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Endotoxin Induction of Plasminogen Activator and Plasminogen Activator Inhibitor Type 1 mRNA in Rat Tissues *in Vivo**

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The tissue-specific distribution of tissue-type and urokinase-type plasminogen activator (t-PA and u-PA) and their inhibitor type 1 (PAI-1) was analyzed at mRNA level in five major rat organ tissues. t-PA mRNA was detected in lung, kidney, heart, and liver. u-PA mRNA was detected in kidney and lung. Presence of PA mRNA correlated with the detection of PA activity in extracts of these tissues. PAI-1 mRNA was detected predominantly in heart and lung. Although PAI activity could not be measured directly in tissue extracts, the presence of PAI-1 mRNA correlated with the occurrence of PA·PAI complex in fibrin autography of tissue extracts.

Endotoxin injection caused a very large increase in plasma PAI activity. This increase correlated with a marked increase in PAI-1 mRNA in nearly all tissues studied. The increase in PAI-1 mRNA is most pronounced in lung and liver. Endotoxin injection also caused an increased level of t-PA mRNA in heart and kidney, and an increased u-PA mRNA level in kidney. mRNA analysis of freshly isolated and separated subfractionated liver cells showed that the marked increase in PAI-1 mRNA in the liver after endotoxin injection may be due mainly to a strong increase of PAI-1 mRNA in the liver endothelial cells.

Plasminogen activators are serine proteases that can activate the zymogen plasminogen to the active serine protease plasmin. The protease plasmin has a broad spectrum of activities and is thought to be involved in many processes where extracellular proteolysis occurs. Its role in fibrinolysis has been studied most extensively but plasmin may also play a role in processes such as cell migration, tissue remodeling, tumor development and metastasis, and angiogenesis (1).

Two types of plasminogen activators can be distinguished, tissue-type plasminogen activator (t-PA)[1] and urokinase type plasminogen activator (u-PA), which differ in immunological reactivity and are encoded by different genes (2–4).

The activity of plasminogen activators can be inhibited by specific inhibitors. At least two types of inhibitor exist, plasminogen activator inhibitor type 1 (PAI-1) and type 2 (PAI-2), encoded by different genes (5). Both PAI-1 and PAI-2 can form proteolytically inactive stable complexes with t-PA or u-PA.

It has previously been reported that treatment of rats with endotoxin or interleukin 1 increased plasma PAI activity rapidly (6, 7). A similar effect has been observed in cultured endothelial cells treated with endotoxin. In this case an increase in PAI-1 mRNA was observed (8). In contrast to the situation in cultured endothelial cells, no effect on the PAI-1 level has been found in endotoxin-treated cultured hepatocytes (6, 8). PAI-1 production by hepatocytes can be induced by other mediators such as glucocorticoids or cyclic AMP, however (9–11). Although the tissue distribution of plasminogen activator activity has been investigated (12, 13) as well as the t-PA mRNA levels in several murine tissues (14), the combination of the mRNA levels and activity of PA has to our knowledge not yet been demonstrated.

In this paper we have analyzed the mRNA levels of t-PA, u-PA, and PAI-1 and the plasminogen activator activities in several tissues of the rat. In addition the effect of endotoxin treatment of the rat on these mRNA and activity levels was studied. The effect of endotoxin treatment on the PAI-1 mRNA level in the liver was studied in more detail. The PAI-1 mRNA levels were determined in endothelial cells, Kupffer cells and parenchymal cells (hepatocytes) isolated from the liver of endotoxin-treated rats.

MATERIALS AND METHODS

Animal Experiments—For all animal experiments male Wistar rats (280–320 g), obtained from the Broekman Institute, Helmond, The Netherlands, were used. All experiments were performed under Nembutal (Sanofi, France) anesthesia (60 mg/kg; intraperitoneally).

Endotoxin Treatment—Rats were intravenously injected with endotoxin (*Escherichia coli* lipopolysaccharide, serotype 0128:B12, prepared by phenol extraction, Sigma) 50 μg/kg in a volume of 1 ml/kg. At the times specified, blood was obtained by aortic puncture, and anti-coagulated with 0.1 volume of trisodium citrate (0.13 M). Platelet-free plasma was prepared at 4 °C and stored at −20 °C. Tissues were obtained immediately after bleeding, briefly washed in saline, and snap-frozen in liquid nitrogen. Tissues were stored in air-tight containers at −70 °C.

Plasma PAI Activity—Plasma PAI activity was determined by titration of (diluted) citrate-treated plasma with human two-chain melanoma t-PA, followed by the spectrophotometric determination of residual t-PA activity, as described (15).

Tissue PA Activities—Frozen tissues, pooled from two rats, were weighted, triturated in liquid nitrogen, and resuspended (at 40 mg/ml) in extraction buffer (16) for all tissues except kidney, which was resuspended in 0.5 M KSCN. The tissue suspensions were then homogenized at 4 °C, using a Polytron PTA7 homogenizer for 1 min at full speed, and the extracts were centrifuged. PA activities in the supernatant were then determined spectrophotometrically essentially

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[1] The abbreviations used are: t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; PA, plasminogen activator; PAI, plasminogen activator inhibitor; kb, kilobase(s).

as described (17), using human t-PA as a standard. The plasminogen activator activity was determined in the presence and absence of anti-rat t-PA IgG. The difference between these activities was regarded as t-PA activity. Fibrin autography of tissue extracts was performed as described by Granelli-Piperno and Reich (18).

Liver Cell Isolation—Parenchymal liver cells were isolated from rats after a short collagenase (type IV, 0.05%, Sigma) perfusion (5 min) of the liver by the method of Seglen (19), modified as described by Kuiper *et al.* (20).

Endothelial and Kupffer cells were isolated after collagenase (type I, 0.05%, Sigma) perfusion of the liver at 37 °C and subsequent counterflow centrifugation, essentially as described by Nagelkerke (21) except for a replacement of the first elutriation step by a centrifugation step of 2 min, 75 × *g* (20). The purity of the cell fractions was routinely checked using light-microscopy. 3,3′-Diaminobenzidine peroxidase staining followed by Papanicolaou counterstaining was used to discriminate between Kupffer cells and endothelial cells (21). Staining with 1,1′-dioctadecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate acetyl low density lipoprotein was used to identify the endothelial cells (22). Using these methods it was found that the parenchymal cell fraction was pure for more than 99.5%, the Kupffer cell fraction was pure for more than 90%, and in the endothelial cell fraction no other cells could be detected.

The validity of these staining methods for determination of the purity of the isolated cell fractions has been demonstrated previously by the identification of the pattern of eicosanoid production in the liver cell fractions (23).

RNA Isolation—Tissues or cells were homogenized in 4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.5, 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol. RNA was isolated from the homogenate according to Chomczynski and Sacchi (24). Total RNA was quantified by absorbance at 260 nm. Poly(A$^+$) RNA was prepared by affinity chromatography, using oligo(dT)-cellulose (type 77F, Pharmacia, Sweden).

mRNA Analysis—The following cDNA fragments were used as probes in the hybridization experiments: a 1.9-kb *Bgl*II fragment of the human t-PA cDNA (25), a 1.0-kb *Eco*RI-*Pst*I fragment of the human u-PA cDNA (26), a 0.9-kb *Pvu*II fragment of the rat PAI-1 cDNA (27), a 1.2-kb *Eco*RI fragment of the human PAI-2 cDNA (kindly provided by Dr. E. K. O. Kruithof) (28), and a 1.2-kb *Pst*I fragment of the rat glyceraldehyde-3-phosphate dehydrogenase cDNA (kindly provided by Dr. R. Offringa, Ref. 29). The cDNA fragments were labeled with [^{32}P]dCTP (Amersham, United Kingdom) using the random primer method (Multiprime, Amersham, UK).

RNA samples were electrophoresed on a 1.2% agarose gel containing 7.5% formaldehyde. The RNAs were transferred to a nylon membrane (Hybond N, Amersham, UK) using a Vacugene system (Pharmacia, Sweden).

The membranes were hybridized at 60 °C in a solution containing 7% sodium dodecyl sulfate, 0.5 M $NaHPO_4$, pH 7.2, 10 mM EDTA with labeled cDNA fragments. The blots were routinely washed with 2 × SSC, 1% sodium dodecyl sulfate for 1 h at 60 °C (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate). Autoradiograms were prepared using Kodak XAR-5 film and intensifying screens at −70 °C.

RESULTS

Effect of Endotoxin on Plasma PA Inhibitor Activity—As reported previously (6), the injection of rats with endotoxin induced a rapid and dosage-dependent increase in plasma PAI activity. Here, the plasma PAI activity was analyzed at an endotoxin dosage of 50 μg/kg, in order to determine the optimal time after endotoxin injection for mRNA analysis. Peak plasma levels (about 40-fold increased above base-line levels) were seen at 4 h (Fig. 1). As the most rapid increase in PA inhibitor activity occurred between 2 and 4 h, it is likely that mRNA levels reached their maximum during this time period. For this reason tissue samples for mRNA analysis were collected at 3 h after endotoxin injection.

mRNA Level in Organs Before and After Endotoxin Treatment—The mRNA levels for t-PA, u-PA, and PAI-1 were determined in lung, liver, kidney, heart, and skeletal muscle. RNA from these tissues was subjected to electrophoresis on denaturing formaldehyde-agarose gels, transferred to nylon membranes, and hybridized with [^{32}P]dCTP-labeled cDNA

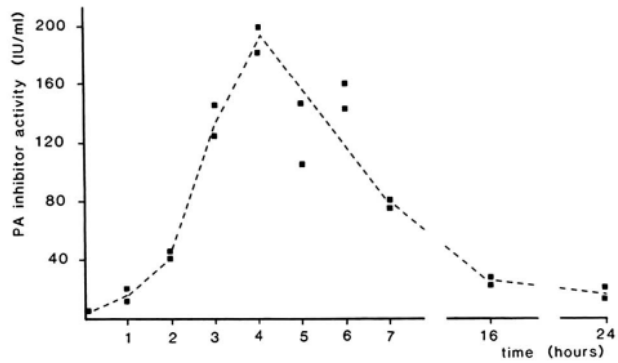

FIG. 1. **PAI activity in plasma of endotoxin-treated rats.** At $t = 0$ endotoxin was injected at a dose of 50 μg/kg. During 24 h after endotoxin injection PAI activity was determined as described under "Materials and Methods" and expressed in IU/ml.

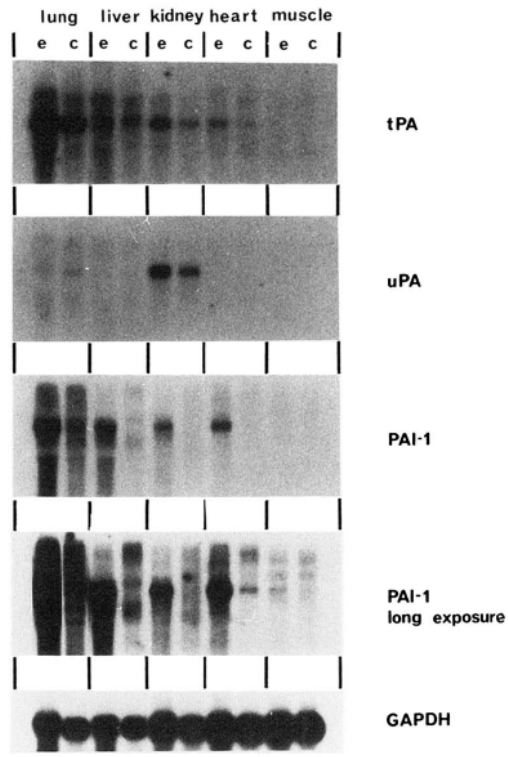

FIG. 2. **Northern blots of RNA extracted from different tissues of control (*C*) rats and rats treated with endotoxin (*E*).** Endotoxin (50 μg/kg) was injected 3 h before killing and RNA extraction. Poly(A$^+$) RNA isolated from 50 μg of total RNA was used in every lane. The filters were subsequently hybridized with DNA fragments of human t-PA, human u-PA, rat PAI-1, and rat glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

probes. In Fig. 2 the results are shown. No significant degradation of RNA had occurred and good hybridization signals could be detected, although for t-PA and u-PA heterologous probes were used. No cross-hybridization occurred between the various probes.

Under basal conditions (*lanes C* in Fig. 2), t-PA mRNA was detected in lung, liver, kidney, and heart tissue. The strongest hybridization signal was found in lung tissue. Intermediate signals were detected in liver and kidney tissue. In heart tissue only a very weak signal could be detected. For u-PA mRNA a strong signal was detected in kidney and a very weak signal in lung.

PAI-1 mRNA could be detected in lung and heart tissue (Fig. 2). PAI-2 mRNA could not be detected in the tissues

studied using a human cDNA fragment as a probe. The possible origin of the observed increase of plasma PAI activity after endotoxin injection was studied by analysis of the mRNA levels of PAI-1 in the various tissues. In Fig. 2, *lanes E*, it is shown that 3 h after endotoxin injection the PAI-1 mRNA level increased in all the tissues studied. The increase was most pronounced in lung and liver tissue. These results indicated that the increase of plasma PAI activity is paralleled by a detectable increase in PAI-1 mRNA levels. PAI-2 mRNA was not detected after endotoxin treatment in the studied tissues.

When analyzed at the same time interval after endotoxin injection, surprisingly, t-PA mRNA levels also appeared to be increased in kidney and heart tissue, while the u-PA mRNA in kidney tissue was also slightly increased, although the control glyceraldehyde-3-phosphate dehydrogenase mRNA showed little variations.

Plasminogen Activator Activity in Tissue Extracts Before and After Endotoxin Treatment—Plasminogen activator activity was determined in extracts from six tissues (lung, liver, kidney, heart, skeletal muscle, and aorta), obtained from control and endotoxin-treated (50 μg/kg, 3 h) rats (Table I). We were not able to isolate sufficient quantities of RNA from the aorta for mRNA analysis. In all other tissues, except kidney, the predominant plasminogen activator was t-PA, as demonstrated by antibody-quenching experiments (85–97%) (data not shown) and by fibrin autography (Fig. 3). After endotoxin treatment, in lung a small increase in t-PA activity was observed, while in all other tissues the t-PA activity was markedly decreased. Non-t-PA activity, as determined using anti t-PA antibodies in the assay, remained fairly constant (data not shown).

PAI-1 mRNA in Different Liver Cell Types Before and After Endotoxin Treatment—In the liver a strong increase in PAI-1 mRNA was observed after endotoxin injection (Fig. 2). As no influence of endotoxin on PAI-1 expression on cultured hepatocytes was found (6), we investigated which cell type in the liver is responsible *in vivo* for the endotoxin effect on PAI-1 expression. The presence of PAI-1 mRNA was determined in the various liver cell types of control rats and of rats after endotoxin injection (Fig. 4). Under normal conditions PAI-1 mRNA could be detected in liver endothelial cells only. After endotoxin injection a very strong increase of the PAI-1 mRNA level was seen in the endothelial cell fraction and, moreover, PAI-1 mRNA could be detected in the parenchymal (hepatocytes) cell fraction and in the Kupffer cell fraction. These results suggest that mainly the endothelial cells are responsible for the effect of endotoxin injection on the increase of PAI-1 mRNA in the liver. t-PA mRNA was below the detection limit in these RNA samples.

TABLE I

t-PA activity in tissue extracts (IU/g wet weight)

t-PA activity in tissue extracts of control rats and endotoxin-treated rats. Endotoxin (50 μg/kg) was injected 3 h before killing of the rat and isolation of the tissues. t-PA activity was determined spectrophotometrically. Human two-chain melanoma t-PA was used as a standard. t-PA activity was determined by the difference in PA activity in the presence and the absence of anti-rat t-PA antibodies.

	Controls	Endotoxin-treated	Change in activity
Lung	1826	2165	+19%
Liver	28	3	−89%
Kidney	231	93	−60%
Heart	238	115	−52%
Muscle	33	20	−39%
Aorta	95	28	−71%

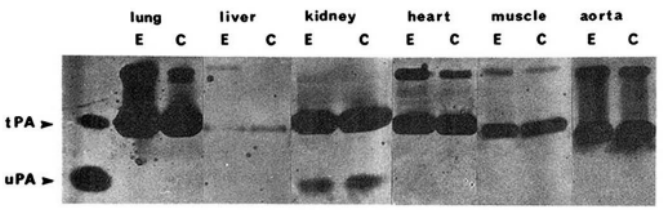

FIG. 3. **Fibrin autography of extracts from different tissues of control (*C*) and endotoxin-treated (*E*) rats.** Endotoxin (50 μg/kg) was injected 3 h before killing and tissue extraction. Equal quantities of sample for each tissue before and after endotoxin were used but sample volumes of different tissues were adjusted to give lysis zones of comparable intensities. Rat urine containing t-PA and u-PA was used as a standard.

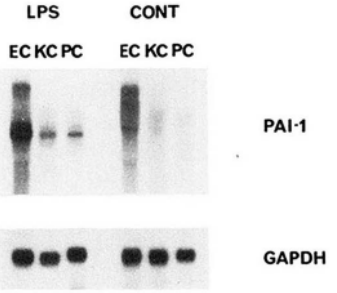

FIG. 4. **PAI-1 mRNA in various cell types of rat liver.** Three h after endotoxin (*LPS*) injection in rats, the livers were perfused and various cell types were separated: endothelial cells (*EC*), Kupffer cells (*KC*), and parenchymal cells (*PC*). RNA was isolated and hybridized with rat PAI-1 cDNA. The *lower panel* shows the same blot hybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA.

DISCUSSION

The specific mRNAs of the plasminogen activators, t-PA and u-PA, and their inhibitors, PAI-1 and PAI-2, were determined in major rat organ tissues. t-PA mRNA was found in the lung, the liver and kidney, and the heart. u-PA mRNA could only be detected in kidney and lung tissue. In muscle neither t-PA nor u-PA could be detected. PAI-1 mRNA could, under basal conditions, be detected in lung and heart tissue. PAI-2 mRNA could not be detected in the tissues studied. It cannot be excluded that this is due to a lack of homology between rat PAI-2 mRNA and the human cDNA probe used. The difference in t-PA and PAI-1 mRNA occurrence in rat and human liver is striking. In rat liver t-PA mRNA was detected but no PAI-1 mRNA while in human liver PAI-1 mRNA but no t-PA mRNA was detected (9, 30). Analysis of the PAI-1 mRNA in the rat showed only one transcript for PAI-1, in contrast to human and bovine PAI-1 (5, 31). This is in accordance with the PAI-1 RNA analysis in cultured rat HTC cells (10).

Rat tissues were also analyzed for the presence of PA activity. The tissue-specific distribution of PA and PAI mRNA is consistent with the presence of PA activity or PA·PAI complexes (as detected using fibrin autography). The presence of the mRNA may give a good indication for the occurrence of the corresponding protein. But as can be seen from our data, Table I and Fig. 2, in tissues with comparable mRNA levels for t-PA (liver and kidney), very different t-PA activities were observed. This indicates that besides regulation of mRNA levels other regulatory mechanisms are involved, such as proenzyme activation (2), receptor binding (32, 33), complex formation with inhibitors (5) or translational control of the production of t-PA, u-PA, PAI-1,and PAI-2 (34).

At 3 h after endotoxin injection the plasma PAI activity

was steeply increasing and had not yet reached its maximum. PAI-1 mRNA analyzed at the same time point was markedly increased in nearly all the tissues studied. This increase was most pronounced in lung and liver tissue. Thus, the increase in plasma PAI activity after endotoxin injection is most likely caused by increase of PAI-1 mRNA.

Unexpectedly, the mRNA levels for t-PA (in kidney and heart) and to a lesser extent u-PA (in kidney) also increased after endotoxin injection. The increase in PAI-1 mRNA levels is in agreement with the detection of more PA-PAI complexes in the fibrin autography experiments. These observations, increase of PAI-1 mRNA and increase of t-PA and u-PA mRNA after endotoxin injection *in vivo* have, to our knowledge, not been reported before, although recently a rapid increase in plasma t-PA antigen level after endotoxin injection was described (35).

It is striking that the PAI-1 mRNA increase is most pronounced in those organs which are known to be extensively vascularized, such as the lung and the liver. This might suggest that endothelial cells are involved in the PAI-1 increase after endotoxin injection.

This possibility was studied in more detail by subfractionation of the various liver cell types and analysis of the increase in PAI-1 mRNA levels in these liver cell types. Subfractionation of the liver in parenchymal cells (hepatocytes), endothelial cells and Kupffer cells provides a powerful tool to locate the cellular sites of mRNA synthesis *in vivo*. After the rat was injected with endotoxin, the liver cells remain in their natural environment, and are able to exert their cell to cell contacts, until they are harvested for RNA extraction.

A marked increase in PAI-1 mRNA was seen in the liver endothelial cells but some PAI-1 mRNA could also be detected after endotoxin injection in the Kupffer and parenchymal cells. The detection of PAI-1 mRNA in the Kupffer cell fraction is probably due to a slight contamination (<10%) with endothelial cells. The parenchymal cell fraction was nearly 100% pure, therefore the detected PAI-1 mRNA is likely to be produced in these cells (20–23). The experiments showed that *in vivo,* at least in the liver, the endothelial cells are mainly responsible for the strong increase in PAI-1 after endotoxin injection (Fig. 4), but a minor contribution of the hepatocytes cannot be excluded. In contrast to the *in vivo* situation, both cultured liver endothelial cells and hepatocytes produce PAI-1 (9, 36). It cannot be concluded from our data that the results obtained with liver endothelial cells can be extrapolated to vascular endothelial cells, although the data might suggest this. The rapid increase in plasma PAI activity after endotoxin injection suggests an acute phase response. A classical acute phase response is thought to be restricted to hepatocytes (37). The increase in PAI-1 mRNA is seen in many tissues and is not limited to the liver. Furthermore, in the liver the increase is mainly due to endothelial cells and not hepatocytes. Therefore the increase of PAI-1 after endotoxin injection cannot be regarded as a classical acute phase response.

In this study we have demonstrated that the tissue-specific distribution of t-PA, u-PA, and PAI-1 mRNA correlates with the presence of PA activity and PA·PAI complexes in these tissues. We also showed that the sharp increase in plasma PAI activity after endotoxin injection is due to a sharp increase in PAI-1 mRNA, while t-PA and u-PA mRNA levels are increased as well. The increase in PAI-1 mRNA in the liver *in vivo* can be mainly ascribed to an increase of PAI-1 mRNA in the endothelial cells.

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