Analysis of Changes in Pro (Gadd153) and Anti Apoptotic (Grp78) Gene Expression after Ischemic-reperfusion Injury of the Small Intestine

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Abstract: Analysis of changes after ischemia-reperfusion (IR) attack to the small intestine leads to multiple organ dysfunction (multiple organ dysfunction syndrome, MODS) and the subsequent death of patients is a topic for discussion. IR stress affects the endoplasmic reticulum (ER). ER dysfunction induces responses through kinases activation that stimulate anti-apoptotic mechanism, for example Grp78 (Bip) (Yeung et al., 2008) and pro-apoptotic mechanism, for example, activation Gadd153 (Chop) (Allyson et al., 2007). We analyzed the impact of IR damage of epithelium of the small intestine of rats after 1 h ischemia and subsequent 1 h, 24 h and 30 days of reperfusion on the level of apoptotic genes expression (Gadd153) and (Bip). In this study we used RT-PCR for detection of changes in gene expression. Significantly increased levels of mRNA for Gadd153 gene were detected after 1 h ischemia and 1 h reperfusion. The mRNA level of Grp78 gene was increased 24 h after ischemia comparing with the control groups. After 30 days of reperfusion Grp78 was at the level of control groups. Still, it is necessary to analyze the changes in the damaged tissue at the molecular level to define possible pathways leading to the tissue protection.

Introduction

Analysis of pathological changes after ischemia-reperfusion (IR) attack of the small intestine, which usually leads to multiorgan dysfunction syndrome (MODS) and death of patients, is a great challenge. It usually involves two or more organ systems (Bone et al., 1992). The stimulating moment of MODS is usually trauma, sepsis and other forms of shock. It is assumed the small intestine plays the key role in MODS. Various forms of shock like ischemia and reperfusion can contribute to redistribution of blood to vital organs and consequent hypoperfusion of the intestine. At the beginning, enterocytes are impaired by the reversible process, which could lead to the failure of compensatory mechanisms and initiation of MODS. It depends on the intensity and duration of the IR attack; the ischemic injury manifests the process of uncontrolled cells death (necrosis) or programmed cell death (apoptosis). Necrosis is characterized by cell oedema followed by rupture of the plasma membrane and release of the catalytic enzymes to the cell's environment. In opposite to this is the apoptosis, a programmed process which is strictly regulated. It is characterized by shrivelled cell and condensation of chromatin. In the normal small intestine, apoptotic cells are seen once in every 5th–10th crypt section, which implies that less than 1% of all crypt cells are apoptotic at any given time. However, this spontaneous apoptosis is localized at the stem cell positions, so that approximately 10% of stem cells are undergoing apoptosis at any given time (Merritt et al., 1995). The main aim of apoptosis is to remove cells, which inhibit the standard physiological functions of the body. Cystein caspases play the key role in the initiation of apoptosis.

Apoptosis can be triggered by intrinsic or extrinsic signals (Li et al., 1998). The example of the extrinsic pathway is activation through Fas ligand. Crosstalk exists between the extrinsic and intrinsic pathways. Bid, a pro-apoptotic member of Bcl-2 family, represents the way by which extrinsic signals can activate the intrinsic pathway. Caspase-8, activated by extrinsic signals, can cleave Bid at a specific site, generating a fragment containing a BH3 domain. This fragment can be translocated to mitochondria, causing cytochrome c release and subsequent activation of the intrinsic pathway (Li et al., 1998).

Another type of intrinsic pathway (alternative way) started with activation of defensive response of the ER (unfolded protein response – UPR and EOR) and subsequent activation of caspase-12. IR stress significantly affects the endoplasmic reticulum (ER), which reflected an increase in the levels of gene expression for folding proteins. ER dysfunction induced responses through activation of kinases that stimulate anti-apoptotic (Grp78 (Bip)) (Yeung et al., 2008), or pro-apoptotic mechanisms (Gadd53 (Chop)) (Allyson et al., 2007). Ischemia activates RNA-dependent $eIF2\alpha$ kinase (Perk) (Zu et al., 2006), which phosphorylates subunit $eIF2\alpha$, that activates transcription factor 4 (Atf-4). Protein Atf-4 induces Chop (Gadd153) and triggers apoptosis by increasing expression of caspases. Atf-4 and Chop activation is an important step which leads to cell apoptosis after IR injury.

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The results of Pritchard and Watson (1996), detected by TUNEL method, suggested that I/R of the small intestine caused significant cellular apoptosis after 1 hour of ischemia and at 1 and 6 hours after initiation of reperfusion. In consequence to that we decided to choose one hour reperfusion time (point) mainly for the ability to observe the first early signs of apoptosis at the mRNA level. The 24 hours long reperfusion was determined for consecutive measurements of histological and protein changes, which are in this later period more visible. After 30 days long reperfusion we assumed that the MODS will develop. For that we were analyzing the influence of one hour ischemia and 1 h, 24 h and 30 days reperfusion by detecting the gene expression changes of the pro (Gadd153) and anti-apoptotic (Grp78) genes by real-time PCR (RT-PCR).

For better understanding of the activation of apoptosis alternative pathway it is necessary to analyze the molecular and biochemical changes after ischemia and reperfusion, and determine the contribution of small intestine damage and recovery to the others vital organs and MODS spreading.

Material and Methods

Animal model of ischemia and experimental design

Adult male Wistar rats (mean body weight ± 320 g, total n=36) used for the experiments were housed in standard conditions with a temperature set at 22 ± 2 °C, and with dark-light cycles of 12 hours. Food and water were provided *ad libitum*. Ischemia of the small intestine was induced by the occlusion of *artheria mesenterica cranialis* for 60 minutes by small atraumatic clip under anaesthesia. Normothermic conditions (37 °C) were monitored by a microthermistor placed in the ear. Temperature was maintained using a homoeothermic blanket. Sham control animals (C, n=4 for each time interval) were prepared in the same way without the occlusion of *a. mesenterica*. The rats then underwent 60 minutes long ischemia followed by 1 h, 24 h and 30 days of reperfusion (R1, R24 and R30, respectively, each group n=8). Under anaesthesia 2 cm long parts of jejunum were isolated from each animal, washed in RNAse free water and weighed and stored at –80 °C.

RT-PCR

For the proof of changes in mRNA levels, we decided to use RT-PCR. We performed four analyses for each gene per animal. The total RNA was harvested by total RNA purification kit (Quiagen). First-strand cDNA synthesis was performed using superscript II (Invitrogen). PCR was made using primers (Table 1).

Table 1 – Sequences of primers used for PCR

Gene	Forward primer sequence	Reverse primer sequence
Gadd153	GCAGCGACAGAGCCAAAATAACA	GGGCTGCGCACTGACCACTCT
Grp78	CGTCCCGTGGCATCAACC	CAGCAAACTTCTCGGCGTCAT
Gapdh	TGGGGCCAAAAGCATCATCTC	GCCGCCTGCTTCACCACCTTCTT

The PCR was conducted for 30 cycles (94 °C for 5 min, cycles: 94 °C for 30 s and 58.4 °C for 30 s and 72 °C for 45 s) by PCR cycler TC-3000 (Barloworld scientific). Each sample was loaded at least four times. Individual fragments of cDNA were divided by electrophoresis on agarose gel. The change in expression under different intensity of each band of cDNA was detected by G-BOX detection system (Syngene). Numerical quantification of changes in expression levels was evaluated using DataSyngene. For normalization of the results housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (Gapdh) were used.

Statistics

One-way ANOVA with the paired Student's *t*-test were used to compare results between different times of reperfusion against controls (GraphPad InStat, GraphPad software). Data were presented as mean percent ± SEM.

Results

We analyzed the effects of IR injury of the small intestine epithelium of Wistar rats after 1 hour ischemia and subsequent reperfusion times in periods 1 h, 24 h and for 30 days. After isolation of RNA and reverse transcription into cDNA, we measured changes in gene expression by PCR. We studied expression of pro (Gadd153) and anti (Grp78) apoptotic genes.

All values from control animals for each gene in different times of reperfusion were almost the same with only very small SD. We found that after 1 h of ischemia and 1 h reperfusion levels of mRNA for gene Gadd153 were significantly increased (38.3% higher than control), whereas the level of Grp78 was significantly lower (32.6% lower than control) (Figure 1).
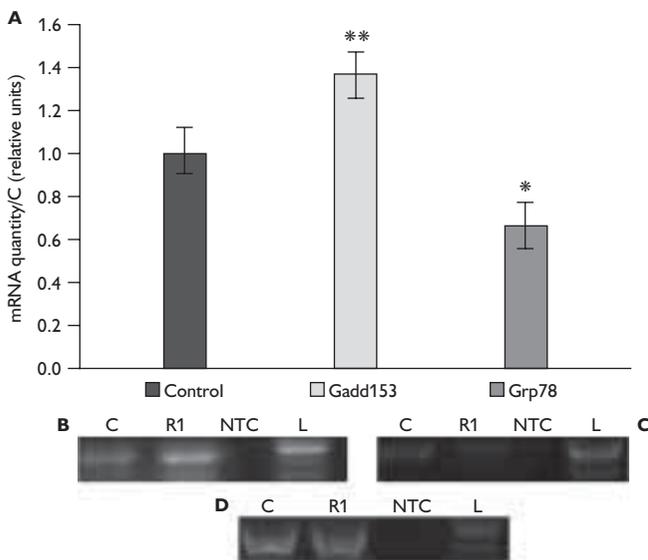

Figure 1 – mRNA levels of Gadd153 and Grp78 in rat small intestine after 1 h ischemia and 1 h reperfusion (R1). a) Semiquantification graph of gene expression. Results are presented as mean ± SEM, normalized to control levels (C). *$P<0.05$; **$P<0.01$ significantly different as compared to controls; b) detected bands for Gadd153; c) detected bands for Grp78; d) detected bands for Gapdh. For PCR reaction was used negative control (NTC) and band position determined against ladder (L).

Surprisingly the levels of Gadd153 after 24 hours of reperfusion were significantly decreased in comparison with controls (49% lower than control). In contrast to that, the mRNA level of Grp78 showed a huge increase compared to the controls (more than 250% higher than control) (Figure 2).

After 30 days of reperfusion there was partial restoration of the epithelium as demonstrated by the levels of Grp78 at the level of control. At this time point we also found an increased mRNA level of Gadd153 (41.6% higher than control) (Figure 3).
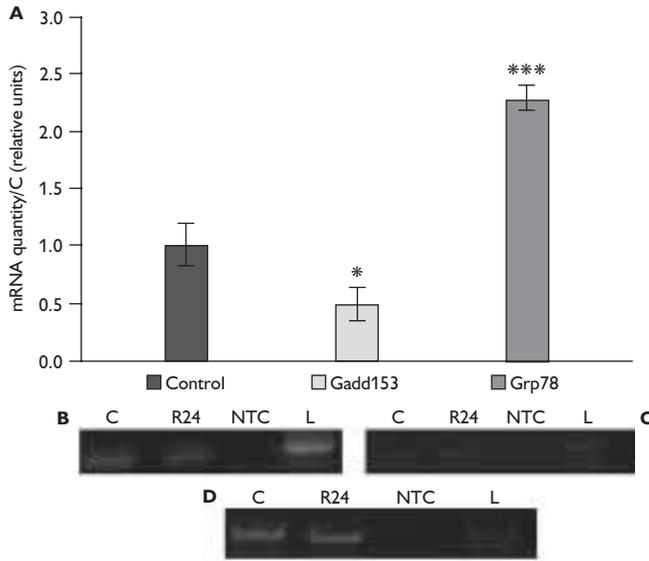

Figure 2 – mRNA levels of Gadd153 and Grp78 in rat small intestine after 1 h ischemia and 24 h reperfusion (R24). a) Semiquantification graph of gene expression. Results are presented as mean ± SEM, normalized to control levels (C). *P<0.05; ***P<0.001 significantly different as compared to controls; b) detected bands for Gadd153; c) detected bands for Grp78; d) detected bands for Gapdh. For PCR reaction was used negative control (NTC) and band position determined against ladder (L).
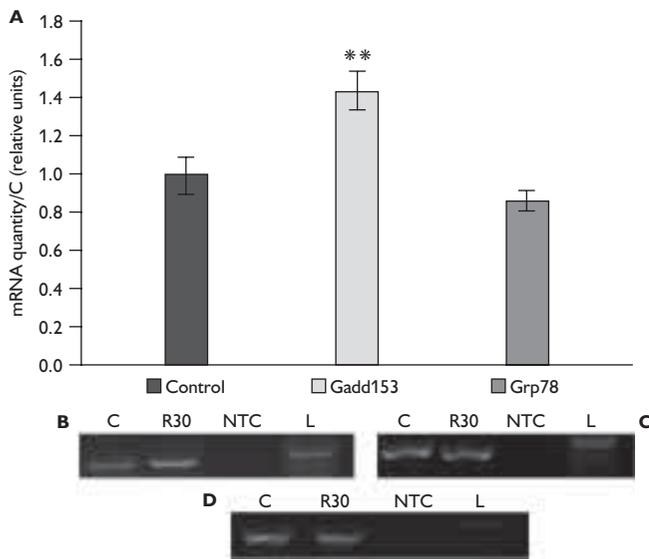
Figure 3 – mRNA levels of Gadd153 and Grp78 in rat small intestine after 1 h ischemia and 30 days of reperfusion (R30). a) Semiquantification graph of gene expression. Results are presented as mean ± SEM, normalized to control levels (C). **P<0.01 significantly different as compared to controls; b) detected bands for Gadd153; c) detected bands for Grp78; d) detected bands for Gapdh. For PCR reaction was used negative control (NTC) and band position determined against ladder (L).

Apoptotic Genes Expression after IR Injury of the Bowel

Discussion

Although endoplasmic reticulum (ER) stress responses contribute to the initiation and regulation of various cellular stress mechanisms including the induction of inflammatory processes and apoptosis, the contribution of ER stress with respect to the glucose regulated protein 78 (Grp78) is completely unknown under the pathologic conditions of experimental I/R injury (Shkoda et al., 2007). Such intestinal I/R injury will damage the mucosa, impairing its barrier function and leading to bacterial translocation (Kim et al., 2005). Although reperfusion is essential to prevent anoxic cell death after ischemia, it may be associated with additional and severe cellular damage (Duchmann et al., 1995). Similarly, I/R injury also induces apoptosis in the small intestine epithelium, with apoptosis considered the principal mode of cell death after this type of insult (Asseman et al., 1999).

Stress condition (such as glucose starvation, energy deficiency, chemical toxicity, acidosis and hypoxia, oxidative stress, ER Ca^{2+} depletion, and inhibitors of glycosylation are also increasing damage of the small intestine and the induction of the glucose regulated protein family members (Schroder and Kaufman, 2005). Grp78 was identified as a prototypic ER stress marker and master regulator of UPR (Zhang and Kaufman, 2006). The accumulation of misfolded proteins in the ER because of environmental and/or metabolic stress conditions triggers Grp78 liberation from transmembrane ER signalling proteins to initiate Grp78 mRNA resynthesis.

In correlation with apoptosis, the Grp78 protein in normal conditions prevents the release of Atf6 from the ER membrane, which acts as its own negative regulator. There is a reduction in post-ischemic protein aggregation and redistribution conjugates of ubiquitine.

There is no evidence about Grp78 mRNA levels and I/R injury yet. Apart from examine relations between Grp78 and apoptosis we were also studying its possible relationship to inflammatory processes leading to MODS. Very similarly to us, Skhoda et al. (2007) observed that after 1 hour stimulation the Grp78 expression levels seemed to remain constant in the ER compartment, and TNF (tumor necrosis factor) did not trigger significant Grp78 protein expression, suggesting that the cellular redistribution of Grp78 at early stimulation time points seemed to be independent from Grp78 protein resynthesis. Similar to that we found the maximal expression of Grp78 at 24 hours after ischemia (250% higher level than controls). These findings suggest the involvement of anti-apoptotic signalling in later phases and effort for reconstruction after unsuccessful reparation processes in the intestinal epithelium.

It is expected that the level of Grp78 mRNA induced with reaction to unfolded proteins leads to a reduction in activity Perk, which indirectly activates the expression of Gadd153. Nagotani et al. (2005) in their work suggest that higher expression of Grp78 delays expression of Gadd153. Level of gene transcription of Gadd153 may influence several transcription factors such as Atf4 and Atf6/Xbp1.

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The presence of Gadd153 mRNA is therefore one of the indicators for the degree of ER stress and for activation of UPR. According to that Merritt et al. (1995) found that apoptosis is significantly elevated in small intestine 3 hours after irradiation, as a form of ER stress. The results of Pritchard and Watson (1996), obtained using TUNEL method, also suggested that I/R of the small intestine caused significant cellular apoptosis after 1 hour of ischemia and at 1 and 6 hours after initiation of reperfusion. Our findings support mentioned facts. We determined increased levels of Gadd153 (38.3% higher than control) after one hour reperfusion, what could mean that there is a sharp increase pro-apoptotic signalling in the early stages of reperfusion. In comparing to other apoptotic pathways, total I/R of the small intestine suppressed the expression of p53 and Bcl-2 without any change to Bax (decreased Bcl-2 to Bax ratio) during the reperfusion stage (Hung et al., 2004). In addition to that, their results also indicated increased caspase-3 activity in the small intestine after I/R at 1 and 6 hours after ischemia. Activation of caspase-3 has been found to induce Bcl-2 protein cleavage, promoting the release of cytochrome c and leading to further cellular damage (Kirsch et al., 1999). Till now don't exist any results about the delayed cell death in correlation with later time intervals of reperfusion after ischemia of small intestine exist until now. We found that the levels of Gadd153 mRNA after 30 days long reperfusion were increased against controls (41.6% higher), what could indicates failure of compensatory mechanisms after ischemia and increased apoptosis, probably due to emerging MODS from other secondary injured organs. From the fact that the levels of Grp78 mRNA at this time point were similar to control levels, we assumed that the small intestine is after long reperfusion more directed to processes of apoptosis than inflammation and necrosis.

Conclusion

There is still no effective approach to the treatment of affected ischemic intestine tissue and it is therefore necessary to study changes in the damaged tissues at the molecular level and try to suggest possible therapeutic defined routes to the protection of tissue.

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