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Heat Shock Protein 70 Messenger RNA in Rat Leukocytes Elevates After Severe Intestinal Ischemia–Reperfusion

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ABSTRACT

Background: Heat shock protein 70 (HSP70) confers protection against heat shock, oxidative stress, infection, and inflammation in many cell types. A recent study reported that the induction of HSP70 was associated with morphologic protection against ischemia–reperfusion injury (IRI) in the rat small intestine. This study investigated the dynamics of HSP70 in leukocytes during intestinal IRI in a rat model.

Materials and methods: Serial blood samples were collected at 60-minute intervals up to 240 min from male Wistar rats ($n = 15$). The rats were divided into three groups of five each: the control group, the nonlethal IRI group, and the lethal IRI group. Rats belonging to the control group underwent a sham operation, and laparotomy was performed on rats in the lethal and nonlethal IRI groups. The nonlethal group experienced a 30-minute clamping of the superior mesenteric artery, and the lethal group experienced a 75-minute clamping of the superior mesenteric artery. The expression of HSP70 messenger RNA (mRNA) in leukocytes was measured by real-time quantitative polymerase chain reaction. Mixed-effects modeling of repeated measures was used to carry out the statistical analysis. The Bonferroni correction was applied to multiple comparisons. A P value < 0.0167 was considered to indicate statistical significance.

Results: The expression of HSP70 mRNA in leukocytes increased 60 min after reperfusion in both IRI groups, and it was 12.8 times higher in the lethal group and 3.6 times higher in the nonlethal group compared with the control group. The expression of mRNA in the lethal group was significantly increased compared with the nonlethal group and the control group at 120 and 180 min after reperfusion. At 120 min after reperfusion, the expression of HSP70 mRNA was 6.1 times higher in the lethal group than in the nonlethal group ($P = 0.0075$) and 17.7 times higher than in the control group ($P = 0.0011$). At 180 min after reperfusion, the expression of HSP70 mRNA was 6.8 times higher in the lethal group than in

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the nonlethal group ($P = 0.0007$) and 4.3 times higher than in the control group ($P = 0.0032$). Although the expression of HSP70 mRNA in the nonlethal group was elevated in the early stages of reperfusion, there was no difference between the nonlethal group and the control group ($P = 0.0212$ at 60 min).

Conclusions: The expression of HSP70 mRNA in leukocytes may be a clinically useful indicator for evaluating pathologic conditions in intestinal IRI.

Introduction

Ischemic bowel diseases (IBDs) result from inadequate flow of oxygenated blood to the intestines. The morbidity and mortality rates are high. Common IBDs are acute mesenteric ischemia, nonocclusive mesenteric ischemia, and strangulated intestinal obstruction. Intestinal mucous ischemia, an IBD condition, results in inflammation and ulcers due to insufficient supplies of oxygen and nutrients.[1] Ischemia–reperfusion injury (IRI) results in the activation of inflammatory cascades that bring about tissue damage.[2] Reversible damage is categorized as ischemia, and irreversible damage is categorized as necrosis. There is a consensus that the presence of intestinal necrosis requires surgery to remove any nonviable intestine.[3,4] IBD is IRI occurring in the intestines. Although the diagnosis of intestinal necrosis is usually based on clinical, radiologic, and serologic findings, these alone are not enough to evaluate the viability of the intestines.[5] Exploratory laparotomy is frequently required for accurate diagnosis; however, it is extremely invasive. Recent studies investigated specific biomarkers reflecting IBD and IRI: intestinal fatty acid–binding protein, smooth muscle actin, and glutathione S-transferase.[6-11] However, a practical biomarker for intestinal IRI has yet to be clinically established. Increased understanding of the pathologic and chronologic conditions after IRI may lead to the development of an effective indicator for the determination of reversible and irreversible intestinal ischemia.

Some authors have examined the effects of various periods of ischemia and reperfusion in rat intestinal IRI models. For example, they established a rat model with a 24-hour reperfusion time and 30 min of ischemia, and another model with a 60-minute reperfusion time and 120 min of ischemia.[12] However, no clear relationship was shown between intestinal ischemic time and length of survival. To examine this relationship, we created a lethal rat model that dies within 6 h and a nonlethal model that survives up to 24 h after reperfusion. This study may potentially help identify a new indicator for IRI.

Nonocclusive mesenteric ischemia and acute mesenteric ischemia are included in IRI. Ischemia creates a hypoxic condition in which adenosine triphosphate is converted to adenosine monophosphate, and the accumulated adenosine monophosphate is catabolized to hypoxanthine. Simultaneously, xanthine dehydrogenase is converted to xanthine oxidase, which catalyzes the formation of xanthine from hypoxanthine, with the reintroduction of molecular oxygen during reperfusion.[13,14] The overproduction of superoxide causes damage to the tissues and microcirculation.[15] Sometimes, intestinal IRI can lead to multiple organ failure. The cause of multiple organ failure is disturbance of the microcirculation with vascular endothelial cell damage following the activation of leukocytes and the production of many mediators.[16] Adhesion factors, such as intercellular adhesion molecule 1, are activated on the surface of vascular endothelial cells in the intestinal mucosa after reperfusion, and leukocytes are also activated in the mucosa.[2] Expression of the genes for C-fos, heat shock protein 70 (HSP70), interleukin-6 (IL-6), and IL-8 is induced by ischemia. L-selectin, matrix metallopeptidase-2 (MMP-2), MMP-9, nicotinamide adenine dinucleotide phosphate oxidase 1 (NOX1), myeloperoxidase (MPO), integrin α L (ITGAL), and ras-related C3 botulinum toxin substrate 1 (Rac1) are also expressed in neutrophils activated by IRI.[17,18] We used the models we established to investigate the expression of these genes. HSP70 has been well studied and has an important role in the self-protection of cells and inflammation and can also modulate cytokine protection in mononuclear cells. Cytokines activate human peripheral blood mononuclear cells, associated with the intracellular induction of HSP70 expression. It has been demonstrated that the neutrophils of critically ill patients express HSP70 spontaneously. In addition, HSP70 is expressed in the lungs and brain in the early stages of ischemic injury,[19,20] and the expression of HSP70 protein *in vivo* is also associated with mucosal protection in the rat model of small intestinal IRI.[21,22] However, there has been no report on the time course of HSP70 gene expression by neutrophils in the systemic circulation after reperfusion of intestinal ischemia. There are a few reports describing the kinetics of expression of the gene when activated in neutrophils to evaluate the severity of IRI.

The aim of this study was to investigate the expression of HSP70 messenger RNA (mRNA) during IRI using our rat models.

Materials and methods

Animals

The animal experiments were performed at the University of Nagasaki after approval by the Institutional Animal Care and Use Committee of the university (approval number 1505201228-3). All the animals were treated according to the Guidelines for Animal Experiments of Nagasaki University.

Male Wistar rats weighing between 350 and 400 g were kept under standard conditions at 22.8°C and 50% humidity on a 12-hour/12-hour light–dark cycle, with access to food and water ad libitum.

Intestinal IRI rat model

The rats were divided into three groups of five each: the control group, the nonlethal IRI group, and the lethal IRI group. The rats were anesthetized with 1%-3% isoflurane mixed with oxygen. Laparotomy was performed on the rats with a midline incision. A microvascular clip was placed across the root of the exposed superior mesenteric artery (SMA), with the accompanying vein remaining intact. The removal of the clamp after each defined duration of clamping allowed reperfusion in the lethal and nonlethal IR groups ($n = 10$). Time-matched, sham-operated rats served as controls ($n = 5$). The controls underwent laparotomy without clamping.

Configuration of ischemic time

We set the SMA clamping time at 30-120 min and measured the duration of survival after reperfusion. The clamping time was 30 min for 10 rats, 60 min for seven rats, and more than 75 min for five rats.

Collection of blood and intestinal tissue samples

We collected 600 μL blood samples from the tail vein. We collected blood from the controls before laparotomy, at the start of reperfusion ($t = 0$ min), and 60, 120, 180, and 240 min after reperfusion. In the control group, we began collecting blood samples after closing the abdomen.

The blood samples were centrifuged at 5000 rpm for 10 min immediately after collection, and 100 μL of buffy coat was collected. Total RNA was extracted from the buffy coat of each blood sample using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA samples were stored at −30°C until use.

Tissue samples from the small and large intestines were collected at the time of death in the lethal group and were also collected from the rats in the control and nonlethal groups, which were anesthetized 24 h after reperfusion. The tissue samples from the small intestine were collected approximately 5 cm distal from the pyloric ring of the stomach and approximately 3 cm proximal from the cecum. The tissue samples from the large intestine were collected approximately 1 cm distal from the cecum.

Histopathologic assessment

The tissue samples from the small and large intestines were promptly rinsed in cold saline solution and were immediately fixed in 10% buffered formalin phosphate. Then, they were embedded in paraffin, were sectioned transversely, and were stained with hematoxylin and eosin.

Reverse transcription and quantitative real-time polymerase chain reaction

Total RNA (1500 ng) was reverse-transcribed using the PrimeScript RT Reagent Kit (Takara Bio Inc, Otsu, Japan) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction was performed in a 10-μL reaction mix using SYBR Premix Ex Taq II (Takara Bio Inc) with the Thermal Cycler Dice Real Time System (Takara Bio). The contents of the amplification mix and thermal cycling conditions were set according to the manufacturer's instructions. The primers for C-fos, HSP70, tumor necrosis factor α (TNF-α), IL-6, IL-8, L-selectin, MMP-2, MMP-9, NOX1, MPO, ITGAL, and Rac1 were purchased from Takara Bio. The second-derivative maximum method was used to perform the relative quantification of mRNA transcripts. The expression levels of the target transcripts were described as the ratios of the targets normalized to the endogenous reference (glyceraldehyde 3-phosphate dehydrogenase).

Statistical analysis

JMP Pro 11 (SAS Institute Inc, Cary, NC) was used to analyze the data. Mixed-effects modeling for repeated measures, Kaplan–Meier, and log-rank analyses were used for the statistical analyses. Bonferroni adjustment for multiple comparisons was applied. $P < 0.0167$ was considered to indicate a significant difference, since the number of groups was three, and the significance level was 5%.

Results

Configuration of ischemic times

We investigated the relationship between intestinal ischemic time and duration of survival (Fig. 1). All the 10 rats that underwent the 30-minute ischemia treatment survived 24 h after reperfusion. Four of the seven rats (57%) that underwent the 60-minute ischemia treatment survived for more than 24 h. The remaining rats, which had received more than 75 min of ischemia treatment (i.e., 75, 90, and 120 min), all died within 24 h after reperfusion. They showed similar macroscopic findings such as necrosis and intestinal enhancement. We decided to categorize these rats as >75 min group (see

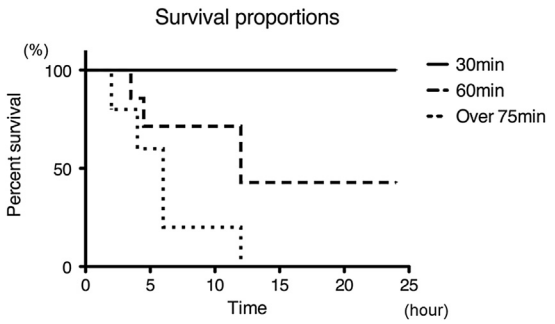

Fig. 1 – Relationship between intestinal ischemic time and survival rate after reperfusion. The rats that underwent the 30-minute ischemia treatment survived for 24 h after reperfusion. Four out of the seven rats that underwent the 60-minute ischemia treatment survived 24 h after reperfusion, and all the rats that underwent more than 75 min of ischemia treatment died within 6 h after reperfusion.

Appendix Table A). The survival curves showed a significant difference among these three groups and, therefore, we assigned the rats that underwent 30 min of ischemia to the nonlethal group and the rats that underwent more than 75 min of ischemia to the lethal group.

Animals for sample collection

All the rats in the sham ($n = 5$) and nonlethal ($n = 5$) groups survived for 24 h after reperfusion. All the rats in the lethal group ($n = 5$) died within 6 h after reperfusion.

Histologic findings of small and large intestine

Tissue damage of the small intestine after reperfusion was evaluated with hematoxylin and eosin staining. There were obvious changes in the oral and anal sides of the small intestine in the lethal group (Fig. 2A and B). The villi of the small intestine were shrunken and exfoliated, and the lamina propria were exposed.

In the nonlethal group, an expansion of the subepithelial space, with the epithelial layer lifted up in sheets from the lamina propria, and local enucleation in the oral side of the small intestine were observed. In contrast, the anal side of the mucosa of the small intestine did not show much change. In the control group, both the oral and the anal sides of the small intestine showed a normal mucosa. In all the groups, a normal mucosa was observed in the large intestine.

mRNA expression

Expression of HSP70 mRNA in neutrophils increased immediately and continued to increase until 180 min after reperfusion, then decreased. There were no significant differences in the expression of HSP70 mRNA in the sham, nonlethal, and

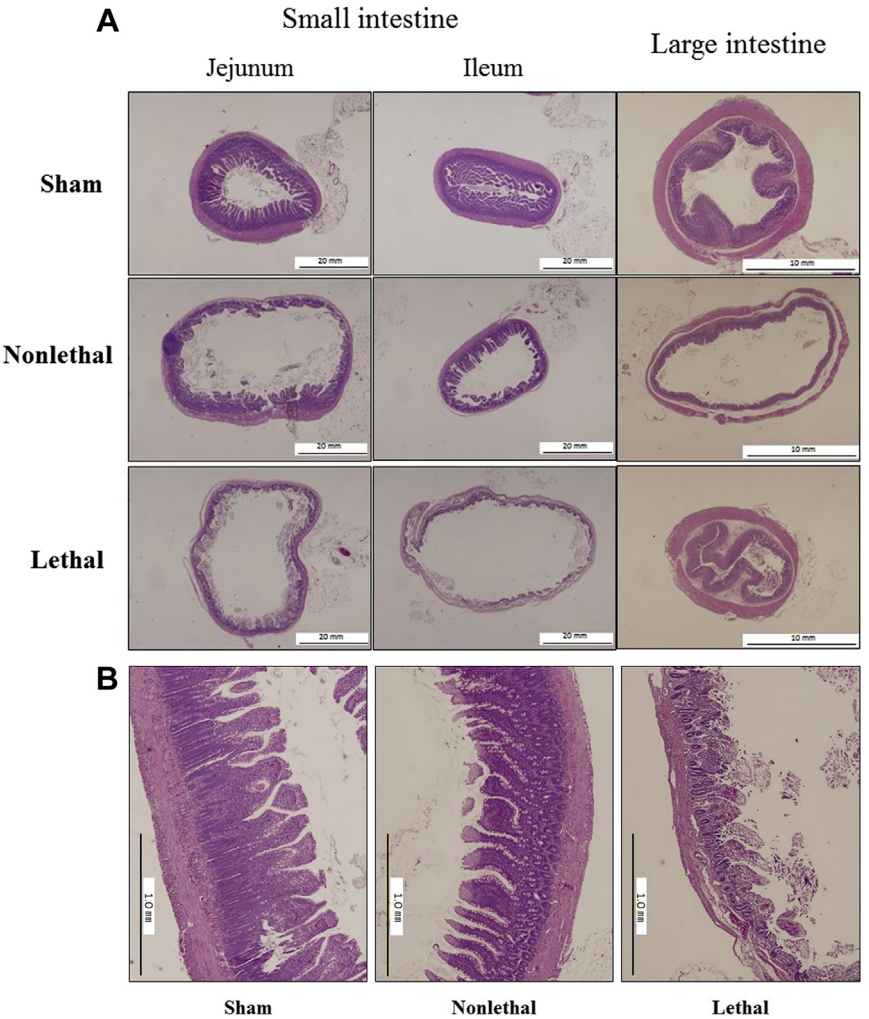

Fig. 2 – (A) Low magnification of small and large intestinal specimens stained with hematoxylin and eosin. The mucosa of the small intestine is thinner in the lethal group than in the nonlethal and control groups. The thickness of the mucosa in the large intestine does not differ much among the three groups. (B) High magnification of the jejunum after ischemia–reperfusion treatment. In the lethal group, the villi of the small intestine were widely shrunken and exfoliated, and the lamina propria was observed. An elevated epithelial layer and local enucleation were observed in the nonlethal group. (Color version of figure is available online.)

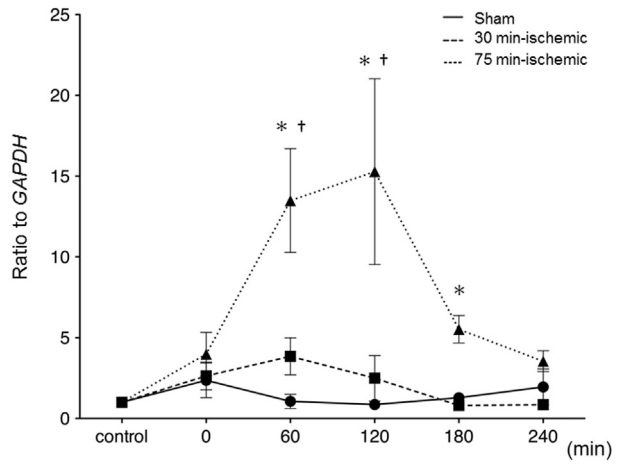

Fig. 3 – Expression of HSP70 mRNA in neutrophils. Expression of mRNA in the lethal group significantly increased between 60 and 180 min after reperfusion relative to the sham group. *P < 0.0167 (lethal group *versus* sham group). Compared with the nonlethal group, expression of mRNA in the lethal group significantly increased between 120 and 180 min after reperfusion. †P < 0.0167 (lethal group *versus* nonlethal group).

lethal groups before laparotomy (control) and immediately after reperfusion ($t = 0$ min; Fig. 3).

In the lethal group, the expression of HSP70 mRNA was significantly higher between 60 and 180 min after reperfusion compared with the sham group (at 60 min after reperfusion, lethal group *versus* control group, 12.8 times higher, $P = 0.0004$; at 120 min, 17.7 times higher, $P = 0.0011$; at 180 min, 4.3 times higher, $P = 0.0032$). The expression of HSP70 mRNA between 120 and 180 min after reperfusion was significantly higher in the lethal group than in the nonlethal group (at 120 min, lethal group *versus* nonlethal group, 6.1 times higher, $P = 0.0075$; at 180 min, 6.8 times higher, $P = 0.0007$; Fig. 3). The expression of HSP70 mRNA was higher in the nonlethal group than in the sham group in the early stages after reperfusion; however, the difference was not statistically significant for any time point. Although the expression of mRNAs for c-fos, TNF-α, IL-6, IL-8, L-selectin, MMP- 2, MMP-9, NOX 1, MPO, ITGAL, and Rac 1 was measured, expression of mRNA for IL-6, IL-8, c-fos, NOX-1, MMP-2, and MPO was not detected. TNFα, L-selectin, Integrin, Rac-1, and MMP-9 showed gene expressions but did not show significant differences among the three groups. We observed similar gene expressions of MMP-9 in both lethal and nonlethal groups (see Appendix Figure A).

Discussion

When intestinal ischemic injury occurs, physicians need to diagnose immediately and accurately whether it is reversible or irreversible so that they can decide what steps to take during operation. Some researchers have studied the relationship between ischemic time and duration of survival after reperfusion in rats; however, no one has generated a lethal rat model (in which the injury is irreversible, and the affected area needs to be removed) and a nonlethal rat model. In our study, we generated new lethal and nonlethal rat models of intestinal IRI to understand the pathologic and chronologic conditions after IRI. In addition, we investigated a possible practical biomarker for intestinal IRI. Rats that underwent only 30 min of ischemia treatment survived. The survival time of rats that underwent between 60 and 75 min of ischemia varied, and four of seven rats survived. The rats that underwent more than 75 min of ischemia died within 6 h after reperfusion. The survival curves showed significant differences among the three groups and, therefore, we assigned the rats that underwent 30 min of ischemia to the nonlethal group and those that underwent more than 75 min of ischemia to the lethal group.

Kanda *et al.* reported that a mucosal change was observed 60 min after ischemia by SMA ligation in their rat model.[22] The change in the deep layer occurred as time proceeded, and full-thickness necrosis of the small intestine was confirmed in 8 h. IRI caused severe histologic changes, ranging from villous denudation to focal necrosis, ulceration, hemorrhage, and architectural disintegration at 240 min after reperfusion following 60 min of ischemia.

In another report, strong histologic changes, hemorrhage, and mucosal vas congestion were observed in the intestinal tissue 120 min after reperfusion in a 40-minute ischemic model.

In our study, mild histologic injury was observed on the oral side of the small intestinal tissue 24 h after reperfusion in the nonlethal model. The anal side exhibited minimal change compared with the oral side, and the findings were essentially normal. This result strongly indicated that reversible disorder may have occurred. In contrast, the villi of the small intestine were widely shrunken and exfoliated, and the lamina propria was observed on both the oral and anal sides of the small intestine in the lethal group. Our histopathologic findings strongly support our rat model as being appropriate for IRI because these findings were consistent with those in the reports mentioned previously.

C-fos is one of the immediate early genes because its induction is transiently observed early in response to cell stimulation.[23] TNF-α, IL-6, and IL-8 are inflammatory cytokines whose expression is induced early on stimulation.[24] In addition, L-selectin, MMP-2, MMP-9, NOX 1, MPO, ITGAL, and Rac 1 are involved in the expression and migration of neutrophils. In this study, we examined the expression of these genes because we assumed that their mRNA expression might be induced by activated neutrophils during ischemia. Surprisingly, there was no significance about the expression of mRNAs mentioned previously among control group, lethal group, and nonlethal group at any time points which we observed. We suggest that expression of mRNAs for the cytokine group might be increased by stimulation from laparotomy rather than by IRI.

The expression of HSP70 mRNA significantly increased in the lethal group compared with the control group at 60, 120, and 180 min after reperfusion. HSP70 is hardly expressed in the plasma of unstressed, healthy individuals; however, its protein is produced under various stress conditions. Overexpression of HSP70 is protective against heat shock,

oxidative stress, infections, and toxic molecules in many cell types.[21,25,26] Stojadinovic *et al.* reported that HSP70 induced under hyperthermia provided mucosal protection in a rat small intestinal IRI model.[21] We showed that HSP70 mRNA in leukocytes was induced in the early stages of small intestinal IRI. The HSP family are among the danger-associated molecular pattern molecules (DAMPs). The DAMP theory suggests that the innate and the adaptive immune systems are activated by endogenous signals that originate from stressed, injured, or necrotic cells, signifying "danger" to the host.[27] DAMPs bind to pattern recognition receptors and toll-like receptors of immune cells and promote the release of inflammatory cytokines such as IL-6 and IL-8. Because the HSP family is included in the DAMPs, the elevation of DAMPs might be induced by a similar mechanism.[28,29]

Ren *et al.*[30] suggested that the serum levels of HSP70 and other DAMP proteins could be used to predict the severity of traumatic injury. They indicated that HSP70 could serve as a marker of the degree of injury suffered by trauma patients and for prediction of secondary infection. Nowak *et al.*[31] suggested that the degree of expression of HSP70 was related to the severity of reperfusion injury, as HSPs might be established markers of the stress response following IRI. Although the authors did not mention intestinal IRI, HSP70 and DAMPs may also be related to the severity of IRI.

In this study, we showed that the expression of HSP70 mRNA was significantly higher in the lethal group than in the nonlethal group between 120 and 180 min after reperfusion. Hence, examining HSP70 expression at 120- and 180-min reperfusion times in rats will allow us to determine life or death of rats. For further investigation, we will examine HSP70 expression at 120- and 180-min reperfusion times with different ischemic times including 60 min and investigate any correlation between HSP70 expression and rat's life or death. We postulate that the expression level of HSP70 mRNA may reflect the severity of IRI. In addition, the expression levels of HSP70 and DAMPs could be markers for the assessment of whether intestinal IRI is lethal or nonlethal.

In nonlethal IRI, we can perform minimally invasive treatments such as anticoagulation therapy and interventional radiology. In lethal IRI, this reversibility is lost, and necrosis and death will occur. In such cases, it is necessary to resect the necrotic intestinal tract immediately. We suggest that expression of HSP70 mRNA, which reflects the severity of IRI, may be a useful indicator for the decision of what treatment to apply in a patient with intestinal ischemia.

This study has some limitations. The time interval that can be applied for determining the severity of IRI is limited because increased expression of HSP70 mRNA occurs for only a short period of time, even in lethal IRI. In addition, HSP70 expression increases in response to heat shock, infection, toxic molecules, and so on, which making it more difficult to decide whether or not the expression of HSP70 is due to IRI. However, there has been no report on the expression of HSP70 mRNA using rat small intestinal IRI models, and our study has suggestive results.

Surgeons who respond in emergency situations need some markers to evaluate and estimate the severity of IRI as soon as possible. In ordinary protein synthesis, DNA genetic information is transcribed into mRNA, and proteins are synthesized by joining amino acids by translation, a process that takes several minutes to hours. mRNA responds to stimulation faster than proteins. Therefore, for a faster diagnosis of the severity of IRI in a patient, the measurement of the expression of HSP70 mRNA could be more useful than the measurement of the expression of its protein.

Conclusions

HSP70 mRNA in leukocytes can be a clinically useful indicator for the evaluation of pathologic condition of intestinal IRI. The determination of the severity of intestinal ischemia by evaluating the expression of HSP70 mRNA as an indicator of IRI may lead to the establishment of a therapeutic policy for the early stages of ischemia and may contribute to improving the prognosis of IRI.

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Disclosure

The authors declare that they have no conflict of interests regarding the publication of this article.

Supplementary data

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