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RESEARCH ARTICLE

Evaluation of Immunohistochemical Expression of GSTA1 and GSTP1 Isoenzymes before and after Treatment of Trx and L-NAME in Experimental Hepatic Ischemia/Reperfusion Model

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Summary

Ischemia/reperfusion (I/R) causes formation of Reactive Oxygen Species (ROS) in tissues, in response to which injured cells improve a number of defense mechanisms including Glutathione S-Transferases (GSTs). The aim of this study was to investigate the expressions of GSTA1 and GSTP1 following Thioredoxin (Trx) and N-nitro-L-arginine methyl ester (L-NAME) treatment in a rat model of hepatic I/R model. A total of 50 Wistar rats were randomly allocated into 5 groups: sham (n = 10), control (I/R) (n = 10), Trx (n = 10), L-NAME (n = 10), and Trx+L-NAME (n = 10). With an exception to those in sham group, all rats were subjected to a hepatic ischemia process for an hour and then subsequent reperfusion. GSTA1 and GSTP1 expressions in the liver tissues were determined by immunohistochemical method. The GSTA1 expression was absent in sham group while varying degrees of expression occurred in other groups. The GSTA1 expression was significantly higher in Trx/L-NAME group compared to other groups ($p < 0.05$). GSTP1 expression was no difference between groups ($p > 0.05$). As a result, we think that GSTA1 expression may have increased in response to I/R as a part of the liver oxygen radical scavenging process.

Key Words: *Hepatic Ischemia/Reperfusion, GSTA1 and GSTP1.*

Deneysel Karaciğer İskemi/Reperfüzyon Modelinde Trx ve L-NAME Tedavi Öncesi ve Sonrası GSTA1 ve GSTP1 İzozimlerinin İmmunohistokimyasal İfadelerinin Değerlendirilmesi

Özet

İskemi/Reperfüzyon (I/R) dokularda Reaktif Oksijen Türleri (ROS) oluşumuna neden olur ve buna cevaben hasarlı hücreler, Glutatyon S-Transferaz'ların (GST) da dahil olduğu bir dizi savunma mekanizması geliştirir. Bu çalışmanın amacı, karaciğer I/R modeli oluşturulmuş ratlarda, Thioredoxin (Trx) and N-nitro-L-arginine methyl ester (L-NAME) tedavisi sonrası GSTA1 ve GSTP1 ifadelerinin incelenmesidir. Toplamda 50 Wistar rat rastgele, sham (n = 10), kontrol (I/R) (n = 10), Trx (n = 10), L-NAME (n = 10) ve Trx/L-NAME (n = 10) şeklinde 5 gruba ayrıldı. Sham grubu dışındaki bütün ratlar bir saatliğine karaciğer iskemi işlemine tabi tutuldu ve daha sonra reperfüzyon edildi. Karaciğer dokularında, GSTA1 ve GSTP1 ifadeleri immunohistokimyasal yöntemle belirlendi. Diğer gruplarda, değişen derecelerde GSTA1 ifadesi görülürken, sham grubunda GSTA1 ifadesi yoktu. GSTA1 ifadesi diğer gruplarla karşılaştırıldığında Trx+L-NAME grubunda istatistiksel olarak daha yüksekti ($p < 0,05$). GSTP1 ifadesi gruplar arasında farklılık göstermemiştir ($p > 0,05$). Sonuç olarak, karaciğer oksijen radikal temizleyicinin bir parçası olarak, I/R cevaben GSTA1 ifadesinin artmış olabileceğini düşünmekteyiz.

Anahtar Kelimeler: *Karaciğer İskemi/Reperfüzyon, GSTA1 ve GSTP1.*

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INTRODUCTION

Progressive production of membrane bound NADPH oxidase-mediated reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) as well as reactive nitrogen species (RNS) such as nitric oxide (NO) resulting from activity of nitric oxide synthetase (NOS) causes oxidative stress and subsequently tissue injury (1,2). Cell and tissue injuries resulting from oxidative stress and generation of oxygen radicals have also been implicated in the liver damage (4-7). The cell and total organ responses of the liver to ischemia and reperfusion (I/R) have been a subject to numerous scientific studies, many of which suggested that availability of antioxidative enzymes is important in counteracting and/or alleviating the oxidative burden (5-13). As a part of oxidative mechanism in cells, subcellular compartments have mechanisms to generate local ROS (9-16) and the oxidative modification of critical cell components including membrane lipids, proteins and nucleic acids impairs important cellular functions (17,18). In the absence of a proper antioxidant scavenging mechanism, ROS cause cellular dysregulation, permanent cell injury, and even cell death (18).

In response to oxidative stress, most tissues elaborate various antioxidative defense mechanisms including enzymatic components (superoxide dismutases, glutathione peroxidase, glutathione reductase, glutathione *S*-transferases, quinone reductase and catalase) and nonenzymatic components (e.g. glutathione, b carotene, a-tocopherol, ascorbic acid, and urate among others) (9, 19-22). Having such anti-oxidative propertites, subcellular organelles have been shown to have an intrinsic and possibly specific antioxidant mechanism capable of combating oxidative stress (14,15).

Among the important enzymatic anti-oxidants are cytosolic glutathione *S*-transferases (GSTs), an important family of detoxication enzymes present in the cytosol of most cells. Isoenzymes of the cytosolic GSTs are classified by amino acid and gene sequences, substrate specificities, and affinity for nonsubstrate ligands as alpha (GSTA), pi (GSTP), theta (GSTT), and mu (GSTM) (16, 23). Different cell types may have different GST isozymes in various amounts and combinations. The GSTs are believed to play a crucial role in cellular metabolism and detoxification electrophilic compounds by conjugation with glutathione. The GSTs play a crucial role in protecting cells against injury through toxic electrophiles and especially carcinogens (16). The GSTs are implicated to play roles in the liver including antioxidant defense, leukotriene biosynthesis, intracellular transport, drug metabolism, cell survival, and drug multiple resistance (15). Recent studies indicated that GSTA and GSTP isoenzyme activities increase in response to oxidative stress caused by superoxide radicals resulting from lipid peroxidation (24-26, 29).

Among the cellular defense mechanism is elaboration of anti-oxidative molecules such as thioredoxin (Trx). Thioredoxin is a cellular protein implicated in the cellular defense mechanism through prevention of apoptosis and inhibition of excessive ROS formation. N-nitro-L-arginine methyl ester (L-NAME) inhibits nitric oxide synthase (NOS). Subsequently, it inhibits production of nitric oxide (NO) and indirectly suppresses generation of peroxynitrite and hydroxyl radicals (24).The liver injury resulting from I/R is associated with oxidative stress in most parts. However, information is limited describing the *in vivo* subcellular organelle antioxidant enzyme response of the liver to I/R (14,15). Thus, the present study aimed to investigate expression of GSTA1 and GSTP1 isozymes following I/R in the rat liver and to evaluate effects of anti-oxidative drugs Trx and L-NAME on their expressions.

MATERIALS AND METHODS

Animals Model

Archival tissues of the study by Akin and coworkers (2010) (27) were used with an approval of the Animal Research Committee at Gazi University, Turkey. A total of 50 rats weighing 235-275 g were used in the study. The animals were kept under cycles of 12 h of light and 12 h of dark in individual cages, and they were allowed free access to standard rat chow and water. The rats were randomly allocated into five groups: a sham (n = 10), positive control (n = 10), Trx (n = 10), L-NAME (n = 10), Trx and L-NAME (n = 10) groups. Except in sham group, all rats were subjected

to ischemia and reperfusion. In sham group, rats were subjected only to laparotomy to under general anesthesia. In positive control, immediately after reperfusion 1 ml of vehicle (phosphate-buffered saline solution) was infused into the portal vein for 10 minutes immediately upon perfusion. In Trx group, immediately after reperfusion recombinant Trx (10mg/kg) (Promega Corporation, WI, USA) was infused for 10 min via the portal vein. In L-NAME group, immediately after reperfusion L-NAME (10mg/kg) (Cayman Chemical, USA) was infused for 10 min via the portal vein. In Trx and L-NAME group, immediately after reperfusion, L-NAME (10mg/kg) and recombinant Trx (10mg/kg) was infused for 10 min via the portal vein (27).

Preparation of the Drugs

One hundred microgram L-NAME was dissolved in 40 ml phosphate–buffered saline solution. For Trx, 50 mmol/L Tris-HCl (pH = 7,5) was dissolved in 40 ml solution including 1 mmol/L EDTA (27).

Ischemia and Reperfusion

Prior to surgery, rats had fasted for 12 h before surgery. Rats were anaesthetized with intra-peritoneal ketamine (100 mg/kg body weight [BW]) and xylazine (20 mg/kg) and prepared for aseptic surgery. A midline incision extending from the xiphisternum to the pubis was made. A polyethylene catheter (PE-50, ID 0.28, OD 0.61; Portex, Hyte, UK) was inserted from the ileocecal vein to the portal vein to infuse the drugs. For ischemia, the liver was exposed with retractors placed in the flank, and a clamp was attached to the xiphisternum and elevated. The ligamentous attachments between the liver and the diaphragm were freed. In order to avoid splanchnic congestion, we used a model of partial liver ischemia. Partial liver ischemia was induced by selective clamping of the portal vein and hepatic artery, which supply the left lateral and median lobes of the liver (segments II–IV), using an atraumatic vascular clamp (Harvard Apparatus Inc., Hollinston, MA, USA) for 60 min; followed by 2 hours of reperfusion 10 minutes of which were performed with the studied solutions. To avoid the influences arising from major fluid loss or drying of the liver, the abdominal cavity was covered with wetted gauze (27).

Immunohistochemical Staining

Tissues were fixed in 10% buffered formalin and embedded in paraffin blocks. Sections that were 4µm thick were cut, and one section was stained with hematoxylin-eosin to observe the tissue morphology. For immunohistochemistry (23, 28), endogenous peroxidase activity was blocked by incubating the sections in 1% hydrogen peroxide (v/v) in methanol for 10 minutes at room temperature (RT). The sections were subsequently washed in distilled water for 5 minutes and antigen retrieval was performed for 3 minutes using 0.01M citrate buffer (pH 6.0) in a domestic pressure cooker. The sections were transferred in 0.05M Tris-HCl (pH 7.6) containing 0.15M sodium chloride (TBS). After washing in water, the sections were incubated at RT for 30 minutes with either normal swine serum (for anti-GSTA1 and GSTP1) (1:20) diluted in TBS to block nonspecific binding. The sections were then covered with the primary antibodies diluted 1:100 for anti-GSTA1 and 1:100 for anti-GSTP1 in TBS at 4°C overnight (Polyclonal antibodies against GSTP1 and GSTA1 raised in rabbit were purchased from Lab Vision Thermo Scientific, USA). After washing in TBS (15 minutes) sections were incubated at RT for one hour with secondary antibody (swine-anti-rabbit Ig-biotinylated) at a dilution of 1:100. Then treatment followed with avidin-biotin peroxidase complex (Dakopatts, Denmark). Diaminobenzidine was used to visualize peroxidase activity in the tissues. Nuclei were lightly counterstained with hematoxylin and then the sections were dehydrated and mounted. Negative controls were included in each run. TBS was used in place of the primary antibody for negative controls.

Light microscopy of immunohistochemically stained sections was performed by a pathologist and a biologist who were blinded to the treatment groups. Distribution, localization and characteristics of immunostaining were recorded. Brown color in cytoplasm of the epithelial cells was evaluated as positive staining. Scoring differences between observers were resolved by consensus. For each antibody, two features were determined using a semi-quantitative scale in order to describe the immunoreaction: the intensity and the number of positive staining cells of the reaction (negative (-), weak (1+), moderate (2+), and strong (3+).

Statistical Analyses

Statistical analyses were performed with SPSS software (Statistical Package for the Social Sciences, version 15.0, SSPS Inc, Chicago, IL, USA). The differences between the expression of GSTA1 and GSTP1 among groups were analyzed by the Post Hoc Tests. A *p*-value of less than 0.05 was considered as statistically significant.

RESULTS

According to the immunohistochemical staining; there was no GSTA1 isoenzyme expression in Sham group. In control and L-NAME treatment groups two cases showed negative staining (20%) and eight cases (80%) showed weak GSTA1 expression. In Trx treatment group, four cases (40%) showed negative staining, but six cases (60%) showed weak GSTA1 expression. All tissues of Trx+L-NAME groups showed GSTA1 expression, six cases (60%) showed weak expression, four cases (40%) showed strong expression (Table 1). GSTA1 expression in control, L-NAME and Trx groups was similar and significantly higher compared to sham group (*p = 0,035; 0,023; 0,000 <0,05*) (Table 2). Although GSTA1 expression in Trx treatment group was higher than sham group, this difference was not statistically significant (*p* >0,05) (Table 2). Importantly, GSTA1 expression was stronger in Trx+L-NAME treatment group compared to sham, control, Trx and L-NAME groups (*p = 0,000; 0,003; 0,000; 0,003 <0,05*) (Figure 1A.) (Table 2). There was weak GSTP1 isozyme expression in all cases in all treatment groups (Figure 1C.) (Table 1), and there were no statistically significant differences in GSTP1 expression between treatment groups (*p >0,05*).

Discussion

In this study, we investigated expression profiles of detoxification enzymes, GST in injured liver tissues resulting from I/R models in Rats. In addition, we investigated how the expression profiles changes following administration of Trx, and L- NAME drugs, which are commonly used in liver injuries.

The mechanisms of physiopathology of I/R injury is not clearly understood. Everyday new molecules and genes along with various factors are determined that even makes even more confusion to determine the related mechanisms. By enlarge, the I/R injury results from a complex interaction among ROS complement system, hemoxygenase system, endothelial cells and neutrophils. Reactive oxygen species (ROS) are the most important elements to induce cells and tissue injury in I/R. During the I/R process, kupffer cell, polymorphonuclear leukocytes, endothelial

Table 1. According to intensity and prevalence of immunohistochemical staining of GSTA1 and GSTP1 isoenzymes expression in Rat Groups.

Groups	GSTA1				GSTP1			
	0/n% *	+1/n%	+2/n%	+3/n%	0/n%	+1/n%	+2/n%	+3/n%
sham (n = 10)	10/100	0/0	0/0	0/0	0/0	10/100	0/0	0/0
control I/R[a] (n = 10)	2/20	8/80	0/0	0/0	0/0	10/100	0/0	0/0
Trx[b] (n = 10)	4/40	6/60	0/0	0/0	0/0	10/100	0/0	0/0
L-NAME[c] (n = 10)	2/20	8/80	0/0	0/0	0/0	10/100	0/0	0/0
Trx+L-Name[d] (n = 10)	0/0	6/60	0/0	4/40	0/0	10/100	0/0	0/0

Staining scores was calculated according to the intensity of positively stained liver epithelial cells. 0: negative expression, +1 weak expression, +2: moderate expression, +3: strong expression.
*: Percentages are given by rows.
a: Ischemia/reperfusion
b: Thioredoxin
c: N-nitro-L-arginine methyl ester
d: combination of Trx and L-NAME

Table 2. Statistical comparisons between groups of GSTA1 expression

GSTA1		sham (n = 10)	control I/R (n = 10)	Trx (n = 10)	L-Name (n = 10)	Trx+L-Name (n = 10)
sham (n = 10)	0,0 ±0,0[a] (0-0) [b]		**0,035***	0,144	**0,023**	**0,000**
control I/R[c] (n = 10)	0,800 ±0,26 (0-1)	**0,035**		0,963	1,000	**0,003**
Trx[d] (n = 10)	0,600 ±0,25 (0-1)	0,144	0,963		0,938	**0,000**
L-NAME[e] (n = 10)	0,800 ±0,26 (0-1)	**0,023**	1,000	0,938		**0,003**
Trx+L-Name[f] (n = 10)	1,800 ±0,25 (1-3)	**0,000**	**0,003**	**0,000**	**0,003**	

Staining scores was calculated according to the intensity of positively stained liver epithelial cells. 0: negative expression, +1 weak expression, +2: moderate expression, +3: strong expression. The differences between the expressions of GSTA1 among groups were analysed by the Post Hoc Tests.
*: A *p*-value of less than 0,05 was considered as statistically significant.
a: Mean ±SE.
b: min-max staining intensity
c:Ischemia/reperfusion,
d: Thioredoxin
e: N-nitro-L-arginine methyl ester
f: combination of Trx and L-NAME

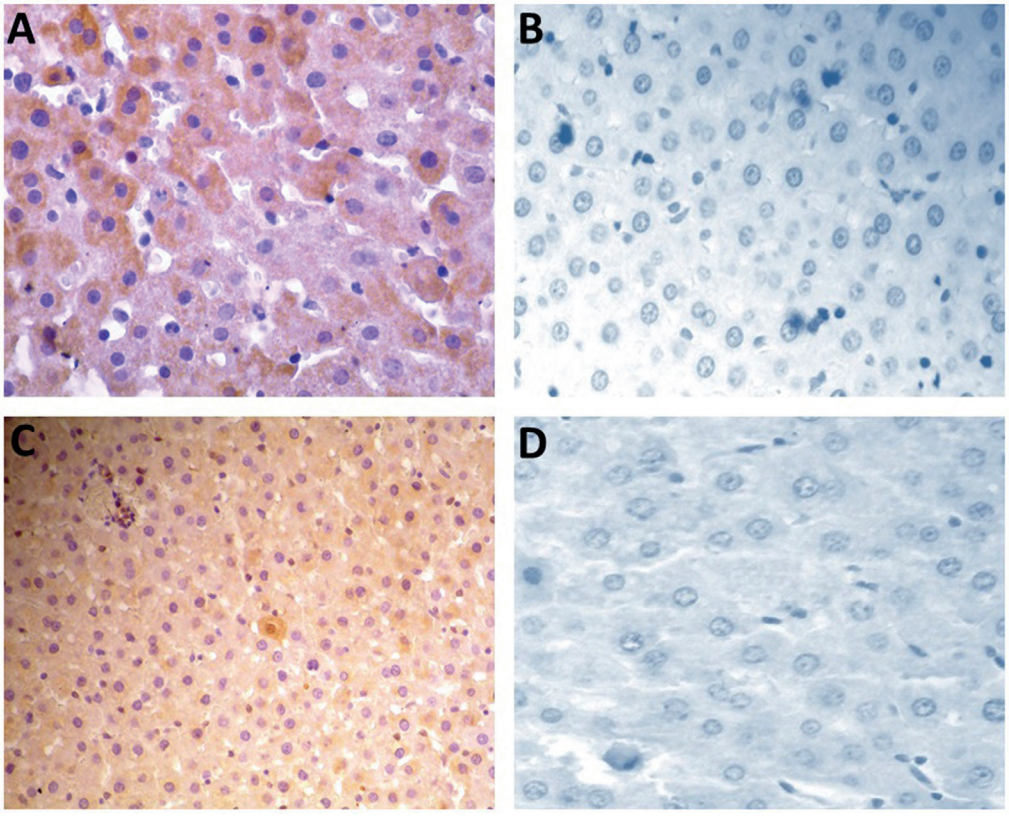

Figure 1. Immunohistochemical expressions of GSTA1 and GSTP1 isoenzymes. **A)** strong expression of GSTA1 in Trx+L-NAME group (400X), **B)** negative control staining of GSTA1 without antibody (400X), **C)** weak expression of GSTP1 in Trx group (400X), **D)** negative control staining of GSTP1 without antibody (400X).

cells, hepatocytes may have oxidative damages. In the same time, cells have many defense mechanisms to prevent oxidative damages. Such mechanisms are called antioxidative defense system or antioxidant (27). Our data generated in this study suggest that oxidative stress mechanism and subsequent scavenging system involve I/R process as GSTA1 expression occurred in hepatocytes.

I/R injury causes liver damage that is a very important issue during surgical procedures such as hepatic resections and liver transplantation. The total organ response of the liver and in part, the cell response to ischemia/reperfusion has been extensively investigated in hepatic tissues, and several recent studies suggest the availability of antioxidant enzymes that counteract and/or alleviate the oxidant burden observed under these conditions. However, limited information is available describing the *in vivo* subcellular organelle antioxidant enzyme response of liver to ischemia/reperfusion mediated oxidative stress. Oxidative stress and the resulting generation of oxidants have been implicated as putative mediators of injury in multiple diseases including hepatic ischemia and allograft dysfunction, bowel ischemia, acute renal failure, myocardial ischemia, hyperoxia induced lung injury, adverse reactions to xenobiotics, as well as accelerated aging. Oxidative stress can be defined as a condition, in which generation of potentially harmful reactive oxygen species (ROS) over- whelms the antioxidant defense mechanisms. At cellular level, the balance of reduction and oxidation (redox) is regulated various antioxidant systems. Among these systems, thiol system is one of them. The thiol system composed of thioredoxin, glutathione, and glutaredoksin (4).

Trx takes partial role on hydrogen donor to ribonucleotide reduction, regulation of photosynthetic enzymes and transcriptional factors in cells. Studies conducted by different research proved that L-NAME inhibits Nitric oxide synthase (NOS), which in turn reduce Nitric oxide (NO) production. Reduction of NOS, in turn, by indirect inhibition of L-NAME protects these cells from generating peroxynitrite and hydroxyl radicals. As a result, inhibition of inducible NOS (iNOS) brings in reduction of NO production, supporting the antioxidant defense system (27).

In a previous study conducted by Branum and coworkers (1998) expression of GSTA1/A2 increased in ischemic and nonischemic liver lobs and they found no statistical significance compared to sham animals. Chouker and coworkers (2005) reported that alpha-GST can be completely prevented by ischemic preconditioning. They also stated that only alpha-GST concentrations (>490 g/L) determined early after resection (2 hours) predict postoperative liver dysfunction (24 hours PT <60%) with a positive predictive value of 74% and a negative predictive value of 76%. As we also suggest that alpha-GST seems to be a sensitive. Also, they suggest that a-GST can be considered as a predictive marker of ischemia/reperfusion-induced hepatocellular injury and postoperative liver dysfunction (3,29).

In the present study, the GSTP1 isozyme expression in the rat liver is not different among groups. However, GSTA1 isozyme expression is significantly higher in the rat liver with I/R compare to sham group. It is also higher in all drug applied groups: Trx, L-NAME and their combine use (Table 1,2). These results indicate a possible role of GSTA1 isozyme in repair of oxidative stress-mediated liver injury. In the meantime, the GSTA1 expression profile is similar (80%) between I/R and L-NAME groups indicating that application of the anti-oxidative drug L-NAME did not change GSTA1 isozyme expression in the rat liver. However, GSTA1 expression profile is lower (60%) in Trx applied group compared to I/R and L-NAME applied groups (Table 1). Based on this result, it can be said that compare to L-NAME the anti-oxidant drug Trx is more effective in the liver injury resulting from I/R and this effect is possibly due to reduction of GSTA1 isozyme substrate load. However, GSTA1 expression was observed in all liver tissues of the combined drug (Trx+L-NAME) applied group, 40% of which exhibited overexpression of GSTA1. Thus, this situation may be related to a cellular defense mechanism against oxidative stress resulting from use of various cellular pathways to detoxify two different drugs.

CONCLUSIONS

In conclusion, the GSTA1 isozyme expression increases in the liver in response to oxidative damage resulting from I/R, indicating a possible role of the GSTA1 isozyme on pathophysiology and subsequent repair process. The anti-oxidant drug Trx is more effective when compared to L-NAME in the liver I/R by reducing the oxidant loads. However, overexpression of GSTA1 isozyme may have been the result of increased oxidative stress caused by the combined use of Trx with L-NAME.

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