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Monoamine oxidase (MAO) activity and MAO-derived hydroxyl radical production in mouse model of hepatic ischemia-reperfusion injury

[Fare modeli karaciğer iskemi-reperfüzyon hasarında monoamin oksidaz (MAO) aktivitesi ve MAO kaynaklı hidroksil radikali üretimi]

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ABSTRACT

Objective: There are various studies that have been published indicating that reactive oxygen species are produced in large quantities in post-ischemic reperfusion and this oxidative burst mediates the severity of the damage. It has been previously suggested that monoamine oxidase (MAO) is a potential source of hydrogen peroxide (H_2O_2) in early reperfusion; and mitochondrial hydroxyl radicals generated from H_2O_2 during MAO metabolism serve as a contributor to tissue injury. The aim of this study was to investigate the possible contribution of elevated activities of MAO isoforms to the generation of reactive oxygen species and lipid peroxidation in hepatic ischemia-reperfusion injury in mice.

Methods: After 45 minutes of partial ischemia followed by 5 hours of reperfusion performed on mouse liver. Serum lactate dehydrogenase and transaminase activities were measured as indices of hepatic injury. Lipid peroxidation, glutathione content and redox state, antioxidant enzyme activities, total MAO, MAO-A and -B activities and MAO-dependent H_2O_2 release in liver tissue were determined.

Results: MAO-A and -B activities, lipid peroxidation, oxidized glutathione content and H_2O_2 release were found to be increased, while reduced glutathione content, reduced/oxidized glutathione ratio as indice of redox state and antioxidant enzyme activities were decreased in liver tissues of ischemia-reperfusion group when compared to those of control group. A strong positive correlation was found between MAO isoform activities and H_2O_2 release in ischemia-reperfusion group, suggesting that MAO is a potential source of H_2O_2 generation during ischemia-reperfusion.

Conclusion: Our study suggests that both MAO isoforms may contribute to reactive oxygen species generation during ischemia-reperfusion, and MAO inhibitors may be used against liver ischemia-reperfusion injury.

Key Words: Ischemia-Reperfusion, reactive oxygen species, monoamine oxidase (MAO), mouse.

ÖZET

Amaç: İskemiyi takip eden reperfüzyonda fazla miktarda reaktif oksijen türevleri üretildiğine ve bu oksidatif patlamanın, hasarın şiddetini belirlediğine dair çok sayıda çalışma bulunmaktadır. Daha önceki çalışmalarda monoamin oksidaz (MAO)'ın erken reperfüzyon evresinde potansiyel bir hidrojen peroksit (H_2O_2) kaynağı olduğu önerilmiştir ve MAO metabolizması sırasında salınan aşırı H_2O_2 ile ortaya çıkan mitokondri içi hidroksil radikallerinin doku hasarına katkıda bulunabileceği öne sürülmüştür. Bu çalışmanın amacı fare karaciğer iskemi-reperfüzyonunda reaktif oksijen türevlerinin üretimi ve lipid peroksidasyonuna MAO izoformlarının olası katkılarını araştırmaktır.

Yöntemler: Fare karaciğerinde 45 dakikalık kısmi iskemi gerçekleştirilmiş ve takibinde uygulanan 5 saatlik reperfüzyon süresi sonunda serum transaminaz, laktat dehidrogenaz, MAO-A ve -B aktiviteleri, lipid peroksidasyonu, doku glutatyon düzeyleri, H_2O_2 salınımı ve antioksidan enzim aktiviteleri ölçülmüştür.

Bulgular: Kontrol grubuyla karşılaştırıldığında, iskemi-reperfüzyon grubunun karaciğer dokularında MAO izoformlarının aktiviteleri, lipid peroksidasyonu, okside glutatyon içeriği ve H_2O_2 salınımının artmış olduğu saptanırken; redükte glutatyon içeriği, redükte/okside glutatyon oranı ve antioksidan enzim aktivitelerinde azalma görülmüştür. İskemi-reperfüzyon grubunun MAO izoform aktiviteleri ve H_2O_2 salınımları arasında kuvvetli pozitif bir korelasyon bulunmuştur ki bu bulgu MAO'ın, reperfüzyon sırasındaki H_2O_2 üretimi için potansiyel bir kaynak oluşturduğunu düşündürmektedir.

Sonuçlar: Bu çalışmada her iki MAO izoformunun da H_2O_2 üretimine katkıda bulunabileceği gösterilmiş ve iskemi reperfüzyon hasarının zararlı etkilerinin ortadan kaldırılmasında MAO inhibitörlerinin etkili olabileceği ortaya konmuştur.

Anahtar Kelimeler: İskemi-Reperfüzyon, reaktif oksijen türleri, monoamin oksidaz (MAO), fare.

Introduction

Ischemia-reperfusion (I/R) injury is a complex phenomenon often seen in surgical practice or transplantation, and is associated with both local injury and induction of systemic inflammatory response [1]. Hepatic ischemia followed by reperfusion leads to tissue injury that contributes mainly to morbidity and mortality associated with shock, thermal injury, liver transplantation and resectional surgery. In organ transplantation, minimizing I/R injury is shown to improve the success rate of transplantation. Although the phenomenia that occurs during obstruction and restoration of blood flow during ischemia and subsequent reperfusion are complex, it has been proposed that reactive oxygen species (ROS) which are excessively formed during I/R may cause direct cell membrane damage, protein damage, causes oxidation of enzymes and some irreversible DNA changes, leading to cell death [2,3].

Monoamine oxidase (MAO) is a flavoenzyme which deaminates biogenic amines such as 5-HT (serotonin), adrenaline, noradrenaline and dopamine, in both the central nervous system and peripheral tissue [4]; it also catalyzes the oxidation of xenobiotic amines [5]. MAO is found in two different forms as MAO-A and MAO-B, which are encoded by two different genes [6] and distinguished by different substrate specificities and sensitivities to the selective inhibitors [7]. It has been shown that MAO catalyzes the oxidative deamination of biogenic amines to their corresponding aldehydes. This is accompained by the reduction of molecular oxygen to hydrogen peroxide (H_2O_2), which can not be fully scavenged by endogenous antioxidants [8]. Although H_2O_2 is only toxic at high concentrations, it can turn into more reactive species therefore its cellular concentration should be taken under control. H_2O_2 can be inactivated by a further reduction to water by catalase or the glutathione system. The toxicity of H_2O_2 is suggested to originate from its ability to induce oxidative damage to the proteins directly or through its conversion into hydroxyl radicals via Fenton reaction [9]. It has been postulated that intramitochondrial hydroxyl radicals from H_2O_2 generated during MAO metabolism serves as a major contributor to tissue injury in the brain [10].

Since the mouse model of I/R injury has proven to be valuable to exhibit the role of ROS involved in post-ischemic tissue injury, this study was planned to investigate the possible contribution of MAO to ROS generation and lipid peroxidation in induced partial liver ischemia followed by reperfusion in mice.

Materials and Methods

Liver I/R Protocol in Mice

This study was approved by the Animal Ethics Committee of Hacettepe University (2006/15-13) and was performed in compliance with the Ethical Guidelines for Animal Studies. Study groups were randomly assigned as control, sham and I/R protocol. Each group consisted of 6 mice. Male albino mice (8-10 weeks) with body weights of 25–30 g were obtained from the Animal House of Hacettepe. The mice were maintained under conditions of a 12 h light/dark cycle and had free access to food and water. Partial mouse liver ischemia was performed according to the method of Abe et al. [11]. Liver ischemia was induced in anesthetized mice by placing an atraumatic clip across the portal vein, hepatic artery and bile duct just above the branching to the right lateral lobe. The median and left lateral lobes (approximately 70% of the liver) showed significant blanching after 45 minutes of ischemia period (Figure 1). Abdomen was closed and the animal was allowed to recover for 5 hours of reperfusion period**.** Immediately after the reperfusion period, the blood and tissues were collected from the anesthetized mouse. The right atrium was punctured and blood was aspirated with a syringe. Blood samples were collected; centrifuged at 3500 x g for 10 min, and the obtained serum was stored at −20 °C. The hepatic lobes were promptly resected, weighted, washed in cold saline, and kept at −80°C. Liver tissues were homogenized in cold potassium phosphate buffer (50 mM, pH 7.4); centrifuged at 3500 x g for 10 min at 4°C, and the supernatant was used for the determination of lipid peroxidation, glutathione content, antioxidant enzyme levels and MAO activities.

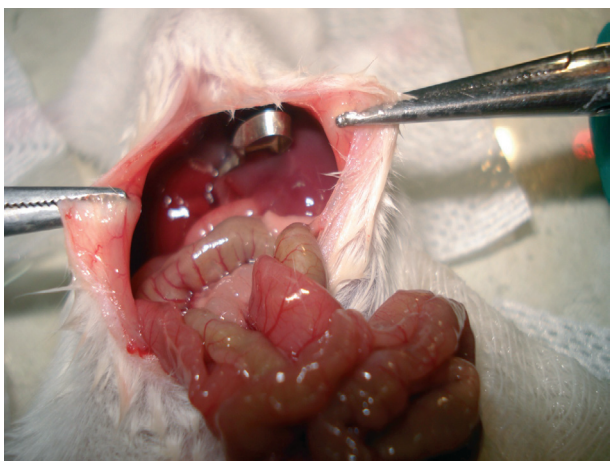

Figure 1. Liver I/R protocol in mouse.

Chemicals

All chemicals were purchased from Sigma Chemical Co. (Germany).

Determination of Serum Transaminase and Lactate Dehydrogenase (LDH) Activities

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined as quantitative indices of liver damage according to the colorimetric method of Reitmann [12]. Serum LDH ac-

tivity was determined according to a method based on monitoring the conversion of NADH to NAD^+ at 340 nm [13]. Activity was expressed as $U.L^{-1}$.

Determination of Tissue Lipid Peroxidation

MDA content in liver tissue was determined using the thiobarbituric acid method [14] with little modification. In brief, 5 µl supernatant was mixed with 25 µl 3.0% sodium dodecylsulfate,75 µl acetic acid buffer (pH=3.5), 75 µl 0.8% 2-thiobarbituric acid and 20 µl deionized water, followed by heating in a water bath at 95°C for 1 h. After cooling, 250 µl n-butanol and pyridine (15:1) was added and the mixture was centrifuged at 1400 x g for 15 min. The organic phase was collected and the fluorescence intensity was monitored with excitation at 515 nm and emission at 553 nm. 1,1,3,3-tetramethoxypropane was used as standard. MDA content was expressed as nmol.mg $protein^{-1}$.

Determination of Tissue Reduced (GSH) and Oxidized (GSSG) Glutathione Levels

GSH and GSSG levels in liver tissues were determined according to the method of Sanchez-Alvarez [15]. Glutathione level was expressed as nmol.mg $protein^{-1}$.

Determination of Tissue Antioxidant Enzyme Activities

Catalase (CAT) activity in liver tissues was determined according to the spectrophotometric method of Ueda et al. [16] and activity was expressed as nmol.mg $protein^{-1}$. Tissue glutathione S-transferase (GST) activity was determined spectrophotometrically by monitoring the formation of 1-cloro-2,4-dinitrobenzene (CDNB) and GSH conjugate at 340 nm [17] and activity was expressed as U.mg $protein^{-1}$. Glutathione reductase (GR) activity was determined according to the method of Carlberk and Mannervik [18], and activity was expressed as nmol.mg $protein^{-1}$. Glutathione peroxidase (GPx) activity was determined by Glutathione Peroxidase Assay Kit (Cayman Chemicals, Ann Arbor, Mich.) and activity was expressed as U.mg $protein^{-1}$. Superoxide dismutase (SOD) activities were determined by Superoxide Dismutase Assay Kit (Cayman Chemicals, Ann Arbor, Mich.) and activities were expressed as U.mg $protein^{-1}$.

MAO Isolation from mouse liver and Determination of tissue MAO Activities

MAO was obtained from mitochondrial pellet of liver homogenates. Total MAO activity was determined spectrophotometrically according to the method of Holt [19]. In order to determine selective MAO-A and MAO-B activities, liver homogenates were incubated with selective MAO-A inhibitor clorgyline for determination of MAO-B activity or selective MAO-B inhibitor pargyline for determination of MAO-A activity for 1 hour at 37°C. Enzyme activities were determined according to the previous method [19] and expressed as nmol.h^{-1}.mg $protein^{-1}$.

Determination of MAO-Derived Hydroxyl Radical Formation in Liver Tissue

Hydroxyl radical formation in liver homogenates was determined according to the spectrofluorimetric method of Barreto et al. [20]. Results were expressed as nmol.mg $protein^{-1}$.

Protein Determination

The protein content of the liver tissue was measured by the method of Bradford [21] using bovine serum albumin as standard protein.

Statistical Analysis

All results were expressed as mean±SEM of three independent experiments and analyzed by SPSS for windows version 16.0. Mann-Whitney U test and one-way analysis of variance (ANOVA) were used for comparison of groups of the variables. Correlations between variables were assessed with Pearson's correlation coefficients (r), and $p<0.05$ was considered as statistically significant.

Results

Deprivation of blood flow in mice liver for 45 minutes followed by 5 hours of reperfusion caused a dramatic increase in liver injury assessed by significantly elevated serum ALT, AST and LDH levels (Table 1). Lipid peroxidation, the hallmark of ROS-induced injury, commences when a radical removes a hydrogen atom from an unsaturated fatty acid. In this study, malondialdehyde (MDA) was used as a marker of lipid peroxidation. After 5h of reperfusion, liver MDA content was significantly elevated in liver I/R-subjected mice (Table 2). A strong positive correlation was found between the serum LDH activity and liver MDA concentration ($r=0.90$, $p<0.001$) and between the serum transaminase activities and liver MDA concentration ($r=0.83$ for AST, $r=0.79$ for ALT, $p<0.001$) in I/R group.

Liver GSH content and the GSH/GSSG ratio were found to be significantly decreased whereas GSSG content was found to be increased in I/R group (Table 2). Tissue GSH content strongly and negatively correlated with tissue MDA content ($r=-0.85$, $p<0.001$) whereas tissue GSSG content positively correlated with tissue MDA content ($r=0.79$, $p<0.001$) in I/R group.

Antioxidant enzyme activities were found significantly lowered in I/R group (Table 2). Negative correlation was found between the tissue CAT, GST and SOD activities and tissue MDA content ($r=-0.80$, $r=-0.70$ and $r=-0.82$ respectively, $p<0.001$) in I/R group. These antioxidant enzyme activities positively correlated with tissue GSH content ($r=0.77$, $r=0.60$, $r=0.71$ respectively, $p<0.001$) in I/R group.

Furthermore, compared to control and sham groups, total MAO, MAO-A and MAO-B activities were significantly increased in the liver tissues of I/R group (Table 3). H_2O_2 release in liver tissues significantly increased in I/R group compared to control and sham groups (Table 3).

Table 1: Serum ALT, AST and LDH activities as indices of liver damage in study groups

PARAMETERS	CONTROL GROUP	SHAM	I/R GROUP
Serum ALT Activity ($U.L^{-1}$)	45.40±0.30	45.51±0.51	5938.00±31.05 ***
Serum AST Activity ($U.L^{-1}$)	51.53±0.29	51.70±0.20	6117.00±33.28 ***
Serum LDH Activity ($U.L^{-1}$)	486.70±9.28	503.40±3.14	6300.00±209.40 ***

Each group consists of 6 mice and values represent the mean±SEM of three independent experiments (***p<0.001 vs control and sham).

Table 2. Lipid peroxidation, glutathione content and antioxidant enzyme levels in liver tissues of the study groups.

PARAMETERS	CONTROL GROUP	SHAM	I/R GROUP
MDA (nmol.mg $protein^{-1}$)	4.03±0.27	4.00±0.40	16.50±1.12 ***
GSH (nmol.mg $protein^{-1}$)	73.00±4.01	72.80±4.50	30.26±5.11 ***
GSSG (nmol.mg $protein^{-1}$)	1.30±0.09	1.31±0.09	8.94±3.99 ***
GSH/GSSG	56.55±2.30	55.60±3.65	2.42±0.25 ***
CAT (nmol.mg $protein^{-1}$)	69.90±3.21	69.00±4.15	30.12±2.00 ***
GST (U.mg $protein^{-1}$)	3.78±0.19	3.80±0.20	1.10±0.09 ***
GR (nmol.mg $protein^{-1}$)	31.45±2.00	30.93±2.00	14.26±1.11 ***
GPx (U.mg $protein^{-1}$)	1.31±0.09	1.30±0.09	0.35±0.02 ***
SOD (U.mg $protein^{-1}$)	29.80±1.30	28.70±1.22	10.90±3.10 ***

Each group consists of 6 mice and values represent the mean±SEM of three independent experiments (***p<0.001 vs control and sham).

Table 3. MAO activities and MAO-derived hydroxyl radical level in liver tissues of study groups

	Total MAO (nmol.h-1.mg protein-1)	MAO-A (nmol.h-1.mg protein-1)	MAO-B (nmol.h-1mg protein-1)	MAO Derived Hydroxyl Radical Level (nmol.mg protein-1)
CONTROL GROUP	266.60 ±1.79	168.40±5.48	83.73±3.84	1.21±0.01
SHAM	272.00±7.00	168.10±12.22	82.66±6.34	1.22±0.01
I/R GROUP	791.90±22.67*	545.30±19.34**	414.10±11.98***	17.16±1.72****

Each group consists of 6 mice and values represent the mean±SEM of three independent experiments.
*p<0.001 vs control and sham of total MAO
**p<0.001 vs control and sham of MAO-A
***p<0.001 vs control and sham of MAO-B
****p<0.001 vs control and sham of MAO-derived hydroxyl radical level

A strong positive correlation was found between the liver MAO isoform activities and H_2O_2 release (r=0.83 for total MAO, r=0.93 for MAO-A and r=0.80 for MAO-B, p<0.001) in I/R group. MAO-derived H_2O_2 release was positively correlated with tissue MDA content (r=0.80, p<0.001) in I/R group.

Discussion

The imbalance between formation and removal of ROS which is termed oxidative stress, plays a major role in many diseases. In this respect, major attention has been focused on the relationship between oxidative stress and I/R injury [22]. Although mitochondria are considered the most relevant site for the formation of ROS, and respiratory chain is generally indicated as a main site for ROS formation, other mitochondrial components are believed to contribute to ROS generation especially in I/R injury. Recent reports highlight the relevance of MAO [23].

MAO is a flavoenzyme located within the outer mitochondrial membrane, responsible for the oxidative deamination of neurotransmitters and dietary amines. It exists

in two isoforms, MAO-A and -B that differ for substrate specificity and inhibitor sensitivity [24]. In peripheral tissues, MAO is involved in the oxidative catabolism of amines from the blood and in preventing the entry of dietary amines into the circulation. MAO-A and B knockout mice showed increased reactivity to stress, similar to that observed after administration of non-selective MAO inhibitors [23]. Considering the possible important role of MAO as a source of H_2O_2, the present study was planned to investigate the contribution of MAO isoform activation and MAO-mediated H_2O_2 or hydroxyl radical production to oxidative damage occuring during I/R in Mouse I/R model.

Following 45 minutes of ischemia and 5 hours reperfusion, the increase in serum ALT, AST and LDH levels of I/R group compared to control and sham groups (Table 1, $p<0.001$) affirmed the hepatic I/R injury.

Activation of macrophages and neutrophils during reperfusion was suggested to cause the excess oxygen radicals via NADPH oxidase [23]; therefore, ROS are believed to be mainly produced on reperfusion phase of I/R. In the present study, MDA concentration (as the indicator of lipid peroxidation) was found to be significantly increased in I/R group (Table 2, $p<0.001$) which is in agreement with the previous reports [2,25]. The strong positive correlations found between the serum LDH activity and liver MDA content, and between the serum transaminase activities and liver MDA content in I/R group suggest that increased lipid peroxidation during I/R causes hepatic injury during I/R in mice.

The decreased GSH/GSSG ratio is a marker for oxidative stress in ischemia. GSH can directly react with ROS or act as a cofactor with the enzyme GPx to detoxify H_2O_2 and lipid peroxides in tissues. Decreased hepatic GSH levels induced an increase in hepatocyte susceptibility to anoxic damage and oxidative stress. In contrast, an optimal level of glutathione has been reported to reduce hepatic necrosis and improves liver function after cold storage [26]. During I/R, sudden bursts of ROS, however, can not be handled by the endogenous antioxidant systems and the accumulation of ROS leads to cellular membrane, protein and DNA oxidative damage [26].

In the present study, liver GSH content and the GSH/GSSG ratio were found to be significantly decreased whereas GSSG content was found to be increased in I/R group (Table 2) in a good accordance with the previous reports [1,2,22,27]. Tissue GSH content strongly and negatively correlated with tissue MDA content whereas tissue GSSG content positively correlated with tissue MDA content in I/R group. Since a reduced GSH/GSSG ratio is commonly used as an indicator of oxidative stress and oxidative damage [27], and our data indicated that GSH/GSSG ratio in I/R group markedly reduced parallel to the decrease in tissue GSH content, we conclude that GSH depletion in 45 min of ischemia followed by 5 h of perfusion in mouse I/R model might be resulted from the consequence of consumption of free radicals produced mainly in the perfusion phase by GSH during I/R injury. There have been some reports indicating that livers subjected to 60 min ischemia recovered from exposed oxidative stress during ischemia by reperfusion, whereas the 120 min ischemia group continued to undergo further severe oxidative stress by reperfusion [28].

Hepatic total glutathione content is generally regulated by a balance between the rate of introduction, the rate of elimination, formation of protein glutathione mixed disulfide (protein–S–SG), and efflux to bile and blood. The redox status of glutathione (GSH/GSSG) is also regulated by the rate of conversion from GSH to GSSG through detoxification of H_2O_2 and lipid hydroperoxides by c-GPx and PHGPx and by the rate of recycling from GSSG to GSH by GSSG reductase at the expense of NADPH. Because ATP is necessary for GSH synthesis through reactions with S-adenosylmethionine synthetase, g-glutamylcysteine synthetase, and GSH synthetase, decrease of total and reduced glutathione and GSH/GSSG would occur during ischemia [29]. A long with our data designated the increase of lipid peroxidation products, changes in total glutathione, GSH, and GSH/GSSG and negative correlation between the tissue MDA and GSH contents, and positive correlation between the tissue MDA and GSSG contents indicate the excess generation of ROS in ischemic liver can not be totally neutralized by endogenous glutathione.

Decreased antioxidant enzyme activities in I/R group (Table 2) was possibly result from the depletion of the antioxidant pool, which is used to remove excess ROS produced during I/R. Since our data indicated that there has been a negative correlation between the tissue CAT, GST and SOD activities and tissue MDA content and a positive correlation between these enzymes and GSH content in I/R group, it was concluded that the enhancement of antioxidant content may have only limited protective effect against H_2O_2-mediated tissue injury. Possible usage of MAO inhibitors together with antioxidants may be more effective in terms of protection against I/R injury in the liver.

It has been previously reported that ischemia-induced MAO and MAO-derived hydroxyl radicals contribute to excess ROS production in I/R [30,31]; ROS generated by MAO in the presence of elevated 5-HT concentrations induced release of cytochrome c, up-regulation of proapoptotic Bax and down-regulation of antiapoptotic Bcl-2 proteins and I/R injury is associated with increased interstitial 5-HT released by activated platelets [32,33]. Our results, indicating the marked increase in tissue total MAO, MAO-A and -B activities during I/R in the liver tissue of mice (Table 3), were in accordance with earlier reports. However, a detailed assay protocol and time course is needed to determine whether MAO is depressed or activated in ischemia and reperfusion phases of the protocol as well as early and late phases of reperfusion.

Since H_2O_2 release in liver tissues significantly elevated and a positive correlation was found between the liver MAO isoform activities and H_2O_2 in I/R group and MAO-derived H_2O_2 release was positively correlated with tissue MDA content in I/R group, we suggest that activated MAO isoforms possible contribute the oxidative damage occurs during I/R in mouse liver. In the smooth muscle cells, oxidation of decreased 5-HT level or tyramine by MAO-A triggers MMP2, neutral sphingomyelinase-1 and sphingosine kinase resulting in cell proliferation [34]. Considering that MAO-A controls the activity of sphingosine kinase and the levels of proapoptotic ceramide and antiapoptotic S1P, it may be an important drug target for regulating I/R injury in the tissue remodelling. Although the present study suggested both MAO isoform activites were elevated during hepatic I/R injury in mice in correlation with the increased MAO-derived hydroxyl radical release, we suggest that further investigations are needed to see whether the both isoforms are involved in this process.

Therefore we intend to design a new study based on mouse I/R model including a time course for both ischemia and reperfusion periods in order to determine the MAO-derived hydroxyl radical release during the different periods of I/R as well as to investigate the possible protective effects of selective MAO inhibitors applied prior to I/R on hepatic I/R injury mostly caused by MAO-derived ROS production.

In summary, our data demonstrated the significant ROS-mediated tissue injury in hepatic I/R and suggested that MAOs may be one of the potential sources of excessive H_2O_2 generation possibly in the reperfusion period of I/R in mouse liver. Pretreatment with specific MAO inhibitors prior to or together with antioxidant treatment may have potential clinical relevance.

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The authors declare no conflict of interest.

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