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IL-1β Down-Regulates Tissue-Type Plasminogen Activator by Up-Regulating Low-Density Lipoprotein Receptor-Related Protein in AML 12 Cells[1]

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Interleukin-1 (IL-1) regulation of tPA in hepatocytes was studied in mouse hepatocyte line AML12. IL-1 induced transient accumulation of tPA mRNA as high as threefold by 2 h after the start of treatment. The cytokine also induced the mRNA for serum amyloid A, a typical acute-phase protein in mice, with more sustained kinetics in a time-dependent manner. In contrast to the induction of mRNA, tPA activity and protein levels in the harvested medium were dramatically diminished by IL-1. IL-1 stimulated the uptake of ^{125}I-tPA by AML 12. This uptake was inhibited by 39-kDa receptor-associated protein (RAP), but not by the sugar mannan. These results revealed that low-density lipoprotein receptor-related protein (LRP), which is known to be a receptor for tPA and to be blocked by RAP, was up-regulated by IL-1. We also demonstrated, for the first time, that IL-1 transiently increased the mRNA level of LRP threefold by 30 min after the start of IL-1 treatment of AML 12. The receptor-mediated endocytosis of tPA by hepatocytes may thus play a crucial role in the down-regulation of fibrinolysis during the acute-phase response.

Key Words: **interleukin-1; tissue-type plasminogen activator; 39-kDa receptor-associated protein; low-density lipoprotein receptor-related protein; mouse hepatocytes.**

Plasminogen activator (PA) is a serine proteinase that converts the inactive proenzyme plasminogen to its active form, plasmin (1). The PA–plasmin system is not only involved in fibrinolysis, but also in other physiological and pathological processes such as ovulation, tissue remodeling, malignant invasion, and metastasis, in which the breakdown of extracellular matrices is evoked topically (2).

There are two types of PA, tissue-type PA (tPA) and urokinase-type PA (uPA), distinguished by their immunological and functional characteristics in the mammalian fibrinolytic system (3). Because tPA has higher affinity for fibrin than uPA (4), tPA is thought to be responsible for the activation of the fibrinolysis system in blood vessels. Human tPA is also of particular pharmacological interest because of its values in the treatment of thromboembolic disorders (5).

The activity of the PA–plasmin system is controlled at different stages including (1) the regulation of PA gene expression/production, and (2) the regulation of PA activity by a specific inhibitor, type-1 PA inhibitor (PAI-1). Rapid hepatic clearance of tPA is also one of the principal mechanisms for the regulation of the tPA concentration in the bloodstream. There are two distinct pathways for the clearance of tPA by the liver (6); Kupper cells and endothelial cells take up tPA by their mannose receptor (7–10), whereas liver parenchymal cells (hepatocytes) internalize tPA via a specific receptor, one identical with the low-density lipoprotein receptor-related protein (LRP) (7, 10). Taken the population of liver cells (hepatocytes and nonparenchymal cells; Kupper and endothelial cells) into account, LRP should be a major receptor responsible for the clearance of tPA by the liver; 55% of the tPA infused into the rat was cleared by hepatocytes (6, 7). LRP, a multiligand clearance receptor (11–14), is also identical with the α2-macroglobulin receptor (15, 16) purified from rat liver and human placenta (17–19). Receptor-associated protein (RAP) is a 39-kDa protein that is copurified with LRP (16, 19). RAP inhibits the LRP-mediated endocytosis of PAs or PA/PAI-1 complexes, and thus RAP is thought to be involved in regulating the clearance of tPA by the liver (20–22).

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PAI-1, a 52-kDa glycoprotein, is a major physiological inhibitor of PA. PAI-1 regulates the net fibrinolytic activity *in vivo* (23). Both tPA and PAI-1 are synthesized by the several types of cells *in vitro*, and their expression has been shown to be regulated by growth factors, cytokines, and hormones (23–27). PAI-1 is also an important component of the hepatic acute-phase (AP) response in mice and humans (28–32).

The mediators of the AP response are primarily cytokines including IL-1, IL-6, and tumor necrosis factor α (TNFα), released by inflammatory cells (33, 34). Previously we reported that (1) IL-1 was a potent inducer of hepatic PAI-1 gene transcription in mouse hepatocytes in tissue culture (26), and (2) the AP responses including PAI-1 production in response to local tissue injury *in vivo* was IL-1 dependent (35).

On the other hand, the AP regulation of tPA, a target enzyme of PAI-1, has not yet been characterized. Thus, in the present study, we characterized the IL-1 regulation of tPA gene expression as well as that of tPA activity/protein in the mouse hepatocyte line AML12. We now report that IL-1-mediated down-regulation of tPA in AML 12 cells and showed this effect to be due to the up-regulation of tPA clearance by LRP.

MATERIALS AND METHODS

Materials. Recombinant human IL-1β and tPA were provided by Otsuka Pharmaceutical Co. Ltd. (Molecular Medical Science Institute, Tokushima, Japan) and by Dr. Osamu Matsuo (Kinki University School of Medicine, Osaka, Japan), respectively. 39-kDa receptor-associated protein (RAP) was provided by Dr. Dudley K. Strickland (American Red Cross, Rockville, MD). Monoclonal antibody against mouse tPA was a generous gift of Dr. Paul J. Declerk (36, 37). Other chemicals used in this study were commercial grade.

Cell culture. AML 12 cells (provided by Dr. Nelson Fausto, University of Washington, Seattle, WA; Ref. 38) were plated on 35- or 100-mm tissue culture dishes (Falcon, Lincoln Park, NJ) and grown as monolayers in DMEM/Ham's F12 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS: Sigma Chemical Co., St. Louis, MO), 10^{-7} M dexamethasone (Sigma), 5 μg/ml of insulin (Sigma), 5 μg/ml of holo-transferring (Sigma) and 5 ng/ml of selenium (Sigma) at 37°C in a 5% CO_2–95% air atmosphere. All experiments used cells in passages 35–39 at 70–100% confluence. The cells were incubated in the 0.1% BSA-DMEM/Ham's F12 for 20 h prior to the incubation with IL-1 β and/or other agents.

Northern blot analysis. Total cellular RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform method (39). Northern blot analysis was carried out by using 20 μg of RNA/lane as described earlier (40). The radioactivity bound specifically on the blot was measured quantitatively by a STORM apparatus (Model 860, Molecular Dynamics Inc., CA), and the amounts of tPA, serum amyloid A (SAA) and albumin mRNA were normalized to that amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, which did not change under the conditions of these experiments.

cDNA probes. Mouse tPA (41), SAA (42), albumin cDNA probes and one for rat GAPDH (43) were labeled by the random primer method using the large fragment of Klenow DNA polymerase I and ^{32}P-dCTP (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan).

Analysis of tPA activity. Zymographic analysis of tPA in the conditioned medium was performed by the method of Heussen (44) with minor modification as described previously (45).

ELISA of tPA. Amounts of tPA and tPA secreted into the medium were measured by ELISA using monoclonal antibody against murine tPA. After incubation of the cells in serum-free fresh medium for 20 h, IL-1 (500 U/ml) was added to the AML 12 cells and incubated for the times indicated in the figures. For the assay of intracellular tPA, AML 12 cells were washed twice with ice-cold PBS, and collected with a rubber-policeman. The cell pellet was suspended in 250 μl of PBS and lysed by 3 cycles of freezing and thawing. ELISA was performed on 180 μl of the cell lysate or harvested medium as described (36, 37). Intracellular tPA protein was normalized to the total cellular protein measured by using a commercial kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, CA).

Labeling of tPA. Recombinant tPA was labeled with ^{125}I by the method using iodogen (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan) as described before (46, 47). The labeled tPA was separated from free ^{125}I by using a Sephadex G-25 (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) column (230 mm × 8 mm) equilibrated with 50 mM Tris–HCl (pH 8.0) containing 0.01% (vol/vol) Tween 80 and 0.1% (wt/vol) BSA. The labeling resulted in approximately 1.8×10^6 cpm/μg protein.

Uptake of ^{125}I-tPA by AML 12 cells. The ^{125}I-tPA (6560 cpm/dish) was added to the AML 12 cells, and the cells were cultured for the times indicated in the figures. After having been washed twice with PBS containing 0.1% (wt/vol) BSA, and twice with PBS, the cells were lysed with 1% (vol/vol) Triton X-100 for 15 min at room temperature; and the radioactivity of the lysate was then measured with an AUTO-GAMMA COBRA 5002 (Packard Instrument Co., Meriden, CT).

Reverse transcription and PCR. Reverse transcription-polymerase chain reaction (RT-PCR) was used to measure the changes in the level of LRP. The first strand of cDNA was synthesized by use of a RT-PCR kit (RT-PCR high, TOYOBO, Tokyo, Japan), in a 5 μl of volume reaction mixture containing 0.25 ng of total cellular RNA, and the subsequent PCR was carried out by using the RT-PCR kit and a thermal cycler (GeneAmp PCR System 9700, Perkin–Elmer, Foster, CA) under the suitable conditions we had established for LRP. The LRP sense primer, 5′-GCAGTGTCTA-CCGCTTGGAA-3′ and the antisense one, 5′-TGGACTCATCTT-CACTGTTC-3′ were applied for amplification of LRP cDNA with the RT-PCR cycle program set at 34 cycles (annealing at 56°C for 1 min, extension at 72°C for 1 min, and denaturation at 94°C for 1 min). The primers for GAPDH were 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′; and 30 cycles were used for the amplification of GAPDH cDNA (annealing at 60°C for 1 min, extension at 72°C for 1 min, and denaturation at 94°C for 1 min). The length of the PCR product was checked by the use of ethidium bromide (0.6 mg/ml)–gel electrophoresis of the products along with DNA molecular weight markers (Smart Ladder, NIPPON GENE, Toyama, Japan) in a 1.5% agarose gel.

Statistical analysis. The data were analyzed by Student's *t* test, and expressed as the mean ± SE.

RESULTS AND DISCUSSION

The liver has been well recognized as an organ responsible for the clearance and metabolism of coagulation and fibrinolytic factors including PAs (48, 49). Earlier, we demonstrated that the hepatocytes have a potential to express and produce tPA, although tPA expression in the normal liver is quite low or undetectable (31, 40). Thus the liver has been viewed to be a

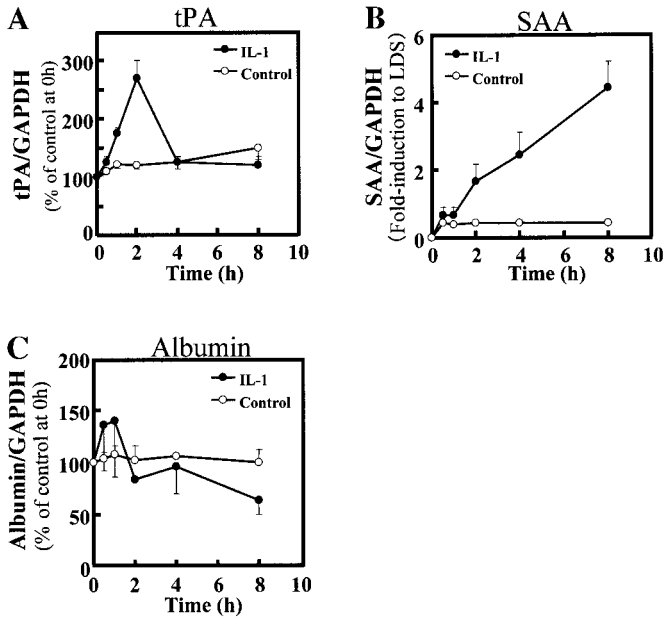

FIG. 1. Time course of the effect of IL-1 on tPA, SAA, and albumin mRNA accumulation in AML 12 cells. AML 12 cells were incubated with 500 U/ml of IL-1 for the times indicated. Total RNA specimen (20 μg) was subjected to Northern blot analysis as described under Materials and Methods. The amount of tPA (A) and albumin (C) mRNAs was normalized to the amount of GAPDH mRNA and presented as a percent of control at 0 time. SAA (B) was expressed as fold induction to the lowest detectable signal (LDS) which was defined as a value three times higher than the background signal. Values represent means ± SE of 4 different cultures.

bifunctional organ in terms of tPA regulation; i.e., it is responsible for both production and degradation of tPA. In this study, we employed mouse hepatocyte line AML 12 to examine the effect of IL-1 on tPA production. The acute-phase (AP) response is well conserved among mammalian organisms, but interspecies differences in the AP response are known (33, 34). Indeed PAI-1 is an AP reactant in both humans and mice, but not in rats (50–52). We decided to use the immortalized mouse hepatocyte line AML12, which was derived from the liver of a TGFα transgenic mouse, to characterize the IL-1 regulation of tPA in mouse hepatocytes, because of the difficulties in getting enough mouse primary hepatocytes to pursuit a series of these studies.

Figure 1 shows the effect of IL-1 on the tPA gene expression in AML 12 cells. Incubation of AML12 cells with 500 U/ml of IL-1 caused transient increase in the level of tPA mRNA, and an approximately threefold accumulation was observed at 2 h after the IL-1 treatment compared with the base line level (Fig. 1A). No change in the tPA mRNA level was observed in the cells treated with vehicle alone (control). SAA, a typical AP reactant in mice (53), was also induced by IL-1, but this induction showed more sustained kinetics than that of tPA; and its mRNA increased for 8 h in a time-dependent manner. On the other hand, the level of the mRNA of albumin, a negative AP reactant, was gradually decreased for up to 10 h by the cytokine.

To assess changes in tPA activity, we harvested the medium from IL-1-treated AML12 cells and analyzed it by zymography (Fig. 2). In contrast to the transient accumulation of tPA mRNA, tPA activity was dramatically diminished by IL-1. Earlier we reported that IL-1 caused a transient fourfold accumulation of PAI-1 mRNA over the base-line value at 1 h and also augmented the level of PAI-1 protein in the IL-1 treated AML 12 cells (26); PAI-1 induced by IL-1 may contribute to the dramatic decrease in the tPA activity in IL-1-treated AML 12 cells. To know if the decrease in tPA activity was due to the inactivation of tPA by PAI-1, we also measured the tPA protein levels in the IL-1-treated AML12 cells (Fig. 3). In AML 12 cells,

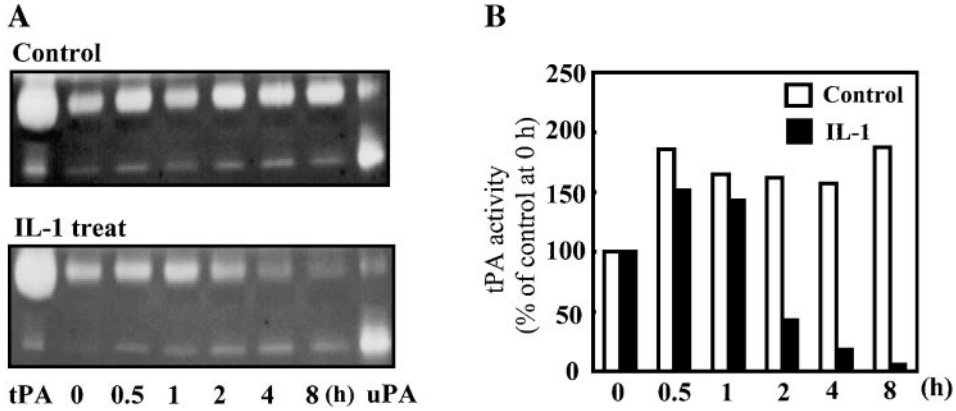

FIG. 2. Zymography of tPA activity in the conditioned medium of IL-1-treated AML12 cells. The condition medium (5 μl) harvested from AML 12 cells was subjected to zymography on 7.5% SDS–polyscrylamide gel containing 1.6 mg/ml of plasminogen-rich fibrinogen as described under Materials and Methods. tPA and uPA-rich fractions used as standard were obtained from rat hepatocytes in primary culture and mouse urine, respectively. The tPA activity was quantified by a computer-assisted analyzer and presented as a percent of the control at 0 time (B).

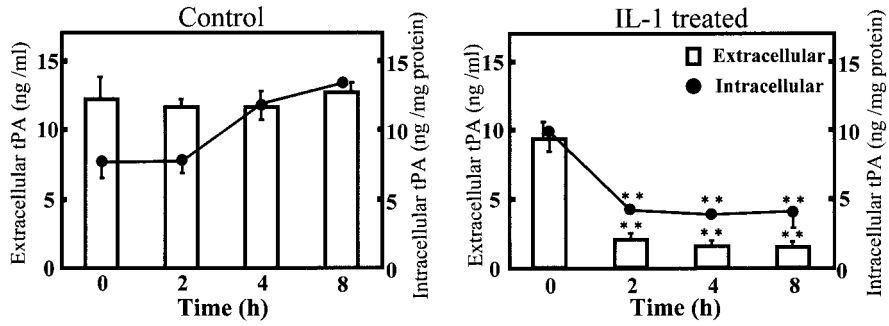

FIG. 3. Effect of IL-1 on the production of tPA in AML 12 cells. AML 12 cells were incubated with 500 U/ml of IL-1 for the times indicated. Intracellular (●) and extracellular (□) tPA proteins were measured by ELISA using anti-mouse tPA monoclonal antibody as described under Materials and Methods. Intracellular tPA protein was normalized to the total cellular protein concentrations in the cell lysate. Each bar represents the mean ± SE of 3 different cultures. $^{**}P < 0.01$ vs control at each time point, by Student's paired *t* test.

constitutive tPA expression and secretion were observed. Extracellular tPA concentration (i.e., tPA secreted into the harvested medium) seems to be regulated precisely after reaching the maximum concentration (approximately 12 ng/ml), and thus the intracellular tPA level was gradually increased (Fig. 3). Levels of both intracellular tPA protein and the tPA protein secreted into the harvested medium were dramatically lowered by IL-1. The down-regulation of tPA protein was well correlated with the tPA activity in the culture medium.

Because both tPA activity and tPA protein present in the culture medium were rapidly decreased by IL-1, we examined the effect of IL-1 on the internalization of tPA in the AML12 cells (Fig. 4). Exogenous ^{125}I-tPA added to the culture medium was lost rapidly from the medium in the presence of IL-1. At the same time, ^{125}I-tPA incorporation into the cellular fraction was accelerated in the presence of IL-1.

To examine which cellular receptor(s) is responsible for the tPA incorporation into the AML12 cells, we challenged the cells with mannan (antagonist for mannose receptor) or RAP (for LRP), as shown in Fig. 5. IL-1 increased ^{125}I-tPA incorporation 2.6-fold at 2 h. Mannan influenced neither the basal tPA uptake nor the IL-1-stimulated tPA uptake by the cells. In marked contrast, RAP almost completely inhibited both IL-1-stimulated tPA uptake and the basal tPA uptake by AML12 cells. Thus we could conclude that LRP was the functional cellular receptor responsible for the uptake of tPA by AML12 cells. There are several possible hypotheses for the up-regulation of tPA clearance in IL-1-treated AML12 cells: (1) IL-1 up-regulates the number of LRPs, (2) IL-1 up-regulates the turnover/recycling of LRP, (3) IL-1 down-regulates RAP. For further elucidation of the mechanism(s) responsible for the increased tPA clearance, we measured the LRP mRNA level in IL-1-treated AML12 cells. As expected (Fig. 6), LRP mRNA rapidly and transiently accumulated at 30 min after the addition of IL-1. The up-regulation of LRP transcription should contribute to

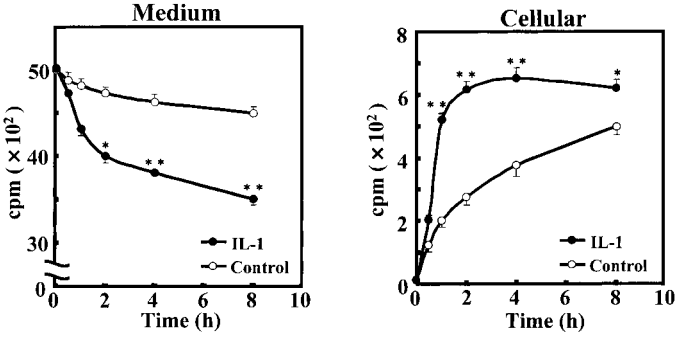

FIG. 4. Effect of IL-1 on the incorporation of ^{125}I-tPA by AML12 cells. AML12 cells were treated with 500 U/ml of IL-1 in the presence of 50 pM ^{125}I-tPA. Radioactivity incorporated into the cellular fraction and that remaining in the medium were measured. Each value represents the mean ± SE of 4 different cultures. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs control at each time point, by Student's paired *t* test.

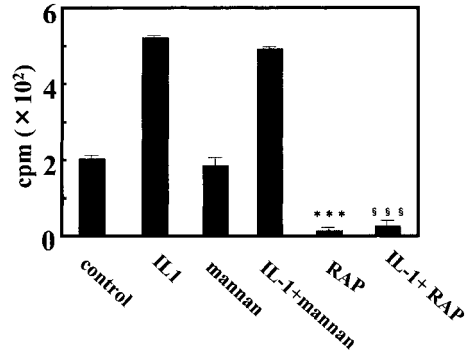

FIG. 5. Effects of receptor antagonists on the incorporation of ^{125}I-tPA by AML 12 cells. AML 12 cells were preincubated with mannan (10 mg/ml) or RAP (50 nM) for 30 min. After the preincubation, ^{125}I-tPA and IL-1 were added sequentially in this order, followed by incubation for 2 h. Radioactivity incorporated into the cellular fraction was then measured. Values represent the mean ± SE of 4 different cultures. $^{***}P < 0.005$ vs control at each time point. §§§$P < 0.005$ vs IL-1-treated-cells, by Student's paired *t* test.

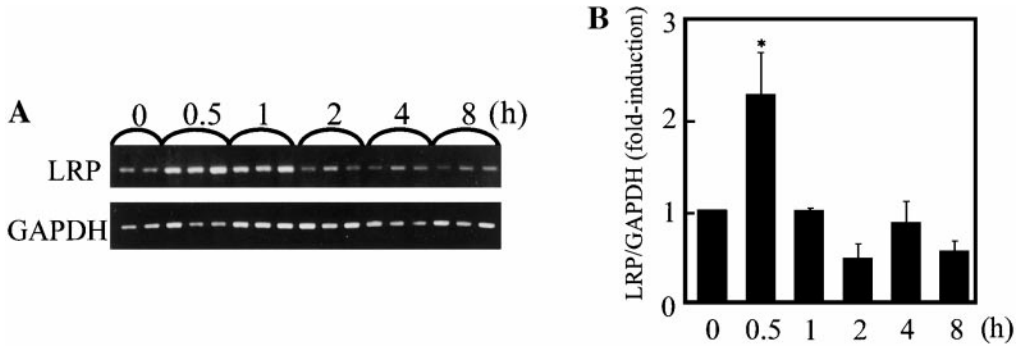

FIG. 6. Effect of IL-1 on LRP mRNA accumulation in AML12 cells. Equal amounts of total RNA (2.5 μg) extracted from AML 12 cells cultured for up to 8 h were subjected to RT-PCR. Typical ethidium bromide staining of a 476-bp PCR product of the LRP mRNA (A) and of GAPDH mRNA was used to quantify the products by a computer-assisted analyzer, and the amount of LRP mRNA was normalized to that of GAPDH mRNA (B). Each value represents the mean ± SE of 4 different cultures. $*P < 0.05$ vs control at each time point, by Student's paired *t* test.

the acceleration of tPA uptake in IL-1-treated AML 12 cells.

There are only a limited number of studies on the regulation of LRP so far. Moriyama *et al.* (54) reported the IL-1-mediated down-regulation of LRP mRNA in mesangial cells. Sitter *et al.* (47) reported that the LRP mRNA level in mesothelial cells was down-regulated by TNFα, which is believed to share many properties with IL-1. Thus the IL-1 may have different effects on renal cells and hepatocytes. RAP is an endoplasmic reticulum resident protein and the physiological role of RAP is thought to be a molecular chaperon for LRP (55). Although we are currently pursuing the examination of IL-1 regulation of RAP expression and of the LRP turnover, the down-regulation of RAP may cause the decrease in tPA clearance through the inhibition of intracellular maturation of LRP.

IL-1 is also known to decrease tPA activity/production in human cultured endothelial cells (56, 57). Fibrinolytic shut-down is often observed in the acute-phase response (29). Thus the down-regulation of tPA in both endothelium and hepatocytes as well as up-regulation of hepatic tPA clearance by IL-1 may contribute to the fibrinolytic shut-down. In preliminary experiments using IL-1β knockout mice, we found that plasma tPA protein level was decreased in turpentine-treated wild–type mice, but not in the turpentine-treated IL-1β deficient ones (Noguchi, T., Seki, T., and Ariga, T., unpublished data). Taken together, these results indicate that IL-1 down-regulates tPA in several cell types including endothelial cells and hepatocytes *in vitro* and *in vivo.*

In summary, we have demonstrated, for the first time, the down-regulation mechanism of tPA by IL-1 in hepatocytes. As the liver is the major organ responsible for the clearance of plasma PAs, the receptor-mediated endocytosis of tPA there may play a crucial role in the regulation of fibrinolysis.

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