Original

Detection of Nitric Oxide Generation by Electron Spin Resonance Spectroscopy after Partial Hepatectomy

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Abstract: Nitric oxide (NO[•]), a paramagnetic molecule, has a very short half-life of less than five seconds. The direct measurement of NO[•] is therefore difficult and is not commonly performed. The liver is one of the organs in the body that is clearly influenced by NO[•] which is generated in response to septic shock, portal hypertension and liver surgery such as, in the case presented here, partial hepatectomy. To detect time-dependent changes in NO[•] after hepatectomy and regeneration of the liver, we used *ex vivo* electron spin resonance (ESR) spectroscopy by injection of an *N*-methyl-D-glucamine dithiocarbamate (MGD) and Fe²⁺ complex ($[(\text{MGD})_2\text{Fe}]$). After injection of this complex, NO[•] signals were detected by ESR in both regenerating and sham-operated livers. NO[•] generation had increased significantly at 36 h and peaked at 72 h after partial hepatectomy. The expression of inducible nitric oxide (iNOS) mRNA was analyzed by real-time PCR and its maximum level was detected at 72 h after partial hepatectomy. These results indicate that NO[•] may play an important role in regulating liver regeneration after partial hepatectomy.

Key words: nitric oxide (NO[•]), electron spin resonance (ESR), partial hepatectomy, liver regeneration, inducible nitric oxide (iNOS)

Introduction

Liver regeneration refers to the ability to restore liver mass and function after injury or partial hepatectomy. But the removed lobes or segments of this organ are usually not recovered. On the contrary, the restoration of the liver mass occurs with compensatory hyperplasia of the hepatocytes in the remaining lobes. In human liver partial transplantation, a mismatch of liver size often occurs after transplantation. Therefore, the liver has to regenerate sufficiently to fit the body¹⁾ and to do this the liver needs to know when to initiate and when to cease regeneration. It is recognized that the portal blood flow plays a pivotal role in the process of restoration of the liver mass after partial hepatectomy²⁾.

It has been recently demonstrated that the stress from an hepatectomy can induce gene

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expression of *c-fos*, *c-myc*, *c-jun*³⁾ and stimulate nitric oxide (NO[•]) release^{4,5)} which is produced by nitric oxide synthase⁶⁾. These events may in turn trigger the liver regeneration cascade^{5,7-9)}. In addition to liver regeneration, NO[•] protects surviving regenerated hepatocytes and induces tissue regeneration, as reported in transgenic inducible nitric oxide synthase (iNOS) deficient mice¹⁰⁾. Also, NO[•] has been shown to play very important roles in the immediate action after partial hepatectomy. After partial hepatectomy, time dependent production of NO[•] is observed. Obolenskaya *et al*¹¹⁾ have shown that there are three peaks of NO[•] production after partial hepatectomy, the first peak is at 1 h after, the second peak is 4–12 h after and the third peak of NO[•] production is detected at 30 h after the hepatectomy¹¹⁾. Although NO[•] is a relatively short-lived and somewhat reactive free radical *ex vivo*, direct detection of the generated NO[•] is demonstrated enzymatically^{11–14)}. Komarov *et al*¹⁵⁾ and Lai and Komarov¹⁶⁾ have detected NO[•] directly in lipopolysaccharide-induced shocked mice by the use of a *N*-methyl-D-glucamine dithiocarbamate (MGD) and Fe²⁺ complex. This *ex vivo* spin-trapping method is sensitive enough to detect micromolar NO[•] levels produced by iNOS. iNOS has been detected and is readily up-regulated in the liver¹⁷⁾, this contributes to the inflammatory processes through high NO[•] output. Cytokines have been demonstrated to regulate the expression level of iNOS¹⁸⁾.

In the present study we measured time-dependant changes of NO[•] in the liver after partial hepatectomy¹⁹⁾ using an electron spin resonance (ESR) spectrometer. In addition, we also analyzed the time-dependant changes of iNOS mRNA expression by real time PCR in order to determine the function of iNOS during liver regeneration after partial hepatectomy.

Materials and Methods

Animals and partial hepatectomy

Male Balb/c mice between 8 and 10 weeks of age were supplied by Sankyo Labo Service Co. (Tokyo, Japan). Mice underwent a 70% partial hepatectomy according to the method of Higgins and Anderson¹⁹⁾. The partially hepatectomized and sham-operated mice were maintained at a stable depth of anesthesia with sevoflurane. Mice and all animal experiments in the present study were treated in accordance with guidelines outlined in the Animal Care and Use Committee of Showa University.

Assay for radical intensity

Mice were anesthetized with sodium pentobarbital and injected with 200 mM *N*-Methyl-D-glucamine dithiocarbamate sodium salt (MGD; Dojin Co., Kumamoto, Japan) and 200 mM ferrous sulfate (FeSO₄) complex ([3 : 1], 0.4 ml/20 g mice weight) through the jugular vein 2 minutes before liver resection. Mice were sacrificed at 12, 24, 36, 48, 72, and 96 h after partial hepatectomy. Two groups of the mice which were sacrificed 72 h after the partial hepatectomy were injected with *N*-nitro-L-arginine methyl ester (L-NAME; Sigma Chemical Co., Ltd.) dissolved in the saline solution (4 mg/20 g and 2 mg/20 g mice weight) intraperitoneally at 24 h. Once sacrificed, a piece of the remnant right lobe (43 ± 3 mg) was compressed immediately on a Tissue cell and the level of NO[•] measured by a ESR spectroscopy (JEOL JES RE1X, X-band, 100 KHz modulation frequency) at room temperature (22 °C). Instrument settings were as follows: center field, 330 ± 5.0 mT; microwave power, 16 mW; modulation amplitude, 0.5 mT; gain, 5000; time constant, 1 s; scanning time, 8 min. The ESR signal of the [(MGD)₂Fe]-NO complex gave a characteristic 3 peak spectrum, the

height of first peak was measured as the ESR intensity.

RT-PCR

Total RNA was isolated from four regenerated remnant liver tissues, which were frozen at each time points after hepatectomy, using ISOGEN reagent according to the manufacture's instructions (NIPPON GENE, Toyama, Japan). The average RNA yield was approximately 1 μ g RNA/mg mouse liver tissue. Hybridization probes for real-time reverse transcription polymerase chain reaction (RT-PCR) analysis were created using the following gene specific primers and RT-PCR amplification: mouse iNOS: 251 bp-fragment (mouse iNOS forward: 5'-GCA AAC CCA AGG TCT ACG TT-3'; mouse iNOS reverse: 5'-GGA AAA GAC TGC ACC GAA GA -3'; hybridization probes: 5'-TGG TAG CCA CAT CC GAG CCA T-3'-Fluorescein; 5'-LCRed640-CGC ACA TCT CCG CAA ATG TAG AGG-3'-phosphorylation). One microgram of RNA was reverse transcribed for 30 min at 42°C (cDNA-synthesis), followed by 5 min at 95°C (enzyme denaturation) using 1 μ l (0.125 μ M) of oligo-dT-primer (TaKaRa RNA PCR Kit (AMV) ver. 2.1, Kyoto, Japan). The reaction was immediately put on ice and stored at -20°C. PCR analysis was performed using standard protocols. Real time quantitative RT-PCR was undertaken using the Light Cycler from Roche Diagnostics GmbH. Using this, it is possible to detect the amount of template online after each cycle of PCR amplification. Therefore, in addition to the two amplification primers, two further template-specific oligonucleotides, which were fluorescently-labeled according to the FRET-principal (Light Cycler-Fast Start DNA Master Hybridization Probes Kit, Roche Diagnostics GmbH, Germany), were included in the PCR reaction. As a standard reaction, cDNA corresponding to 10⁵, 10⁴, 10³, or 10² copies of total RNA from each sample was examined. Subsequently, cDNAs from different samples were analyzed, each corresponding to 250 ng reverse transcribed, total RNA. To determine the absolute copy number of the target transcript, a cloned iNOS plasmid was used to generate a standard curve. The plasmid DNA was purified according to the manufacture's instructions (Nihon Gene Research Lab's Inc, Miyagi, Japan). The copy numbers for all unknown samples were obtained from the standard curve automatically. The cycling steps were as follows: one cycle of 95°C for 10 min; followed by 40 cycles of 95°C for 10 s; 62°C for 15 s; and 72°C for 9 s.

Statistical examination

All values were expressed as means $\pm$ S.D. Student's t test were used to analyze for significant differences. A P value of less than 0.05 was considered to be significant.

Results

ESR signal of the remnant liver

The [(MGD)₂Fe]-NO complex gave a characteristic 3 peak spectrum (Fig. 1) and the height of the first peak was measured. The NO[•] intensity started to increase at 36 h after the partial hepatectomy and peaked at 72 h (48.4 $\pm$ 5.4 mm). The NO[•] intensity in operated animals at 36, 48, 72, and 96 h was significantly higher than that of the sham-operated ones ($P < 0.05$). The sham-operated animals at each time showed a constant NO[•] intensity (30 $\pm$ 3 mm) (Fig. 2). In the animals (72 h models) which were pre-treated with L-NAME (4 mg/20 g and 2 mg/20 g mice weight) at 24 h after partial hepatectomy, the NO[•] intensity

Fig. 1. ESR spectra of $[(\text{MGD})_2\text{Fe}] \text{NO}$ (a characteristic 3 line spectrum) in mouse liver. (A) Control: NOR-3 (ethyl-2-[hydroxyimino]-5-nitro-3-hexeneamide: $\text{NO}^\bullet$ generator) was trapped with $(\text{MGD})_2\text{Fe}$ in the control liver. (B) 72 h after hepatectomy. (C) 72 h after partial hepatectomy which had been injected at 24 h with L-NAME (4 mg/20 g mice weight).

was a significant difference between 72 h operated group (Fig. 3). There was a significant difference between the L-NAME (4 mg/20 g mice weight) treated and sham operated groups at 72 h ($P < 0.05$). However, there was approximately the same $\text{NO}^\bullet$ intensity between the L-NAME (2 mg/20 g mice weight) treated and the sham-operated groups at 72 h (Fig. 3).

RT-PCR

To investigate the expression of iNOS mRNA in the remnant liver at each time point, four mice in each group were measured. Quantification was performed by real time PCR product analysis using two labeled hybridization probes and light cycler technology. The absolute copy number of iNOS mRNA increased at 72 and 96 h after partial hepatectomy. The highest level of gene expression of iNOS mRNA was observed at 72 h after the operation ($P < 0.05$). However, in the group of animals which were treated with L-NAME (4 mg/20 g mice weight) 24 h after the operation, iNOS mRNA expression was very low and the copy number was significantly less than that of the 72 h group ($P < 0.05$).

Fig. 2. Time-dependant ESR spectra. The first peak of intensity was measured at 12, 24, 36, 48, 72 and 96 h after partial hepatectomy (circles and a solid line: $n=5$) and sham operation (cross and a dotted line: $n=3$) in mouse liver. Each point represents the mean $\pm$ S.D. *, $P < 0.05$ vs. sham operated group.

Fig. 3. ESR spectra of 72 h models after partial hepatectomy. Groups which were injected with L-NAME (4 mg/20 g mice weight) ($n=4$) or L-NAME (2 mg/20 g mice weight) ($n=4$) at 24 h after partial hepatectomy, non treated hepatectomy ($n=5$) and sham operation ($n=3$). Each point represents the mean $\pm$ S.D. *, $P < 0.05$ vs. sham group.

Discussion

In the present study, NO[•] was detected in the mouse liver and moreover this is the first demonstration of time-dependent changes in NO[•] after partial hepatectomy using an *ex vivo* spin-trapping method. NO[•] production after partial hepatectomy was inhibited by L-NAME, a competitive inhibitor of iNOS. *Ex vivo* ESR has been shown to be a useful and reliable tool to study NO[•] generation in a real time¹¹⁻¹⁵. It is reported that NO[•] generation is

Fig. 4. iNOS mRNA expression after partial hepatectomy. Light cycler real time RT-PCR quantification is shown. Each time point, 12, 24, 36, 48, 72 and 96 h and the group injected with L-NAME (4 mg/20 g mice weight) group represents the mean $\pm$ S.D. in four animals. *, $P < 0.05$ vs. 24 h non treated group.

increased three times after partial hepatectomy in accordance with the periodicity of liver regeneration¹¹⁾. The first and second peaks of NO[•] generation are seen at about 1 h and 4–12 h after hepatectomy respectively¹¹⁾. This coincides with the transition of the hepatocytes into the G1 phase of the cell cycle. The third peak of NO[•] generation is observed between 30–37 h after the partial hepatectomy which coincides with the end of the first and the beginning of the second hepatic cell cycle and with the entrance of nonparenchymal cells into proliferation^{11,20)}. We have shown that the peak of NO[•] generation occurs at 72 h after hepatectomy (Fig. 2) and this suggests that the third rise of NO[•] generation is not only the second hepatic cell cycle and nonparenchymal cells proliferation. Soonho *et al*²¹⁾ have shown that portal pressure is significantly elevated in portal branch ligation rats from day 1 after surgery and peaked on day 4. Sato *et al*²²⁾ have shown that portal pressure after 70% partial hepatectomy peaks on postoperative day 3. At this time, it also appears that iNOS mRNA expression has increased to high levels (Fig. 4). We have been unsuccessful at detecting iNOS or other types of NOS's (endothelial and neuronal) by immunohistochemistry, therefore other studies to determine these protein levels are required.

It is shown that inflammatory cytokines such as interleukin-1 β (IL-1 β) induce the expression of iNOS genes in primary cultured rat hepatocytes^{23,24)}. Boulton *et al*²⁵⁾ reported that IL-1 is the major inhibitor of hepatocyte DNA synthesis present in nonparenchymal cells isolated from regenerating liver after partial hepatectomy. These findings strongly suggest that IL-1 plays a pivotal role in suppressing hepatocyte proliferation and terminating the surge of DNA synthesis induced after partial hepatectomy²⁵⁾. Wang *et al*²⁵⁾ have shown that the inhibitory effect of IL-1 on rat hepatocyte DNA synthesis is mediated by NO[•] probably through iNOS gene expression. We have very recently shown in the mouse brain that iNOS immunoreactivity after global ischemia is reduced in IL-1 deficient mice as compared with wild-type mice, suggesting that endogenous IL-1 contributes to brain damage, and this acts through the release of NO[•] produced by iNOS²⁷⁾. The previous and present studies strongly reinforce and extend prior observations on the inhibitory actions of IL-1 on hepatocyte

DNA synthesis acting through NO⁻ production by iNOS expression.

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