Heme breakdown and ischemia/reperfusion injury in grafted liver during living donor liver transplantation

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Abstract. Living donor liver transplantation (LDLT) requires ischemia/reperfusion (I/R), which can cause early graft injury. However, the detailed mechanism of I/R injury remains unknown. Heme oxygenase-1 (HO-1) is a rate-limiting enzyme in heme catabolism and results in the production of iron, carbon monoxide (CO), and biliverdin IXα. Furthermore, in animals, HO-1 has a protective effect against oxidative stress associated with I/R injury. However, in humans, the molecular mechanism and clinical significance of HO-1 remain unclear. We previously demonstrated that exhaled CO levels increase during LDLT, and postulated that this may indicate I/R injury. In this study, we elucidate the origin of increased exhaled CO levels and the role of HO-1 in I/R injury during LDLT. We studied 29 LDLT donors and recipients each. For investigation of HO-1 gene expression by polymerase chain reaction and HO-1 localization by immunohistological staining, liver biopsies from the grafted liver were conducted twice, once before and once after I/R. Exhaled CO levels and HO-1 gene expression levels significantly increased after I/R. In addition, HO-1 levels significantly increased after I/R in Kupffer cells. Furthermore, we found a significant positive correlation between exhaled CO levels and HO-1 gene expression levels. These results indicated that increased heme breakdown in the grafted liver is the source of increased exhaled CO levels. We also found a significant relationship between HO-1 gene expression levels and alanine aminotransferase (ALT) levels; i.e., the higher the HO-1 gene expression levels, the higher the ALT levels. These results suggest that HO-1-mediated heme breakdown is caused by I/R during LDLT, since it is associated with increased exhaled CO levels and liver damage.

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Abbreviations: ALAS-1, nonspecific δ-aminolevulinate synthase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; cDNA, complementary deoxyribonucleic acid; CO, carbon monoxide; HO-1, heme oxygenase-1; I/R, ischemia/reperfusion; LDLT, living donor liver transplantation; MELD, model for end-stage liver disease; mRNA, messenger ribonucleic acid; PBS, phosphate-buffered saline; T.Bil, total bilirubin

Key words: ischemia/reperfusion injury, heme oxygenase, liver damage, living donor liver transplantation

Introduction

Recently, liver transplantation has become a common therapeutic method for end-stage liver diseases such as liver cirrhosis with or without hepatocellular carcinoma and fulminant hepatitis (1). However, a major problem with this method is the shortage of donor organs. One of the solutions to this problem is living donor liver transplantation (LDLT). LDLT requires cold preservation and warm revascularization of liver grafts during transplantation. However, injuries due to ischemia/reperfusion (I/R), which is known to induce oxidative stress, are unavoidable during LDLT (2,3).

The rate-limiting enzyme in heme catabolism, heme oxygenase-1 (HO-1), is induced by not only its substrate heme but also oxidative stress resulting from I/R injury (4-7). HO-1 induction leads to increased heme breakdown, resulting in the production of iron, carbon monoxide (CO), and biliverdin IXα, which is subsequently reduced to bilirubin IXα by biliverdin reductase (7,8). According to our previous reports, HO-1 has a protective effect against oxidative stress, as seen in carbon tetrachloride-induced liver injury models (9,10). Other investigators have also reported similar protective effects of HO-1 in animals (11,12). In humans, patients with acute liver hepatitis showed increased HO-1 and decreased nonspecific δ-aminolevulinate synthase (ALAS-1) gene expression levels, which is the rate-limiting enzyme in heme biosynthesis (13). However, the molecular mechanism responsible for increased HO-1 expression levels and its clinical significance remains unclear.

We previously demonstrated that exhaled CO levels increased in LDLT recipients after I/R injury, and suggested that exhaled CO levels indicate the extent of oxidative tissue

Table I. Primer pairs used for polymer chain reaction.

Target cDNA	Accession no.	Primer sequence (5'→3')	Amplicon size (bp)
β-actin	NM_001101	Forward: GTGGCATCCACGAAACTACC	197
		Reverse: GTACTTGCGCTCAGGAGGAG	
HO-1	NM_002133	Forward: ATGACACCAAGGACCAGAGC	169
		Reverse: GCCACCAGAAAGCTGAGTGT	
ALAS-1	NM_000688	Forward: CACACACCCCAGATGATGAA	108
		Reverse: CCTGCAGAAGTTGCACTCAG	

HO-1, heme oxygenase-1; ALAS-1, nonspecific δ-aminolevulinate synthase.

Table II. Patient characteristics.

Patient characteristics	n[a]
Age of recipients (years)	40±23
Gender of recipients (male:female)	16:13
Primary disease	
Viral liver cirrhosis	12
Biliary atresia	7
Nonalcoholic steatohepatitis	4
Primary sclerosing cholangtitis	2
Fulminant hepatitis	1
Budd-Chiari syndrome	1
Alcoholic hepatitis	1
Hepatoblastoma	1
Pre-operation MELD score	15±7
Age of donors (years)	38±14
Gender of donors (male:female)	16:13

[a]Data represent the number of patient (n) or mean ± SD or ratio. MELD score, model for end-stage liver disease score.

injury in the affected organ (14). However, the origin of exhaled CO remains unknown.

In this study, we hypothesized that the source of increased exhaled CO is related to heme breakdown induced by HO-1 after I/R injury. We also investigated the clinical significance of HO-1 in the grafted liver during LDLT. Therefore, we investigated HO-1 gene and protein expression levels and their relationship with exhaled CO levels and liver injury in 29 grafted livers during LDLT.

Materials and methods

Patients. This study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Okayama University Hospital. We prospectively studied 29 (16 male, 13 female) patients who had undergone LDLT between 2006 and 2008 at Okayama University Hospital. Informed consent was obtained from all participants.

Anesthetic procedure. All surgeries were performed under general anesthesia. Each patient received 2 mg/kg of propofol, 5 μg/kg of fentanyl or approximately 0.3 μg/kg/min of remifentanil, and 0.1 mg/kg of vecuronium to facilitate endotracheal intubation. Anesthesia was maintained using a continuous infusion of remifentanil and vecuronium at a rate of 0.1-0.5 μg/kg/min and 2-4 mg/kg/h, respectively, along with 0.5-1.0% of isoflurane in an oxygen/air mixture.

Transplantation procedure. The donor and recipient hepatectomies were performed using a standard technique (15). The grafted liver was weighted and perfused through the portal vein using University of Wisconsin (UW) solution with methyl-prednisolone. Portal flush was performed by gravity. After perfusion, the graft was immersed in the UW solution and stored at 4˚C. The graft was transplanted using the piggyback technique. Before completion of caval anastomosis, the graft was flushed through the portal vein with about 250 ml of 5% albumin stored at 4˚C. Hepatic artery reconstruction was performed by end-to-end anastomosis and biliary duct reconstruction was performed by duct-to-duct anastomosis (16).

Exhaled CO measurement. Exhaled CO levels were measured using a CO analyzer (Carbolyzer™ mBA-2000, Taiyo Instruments, Inc., Osaka, Japan), as described previously (14). The instrument had a sensitivity of 0.1 ppm CO and was capable of continuous side stream sampling. A sampling adaptor was attached to the respiratory circuit for exhaled air sampling. Anesthetic drugs with muscle relaxants maintained constant respiratory conditions throughout the procedure. Throughout the anesthetic period, we controlled the respiratory rate, partial pressure of oxygen in arterial blood, partial pressure of carbon dioxide in arterial blood, and pH within normal ranges by taking periodic measurements of arterial blood gas samples. Although there were small fluctuations after normalization, CO levels were essentially constant and stable, and time courses were comparable among all individuals. During anesthesia, exhaled CO levels were measured twice, once after induction and once after reperfusion.

Liver biopsy. Liver biopsies were performed from the donors just before graft resection. The same amount of tissue was taken from the grafted liver of recipients just before skin closure to assess the effects of I/R. Just after liver biopsy, we divided the samples into two pieces, one was frozen in liquid nitrogen for

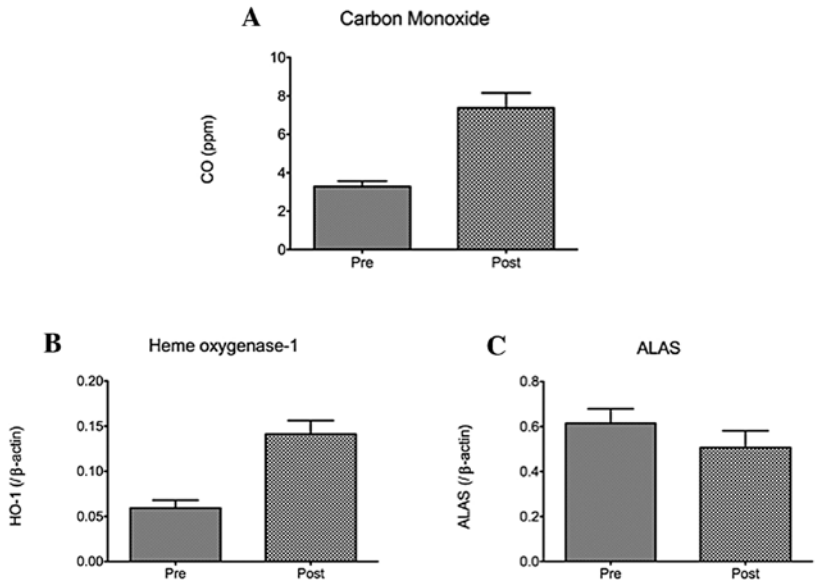

Figure 1. Changes of the indices for heme breakdown after Ischemia/reperfusion. (A) The significant increase of exhaled carbon monoxide (CO) after ischemia/reperfusion (I/R) injury (P=0.00055). (B) The increase of heme oxygenase (HO)-1 gene expression in the grafted liver after I/R injury (P=0.0000013). (C) The tendency of the decrease of nonspecific δ-aminolevulinate synthase (ALAS)-1 gene expression in the grafted liver after I/R injury (P=0.10). These results indicated the increase of heme breakdown during living donor liver transplantation.

assessment of gene expression levels, and the other was fixed in 10% neutral buffered formalin for histological examination.

Measurement of HO-1 and ALAS-1 gene expression levels. HO-1 and ALAS-1 gene expression levels were measured as described previously (17). We extracted messenger ribonucleic acid (mRNA) from the liver samples using the RNeasy Mini kit (Qiagen, Tokyo, Japan) and constructed complementary deoxyribonucleic acid (cDNA) from mRNA using the Ready-To-Go T-Primed First-Strand kit (Amersham Biosciences, Buckinghamshire, UK). Primer sequences are shown in Table I. To analyze HO-1 and ALAS-1 gene expression, real-time polymerase chain reaction was performed using a Light Cycler (Roche Diagnostics, Mannheim, Germany).

Immunohistochemistry. HO-1 exists in nonparenchymal cells, possibly in the Kupffer cells (18). To identify HO-1, immunohistochemical analysis was performed using the indirect immunofluorescence method. The liver samples, which were fixed in 10% neutral buffered formalin, were embedded in paraffin and sectioned at a thickness of 5 μm. Following antigen retrieval in citrate buffer (0.01 M, pH 6.0) using heat treatment by autoclaving, nonspecific binding sites were blocked with 5% normal donkey serum for 60 min. For HO-1 staining, slides were incubated at 4˚C overnight with polyclonal rabbit HO-1 antibody (StressGen Biotechnologies, Victoria, BC, Canada) at a dilution of 1:100 in 1X phosphate-buffered saline (PBS) containing 0.3% Triton X-100. For fluorescent visualization of the bound antibody, the slides were incubated with fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA) and a green fluorescent label for 90 min. To confirm HO-1 localization in the liver, CD68 cells where HO-1 was assumed to exist were stained. After the nonspecific binding sites were blocked by the procedure described above, the slides were incubated at 4˚C overnight with anti-CD68 mouse monoclonal antibody (Calbiochem, San Diego, CA, USA) at a dilution of 1:100 in 1X PBS containing 0.3% Triton X-100. For fluorescent visualization of the bound antibody, the slides were incubated with rhodamine-conjugated donkey anti-mouse IgG (Chemicon International, Billerica, MA, USA) for 90 min. Finally, images were acquired using a Zeiss confocal laser scanning microscope (LSM510; Zeiss, Jena, Germany).

Postoperative biochemical examinations. To estimate the clinical significance of I/R injury, postoperative aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (T.Bil) levels were measured. Tissues for these examinations were obtained as routine laboratory samples at the time of skin closure of the recipients.

Statistical analysis. Data were expressed as mean and standard deviation. The Student's t-test, Pearson's correlation coefficients, or analysis of variance was used for data analysis. We considered $P<0.05$ to be statistically significant.

Results

We prospectively studied 29 (16 male, 13 female) patients who had undergone LDLT between 2006 and 2008. Of these, 12 patients who had viral liver cirrhosis, 7 had biliary atresia, 4 had nonalcoholic steatohepatitis, and 2 had primary sclerosing cholangitis. One patient had Budd-Chiari syndrome, 1 had alcoholic hepatitis, 1 had hepatoblastoma, and 1 had fulminant hepatitis. The mean age of the recipients was 40±23 years. The mean model for end-stage liver disease (MELD) score for these patients was 15±7. The donors were 16 males and 13 females whose mean age was 38±14 years. All donors had normal liver biochemical findings and T.Bil levels. Liver grafts were of the following types: right lobe (n=9), left lobe (n=2), extended right

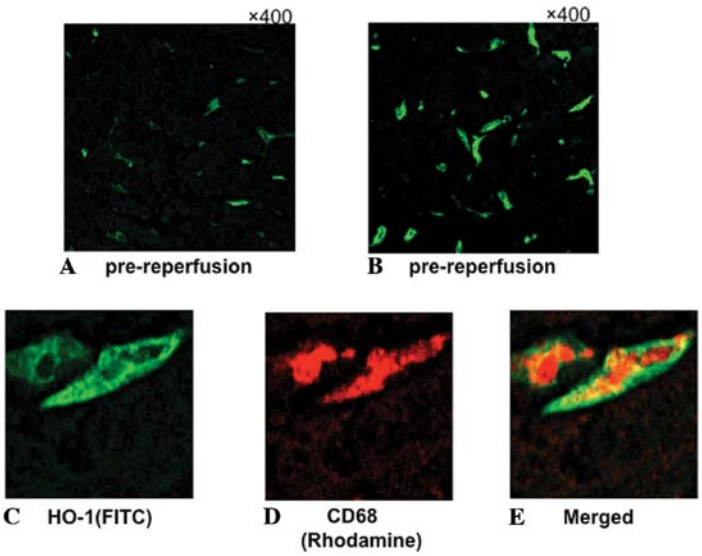

Figure 2. Immunohistochemistry of heme oxygenase-1 and CD68(KP). (A-C) Heme oxygenase (HO)-1 protein in green stained by FTIC. (A and B) HO-1 protein in pre- and post-reperfusion, respectively. These results showed the increase of HO-1 protein in the grafted liver after I/R injury. (C-E) The double staining of HO-1 protein and CH68 positive cells (Kuppfer cells). HO-1 protein stained in green and CD68 stained in red by rhodamine. (E) HO-1 protein existed in Kuppfer cells in the grafted liver in post-reperfusion during living donor liver transplantation.

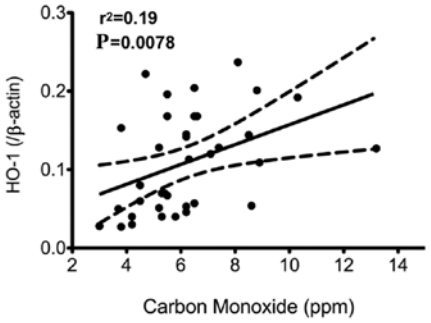

Figure 3. Relationship between exhaled carbon monoxide and heme oxygenase-1 gene expression in post reperfusion. Significant positive correlation was observed between exhaled carbon monoxide (CO) and heme oxygenase (HO)-1 gene expression in post reperfusion (r^2=0.19; P=0.0078). This result indicated that exhaled CO was the product from heme metabolized by HO-1.

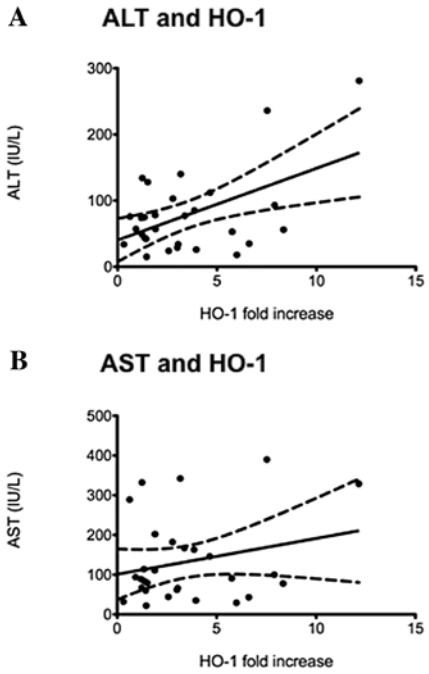

Figure 4. Relationship between heme oxygenase-1 gene expression and early graft function. The analysis of the relationship between heme oxygenase (HO)-1 gene expression and early graft function represented by alanine aminotransferase (ALT) and aspartate aminotransferase (AST). No significant relationship exists between AST and HO-1 (r^2=0.058; P=0.21), but there was a significant positive correlation between HO-1 gene expression and ALT (r^2=0.26; P=0.0052). This result indicated that HO-1 gene expression in the grafted liver would affect early graft dysfunction after living donor liver transplantation.

lobe (n=2), extended left lobe (n=10) and lateral lobe (n=6). The surgery time in the recipients was 559±110 min. Cold and warm ischemia times of the grafts were 68.5±36.8 and 45.6±23.1 min, respectively. The mean graft volume/recipient body weight ratio was 1.23±0.73 and blood loss during surgery in recipients was 98.2±91.0 ml/kg (Table II).

Pre- and post-reperfusion exhaled CO levels were 3.3±0.9 and 7.4±2.5 ppm, respectively. Exhaled CO levels significantly increased after I/R (P=0.00055) (Fig. 1A). This result was almost the same as that in our previous report (14). Pre- and post-reperfusion HO-1 gene expression levels were 0.04±0.05 and 0.13±0.08, respectively. HO-1 gene expression was significantly up-regulated after I/R (P=0.0000013) (Fig. 1B). In contrast, although this result was not statistically significant (P=0.10), ALAS-1 gene expression levels after reperfusion decreased compared to that before reperfusion, from 0.50±0.34 to 0.37±0.40 (Fig. 1C), suggesting that there may be an increase in hepatic free heme levels, which leads to the induction of HO-1, ultimately resulting in heme catabolism.

To confirm that increased HO-1 gene expression levels induce an increase in HO-1, we performed immunohistochemical analysis. Fluorescent analysis revealed that HO-1 levels were increased after I/R (Fig. 2A and B). Additionally, we verified HO-1 localization in the liver using fluorescent double

staining, which showed that HO-1 and CD68 existed in the same cells (Fig. 2C-E). Because the CD68 antigen is specifically expressed by tissue macrophages, including the Kupffer cells, our results verified that HO-1 exist in the Kupffer cells.

Our analysis revealed a significant positive correlation between post-reperfusion exhaled CO levels and HO-1 gene expression levels (r^2=0.19; P=0.0078) (Fig. 3). This result indicated that an increase in exhaled CO levels was caused by increased heme breakdown in the grafted liver. Furthermore, increased HO-1 gene expression levels significantly correlated with postoperative ALT levels (r^2=0.228; P=0.0052) (Fig. 4A), although there were no correlation with AST level (r^2=0.058; P=0.207) (Fig. 4B) or T.Bil level (r^2=0.103; P=0.089) (data not shown). These results suggested that HO-1 gene expression levels indicate the extent of heme breakdown in the grafted liver and results in early graft dysfunction.

Discussion

We conducted a prospective observational study to assess the effect of I/R injury during LDLT on exhaled CO levels and HO-1 mRNA and protein expression. We also assessed the relationship between these indicators and the extent of liver damage. First, we found that exhaled CO levels increased after I/R during LDLT. Second, HO-1 mRNA expression increased in the grafted liver after I/R, and HO-1 increased in the Kupffer cells. In contrast, ALAS-1 mRNA expression did not increase. Third, there was a relationship between increased exhaled CO levels and HO-1 mRNA expression levels. Finally, we found a significant relationship between increased HO-1 gene expression levels and postoperative serum ALT levels.

Increased exhaled CO levels. We found that exhaled CO levels significantly increased after I/R during LDLT. This increase in exhaled CO levels positively correlated with HO-1 gene expression levels in the grafted liver. Previously, we reported that exhaled CO levels significantly increased in critically ill patients in intensive care units and LDLT recipients after reperfusion (14,19). In this study, we again found an increase in post-reperfusion exhaled CO levels. To investigate the source of this increase, we hypothesized that it may indicate heme breakdown caused by oxidative stress caused by I/R injury in humans. In our correlation analysis, the increased exhaled CO levels correlated significantly with increased HO-1 mRNA expression levels in the grafted liver. It is well known that CO is one of the three heme metabolites, and that this heme degradation reaction is mediated by HO-1 (20). It is also well known that this reaction is the only process to produce endogenous CO. Thus, our results and this evidence strongly suggest that the source of increased exhaled CO levels after reperfusion is the increased heme breakdown in the grafted liver, which is mediated by HO-1.

Increased HO-1 mRNA and protein expression levels. HO-1 gene expression levels in the grafted liver increased significantly, but ALAS-1 gene expression levels tended to decrease after I/R. Using immunohistochemistry, we confirmed that the increase in HO-1 gene expression levels led to an increase in HO-1 in the Kupffer cells. To our knowledge, this is the first report that confirms the specific site of HO-1 localization in human liver. Oxidative stress due to I/R injury causes the breakdown of heme proteins and increases free heme levels, which in turn cause cell damage. HO-1 catabolizes free heme into CO, biliverdin, and iron (20,21). According to many animal studies, HO-1 has protective effects against oxidative stress (22-24). We postulated that increased HO-1 levels may be indicated by an increase in free heme levels caused by I/R. Previous reports showed that HO-1 induces heme breakdown in the Kupffer cells in animal models using a hemoglobin-based oxygen carrier injection (18). We found that HO-1 was expressed in the Kupffer cells. These results indicated that the Kupffer cells also play an important role in I/R injury through increased HO-1 expression.

HO-1 and outcome. We also assessed the relationship between HO-1 induction and postoperative early graft dysfunction. We used ALT as a marker of liver injury, since it is believed to be associated with HO-1 (9,10,25). In this study, we found a positive correlation between HO-1 gene expression levels during LDLT and serum ALT levels at the time of skin closure of the recipients. That is, higher HO-1 induction was associated with more severe postoperative early graft dysfunction. Theoretically, if injury is severe, more HO-1 is induced in order to protect cells. Thus, our finding was quite reasonable. However, we were unable to conclude whether HO-1 is effective in protecting the cells. Further studies are needed to elucidate the clinical implications of HO-1 induction in detail. Ideally, future studies should determine whether pharmacological HO-1 induction such as that with glutamine can reduce I/R injury after LDLT.

Limitations. This study has several limitations. First, although we assumed that free heme would increase after I/R, and subsequently HO-1 would be induced, we did not measure free heme levels. However, free heme measurement is not possible at present. Further technological development is required. Second, liver biopsy was only performed twice and graft dysfunction was assessed only once. Thus, we only studied time-specific abnormality rather than the entire process. A study with a larger sample size and longer observation period is required to elucidate the process of HO-1 reaction and its clinical significance.

In conclusion, we demonstrated that increased exhaled CO levels may be due to heme breakdown caused by increased HO-1 after I/R. We also showed that HO-1 is implicated in early graft function during LDLT. Furthermore, a possibility exists that the extent of early graft dysfunction is partly affected by HO-1 expression in the grafted liver.

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