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Changes of Nitric Oxide Synthase Activity and Free Methylarginines Contents in Regenerating Rat Liver After Partial Hepatectomy

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In the present study, liver regeneration rate (%) was increased up to 70% 3 days after partial hepatectomy (PH). Nitric oxide synthase (NOS) activity in liver tissue as well as serum nitrite/nitrate content had no timed response, revealing no significant difference between sham-operated and partially hepatectomized rat liver. Contents of free methylarginines in liver tissue were increased biphasically in a time-dependent manner after PH. However, those in serum did not exhibit the same patterns as in liver. Taken together, the results suggest that N^G-monomethyl-L-arginine (MMA) and N^G, N^G-dimethylarginine (DMA) play a role in inhibiting nitric oxide (NO) synthesis in regenerating rat liver because the increase of their contents was synchronized with NOS expression.

Key words : N^G-Monomethyl-L-arginine, N^G, N^G-Dimethylarginine, Nitric oxide, Partial hepatectomy

INTRODUCTION

Liver regeneration is the capability of a liver to replace tissue mass of its original size after tissue removal (Fausto *et al.*, 1994) or pathologic injury (Hoffman *et al.*, 1994). In general, the adult hepatocyte comprising a large portion of liver is normally growth-arrested and highly differentiated (Schaffner *et al.*, 1991). But, it can begin the process of replication and cell division immediately in response to physiological stimuli including PH.

In regenerating rat liver, NO is released in the early steps of regeneration. Obolenskaya *et al.* (1994a) identified at first NO production by the regenerating rat liver by the electron paramagnetic resonance method. It appeared that the fluctuations in NO production are dominant during liver restoration and has an inverse correlation with DNA synthesis although the absolute values of NO production were significantly smaller (Obolenskaya *et al*, 1994b). Consequently, they estimated NO production of regenerating rat liver from the amount of nitrite accumulated during 24 hour in the culture media of hepatocytes, Kupffer cells and sinusoidal endothelial cells isolated at different times after PH (Obolenskaya *et al.*, 1994a). Hortelano *et al.* (1995) also observed the elevation of partially purified NOS activity in the remnant liver and detected NOS mRNA 4 to 6 hours after PH.

In other hands, recent studies have reported that free methylarginines, especially MMA and DMA, play a crucial role in regulating NO synthesis as endogenous inhibitors *in vivo*. Furthermore, they have suggested that arginine or methylarginine metabolism is changed along with NOS activation in wounds (Albina *et al.*, 1988), renal failure (Vallance *et al.*, 1992), glumerulonephritis (Cook *et al.*, 1994), regenerating endothelial cells (Azuma *et al.*, 1995) and septic shock after LPS treatment (Inoue *et al.*, 1993). Thus, the NOS activity and free methylarginines contents in crude liver extracts must be measured to investigate NO function and its regulatory mechanism in regenerating liver because NOS activity measurement after partial purification (Hortelano *et al.*, 1995) excluded the alterations of the contents in NOS cofactors, substrates and inhibitors in accompany with NOS expression after PH (Morris *et al.*, 1994) and NOS activity measurement in isolated liver cells (Obolenskaya *et al.*, 1994b) ruled out autocrine and paracrine effects of hepatocytes, Kupffer cells, and sinusoidal endothelial cells in process of liver regeneration.

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The purpose of this study is to evaluate the role of free methylarginines as endogenous inhibitors of NOS in regenerating rat liver. Therefore, we measured the time course of NOS activity in liver tissue and nitrite/nitrate content in serum. In addition, we quantitated free methylarginines contents in liver tissue and serum to examine the change of them. Finally, we measured the liver regeneration rate as liver regeneration parameter.

MATERIALS AND METHODS

Materials

[2,3,4,5-^{3}H]-L-Arginine (57 Ci/mmole) were purchased from Amersham Life Science, U.S.A. Triethylamine was purchased from Aldrich Chemical Co., U.S.A. Phenylisothiocyanate (PTC) was purchased from Pierce Chemical Co., U.S.A. Acetonitrile and methanol were purchased from Mallinckrodt AR. HPLC.

Animals

Male Sprague-Dawley rats (150~200 g) were purchased from Ansung Cheil Inc. (Ansung, Korea) and housed under conditions of controlled temperature (22~24°C) and illumination (12-h light cycle starting at 8 A.M.) for 1 week in GLP room.

Operation of PH and calculation of regeneration rate

Male Sprague-Dawley rats were subjected to either PH (70%) or sham operation. PH was performed as described by Higgins and Anderson (Higgins *et al.*, 1931; Lee *et al.*, 1993). Following surgery, animals were kept warm until they regained consciousness and were fed *ad libitum*. At various time points after surgery (0, 6, 12, 18, 24, 48 and 72 h), livers were removed after perfusion with 0.9% NaCl and blood was collected under ether anaesthesia. After PH, regeneration rate (%) of the liver was calculated as described by Igarashi *et al.* (1990).

NOS assay (citrulline forming assay)

The activity of NOS was determined using an assay based on the conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline as described (Duval *et al.*, 1995) with minor modification. Enzymatic reactions were carried out at 37°C for 10 min in 50 mM Tris-HCl, pH 7.4, containing 25 μM L-arginine (approximately 200,000 dpm of L-[2,3,4,5-^{3}H]-L-arginine HCl: 57 Ci/mmole: Amersham Life Science), 1 mM NADPH, 2 μg calmodulin, 1 mM DTT, 1.5 mM $CaCl_2$, 100 μM (6R)-5,6,7,8-tetra hydrobiopterin (H4B), 10 μM FAD, 10 μM FMN, 50 mM valine, 1 mM citrulline, 1 mM ornithine and enzyme source (0.6~1.0 mg liver cytosol protein) in a final incubation volume of 200 μl. Reactions were terminated by the addition of 1 ml of ice-cold Dowex-50W, Na^+ form pre-equilibrated with 20 mM sodium acetate buffer, pH 5.5, containing 1 mM citrulline, 2 mM EDTA and 0.2 mM EGTA (stop buffer), and the reaction mixtures were left in ice at 4°C for 5 min and centrifuged at 11,000×g for 10 min. The supernatant (400 μl) was collected into a suitable water-miscible scintillant and the radioactivity was counted in a Pharmacia 1209 Rackbeta liquid scintillation counter.

Nitrite/nitrate determination in serum

Nitrite/nitrate content was quantitated colorimetically by adding Griess reagent (0.5% naphthylethylene diamine-dihydrochloride, 5% sulfanilamide, 25% H_3PO_4) to the serum following enzymatic conversion of nitrate to nitrite by nitrate reductase (EC 1.6.6.2) (Bories *et al.*, 1995 ; Moshage *et al.*, 1995).

Quantitation of free methylarginines in liver tissue and serum

Contents of arginine, MMA, DMA and D'MA in liver tissue and serum were determined by means of automated high-performance liquid chromatography (HPLC) according to the method described by Kakimoto *et al.* with minor modification (Ueno *et al.*, 1992).

Preparation of free methylarginines fraction

Rat liver tissues (wet weight: 1 g) or serum (1 ml) were homogenized in 9 volumes of ice-cold 5% (wt/vol) trichloroacetic acid (TCA). Homogenates were then centrifuged at 15,000×g for 20 min at 4°C. The supernatants were applied to a column of Dowex-50W column (H^+, 100-200 mesh, 0.4×4.5 cm), previously equilibrated 5% (wt/vol) TCA. The column was washed with 10 ml of distilled water (DW) and afterwards 10 ml of 1 M pyridine. The absorbed basic amino acids were eluted with 10 ml of 3 M ammonia. The eluates were quickly dried under vacuum using a lyophilizer, and these residues were used in the determination of free methylarginines.

Derivatization of methylarginines

PTC derivatization of the fraction was carried out under the conditions described by Bidlingmeyer *et al.* (1984).

Chromatographic conditions and peak identification

The HPLC system was composed of Waters solvent delivery system (Waters 501 pumps and Waters 680 gradient controller), a Shodex C18 5B column (4.6×250 mm, 5 μm), Waters 480 absorbance detector and Gilson autoinjector system (Gilson autoinjector and Gilson 712 control program). The elution solvent sys-

Table I. Gradient program for reverse-phase HPLC for the separation of PTC-derivatized amino acids

Time (min)	Gradient solvent A (%)	Column Temp. (°C)
Initial	100	43
10.00	100	
10.01	97	
20.00		
40.00	95	
45.00		
45.20		
55.00	95	
65.00	85	
69.00	70	
70.00	0	
80.00	0	
85.00	100	
100.00	next injection	

tem consisted of solvent A [2.5% (vol/vol) acetonitrile in 70 mM sodium acetate buffer, pH 6.5] and solvent B [45% (vol/vol) acetonitrile and 15% (vol/vol) methanol in water]. The flow rate was 1.2 ml/min, the gradient used is described in Table 1. and chromatographic analysis was carried by measuring the absorbance at 254 nm and the a.u.f.s. at 0.01.

The calibration curves were made as peak area after the analysis of standard methylarginines (MMA, DMA, D'MA) 0.125 nmole, 0.25 nmole, 0.5 nmole, respectively. MMA, DMA, and D'MA peaks in samples were identified as increase of peak area at identical time following HPLC analysis of samples fortified with standard methylarginines as well as retention time (liver tissue: 250 pmole, serum: 1 nmole).

Protein determination

Protein contents of the enzyme preparations were measured by the Bradford method (Bradford *et al.*, 1976), using bovine serum albumin as a standard.

Statistical analysis

The results were expressed as mean plus or minus the standard error. Statistical analysis was performed by using a two-tailed Student's t test. A difference with a p value of <0.05 was considered statistically significant.

RESULTS

Determination of liver regeneration rate after PH

Liver regeneration rate (%) is the good parameter for liver regeneration, although its calculating equation is not always consistent with all experiments. In our experiment, liver regeneration rate after PH was calculated at various time points (0, 3, 6, 12, 24, 48, and 72 hour) to verify the degree of mass recovery as described in materials and method. Regeneration rate in liver after PH was increased about 70% 3 days later (Fig. 1).

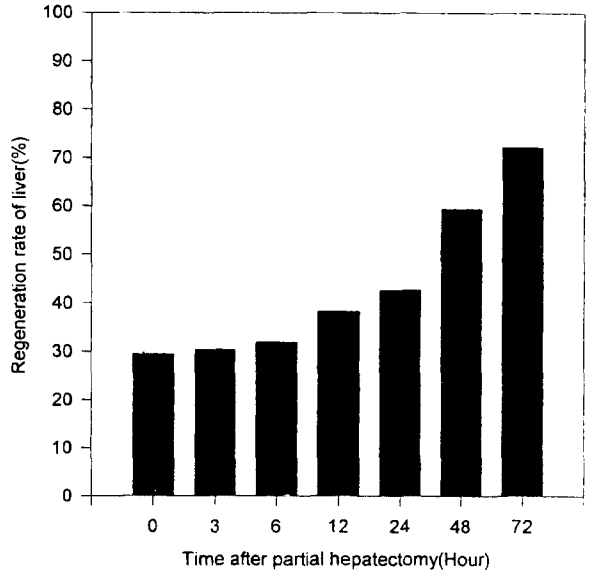

Fig. 1. Determination of liver regeneration rate after PH. Liver regeneration rate (%) was calculated at various time points (0, 3, 6, 12, 24, 48, 72 Hour) as described in materials and methods. Values are the means of 4 independent experiments.

Changes of NOS activity in regenerating rat liver after PH

NOS activity in crude liver tissue after PH exhibited no significant difference between sham-operated rat and partially hepatectomized rat (Fig. 2) This observation could be caused by the fact that NOS activity during liver regeneration is too small to be detected in crude liver tissue in comparison with that of purified liver NOS.

Changes of nitrite/nitrate content in serum from rats that underwent hepatectomy

Nitrite/nitrate content in serum fluctuated in partially hepatectomized rat as well as in sham-operated rat, but difference between two groups was not significant (Fig. 3). On the contrary, nitrite/nitrate content in serum remarkably increased after addition of LPS likewise other previous reports (Data not shown). This result supported the previous data (Fig. 2) that the elevation of NOS activity after PH is very small in intact liver.

Peak identification of arginine, MMA, DMA, and D'MA on HPLC sample chromatograms

The retention time of arginine, MMA, DMA, and D'MA were about 31.5 min, 46.5 min, 61.5 min and 72.0 min, respectively. Peaks for methylarginines were

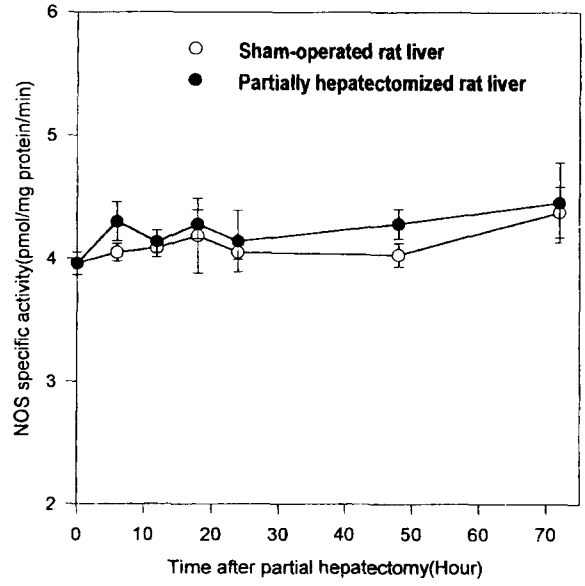

Fig. 2. Change of NOS activity in regenerating rat liver after PH. All reactions were performed at 37°C and the enzymatic activity was followed by the synthesis of L-citrulline from L-[2,3,4,5-^{3}H]-L-arginine. Results are the means±SEM of 4-6 separate experiments, or the means of a representative experiment assayed per duplicate.

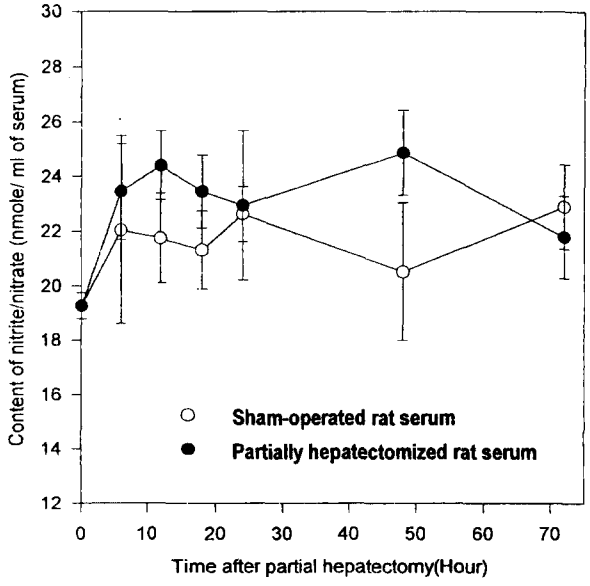

Fig. 3. Change of nitrite/nitrate content in serum from rats that underwent hepatectomy. Nitrite/nitrate content was quantitated colorimetically by adding Griess reagent to serum after enzymatic conversion of nitrate to nitrite by nitrate reductase (EC 1.6.6.2). Results given represent means±SEM of nmole/ml of serum in 5 separate experiments.

identified comparing sample chromatograms with those fortified with known amounts of standards as well as retention time (Fig. 4). Although the biological sample is complicated, this approach can reinforce the accuracy of peak identification.

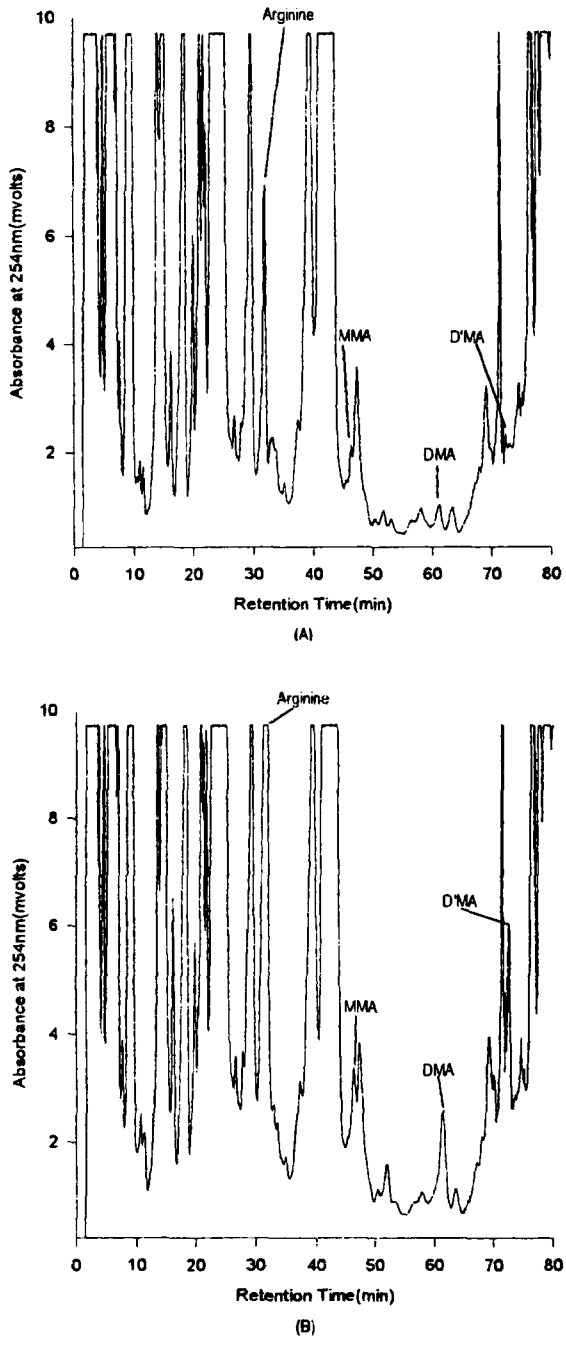

Fig. 4. HPLC chromatogram of PTC-derivatized amino acids in liver tissue (A) and fortified liver tissue with standard methylarginines (B) Arg: 1 nmole MMA: 250 pmole DMA: 250 pmole D'MA: 250 pmole.

The time course of arginine, MMA, DMA, and D'MA contents in regenerating rat liver after PH

Contents of MMA and DMA significantly increased biphasically at early stages of the regenerating liver and the content of arginine also increased up to 6 hour and then decreased after PH. (Fig. 5) In particular, the increase of MMA and DMA contents(two

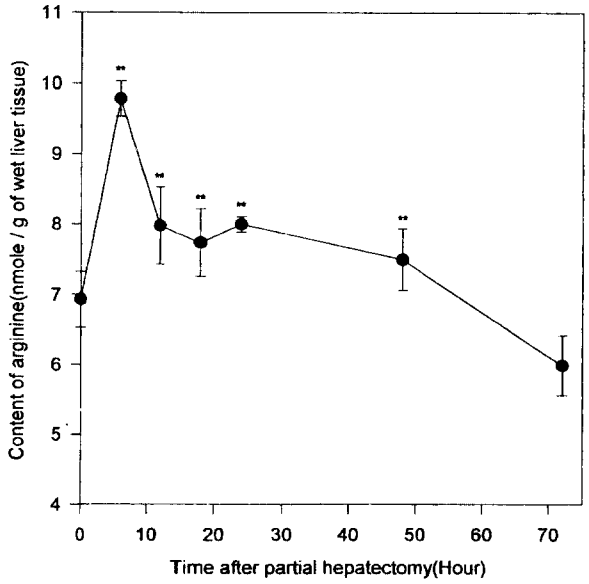

Fig. 5. The time course of arginine content in regenerating rat liver after PH. Control liver tissues were used as the removed liver in operating PH as well as sham-operated liver and regenerated liver tissues were used as remnant liver after PH. Analysis was carried out by means of HPLC. Results are given as means±SEM of 4 separate experiments. Significant difference vs. control at *P<0.05, **P<0.01 (two-tailed Students's t-test).

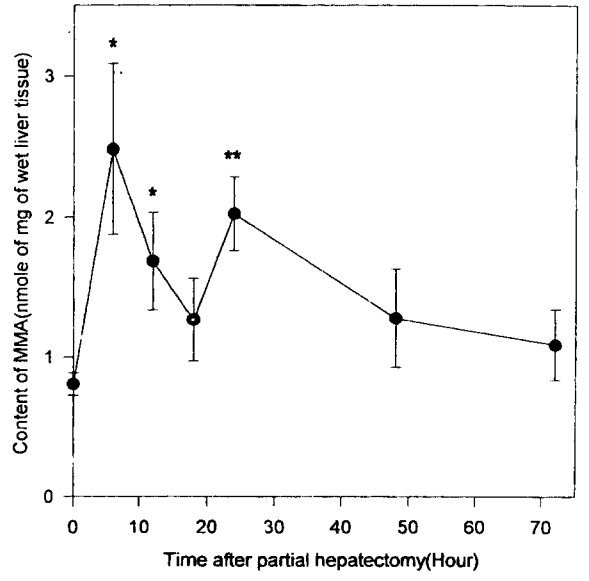

Fig. 6. The time course of MMA content in regenerating rat liver after PH. Control liver tissues were used as the removed liver in operating PH as well as sham-operated liver and regenerated liver tissues were used as remnant liver after PH. Analysis was carried out by means of HPLC. Results are given as means±SEM of 4 separate experiments. Significant difference vs. control at *P<0.05, **P<0.01 (two-tailed Students's t-test) *MMA: N^G-monomethyl-L-arginine.

to three folds) at the period between 6 hour and 18 hour was synchronized with the induction of NOS after PH reported in previous study (Hortelano *et al.*, 1995) (Fig. 6, Fig. 7).

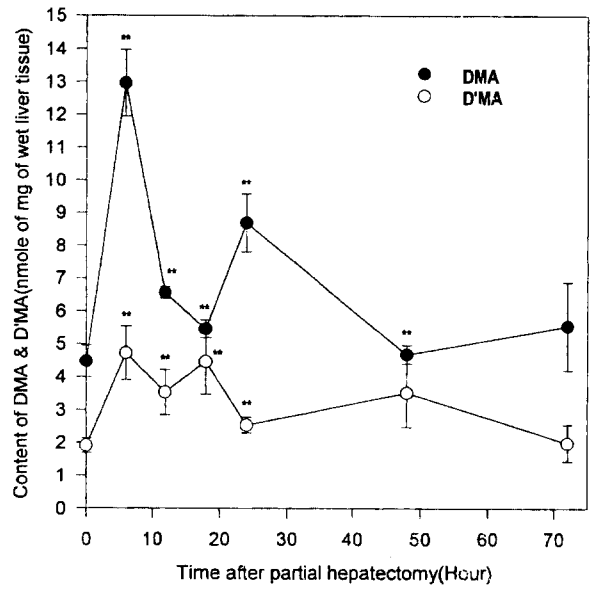

Fig. 7. The time course of DMA and D'MA contents in regenerating rat liver after PH. Control liver tissues were used as the removed liver in operating PH as well as sham-operated liver and regenerated liver tissues were used as remnant liver after PH. Analysis was carried out by means of HPLC. Results are given as means±SEM of 4 separate experiments. Significant difference vs. control at *P<0.05, **P<0.01 (two-tailed Student's t-test) DMA: asymmetrical N^G,N^G-dimethylarginine D'MA: symmetrical N^G,N'^G-dimethylarginine.

The results obtained from this experiment suggest that free methylarginines probably regulate NOS activity expressed in early regenerating rat liver after PH and partially provide the evidence that the change of NOS activity in crude liver tissue after PH is not significant. Finally, these results demonstrate for the first time that the alterations of arginine and free methylarginines metabolism in liver occur in response to PH.

The time course of MMA, DMA, and D'MA contents in partially hepatectomized rat serum

Contents of free methylarginines in serum more or less turned out to be high at 6 hour, particularly DMA, but the change patterns of them were not clear (Table II). The observation suggest that the contents of serum free methylarginines seem not so much to be influenced in response to PH, although the content of MMA and DMA exhibit fluctuation. However, the cellular origin of free methylarginines must be iden- tified when they increase and fluctuate, especially DMA.

DISCUSSION

In the mammalian organ, liver is unique in its ability to regenerate and regain its original functions (Hoff-

Table II. The time course of MMA, DMA, and D'MA contents in the partially hepatectomized rat serum

	Serum Content (nmole/ ml of serum)				
	Hour[a]	N[b]	MMA[1]	DMA[2]	D'MA[3]
Control rat		18	0.30±0.03	0.55±0.04	0.07±0.01
Partially hepatectomized rat serum	6	4	0.45±0.02**	0.86±0.08**	0.18±0.07
	12	4	0.44±0.15	0.80±0.24	0.14±0.05
	18	4	0.49±0.13	0.76±0.15	0.12±0.08
	24	4	0.37±0.15	1.15±0.17**	0.08±0.01**
	48	4	0.34±0.11	1.10±0.12**	0.07±0.01
	72	4	0.57±0.16	0.97±0.09**	0.16±0.07**

In this experiment, control rat serums were used as normal rat serums as well as sham-operated rat serums and partially hepatectomized rat serum were used as serums from rats that underwent hepatectomy. Analysis was carried out by means of HPLC. Results are given as mean±SEM. Significant difference vs. control at *P<0.05, **P<0.01(two-tailed Students's t-test).
1: N^G-monomethyl-L-arginine, 2: N^G,N^G-dimethylarginine, 3: N^G,N'^G-dimethylarginine, [a]: time after PH, [b]: experimental numbers.

man *et al.*, 1994). Restoration of the liver mass after PH occurs as the results of both hypertrophy and hyperplasia (Fausto *et al.*, 1994). In this study, liver regeneration rate (%) increased about 70% 72 hour after PH. This observation indicated that liver is predominantly regenerated during early phase after PH.

In addition to liver mass recovery, regenerating liver regains the liver function by the action of various cytokines responsible for growth control (Steer, 1995). In response to these cytokines, particularly TNF-α, the ability of hepatocyte as well as Kupffer cells to express iNOS is now well accepted despite the controversy (Curran *et al.*, 1990). Accordingly, the synthesis of NO in regenerating liver after PH is probably caused by the combined action of various cytokines containing TNF-α that are released during the early steps in regenerating liver (Hortelano *et al.*, 1995). Our study showed that NOS activity in crude liver tissue had no timed response, revealing no significant difference between sham-operated and partially hepatectomized rat liver. Serum nitrite/nitrate content showed no marked elevation following PH despite its fluctuations. These results explained that the change of NOS activity after PH is too small to be detected in crude liver tissue compared with that under other conditions. Because NOS activity in crude liver tissue homogenates is rarely detected, it is unclear what factors are limiting for NO production by intact liver (Morris *et al.*, 1994).

In this sense, although previous studies detected NOS mRNA 4 to 6 hour and remarkable difference of partial purified NOS activity 4 to 18 hour between sham-operated and partially hepatectomized rat liver after PH, NO production in intact liver can be distinctively smaller by the coordinated action of other factors possibly including endogenous inhibitor, free methylarginines. Therefore, small NO produced in regenerating liver may not act as cytotoxic molecules, but may act as signaling molecules such as activitor of guanylate cyclase (Billiar *et al.*, 1992) or inhibitor of ribonucleotide reductase (Lepoivre *et al.*, 1990) and chemotactic agent for neutrophils (Kalpan *et al.*, 1989) during liver regeneration.

In other sense, the arginase and NOS sharing a common substrate, arginine, are found in the liver after PH and thus the interaction of two pathways or possible regulation of one enzyme by the other as well as the effects of other urea-cycle enzyme, argininosuccinate synthetase and argininosuccinate lyase, have been considered as that result (Beaudet *et al.*, 1986; Nussler *et al.*, 1994). However, the exact relationships of two pathways remain to be elucidated, although a few studies has been reported in other states such as inflammation (Cook *et al.*, 1994; Daghigh *et al.*, 1994).

Since many inhibitors of NOS have been found, MMA and DMA among them are particularly not synthetic, but naturally occurring substances in animal tissues (Vallance *et al.*, 1992; Azuma *et al.*, 1995; Ueno *et al.*, 1992). Thus, it is meaningful to investigate the change of their content after PH for the identification of NOS regulatory mechanism in regenerating rat liver.

In our study, contents of free methylarginines in liver tissue after PH varied biphasically with time-course in hepatectomized rat liver. Remarkably, contents of MMA and DMA as well as arginine were increased at 6 to 18 hour, known as the period of NOS induction after PH by previous reports (Hortelano *et al.*, 1995). However, those in serum did not exhibit this patterns. The results obtained in this work suggest that the alterations of arginine and methylarginines metabolism in regenerating liver occur in prereplicative phase and play a role in NOS regulatory process. In addition, these phenomena may affect the balance between NOS activity and arginase activity in liver after PH, which could be supported by the previous report that the inhibition of NOS with MMA *in vitro* lead to a significant increase in arginine metabolism to urea in nephritic glomeruli by arginase pathway. In this case, MMA does not inhibit arginase activity or serve as substrate for arginase, but inhibit NOS activity (Cook

et al., 1994; Hibbs *et al.*, 1990; Hrabak *et al.*, 1994; Robertson *et al.*, 1993), by which implicate in modulating some aspects of the immune response as well as in the production of ornithine for the synthesis of the polyamines (Terakura *et al.*, 1995; Tabor *et al.*, 1984) and proline (Yamamoto *et al.*, 1995; Smith *et al.*, 1978), a promoter of cell proliferation and a precursor of collagen synthesis, respectively. These findings may suggest that free methylarginines, endogenous inhibitors of NOS, contribute to small release of NO and large production of ornithine for positive role in liver regeneration following PH in intact liver.

In conclusion, we have investigated the possibility of free methylarginines as endogenous inhibitors of NOS in regenerating rat liver after PH. From results of this study, following hypothesis is plausible. NOS and urea-cycle enzymes such as arginase in liver are co-induced after PH and afterwards arginine uptake into liver cells increased for substrate availability of NOS and arginase. Since arginine has a more high affinity for NOS than arginase, arginine easily can be converted to NO via NOS pathway, which may cause the detrimentals for regenerating liver cells by the cytotoxicity of large amount of NO and the deficiency of ornithine essential for cell proliferation. Therefore, it is likely that the elevation of free methylarginines content in liver during NOS induction is beneficial to prevent NOS pathway in part and activate arginase pathway, because methylarginines cannot act as an inhibitor for arginase, but preferably as a substrate, whereas they can act as a strong inhibitor for NOS. As a result of these events, liver can effectively regenerate in prereplicative phase after PH.

However, it is necessary to investigate the arginase activity, ornithine content as well as polyamines content, and free methylarginines metabolism to clarify this putative mechanism. In addition, development of accurate NOS assay method could dissolve the problems with detection in crude liver extractions of little difference of the NOS activity in response to PH.

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