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Norepinephrine Stimulates Interleukin-6 mRNA Expression in Primary Cultured Rat Hepatocytes[1]

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Non-invasive immobilization stress causes an increase in the plasma interleukin (IL)-6 level accompanied by increased IL-6 mRNA expression and IL-6 immunoactivity in the liver [*Biochem. Biophys. Res. Commun.* (1997) 238, 707–711]. In the present study, using rat primary cultured hepatocytes and non-parenchymal liver cells, the effect of norepinephrine (NE) on IL-6 mRNA expression was determined. IL-6 mRNA expression in hepatocytes, but not in non-parenchymal liver cells, increased when the cells were treated with NE. The stimulatory effect of NE was inhibited by the combined use of α- and β-adrenergic antagonists. IL-6 mRNA expression in hepatocytes also increased on incubation with the culture medium of non-parenchymal liver cells treated with NE. The effect of the medium was blocked by an IL-1 receptor antagonist. Moreover, exogenous IL-1β stimulated IL-6 mRNA expression in hepatocytes. IL-1β was present in the medium of non-parenchymal liver cells and increased with NE-treatment. These results suggest that NE released from sympathetic nerve terminals during stress can directly increase IL-6 mRNA expression in hepatocytes and indirectly through IL-1β production from non-parenchymal liver cells.

Key words: hepatocytes, interleukin-1, interleukin-6, norepinephrine, stress.

Interleukin (IL)-6 is a crucial cytokine which induces acute phase protein synthesis in the liver. The IL-6 level in the blood is known to be elevated not only during inflammation elicited by infection and/or injury (*1*, *2*), but also during non-invasive stress such as immobilization and open field tasks (*3–5*). It is thus suggested that IL-6 may play a defensive role against physiological and psychological stress even without any apparent inflammation. Recently, we demonstrated in mice that immobilization stress induced IL-6 mRNA expression in the liver in parallel with elevation of the plasma IL-6 level, and that IL-6 immunoactivity was mainly present in hepatocytes, and more than in non-parenchymal liver cells such as Kupffer cells (*5*).

Takaki *et al.* have reported that partial hepatectomy and hepatic sympathectomy attenuated the increase in the plasma IL-6 concentration upon immobilization (*6*). We have also shown that intracranial injection of IL-1 mimicked the IL-6 responses to immobilization stress, and that these responses were suppressed by ganglionic blockade (*7*). Furthermore, it has been shown that epinephrine enhanced lipopolysaccharide (LPS)-induced IL-6 production in the perfused rat liver and in isolated Kupffer cells (*8*). These findings suggest that the hepatic sympathetic nerve may play a significant role in IL-6 expression in the liver. However, intra-hepatic mechanisms such as hepatocyte and non-parenchymal liver cell interaction in the sympathetic nerve–mediated IL-6 production remain obscure. In the present study, we examined the effect of norepinephrine (NE) on IL-6 mRNA expression in primary cultured rat hepatocytes and non-parenchymal liver cells. We found that IL-6 mRNA expression in hepatocytes, but not in non-parenchymal liver cells, was increased by NE, and also by IL-1β derived from non-parenchymal liver cells.

MATERIALS AND METHODS

Materials—Norepinephrine (NE), epinephrine, phenylephrine, and isoproterenol were purchased from Sigma Chemical (St. Louis, MO, USA). Prazosin and yohimbine were obtained from the Research Biochemical Institute (Natick, MA, USA), and propranolol was from Nacalai Tesque (Kyoto). Recombinant human IL-1β and IL-1 receptor antagonist were purchased from Genzyme (Cambridge, MA, USA) and Innogenetics (Canadastraat, Zwijndrecht, Belgium), respectively. Recombinant human IL-6 and human tumor necrosis factor (TNF) α were kindly provided by Dr. K. Yasukawa (Tosoh, Kyoto), and Dr. M. Kitaura (Dainippon Pharmaceutical, Osaka), respectively.

Primary Cultures of Rat Hepatocytes and Non-Parenchymal Liver Cells—Male Wistar rats (180–230 g) were housed with a 12:12-h light–dark cycle (lights on at 7:00 h–19:00 h), and given free access to laboratory chow and water.

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Abbreviations: IL-1, interleukin-1; IL-6, interleukin-6; LPS, lipopolysaccharide; NE, norepinephrine; TNF, tumor necrosis factor.

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Hepatocytes and non-parenchymal liver cells were separated according to the method of Horiuti *et al.* (*9*). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and then their livers were perfused through the portal vein initially with HEPES-buffered Hanks' solution (pH 7.4) containing 0.4 mM EGTA and 5 mM glucose, and then with HEPES-buffered Hanks' solution supplemented with collagenase (0.05%, type IV; Wako Pure Chemical, Osaka). After the digestion, the cells were separated by gentle agitation and low-speed centrifugation (30 ×*g*). The resulting pellet, which mainly consisted of hepatocytes, was subsequently centrifugated with Percoll at 400 ×*g* to remove residual non-parenchymal liver cells. After washing three times with phosphate-buffered saline (PBS), hepatocytes (>98% in purity) were plated at 6×10^5 cells on 60-mm plates in 5 ml of Williams' E medium (Gibco BRL, Grand Island, NY, USA) supplemented with 5% fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin sulfate, 1 μM dexamethasone (Wako), and 1 μM insulin (Sigma). After 3 h at 37°C, the medium was replaced with fresh medium and cultured further for 48 h at 37°C.

For the non-parenchymal liver cell culture, the supernatant obtained on low-speed centrifugation as described above was pooled and centrifuged at 30 ×*g* to remove residual hepatocytes. After centrifugation for 20 min at 400 ×*g*, the pellet of non-parenchymal liver cells was resuspended in Williams' E medium and cultured for 3 h at 37°C. At 3 h after plating, nonadherent cells were removed and cultured further for 24 h in Williams' E medium supplemented with 5% FCS.

Stimulation of Cells and Sampling—Hepatocytes cultured for 48 h were treated with various stimulants. After 2 h stimulation, cells were washed with PBS and scraped into TRIzol solution (Gibco) for RNA extraction. When inhibitors or antagonists were used, they were added to the culture medium 10 min before the addition of stimulants. In a separate series of experiments, non-parenchymal liver cells were cultured for 24 h, and then cultured in fresh medium in the presence of various concentrations of NE. After 2 h, the medium was collected for the stimulation of hepatocytes, and then the cells were scraped into TRIzol. Hepatocytes were incubated with this culture medium diluted with an equal volume of fresh Williams' E medium for 2 h and then extracted with TRIzol.

RT-PCR and Southern Blotting—Expression of IL-6 mRNA was determined by the combination of reverse transcription-polymerase chain reaction (RT-PCR) and Southern blotting. Total RNA (2 μg) was denatured at 70°C for 10 min, cooled immediately on ice, and then reverse-transcribed using 100 units of Moloney murine leukaemia virus reverse transcriptase (Gibco), 50 pmol of poly (dT) primer and 20 nmol of dNTPs in a total volume of 10 μl at 37°C for 1 h. After heating at 95°C for 5 min, PCR amplification was performed with 2.5 unit of Taq polymerase Gold (Perkin-Elmer, Foster City, CA, USA), 3 mM $MgCl_2$, and 50 pmol of forward and reverse primers for rat IL-6 in a total volume of 50 μl. The primers used were: forward, 5′-ATGAAGTTTCTCTCCGCAA-3′; reverse, 5′-TAGGCATAGCACACTAGGTT-3′. PCR was conducted for 27 to 35 cycles, each cycle consisting of denaturation at 95°C for 45 s, annealing at 55°C for 2 min, and DNA extension at 72°C for 3 min. The PCR product was electrophoresed on a 2% agarose gel, stained with ethidium bromide, and then transferred to a nylon membrane (Amersham, Buckinghamshire, UK). A cDNA probe corresponding to nucleotides 63 to 713 of the published cDNA sequence of rat IL-6 (*10*) was synthesized by PCR, and then labelled with [α-^{32}P]dCTP using a multiprime DNA labeling kit (Amersham). The membrane was hybridized to the labelled probe at 65°C for 20 h in the presence of 0.2 mg/ml salmon sperm DNA (Sigma), and then washed twice at 42°C for 30 min with 2 × SSC (1 × SSC:0.15 M NaCl/0.015 M sodium citrate)/0.1% (w/v) SDS, and subsequently washed twice at 65°C for 30 min with 0.1 × SSC/0.1% (w/v) SDS. The radioactivity present on the membrane was determined with a bioimage analyzer (BAS1000; Fuji Photo Film, Tokyo), and depicted on X-ray films.

For quantitative estimation of IL-6 mRNA, the appropriate number of cycles and the amount of total RNA in the cell extract were determined to confirm the linearity of the RT-PCR assay. In addition, mRNA of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was also analyzed by RT-PCR and Southern blotting, and the level of IL-6 mRNA was expressed relative to that of G3PDH mRNA. The primers used were: forward, 5′-ACCACAGTCCATGCCATCAC-3′; reverse, 5′-TCCACCACCCTGTTGCTGTA-3′ (CLONTEC, Palo Alto, CA, USA), and PCR was conducted for 25 cycles. The cDNA probe for G3PDH was also prepared by PCR using a control template supplied with the primers.

Measurement of IL-1β Concentrations—The IL-1β con-

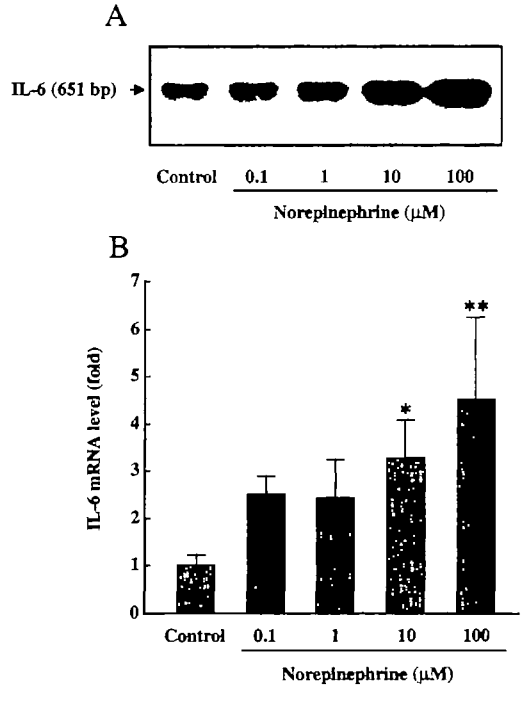

Fig. 1. **Expression of IL-6 mRNA in cultured rat hepatocytes and the effect of NE.** Rat hepatocytes were cultured for 48 h in Williams' E medium supplemented with 5% FCS, and then various concentrations of NE were added to the medium, followed by incubation for 2 h. A: Typical autoradiograms on Southern blotting are shown. B: The amount of IL-6 cDNA amplified by RT-PCR was determined with a bioimage analyzer and expressed as the ratio to the amount of G3PDH cDNA. The results are expressed relative to the control value (mean ± SEM, $n = 5$). $^*p < 0.05$, $^{**}p < 0.01$ compared with the control.

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centration in the medium of non-parenchymal liver cells was measured using a IL-1β enzyme–linked immunosorbent assay (ELISA) kit (Immuno Biological Laboratories, Fujioka) according to the manufacturer's instructions.

Statistical Analysis—Data were expressed as means ± SEM. Statistical significance was evaluated using Fisher's protected least significant difference test or Scheffe's F test. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

To determine whether or not NE stimulates IL-6 expression in hepatocytes and non-parenchymal liver cells, primary cultured rat hepatocytes (>98% in purity) and non-parenchymal liver cells were incubated with NE for 2 h, and then the IL-6 mRNA levels in both types of cells were determined by RT-PCR and Southern blotting. As shown in Fig. 1A, IL-6 mRNA in hepatocytes was detected even in the basal state without any stimulation (control), and markedly increased after NE stimulation. To assess the effect of NE quantitatively, the IL-6 mRNA level was calculated relative to the G3PDH mRNA level, which was also determined by RT-PCR and Southern blotting using the same sample (Fig. 1B). The IL-6 mRNA level thus calculated was increased by NE in a dose-dependent manner (0.1–100 μM). In non-parenchymal liver cells, although expression of IL-6 mRNA was detected, it was not influenced by NE stimulation (Fig. 2).

The effects of adrenergic agonists and antagonists on IL-6 mRNA expression in hepatocytes were examined. Stimulation with an α-adrenergic agonist, phenylephrine (10 μM), a β-adrenergic agonist, isoproterenol (10 μM), or epinephrine (10 μM) also enhanced the IL-6 mRNA expression in hepatocytes (Fig. 3A). The NE-induced IL-6 mRNA expression in hepatocytes was partially inhibited by either an α_1-receptor antagonist (prazosin), an α_2-receptor antagonist (yohimbine), or a β-receptor antagonist (propranolol) (Fig. 3B). Moreover, the combined use of these three antagonists completely inhibited the stimulatory effect of NE (Fig. 3B).

Next, the possible effects of factors derived from non-parenchymal liver cells on hepatocyte IL-6 mRNA expression were examined, because non-parenchymal liver cells are known to produce a variety of cytokines, such as IL-1β, TNFα, and IL-6, all of which influence hepatocyte gene expression (*11*, *12*). When hepatocytes were incubated for 2 h with the culture medium of non-parenchymal liver cells, the IL-6 mRNA level increased (Fig. 4). IL-6 mRNA expres-

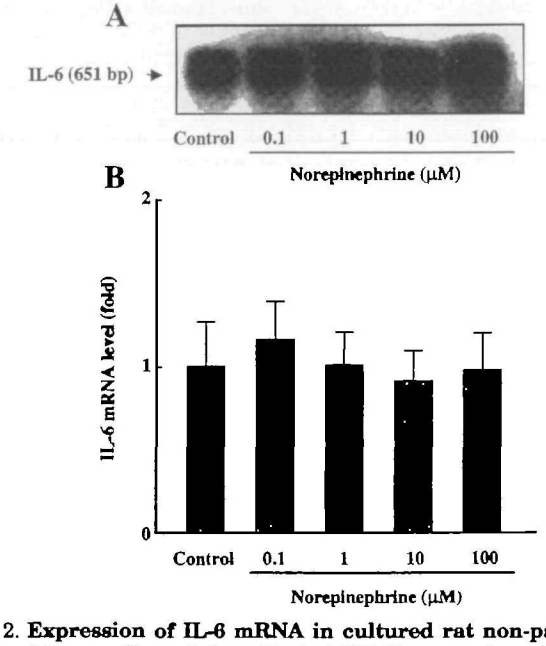

Fig. 2. **Expression of IL-6 mRNA in cultured rat non-parenchymal liver cells and the effect of NE.** Nonparenchymal liver cells were cultured for 24 h in Williams' E medium supplemented with 5% FCS, and then various concentrations of NE were added to the medium, followed by incubation for 2 h. A: Typical autoradiograms on Southern blotting are shown. B: The amount of IL-6 mRNA was expressed relative to the control value (mean ± SEM, n = 4)

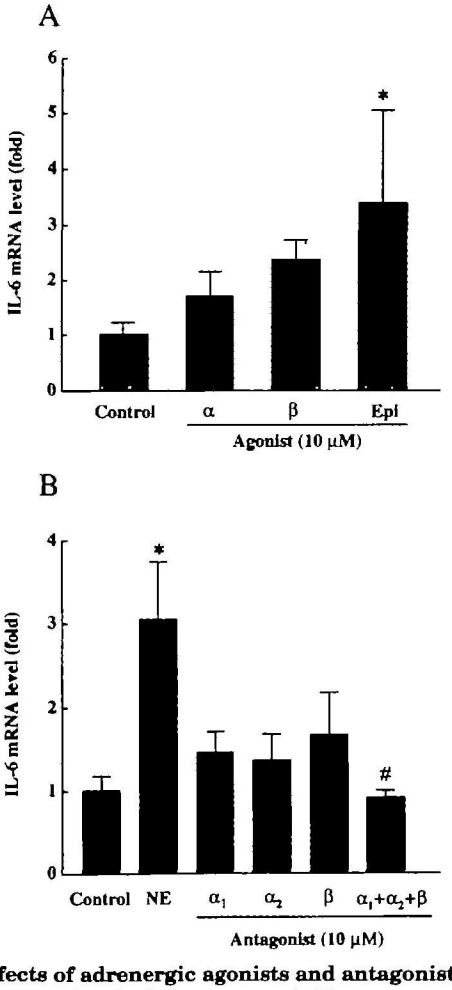

Fig. 3. **Effects of adrenergic agonists and antagonists on IL-6 mRNA expression in hepatocytes.** A: Hepatocytes were incubated with either 10 μM α-adrenergic receptor agonist (phenylephrine, α), β-adrenergic receptor agonist (isoproterenol, β), or epinephrine (Epi) for 2 h (n = 5). B: Hepatocytes were incubated with an α_1-receptor antagonist (prazosin, α_1), an α_2-receptor antagonist (yohimbine, α_2), or a β-receptor antagonist (propranolol, β) for 10 min, and then for a further 2 h with 10 μM NE (n = 4–5). The amount of IL-6 mRNA was expressed as in Fig. 1. $^*p < 0.05$ compared with the control. $^\#p < 0.05$ compared with 10 μM NE stimulation.

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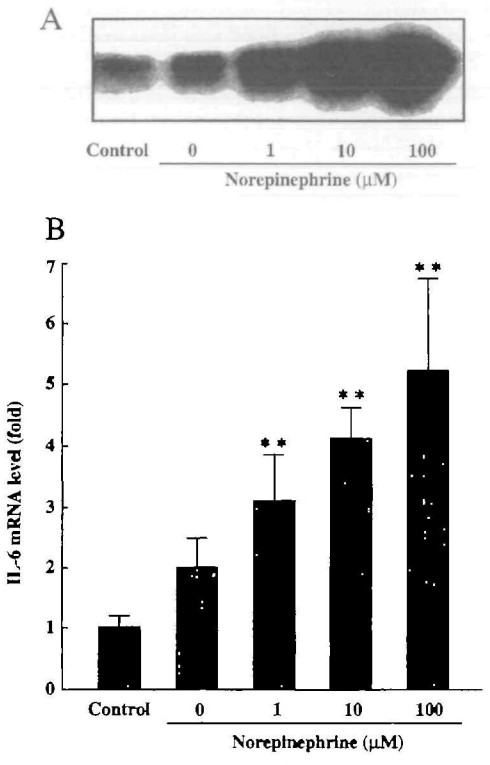

Fig. 4. **Effect of the culture medium of non-parenchymal liver cells on IL-6 mRNA expression in hepatocytes.** Non-parenchymal liver cells were cultured for 24 h and then incubated with increasing concentrations of NE for 2 h. Hepatocytes were cultured for 48 h in Williams' E medium supplemented with 5% FCS and then stimulated with the culture medium of non-parenchymal liver cells diluted with an equal volume of fresh Williams' E medium. Control indicates IL-6 mRNA expression in hepatocytes without the addition of the culture medium of non-parenchymal liver cells. A: Typical autoradiograms on Southern blotting are shown. B: The amount of IL-6 mRNA was expressed relative to the control value (mean ± SEM, $n = 5$). $^{**}p < 0.01$ compared with the control.

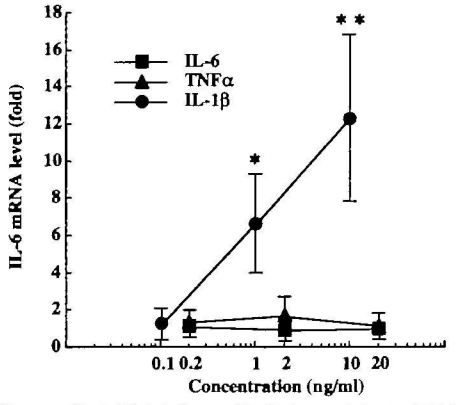

Fig. 5. **Effects of IL-1β, IL-6, and TNFα on IL-6 mRNA expression in hepatocytes.** Hepatocytes were stimulated with various concentrations of each cytokine for 2 h ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control. The amount of IL-6 mRNA was determined as in Fig. 1.

TABLE I. **Effect of an IL-1 receptor antagonist on IL-6 mRNA expression in hepatocytes.**

Stimulation	IL-1 receptor antagonist	
	−	+
Control	1.00 ± 0.20 ($n = 7$)	—
Culture medium of non-parenchymal liver cells without NE-treatment	2.00 ± 0.65 ($n = 6$)	1.37 ± 0.42 ($n = 4$)
Culture medium of non-parenchymal liver cells with NE-treatment	4.09 ± 1.09* ($n = 8$)	1.96 ± 0.38# ($n = 7$)

Hepatocytes were preincubated in Williams' E medium (5 ml) containing the IL-1 receptor antagonist (25 ng/ml) for 10 min, and then the culture medium (5 ml) of non-parenchymal liver cells treated with or without NE (10 μM) was added. The results are expressed relative to the control value obtained without any stimulus (mean ± SEM). $^{*}p < 0.05$ compared with the control, $^{\#}p < 0.05$ compared to the corresponding value obtained without the IL-1 receptor antagonist.

sion increased further on incubation with the culture medium of non-parenchymal liver cells stimulated with increasing concentrations of NE (Fig. 4). IL-1β, TNFα and IL-6 were examined as to whether or not they affect IL-6 mRNA expression in hepatocytes. Recombinant human IL-1β increased IL-6 mRNA expression in a concentration-dependent manner, while TNFα and IL-6 did not have any effect at all (Fig. 5). Thus, IL-1 is suggested to be one of the non-parenchymal liver cell–derived factors which may effectively stimulate IL-6 mRNA expression in hepatocytes. To prove this idea, an IL-1 receptor antagonist (IL-1Ra) was added to the hepatocyte culture medium. In the presence of IL-1Ra, the culture medium of non-parenchymal liver cells, especially those treated with NE, failed to enhance the IL-6 mRNA expression in hepatocytes (Table I). The concentration of IL-1β in the culture medium of non-parenchymal liver cells, estimated using an ELISA kit, was 232 ± 11 pg/ml without NE stimulation, and significantly increased to 277 ± 13 pg/ml after stimulation with 10 μM NE ($n = 6$).

It may be possible that the NE-induced IL-6 mRNA expression in hepatocytes is also mediated *via* autocrine expression of IL-1β. To examine this possibility, hepatocytes were stimulated with 10 μM NE as in Fig. 1 in the presence of IL-1Ra (25 ng/ml). However, the NE-induced IL-6 mRNA expression was not affected by the presence of IL-1Ra (data not shown).

DISCUSSION

In the present study, we showed that rat hepatocytes in primary culture expressed IL-6 mRNA, in accordance with previous observation of IL-6 production by hepatocytes and $HepG_2$ cells (*13*, *14*). Moreover, we have demonstrated for the first time that IL-6 mRNA expression is enhanced by NE. In contrast, IL-6 mRNA expression in non-parenchymal liver cells was not influenced by NE.

Using adrenergic receptor antagonists (Fig. 3), we observed that the action of NE on hepatocytes is mediated by α_1, α_2, and β-adrenergic receptors. Although α-adrenergic receptors are dominant in adult male rat hepatocytes *in vivo*, β-adrenergic receptors have been shown to increase during primary cultures of hepatocytes (*15*). Thus, it is not unexpected that NE-signals can be transduced through α-adrenergic receptors as a Ca^{2+}-signal and also through β-adrenergic receptors as a cAMP signal (*15*). Moreover, it has been reported that the cAMP-response element is pre-

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sent in the IL-6 gene promoter region (*2*), and can be activated in either a cAMP- or Ca^{2+}-dependent manner (*16, 17*).

Based on these *in vitro* observations, it seems rational to conclude that the *in vivo* sympathetically stimulated IL-6 production in the liver is due to the direct action of NE on hepatocytes. However, it also seems possible that NE may stimulate hepatocyte IL-6 production indirectly through some action on non-parenchymal liver cells. In fact, we found that the addition of medium from a non-parenchymal liver cell culture increased the IL-6 mRNA expression in a hepatocyte culture, and that the stimulatory effect of the culture medium was augmented when the non-parenchymal liver cells were cultured in the presence of NE. It is well known that non-parenchymal liver cells produce a variety of cytokines, such as IL-1β, TNFα, and IL-6, all of which strongly influence hepatocyte gene expression (*11, 12*). In fact, we found that recombinant human IL-1β, but neither IL-6 nor TNFα, effectively stimulated IL-6 mRNA expression in hepatocyte primary cultures, and that the production of IL-1β by non-parenchymal liver cells was elevated by NE. Furthermore, the stimulatory effect of the non-parenchymal liver cell culture medium was much attenuated by an IL-1 receptor antagonist. Thus, IL-1 is the most likely candidate among the stimulatory factors derived from non-parenchymal liver cells. Among non-parenchymal liver cells, Kupffer cells and vascular endothelial cells are known to produce IL-1β in response to various stimuli such as LPS (*11, 18, 19*). Collectively, our results suggest that sympathetic activation of hepatocyte IL-6 production *in vivo* is mediated, at least in part, through IL-1β produced from non-parenchymal liver cells.

It is also of interest to note that TNFα is not effective in IL-6 expression in hepatocytes, although it can induce IL-6 expression and production in various cell lines (*12, 20, 21*). This discrepancy is probably due to the difference in cell types and different concentrations of TNFα used. In fact, Busam *et al.* reported that IL-6 production from Kupffer cells was stimulated by IL-1β, but not by TNFα at doses equivalent to those used in the present study (*12*).

In conclusion, NE released from sympathetic nerve endings stimulates hepatocyte IL-6 production in at least two ways: directly acting on the adrenergic receptors of hepatocytes and indirectly through IL-1β production from non-parenchymal liver cells. This sympathetic nerve-mediated hepatic IL-6 production may participate in some hepatic stress-responses, such as acute phase protein synthesis, in an autocrine manner.

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