J. Biochem. 124, 892-899 (1998)

# Post-Isolation Inducible Nitric Oxide Synthase Gene Expression Due to Collagenase Buffer Perfusion and Characterization of the Gene Regulation in Primary Cultured Murine Hepatocytes[1]

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Received for publication, May 6, 1998

**The traditional two-step EGTA/collagenase method is widely used in studying nitric oxide (NO) production in hepatocytes. The present study first revealed that hepatocytes isolated by this method spontaneously express an iNOS mRNA. Thereafter, based on this novel finding, we characterized the expression and regulation of the gene in primary cultured hepatocytes. Using Northern blot analysis, the iNOS mRNA was observed 4 h after isolation, reached peak at 8 h, and declined to an undetectable level after 24 h. iNOS gene expression was shown to be serum-independent and not due to lipopolysaccharide contamination. Time-course analysis of the effects of actinomycin D demonstrated that the increase in iNOS transcripts is the result of an accompanying great increase in iNOS gene transcription and lower iNOS mRNA stability; also blockage by cycloheximide suggests that it is dependent on *de novo* protein synthesis. Inhibition by pyrrolidine dithiocarbamate, a NF-$\varkappa$B/c-rel inhibitor, further implies the involvement of NF-$\varkappa$B/c-rel. To clarify reason(s) for the induction, hepatocytes were isolated with the collagenase buffer perfusion step omitted. As a consequence, iNOS mRNA was undetectable in the hepatocytes. These findings show that the traditional hepatocyte-isolation culture does indeed transiently express a serum-independent but *de novo* protein synthesis-dependent iNOS mRNA due to collagenase (type IV) buffer perfusion.**

**Key words: collagenase buffer perfusion, gene expression, inducible nitric oxide synthase, murine primary hepatocyte, nuclear factor-$\varkappa$B.**

Nitric oxide (NO) is a reactive, gaseous, lipophilic molecule that functions at high concentrations as a defensive cytotoxin against tumor cells and pathogens, and as a signal in many diverse physiological processes including blood flow regulation, neurotransmission, learning, and memory (*1-5*). In some degree, however, NO is a double-edged sword (*6-8*). Constitutive and inducible isoforms of NO synthases exist, which differ in structure and regulation (*2*). The inducible NO synthase (iNOS) isozyme is critical for the immune reponse, but is also implicated in most diseases involving NO overproduction (*3, 4*). The iNOS pathway was first characterized in macrophages activated by LPS and/or IFN-$\gamma$ (*9*). Following the discovery of the NO pathway, a variety of other cell types such as endothelial cells, smooth muscle cells, hepatocytes, and neurons have been shown to produce NO from L-arginine (*2, 10*).

There have been many reports that rat, mouse, and human hepatocytes stimulated with bacterial lipopolysaccharide (LPS), parasite-derived factors, or a mixture of them and/or cytokines including tumour necrosis factor $\alpha$ (TNF-$\alpha$), interleukin 1 (IL-1), and interferon gamma (IFN-$\gamma$) produce large amounts of nitrite and nitrate, the stable end products of NO metabolism (*11-15*). The physiological significance of NO biosynthesis in the liver is beginning to be elucidated. Meanwhile, primary cultured hepatocytes isolated by the two-step EGTA/collagenase method (ECM) are widely used in elucidating hepatocyte NO production and its regulation mechanism (*11-14, 16*). Apart from hepatocytes, it has been shown that shear stress can induce NO synthase gene expression in endothelial cells (*17, 18*), and that some proteases appear to be associated with iNOS expression (*19, 20*). In addition, it is well known that cell-to-cell interactions are important regulators of cell function. However, almost nothing is known about the

[1] This work was supported in part by a Grant-in-Aid for Scientific Research (No. 09670258) and a JSPS Fellowship from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: DMEM/F12, Dulbecco's modified Eagle's medium/Ham's F-12 mixtured medium; ECM, EGTA/collagenase method; FBS, fetal bovine serum; NO, nitric oxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBSS, Hanks' balanced salt solution; iNOS, inducible NO synthase; IFN-$\gamma$, interferon gamma; LPS, lipopolysaccharide; NF-$\varkappa$B/c-rel, nuclear factor-$\varkappa$B/c-rel; PBS, phosphate-buffered saline; PTDC, pyrrolidine dithiocarbamate; PKC, protein kinase C; PNE, post-isolation iNOS gene (mRNA) expression.

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effects of the traditional two-step hepatocyte isolation procedure using EDTA (or EGTA) and collagenase on iNOS gene expression.

The present study was undertaken (a) to determine whether hepatocytes isolated by the traditional ECM express a detectable iNOS mRNA soon after their isolation without addition of iNOS-inducing stimuli such as LPS or IFN-$\gamma$, and if so (b) to characterize the regulation of iNOS mRNA induction, and (c) to investigate the cause(s) of iNOS mRNA expression. We found that post-isolation murine hepatocytes isolated by the traditional ECM do express a serum-independent detectable iNOS mRNA due to collagenase buffer perfusion.

## MATERIALS AND METHODS

*Reagents and Animals*—Dulbecco's modified Eagle's medium/Ham's F-12 mixtured medium (DMEM/F12) was purchased from ICN Biomedicals (CA, USA). Ethylene glycol-bis-($\beta$-aminoethyl)-*N,N'*-tetraacetic acid (EGTA) was obtained from Wako Pharmaceutical (Tokyo). FBS was obtained from Equitech-Bio (TX, USA). Pentobarbital sodium was purchased from Dainippon Pharmaceutical (Osaka). Lipopolysaccharide (LPS, *Escherichia coli* 005: B5) was obtained from Difco Laboratories (Detroit MI, USA). Pyrrolidine dithiocarbamate (PTDC), actinomycin D, cycloheximide, dexamethasone, polymyxin B, type IV collagenase (for hepatocyte isolation), Percoll, and trypsin inhibitors were purchased from Sigma Chemical (St. Louis, MO, USA). Tris and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laboratories (CA, USA). INC Pharmaceuticals (CA, USA) was the source of [$\alpha$-$^{32}$P]-dCTP. The concentrations of LPS in all buffers and media used were less than 0.03 EU/ml (=0.125 ng/ml) as measured by a *Limulus* amebocyte lysate test kit purchased from Associates of Cape Cod (MA, USA).

Eight-week-old male ICR mice weighing 40–45 g were obtained from Shimizu Experimental Animal (Shizuoka Prefecture). The mice were housed under standard lighting conditions (lights on from 0700–2000 h) and maintained in the Laboratory Animal Center, Faculty of Medicine, Tottori University. The animals received humane care in compliance with guidelines of the National Institutes of Health.

*Preparation of Hepatocytes and Their Culture*—Experiment I: Hepatocytes were isolated in the traditional way from male ICR mice using the hepatic portal perfusion technique developed by Seglen (*21*) as modified by Klauning *et al.* (*13, 22*). Briefly, after the mice were anaesthesized with pentobarbital sodium (i.p., 0.5 mg per mouse), the livers were perfused at a flow rate of 8 ml/min first with calcium- and magnesium-free Hanks' balanced salt solution (HBSS) containing 0.5 mM ethylene glycol-bis-($\beta$-aminoethyl)-*N,N'*-tetraacetic acid (EGTA) and 0.05 M *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES, pH 7.35) for 4 min and then with HBSS containing 0.05% type IV collagenase for hepatocyte isolation, 0.005% Turchey-derived trypsin inhibitor (to rule out the possibility of trypsin contamination), and HEPES (pH 7.5) maintained at 37°C for 6 min. Instead of recirculating the perfusate, both the Hanks' and collagenase solutions were allowed to run as waste through a cut made in the subhepatic inferior vena cava. For Experiment II, hepatocytes were isolated by deleting the collagenase buffer perfusion step, while the EGTA buffer perfusion time was extended to 30 min. Afterward, a crude cell suspension was obtained by differential centrifugation at $50\times g$ five times (1 min each, 4°C), and then passed over a 45% Percoll gradient to obtain a highly purified cell population. Hepatocyte purity, examined by microscopy, was greater than 99% and the viability consistently exceeded 98% by trypan blue exclusion. Hepatocytes were plated at a density of $2\times10^6$ cells/60 mm collagen-covered culture dishes (Sumitomo Bakelite, Tokyo). The medium consisted of DMEM/F12 with 10 mM HEPES and 20 mM $NaHCO_3$ supplemented with 5% FBS, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin. The hepatocytes were cultured at 37°C under a humidified atmosphere (5% $CO_2$). After attachment for 4 h, cells were washed three times with PBS (pH 7.2), and then maintained in a serum-free or 5% FBS medium. Thereafter, the media were changed every 24 h. Hepatocytes were stimulated with different reagents, and total RNAs were isolated at the times indicated.

*Preparation of RNA*—The total RNA from cultured murine hepatocytes was prepared by using an ISOGEN Kit according to the manufacture's protocol (*13*) (Nippongene, Tokyo). Briefly, the hepatocyte monolayer was lysed in the ISOGEN solution (1 ml/60 mm culture plate). The homogenates of cell lysates were stored at room temperature for 5 min, and then treated with 0.2 ml chloroform for 3 min. The mixture was centrifuged at $12{,}000\times g$ in a Hitachi refrigerated centrifuge for 15 min at 4°C. The upper aqueous phase was collected and treated by adding 0.5 ml isopropanol for 10 min and then centrifuged for 10 min under the same conditions. The RNA pellet was washed in 75% ethanol and dried after centrifugation. The RNA was dissolved and stored at −20°C. All RNA samples had an $OD_{260}/OD_{280}$ ratio > 1.50.

*Molecular Probes*—A murine macrophage iNOS cDNA consisting of a 823 base pair fragment, cloned into a pGEM plasmid (*23*) was kindly provided by Dr. Thomas A. Hamilton and Dr. Dennis J. Stuehr (Cleveland Clinic Foundation, USA). The iNOS cDNA plasmid (100 ng) was labeled with [$\alpha$-$^{32}$P]dCTP by the random priming method with a Takara BcaBEST labeling kit (Takara Biomedicals, Tokyo); a specific activity of $1\times10^9$ dpm/$\mu$g DNA was obtained. A plasmid containing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, used as a control, was kindly provided by Dr. David Stern (Columbia University, USA). The 18S rRNA probe is a 5.4-kb fragment of rat rRNA genomic DNA isolated from the cloned plasmids by *Hin*dIII and *Eco*RI restriction enzyme digestion as described previously (*24*).

*Northern Blot Analysis*—This was carried out by the method previously described (*25*). Total RNA samples were denatured in 50% formamide and 2.2 M formaldehyde at 55°C for 15 min. Aliquots containing 15 $\mu$g of total RNA were loaded onto a 1% agarose gel containing 2.2 M formaldehyde and electrophoresed using a running buffer containing 50 mM MOPS−acetate (pH 7.0), 10 mM sodium acetate, and 1 mM EDTA. Ribosomal RNAs (28S and 18S) were visualized under UV light after ethidium bromide staining to ensure the integrity of the RNA samples. The RNA was then transferred to MSI nylon membranes (Westborough, MA, USA) by capillary transfer and UV autocrosslinked.



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*Hybridization*—Membranes were prehybridized in 5× SSPE (1×SSPE=180 mM NaCl, 10 mM $NaH_2PO_4$, 1 mM EDTA, pH 7.4), 50% formamide, 5×Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 300 μg salmon sperm DNA/ml, for 20 h at 42°C. The prehybridization solution was then replaced with fresh hybridization solution containing the labeled probe at a final concentration of $1\times10^6$ (cpm/ml) and the membranes were hybridized for 24 h at 42°C. The blots were washed three times at 65°C in 1× SSC (=0.15 M NaCl, 0.015 M sodium citrate, pH 7.2), 0.1% SDS for 10 min. The blots were then exposed to Kodak X-AR film (Eastman Kodak, Rochester, NY, USA) at −80°C for 3-4 days. After hybridization with the iNOS cDNA probe, the membranes were stripped by boiling in 5 mM EDTA/0.1% SDS and rehybridized with probes specific for GAPDH mRNA and/or 18S rRNA as controls for variations in the amount of RNA per lane. Unless otherwise specified, each Northern blot shown is representative of at least three separate experiments performed at different times.

## RESULTS

*Post-Isolation iNOS Gene Expression (PNE) in Traditionally Isolated Primary Cultured Murine Hepatocytes*—It has been reported that LPS, IFN-γ, and some other cytokines (or a combination of them) can induce iNOS gene expression in murine hepatocytes (*13-15*); in the case of endothelial cells, shear stress is also capable of inducing iNOS gene expression (*17, 18*). To identify the effects of the hepatocyte isolation procedure itself (including the reagents used, changes in cell-to-cell contact, possible mechanical damage, and shear stress on the hepatocytes), we first examined iNOS mRNA expression in ECM-isolated primary cultured hepatocytes. We found that the iNOS mRNA was detectable 4 h after isolation in hepatocytes cultured in the medium with 5% FBS. The iNOS mRNA reached a peak at about 8 h, maintained that level for over 4 h, and then declined to an undetectable level 24 h after isolation (Fig. 1A), suggesting that it is expressed in a time-dependent fashion. To further examine the influence of FBS, the experiment was also done using serum-free medium. A similar pattern of iNOS mRNA expression was observed (Fig. 1B). Similar results were obtained when ordinary plastic dishes were used instead of collagen-covered culture ones (data not shown). We failed to detect NO in the hepatocyte incubation medium, which may be due to the low sensitivity of our detection method. These results, taken together, show that ECM-isolated primary cultured murine hepatocytes express a serum-independent post-isolation iNOS mRNA without addition of LPS and/or cytokine, and that collagen covering the dish is not necessary for the gene expression. In the lanes of control murine liver tissues, the iNOS mRNA was undetectable.

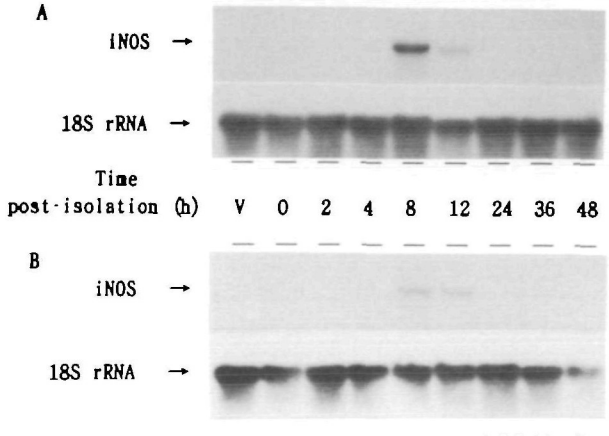


Fig. 1. **Spontaneous iNOS mRNA expression in ECM-isolated primary cultured hepatocytes.** (A) Hepatocytes were cultured in medium with 5% FBS. (B) Hepatocytes were cultured in serum-free medium. Lane V is a normal murine liver tissue control. The times (h) shown are the culture time after isolation. Northern hybridization was performed using an iNOS cDNA probe. The same blot was stripped with strip buffer and rehybridized for18S rRNA as described in "MATERIALS AND METHODS." Blots are representative of a series of four blots, all of which gave comparable results.

*Effect of Polymyxin B on PNE of Hepatocytes*—LPS alone or in combination with IFN-γ, TNF-α, and/or IL-1 has been shown to be capable of triggering iNOS gene expression in hepatocytes (*4, 11, 13*). Hence, the possibility of LPS contamination being involved in the iNOS gene expression should be considered. To rule this out, we first measured the concentration of LPS in the buffers and media used. It was found to be extremely low—less than 0.03 EU/ml as quantified by *Limulus* amebocyte lysate assay. To further ensure there was no possibility of LPS contamination giving rise to the iNOS mRNA induction, a neutralization experiment was performed using the well-known LPS-activity neutralizing agent, polymyxin B (*26, 27*). PNE was not neutralized by polymyxin B (Fig. 2), but it did downregulate the iNOS gene expression of murine hepatocytes induced by LPS (*13, 14*) (data not shown). These results indicate that the iNOS mRNA expression is not due to LPS contamination.

*Regulation of Post-Isolation iNOS mRNA Expression by Dexamethasone, Actinomycin D, Cycloheximide, and Pyrrolidine Dithiocarbamate*—Glucocorticoids have been shown to downregulate iNOS gene expression in both rat and murine hepatocytes, whereas the effect of cycloheximide varies with cell type (*11, 13*). Moreover, the promoter of iNOS contains several well-known *cis* elements, including GAS, NF-κB, AP-1, and ISRE (*28*). To investigate the effects of the above-mentioned reagents and identify the

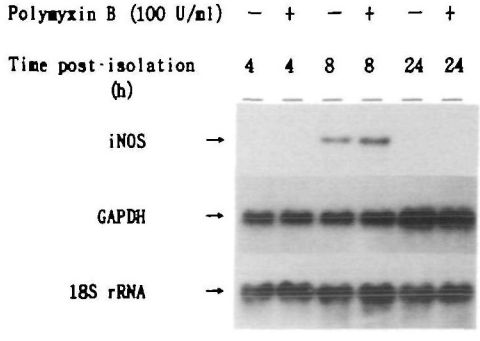


Fig. 2. **Polymyxin B did not suppress the post-isolation iNOS mRNA expression of hepatocytes.** Polymyxin B (100 U/ml) was added soon after hepatocyte isolation. The times (h) shown are the incubation time after isolation. Northern hybridization was performed using an iNOS cDNA probe. The same blot was stripped with strip buffer and rehybridized for GAPDH and 18S rRNA as described in "MATERIALS AND METHODS."

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signal transduction pathways, two sets of experiments were carried out. Figure 3 shows that PNE was suppressed by the glucocorticoid dexamethasone in a dose-dependent manner, and blocked by both the transcription inhibitor actinomycin D and the protein synthesis inhibitor cycloheximide, suggesting that the induction of post-isolation iNOS mRNA is dependent on *de novo* protein synthesis, and its regulation is mainly at the transcription level. Since many reports have shown that PTDC can specifically inhibit activation of the NF-$\kappa$B/c-rel family of transcription factors and their binding with the nuclear element sequence (*29, 30*), PTDC was used to treat hepatocytes. We found that varying with the incubation time and/or PTDC concentration, PNE was either inhibited or blocked by the addition of PTDC, and that the earlier the addition and/or the higher the PTDC concentration to which the hepatocytes were exposed, the stronger was the suppressive effect (Fig. 4). This indicates that the NF-$\kappa$B/c-rel family is likely to play an important role in the PNE of ECM-isolated hepatocytes. These agents did not decrease hepatocyte viability as determined by trypan blue exclusion (data not shown).

*Stability of iNOS mRNA*—The increase in the mRNA level could be due to an upregulation of transcription and/or an increase in mRNA stability. To clarify this point, actinomycin D, a well-known specific gene transcription inhibitor, was used to treat ECM-isolated hepatocytes. As shown in Fig. 5A, the iNOS mRNA level rose gradually in the absence of actinomycin D, whereas after the blockage of transcription by an earlier addition of actinomycin D, its level decreased gradually with incubation time. The time-dependent decrease of the iNOS mRNA level was also recognized in the case of the later addition, but the mRNA level of the housekeeping gene GAPDH remained essentially unchanged (Fig. 5B). While these results further showed that the regulation of ECM-isolated hepatocyte PNE is mainly on the transcriptional level, on the other hand, when the mRNA level of iNOS is compared with that of GAPDH, it is not hard to see that the increase in iNOS transcripts is the result of an accompanying great increase in iNOS gene transcription and low iNOS mRNA stability.

*Regulation of iNOS mRNA by Bacterial LPS in ECM-Isolated Hepatocytes*—Although there have been many reports on iNOS gene induction in rat and human hepatocytes by LPS alone or in combination with certain cytokines (*11, 14-16*), very few studies focusing specially on LPS-induced iNOS gene expression in primary cultured murine hepatocytes have been reported. In addition, there is still some discrepancy in the finding over whether LPS alone can induce a high level of iNOS mRNA (*11, 15*), while the difference in response to LPS among species also needs further study. To clarify these questions, we tested the response of traditionally isolated hepatocytes to LPS. The time course shows that the iNOS mRNA level reached a peak at 4 h and decreased to an undetectable level 8 h after exposure to 10 $\mu$g/ml LPS (Fig. 6A). To observe the dose response, cultured murine hepatocytes were stimulated with LPS at concentrations ranging from 0.1 to 10 $\mu$g per

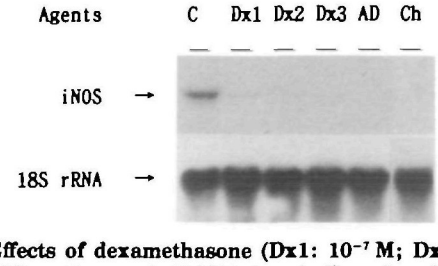


Fig. 3. **Effects of dexamethasone (Dx1: $10^{-7}$ M; Dx2: $10^{-6}$ M; Dx3: $10^{-5}$ M), actinomycin D (AD, 1 $\mu$g/ml), and cycloheximide (Ch, 1.0 $\mu$g/ml) on the iNOS mRNA expression of ECM-isolated hepatocytes.** Hepatocytes were treated with or without the agents 2 h after isolation. Total RNAs were isolated from ECM-isolated primary cultured hepatocytes 8 h after isolation. Northern hybridization was performed using an iNOS cDNA probe. The same blot was stripped with strip buffer and rehybridized for 18S rRNA as described in "MATERIALS AND METHODS."

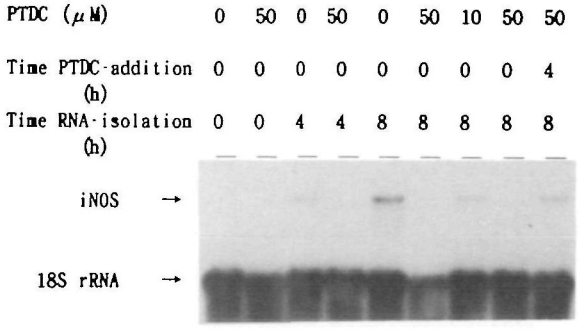


Fig. 4. **PTDC suppressed or blocked the post-isolation iNOS mRNA expression of hepatocytes.** ECM-isolated hepatocytes were exposed to PTDC at various times and concentrations; the total RNAs were isolated 0, 4, and 8 h after isolation as indicated. Northern hybridization was performed using an iNOS cDNA probe. The same blot was stripped with strip buffer and rehybridized for 18S rRNA as described in "MATERIALS AND METHODS."

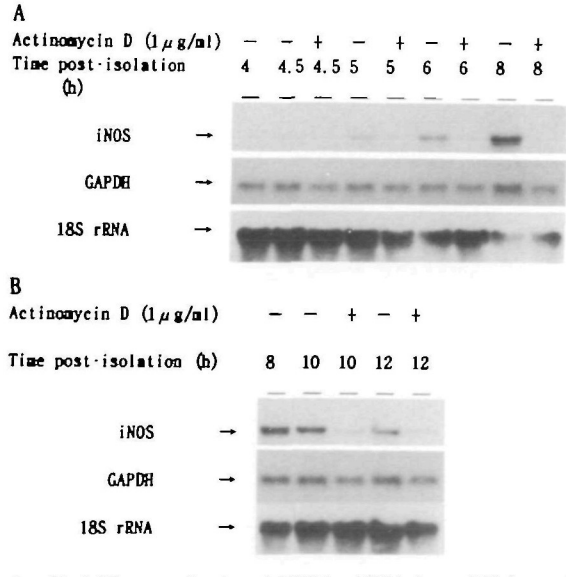


Fig. 5. **Stability analysis of iNOS mRNA by addition of actinomycin D.** Panel A: actinomycin D (1 $\mu$g/ml) was added 4 h after isolation; panel B: actinomycin D was added 8 h after isolation. Total RNAs were isolated at the times indicated. Northern hybridization was performed using an iNOS cDNA probe. The same blot was stripped with strip buffer and rehybridized for GAPDH and 18S rRNA as described in "MATERIALS AND METHODS." Blots are representative for a series of three blots, all of which gave comparable results.



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ml of culture medium. The iNOS mRNA was present after stimulation with LPS at 1 $\mu$g/ml and increased with increasing concentrations of LPS (Fig. 6B). Taken together, these results show that LPS alone can induce hepatocyte iNOS mRNA expression in a time- and dose-dependent manner. In unstimulated hepatocytes, iNOS mRNA was undetectable.

*Identifying the Cause(s) of PNE*—Various types of factors, including bacterial LPS, parasite-derived products, $Ca^{2+}$/mobilizing agonists, and cytokines (*11, 13, 31*), have been shown to be capable of inducing iNOS mRNA expression in hepatocytes and/or macrophages, but none of them seem able to account for PNE in primary cultured murine hepatocytes. In order to identify the cause(s) of PNE, we developed a new one-step EGTA method, in which the collagenase step of the ECM is deleted and 0.5 mM EGTA buffer only is used to isolate hepatocytes. A time course observation experiment showed that there was no detectable PNE in hepatocytes isolated by this EGTA method, whereas expression was observed in the LPS-exposed positive control cells (Fig. 7). To test the direct effect of collagenase on the iNOS gene expression of cultured hepatocytes, we treated ECM-isolated 36-h-cultured hepatocytes with a collagenase buffer identical to that used in the ECM. As shown in Fig. 8, iNOS mRNA was undetectable except in the positive control stimulated with LPS when the primary cultured hepatocytes were kept in a monolayer state. A similar result was obtained with 4-h-cultured hepatocytes isolated by the EGTA method (data not shown). To investigate the inducibility of iNOS gene expression by LPS in EGTA isolated hepatocytes, a dose-response experiment was performed. As shown in Fig. 9, iNOS mRNA was detectable at 5.0 $\mu$g/ml, and its level rose with increasing LPS concentration. When the results are compared with those in Fig. 6B, there does not appear to be any great difference in the responsiveness of hepatocytes isolated by the two methods. In unstimulated hepatocytes, iNOS mRNA was not detectable. Taken together, these results show that the PNE observed in ECM-isolated primary cultured murine hepatocytes is due to collagenase buffer perfusion, but the PNE induction is dependent on

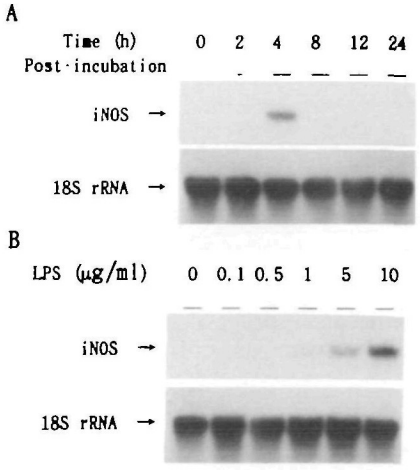


Fig. 6. **Bacterial LPS stimulated iNOS mRNA expression of ECM-isolated 36-h-cultured hepatocytes.** Hepatocytes were isolated, and plated in 5% FBS medium. After 4-h attachment, cells were washed three times with PBS (pH 7.2), and then maintained in serum-free medium. Thereafter the medium was changed every 24 h. (A) Time course of hepatocyte iNOS mRNA level following LPS (10 $\mu$g/ml) exposure. The incubation times after exposure to LPS are shown. (B) Dose response of hepatocyte iNOS mRNA level following LPS exposure. Total RNA was isolated 4 h after exposure to LPS. The LPS concentrations are indicated. Northern hybridization was performed using an iNOS cDNA probe. The same blot was stripped with strip buffer and rehybridized for 18S rRNA as described in "MATERIALS AND METHODS."

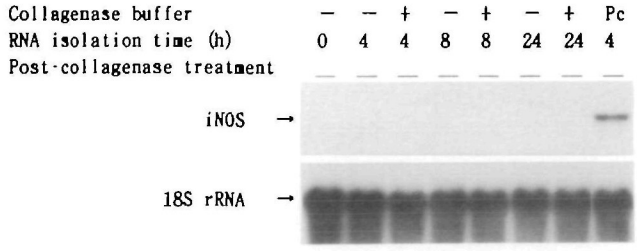


Fig. 8. **Effect of collagenase treatment on iNOS mRNA expression of ECM-isolated 36-h-cultured hepatocytes.** Hepatocytes treated with a collagenase-containing buffer identical to that used in the ECM for 6 min at 37°C, were then washed and cultured in the medium until the RNA isolation times (h) indicated. Pc is a positive control in which hepatocytes were incubated with LPS (10 $\mu$g/ml) for 4 h. Northern hybridization was performed using an iNOS cDNA probe. The same blot was stripped with strip buffer and rehybridized for 18S rRNA as described in "MATERIALS AND METHODS."

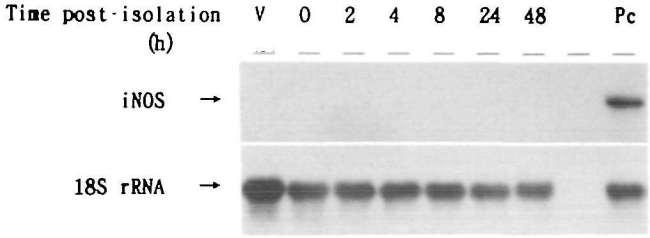


Fig. 7. **Time course analysis of iNOS mRNA expression in hepatocytes isolated by the EGTA method.** Hepatocytes were isolated only by perfusion with the EGTA (0.5 mM)-containing buffer used in the ECM for 30 min at 37°C. The RNA isolation times (h) are indicated. Northern hybridization was performed using an iNOS cDNA probe. The same blot was stripped with strip buffer and rehybridized for 18S rRNA as described in "MATERIALS AND METHODS."

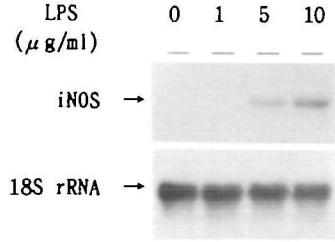


Fig. 9. **Effect of LPS on iNOS mRNA expression in hepatocytes isolated by the EGTA method.** Hepatocytes were stimulated with various concentrations of LPS ($\mu$g/ml) 4 h after isolation. Total RNAs were isolated 4 h after stimulation. The LPS concentrations are indicated. Northern hybridization was performed using an iNOS cDNA probe. The same blot was stripped with strip buffer and rehybridized for 18S rRNA as described in "MATERIALS AND METHODS."

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certain conditions. Among the collagenase buffer components, collagenase itself is most likely to be responsible for the PNE induction either directly and/or indirectly. Moreover, the response to LPS of hepatocytes isolated by the EGTA method is similar to that of ECM-isolated cells.

## DISCUSSION

NO is an important diffusable mediator produced from L-arginine by NO synthase in a range of cells and tissue types and it exerts a variety of physiological and pathophysiological effects (*1, 5*). In the liver, NO regulates hepatic vascular tone (*32*), and modulates various metabolic functions in hepatocytes, such as albumin synthesis (*33*), gluconeogenesis (*34*), mitochondrial respiration (*16, 35*), and cytochrome P450 activity (*36*). In 1976, Seglen developed a very useful two-step hepatocyte isolation assay (*21*), which not only provided a powerful tool for hepatologists and pharmacologists, but also gave rise to a new era of *in vitro* functional research into hepatocytes. Primary hepatocyte culture has become essential for hepatocyte metabolic study and understanding the liver function. As time goes on, the effects of endogenous and exogenous NO in hepatocytes will become clearer and our knowledge will be more extensive. Therefore, it is necessary to re-recognize the importance of the long-used traditional hepatocyte isolation method. The novel finding that PNE is due to collagenase buffer perfusion not only provides us with valuable information for the *in vitro* study of the hepatocyte metabolic mechanism relating to NO, but also widens our knowledge concerning the induction of hepatocyte iNOS gene expression.

Characterization of PNE in hepatocytes may be very useful for studying NO production and related metabolic processes. Our time-course study shows that iNOS mRNA in ECM-isolated hepatocytes is detectable at 4 h, and decreases to an undetectable level 24 h after isolation. This suggests that (i) cell attachment may be required for PNE because hepatocytes generally cannot finish attaching until 4 h after plating; and (ii) when using hepatocytes ECM-isolated for any study relating to NO from 4 to 24 h after isolation, close attention should be paid to PNE, and even 24 h after isolation the possible effects of iNOS mRNA expression and NO production should still be considered. In addition, characterizing the steps and signal transduction pathways involved in iNOS gene expression is important for understanding the regulation of NO production. The fact that PNE depends on *de novo* protein synthesis suggests that a key polypeptide component, which might be a positive regulator of the PNE signal transduction pathway, is labile. Corresponding to this point, hepatocytes are potentially able to produce TNF-$\alpha$, which has been shown to be one of the key mediators in inflammatory response (*37*) and to be capable of mediating iNOS expression (*14*), so endogenous TNF-$\alpha$ might play a role in the PNE of hepatocytes. To confirm this will require further investigation using anti-TNF-$\alpha$ antibodies (*15*). It has additionally been reported that protein kinase C (PKC) is involved in the iNOS expression of macrophages (*38*). Since polymyxin B is also a powerful specific PKC inhibitor, the LPS-neutralizing experiment not only helped rule out the possibility of LPS contamination, but also showed that PKC is not required in the PNE of hepatocytes. With regard to signal-transduction, we also investigated the role of the NF-$\varkappa$B/c-rel family of transcription factors—which is involved in the regulation of many genes in the stimulation of immune and inflammatory processes (*39*)—in the PNE of hepatocytes. Inhibition of PNE by PTDC, a widely used specific NF-$\varkappa$B/c-rel antagonist (*40, 41*), suggests that the NF-$\varkappa$B/c-rel family may well play an important role in the PNE of hepatocytes. Furthermore, direct observation by gel shift assay showed that the NF-$\varkappa$B level in the nuclear extract was indeed suppressed by PTDC (data not shown). Signal transduction for gene expression is a complex network, and final identification of the PNE signal transduction pathway still needs much research.

One important part of this paper is the identification of what could lead to PNE in ECM-isolated primary cultured hepatocytes, but not in cells isolated by the EGTA method. The factors reported to stimulate iNOS gene expression in hepatocytes are not sufficient to explain the occurrence of PNE. The question thus arises whether there are some other factors directly and/or indirectly capable of triggering PNE in hepatocytes. Based on the results obtained in this study (Figs. 1, 4, and 8), we propose the following hypothesis for PNE induction. Specifically, blood and $Ca^{2+}$ are basically removed from the peripheral fluid of the liver by the first-step, EGTA buffer perfusion, causing a loosening of cell-to-cell contact among all liver cells. This loosened cell-to-cell contact may provide a preliminary condition for the action of collagenase and induction of PNE in hepatocytes. During the second-step, collagenase buffer perfusion, several factors which may possibly induce PNE exist: (i) cell-to-cell contact may be completely cut, causing the intrinsic communication among cells to stop; intracellular signal transduction will then inevitably change, including that associated with iNOS expression; (ii) there may be some collagenase-derived damage to hepatocytes, which in turn may spontaneously express some iNOS induction-associated genes or the iNOS gene itself; (iii) collagenase itself may work as a stimulator; (iv) shear stress on hepatocytes due to perfusion buffer flow may be present. Shear stress can also induce iNOS gene expression in blood vessel endothelial cells. Any one of the above four factors may be sufficient to induce PNE or may merely provide a preliminary condition for the final PNE induction. It is known that hepatocytes cannot survive long if they are unable to attach to a plate basement. During and after attachment, hepatocytes not only change morphologically and functionally, but also establish a new communication network among themselves. Although these changes possibly contribute to PNE, the evidence that hepatocytes isolated by the EGTA method do not spontaneously express iNOS mRNA suggests that the changes in hepatocytes after isolation are not crucial to PNE. Taken together, the primary cause of PNE lies in liver perfusion. Because factors 1 and 4 above exist simultaneously when cells are isolated by both the EGTA and ECM methods, PNE can undoubtedly be attributed to collagenase buffer perfusion. With regard to the possible individual differences between lots of type IV collagenase, at least two different lots were examined in liver perfusion and almost identical results were obtained, suggesting that PNE does not change with the lot of collagenase used.

In summary, our results demonstrate that post-isolation iNOS gene expression does indeed occur, and that it is due



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to collagenase buffer perfusion in primary cultured hepatocytes isolated by the traditional method. In addition, the modulation of PNE was characterized on a molecular level. These findings will have important value in studying hepatocyte metabolism, especially that involving NO. However final identification of the inducing factor(s) remains to be made.

The authors thank Dr. K. Miura and other members of the Departments of Medical Zoology and Molecular Biology, Tottori University for their technical assistance and/or valuable discussion.

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