedated mice were treated with citrate, and serum activities of aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase were measured with a commercially available kit.

Western blot analysis

Cell and tissue homogenates were analyzed for protein concentration, and samples were separated on SDS-PAGE followed by transfer to nitrocellulose membrane. Membranes were incubated with primary and secondary antibodies, then chemiluminescent detection was used for detection of protein expression.

Microsome isolation

Liver homogenates were repeatedly ultracentrifuged and microsomal fractions were collected. Microsomes were stored under liquid nitrogen until used.

Glutathione measurement

For the measurement of cellular and microsomal total glutathione, samples were reduced with dithiothreitol (DTT), then free thiol groups were conjugated with monochlorobimane (mCB). For the measurement of reduced glutathione, DTT-treatment was skipped. The glutathione-mCB conjugate was quantitatively measured by HPLC coupled with a fluorimetric detector.

Microsomal glutathione permeability

Microsomal samples were incubated in a buffer containing cold and [^{3}H]-labeled GSH, then rapid filtered. Radioactivity of the filters was detected by a scintillation counter. Alamethicin-permeabilized microsomal samples were also produced in order to differentiate between intravesicular and membrane-bound radioactivity. Intravesicular (i. e. alamethicin-emptiable) radioactivity was obtained by subtracting membrane-bound from total radioactivity. Microsomal glutathione permeability was also measured with light-scattering, a technique based on the fluorimetric detection of osmosis-dependent changes in vesicle size.

Protein redox assay

Microsomal samples were treated with AMS, an alkylating agent reacting with cysteine thiols. Electrophoresis under non-reducing conditions was performed, followed by standard Western blot protocol for the detection of reduced and oxidized forms of microsomal proteins.

RT-PCR

Total RNA was isolated from cell lysates and tissue homogenates. mRNA was transcribed to cDNA and real-time RT-PCR performed.

Apoptosis assay

Apoptotic cells in liver slices were labeled with ApopTag Peroxidase Kit and detected with a light microscope. Apoptotic index was calculated.

RESULTS AND DISCUSSION

1. I observed that a single, sublethal dose of AAP depletes ER glutathione and significantly decreases reduced-to-total glutathione ratio in mouse liver. Glutathione plays a crucial role in ER redox status, thus its depletion threatens luminal redox homeostasis. Oxidative protein folding in the lumen is assisted by redox-sensitive chaperones containing cysteine thiols at the active sites. I was first to observe and publish an oxidative shift in the redox state of ERp72, a luminal chaperon, in an *in vivo* experiment. I observed, thus, an AAP-induced imbalance of ER redox homeostasis.
2. I studied whether the UPR is initiated as a means to recover ER homeostasis. I observed that a sublethal dose of AAP initiates UPR signaling, signs of which were activation of ATF6, phosphorylation of eIF2α and JNK1/2. I interpreted the parallel activation of these branches of the UPR as a clear indication of hepatocellular ER stress.
3. I observed upon the administration of a single, sublethal dose of AAP the induction of the pro-apoptotic transcription factor GADD153, activation of the ER-specific caspase-12 and an increase in apoptotic cell number in liver. I failed, however, to detect the induction of any of the major ER chaperones (GRP78, GRP94, ERp72, protein disulfide isomerase) which could shift the balance of the response from pro-apoptotic to pro-survival. I explain the dominance of pro-apoptotic signaling with the severity of AAP-induced acute stress.
4. I observed *in vivo* that inhibition of *de novo* glutathione synthesis by BSO fails to alter reduced-to-total glutathione ratio, or to induce GADD153, or to initiate apoptosis in hepatocytes. Thus, glutathione depletion *per se* can not make up for AAP in initiating ER redox stress and acute hepatocellular injury. My results are in good accordance with those of others, in that glutathione depletion only sensitizes cells to a further insult.
5. I adapted cell culture models for the study of hypoxia- and glutathione depletion-induced stress. I observed, using a mouse hepatoma cell line, that AAP activates an ER stress response. Contrary to *in vivo* results, however, I did not find

glutathione depletion *in vitro*, a phenomenon explicable with the lack of AAP metabolism in this cell line.

6. My results demonstrate that AAP is capable of inducing ER stress both *in vivo* and *in vitro*, and that some components of the ER stress response are independent of AAP metabolism. As a major result I showed ER stress-dependent signaling in the early phase after AAP-overdose, hence the temporal and functional antecedence of ER to mitochondrial events. From all the obtained results I conclude that AAP-induced ER stress plays a crucial role in the pathomechanism of hepatocellular injury.

LIST OF PUBLICATIONS

Publications directly connected to the thesis

1. Banhegyi G, Csala M, **Nagy G**, Sorrentino V, Fulceri R, Benedetti A (2003) Evidence for the transport of glutathione through ryanodine receptor channel type 1. Biochemical J, 376: 807-812. (IF: 4,101)

2. Kardon T, **Nagy G** *, Csala M, Kiss A, Schaff Z, Nagy PL, Wunderlich L, Banhegyi G, Mandl J. (2006) Influence of BGP-15, a nicotinic amidoxime derivative, on the vascularization and growth of murine hepatoma xenografts. Anticancer Research, 26: 1023-1028. (IF: 1,604)

3. **Nagy G**, Kardon T, Wunderlich L, Szarka A, Kiss A, Schaff Zs, Banhegyi G, Mandl J. (2007) Acetaminophen induces ER dependent signaling in mouse liver. Arch Biochem Biophys, 459: 273-279. (IF: 2,969)

* shared first-authorship with Kardon T.

Further publications

1. Braun L, Coffey M J, Puskas F, Kardon T, **Nagy G**, Conley AA, Burchell B, Mandl J (1998) The molecular basis of bilirubin UDP-glucuronosyltransferase induction in spontaneously diabetic rats, acetone-treated rats and starved rats. Biochem J, 336: 587-592. (IF: 3,855)

2. Csala M, Braun L, Coffey MJ, Puskás F, Kardon T, **Nagy G**, Conley AA, Burchell B, Mandl J. (2000) A bilirubin UDP-glukuroniltranszferáz indukciója diabéteszes, acetonnal kezelt és éhezõ patkányokban. Biokémia, 24: 7-12.

3. Margittai E, Banhegyi G, Kiss A, **Nagy G**, Mandl J, Schaff Z, Csala M. (2005) Scurvy leads to endoplasmic reticulum stress and apoptosis in the liver of guinea pigs. J Nutrit, 135: 2530-2534. (IF: 3,689)