INTERLEUKIN 1 AND LIPOPOLYSACCHARIDE INDUCE AN INHIBITOR OF TISSUE-TYPE PLASMINOGEN ACTIVATOR IN VIVO AND IN CULTURED ENDOTHELIAL CELLS

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In the physiological process of fibrinolysis, the enzymes now considered to be of prime importance are plasminogen and tissue-type plasminogen activator (t-PA)[1] (1). Recently, evidence has been presented (1) for the occurrence in plasma of a fast-acting PA inhibitor. Together with the fast-acting plasmin inhibitor α_2-antiplasmin, the PA-inhibitor determines to a great extent the expression of the fibrinolytic potential of plasma (1). After the discovery of the PA-inhibitor, it has rapidly become clear that the plasma levels of the PA-inhibitor are highly variable, and can be subject to rapid changes, e.g. following trauma, surgery, or infection.

During a study of factors affecting PA-inhibitor levels in rats, we noted that LPS greatly increased plasma PA-inhibitor concentrations. In this paper, we describe the effects of LPS and of IL-1 (a postulated mediator of LPS-induced effects) on PA-inhibitor levels in rat plasma, and on PA-inhibitor synthesis by human endothelial cells, as these cells are known to secrete PA-inhibitor in vitro (2).

Materials and Methods

Rats. Male Wistar rats (200–250 g body weight) were obtained from the Centraal Proefdierenbedrijf TNO (Zeist, The Netherlands). All animal experiments were performed under Nembutal anaesthesia (60 mg/kg i.p.).

Cultured Human Endothelial Cells. Human endothelial cells were isolated from umbilical cord veins and grown to confluency on fibronectin-coated dishes as described (3). Subcultures were obtained by trypsin/EDTA at a split ratio of 3:1.

Lipopolysaccharide. Escherichia coli LPS (serotype 0128:B12), prepared by the phenolic extraction procedure, was obtained from Sigma Chemical Co. (St. Louis, MO). A stock solution of 1 mg/ml in sterile saline was stored at −20°C.

Human IL-1. Human IL-1 was supplied by Genzyme (Haverhill, United Kingdom) as a solution of 100 U/ml in 5% FCS in RPMI 1640. The IL-1 had been prepared by immunoadsorbtion chromatography from *Staphylococcus albus*–stimulated human monocytes.

Recombinant Murine IL-1. rIL-1, lot 14827-48, 3×10^6 U/ml, was a gift from Dr. P. T. Lomedico (Hoffman-La Roche, Inc., Nutley, NJ) (4). Polymyxin B, cycloheximide, and actinomycin D were obtained from Sigma Chemical Co.; saline and human serum albumin from CLB (Amsterdam, the Netherlands). Following the procedure prescribed by the

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[1] *Abbreviations used in this paper:* PA, plasminogen activator; t-PA, tissue-type PA.

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manufacturer (Kabi Vitrum, Stockholm, Sweden) all materials used were tested for endotoxin contamination by the Coatest Endotoxin kit, and found to be endotoxin-free (detection limit 5 pg/ml), except for rIL-1 (see Results).

Animal Experiments. LPS was injected i.v. in a volume of 2 ml/kg. Control rats received saline only. At the times specified, blood was obtained by aortic puncture and anticoagulated with 0.1 volume of trisodium citrate (0.13 M). Platelet-free plasma was prepared and stored at −70°C.

Human IL-1 (diluted to 10 U/ml, using a 1% solution of human serum albumin in saline) was infused for 60 min through the vein of the penis at a dosage of 50 U/kg body weight. rIL-1 (diluted to 1,000 U/ml with albumin/saline) was given similarly at a dose of 5,000 U/kg. Blood samples were obtained from a cannula in the carotid artery before starting of the infusion, and after 1, 2, 3, and 4 h. Controls for human IL-1 received the same volume of 1% human albumin in saline. Controls for rIL-1 received heated rIL-1 (100°C for 60 min).

Cell Culture Experiments. Confluent cultures were used at second or third passage, and were always refed the day before the experiment. Conditioned media were obtained by incubating cells at 37°C for various times (up to 24 h) with culture medium, or (after two washes with serum-free medium) with serum-free medium containing 0.3 mg/ml human serum albumin. Conditioned media were centrifuged to remove cells and cell debris, and stored at −20°C. Protein was determined according to Wang and Smith (5), using BSA as a standard.

PA-inhibitor Assay. The fast-acting inhibitor of t-PA was determined by titration of samples with increasing amounts of t-PA, followed by spectrophotometric measurement of the residual t-PA activity, as described by Verheijen et al. (6). The PA-inhibitor activity of the sample was then determined by graphical extrapolation (6, 7). The PA-inhibitor activity is expressed in IU of t-PA inhibited.

Stability of PA-inhibitor. Samples were incubated at 37°C for 1–24 h, followed by determination of residual PA-inhibitor activity (8).

Results

Rat plasma, like human plasma, contained t-PA inhibitor, as detected by titration with t-PA. In plasma from control rats, the PA-inhibitor activity was 21.6 ± 2.5 IU/ml (mean ±SD; $n = 27$). In parallel experiments, the PA-inhibitor activity of pooled human plasma was 13.3 ± 1.4 IU/ml ($n = 20$). Injection of LPS into rats resulted in a rapid increase in plasma PA-inhibitor activity. At an LPS dosage of 10 μg/kg (Fig. 1 *a*), a significant increase, up to 320% of the control value, was already seen after 2 h, while maximally increased values of ~1,500% were reached 4 h after injection. At later time intervals, PA-inhibitor levels gradually returned to normal. A second injection of LPS (10 μg/kg), 24 h after a first dose of 10 μg/kg, again resulted in increased PA-inhibitor levels 4 h later, although the effect of the second injection was slight diminished (Fig. 1 *a*). Significantly increased PA-inhibitor concentrations were found (at 4 h) after injecting LPS at a dose of as little as 10 ng/kg, increasing responses being obtained up to dosages of 1 mg/kg (Fig. 1 *b*). Rats, bled 4 h after injection with LPS (10 μg/kg), did not differ from control rats with regard to plasma plasminogen (92 ± 11% of controls; mean ±SD; $n = 5$), fibrinogen (97 ± 8%) and α_2-antiplasmin (98 ± 10%) concentrations.

As endothelial cells are known (2, 3, 9) to secrete a PA-inhibitor in vitro, we subsequently studied the effect of LPS on PA-inhibitor synthesis by cultured human endothelial cells. As shown in Fig. 2 *a* and Table I, LPS dose-dependently increased PA-inhibitor synthesis. Cycloheximide (1 μg/ml) suppressed both basal and LPS-induced inhibitor synthesis to 22 ± 6% of the control values after 8 h

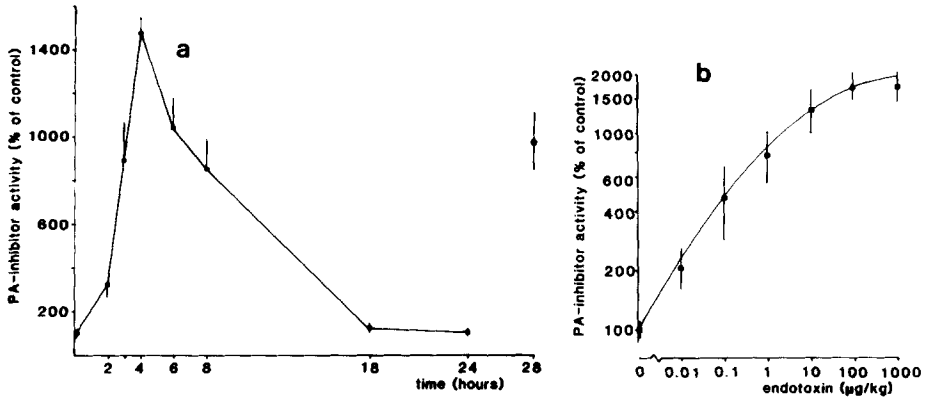

FIGURE 1. PA-inhibitor activity in rat plasma after i.v. injection of *E. coli* LPS. Data shown in *a* are means ±SD (■) (n = 4–7) of PA-inhibitor activity in plasma from rats bled at various times after injecting LPS (10 µg/kg). Also shown is the effect of two injections of LPS (both 10 µg/kg), at 0 and at 24 h; plasma was obtained at 28 h (●) (n = 4). *b* shows means ±SD (n = 4) of PA-inhibitor activity in rat plasma 4 h after injecting increasing amounts of LPS. In both graphs, the 100% value equals 21.6 ± 2.5 IU/ml.

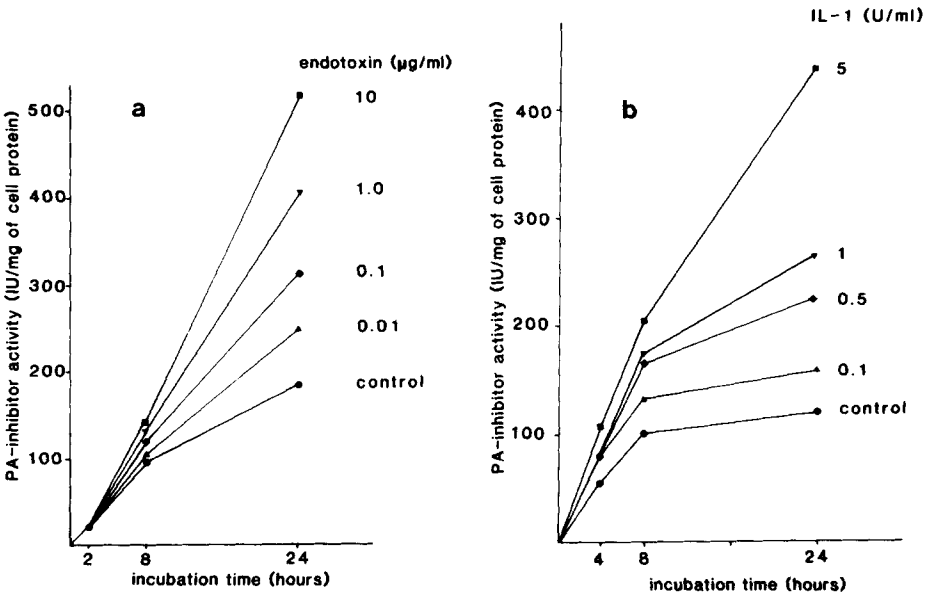

FIGURE 2. PA-inhibitor activity in (secondary) endothelial cell–conditioned medium after incubation for various periods of time in the presence of LPS (0–10 µg/ml) (*a*), or of human IL-1 (0–5 U/ml) (*b*). In this representative experiment (compare Table I), the culture medium contained 20% (vol/vol) of serum.

of incubation. Actinomycin D (40 ng/ml) suppressed the LPS-induced increase in inhibitor synthesis (LPS, 192 ± 61%, LPS plus actinomycin D, 106 ± 21% of control values), showing that the effect of LPS required *de novo* mRNA synthesis. No increased inhibitor synthesis was seen when, before being added to cells, polymyxin B, an antibiotic that inactivates LPS, was mixed with LPS (data not shown).

TABLE I

Effect of LPS or Human IL-1 on PA-inhibitor Activity in Conditioned Media from Cultured Human Endothelial Cells

Stimulant added	PA-inhibitor activity (IU/mg cell protein)*		
	Serum-free medium (8 h)	Serum-free medium (24 h)	Serum-containing medium (24 h)
No addition	32.5 ± 7.3 (4)	40.2 ± 20.6 (5)	87.4 ± 55.4 (5)
LPS (10 μg/ml)	64.8 ± 33.7 (4)	125.8 ± 45.2 (6)	398.0 ± 67.9 (7)
Human IL-1 (5 U/ml)	124.9 ± 9.7 (3)	193.0 ± 61.0 (3)	366.7 ± 23.3 (7)

* Mean ± SD. Number of cultures (n) is shown in parentheses.

TABLE II

Effect of Infusion with IL-1 on PA-inhibitor Activity in Rat Plasma

Time (h)	PA-inhibitor activity (% of time 0 value)			
	Human IL-1	Human IL-1 (controls)	rIL-1	rIL-1 (controls)
0	100 ± 12	100 ± 15	100 ± 14	100 ± 7
1	94 ± 6	104 ± 6	ND	ND
2	104 ± 13	104 ± 16	171 ± 30*‡	118 ± 6
3	132 ± 23	105 ± 8	161 ± 25*	128 ± 24
4	139 ± 16*‡	108 ± 5	163 ± 16*	158 ± 52

Rats were infused for 1 h with human IL-1 (50 U/kg) or with rIL-1 (5,000 U/kg), and blood samples were obtained at hourly intervals thereafter. Data shown are means ±SD of three rats in each group. Time 0 values were 18.1 ± 4.0 IU/ml for the six animals treated with IL-1, and 18.0 ± 3.1 IU/ml for the six control animals.

* Significantly different from time 0 value.

‡ Significantly different from control value at that time (student's t-test; $p < 0.05$).

Also, increased inhibitor synthesis could be induced in cultured endothelial cells by human IL-1. The dose dependency and the time course of the effect of human IL-1 is shown in Fig. 2*b*. As was the case for LPS, the human IL-1–induced effect was suppressed by cycloheximide and actinomycin D (human IL-1 at 5 U/ml, 321 ± 14% of control values; human IL-1 plus cycloheximide, 48 ± 18%; human IL-1 plus actinomycin D, 106 ± 14%). Heating the IL-1 for 15 min at 80°C completely undid its effect, while the same procedure would not affect the LPS-induced increase in PA-inhibitor synthesis (data not shown). Similar results were obtained using rIL-1, although at least 20 times higher rIL-1 concentrations were required to obtain a similar effect. The effect of rIL-1 was again abolished by heating, but was insensitive to polymyxin B (data not shown). The LPS- and IL-1-induced increases in inhibitor level could not be ascribed to a decreased release of t-PA by endothelial cells, as determined by enzyme immunoassay of t-PA antigen (T. Kooistra, unpublished observations).

Infusion of IL-1 (both human and mouse recombinant) into rats resulted in small but significant increases in plasma PA-inhibitor activity (Table II). The slow increase of PA-inhibitor in rats treated with heated rIL-1 can probably be ascribed to the endotoxin content of the infusate (60 pg/ml). Also, in this case rIL-1 proved to be less active than human IL-1.

Both the endothelial PA-inhibitor and the induced PA-inhibitor in rat plasma

were unstable during incubation at 37°C. The in vitro half-life at 37°C was 2.0 ± 0.3 h for the rat plasma inhibitor, and 2.3 ± 0.3 h (mean ±SD; $n = 6$) for endothelial inhibitor.

Discussion

This study defines LPS and IL-1 as potent inducers of PA-inhibitor, both in vivo in rats and in human endothelial cell cultures. That the inhibitory activity measured by the titration assay is indeed due to PA-inhibition and not to plasmin inhibition has been demonstrated before (6, 9). Moreover, plasma levels of the major plasmin inhibitor α_2-antiplasmin were unchanged in LPS-treated rats. A similar effect of LPS on PA-inhibitor activity has recently been described in rabbits in vivo and in cultured human endothelial cells by Colucci et al. (10). Whether the effects observed in vivo are due to increased PA-inhibitor synthesis by endothelial cells remains speculative, since the cellular origin of plasma PA-inhibitor is still unknown. However, LPS and IL-1 did not increase PA-inhibitor synthesis by the human hepatoma cell line Hep G2 or by primary monolayer cultures of human hepatocytes (our unpublished observations), but then the induced plasma PA-inhibitor and the endothelial cell–derived PA-inhibitor are closely related with regard to molecular weight (7), inhibition constant (K_i; 2–5 pM [7]), immunochemical characteristics (8), and instability at 37°C. Therefore it is likely that these PA-inhibitors are homologous proteins, and that the increase in plasma inhibitor is due to increased synthesis by endothelial cells.

The induction of PA-inhibitor synthesis in cultured endothelial cells strongly resembles the induction of procoagulant activity in those cells, also induced by LPS (11) and by IL-1 (12). Although endothelial cells in vitro can synthesize IL-1 (13) and IL-1 synthesis is increased by LPS (14), it remains to be established whether LPS acts by way of increased IL-1 synthesis in endothelial cells.

Murine IL-1 proved less potent in our experiments than human monocyte-derived IL-1, both in the rat in vivo and in the human cell culture system. As the rIL-1 used (4) is an IL-1α (15), and human monocytes contain predominantly mRNA for IL-1β (15), the data suggest that IL-1β might be a better inducer of PA-inhibitor than IL-1α. Verification of this suggestion will, however, require further experiments.

Summary

Human IL-1, recombinant murine IL-1 and *E. coli* LPS were found to be potent inducers of plasminogen activator (PA)-inhibitor activity, both in vivo, in rats, as well as in cultured human endothelial cells.

In vivo, LPS rapidly and dose-dependently (0.01–1,000 μg/kg) increased plasma PA-inhibitor activity. Infusion of IL-1 into rats resulted in a small but significant increase in PA-inhibitor activity in rat plasma.

Likewise, in cultured human umbilical vein endothelial cells, LPS and IL-1 induced increased synthesis of PA-inhibitor.

We suggest that the induced rat plasma inhibitor might be of endothelial origin.

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