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CD39 Modulates IL-1 Release from Activated Endothelial Cells

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The activation of endothelial cells (EC) and monocyte–macrophages (Mϕ) by lipopolysaccharide (LPS) is considered an important element of the vascular injury observed in endotoxemia. Interleukin-1 (IL-1) β release from Mϕ in response to LPS, appears to be mediated by the autocrine/paracrine release of ATP via P2X7 receptor activation. In EC, similar nucleotide-mediated signaling pathways may be influenced by high levels of expression of CD39, the vascular nucleoside triphosphate diphosphohydrolase (NTPDase; ENTPD I). To determine whether CD39 modulates ATP-mediated release of IL-1 from EC, we stimulated human EC with LPS and measured levels of ATP secretion and IL-1 release. LPS triggered ATP secretion from EC that was soon followed by IL-1α release. Overexpression of CD39 following infection with recombinant CD39 adenoviral vectors (AdCD39) abrogated the initial phase of ATP secretion and inhibited IL-1α release; comparable results were obtained with soluble NTPDase. These data demonstrate that CD39/NTPDase modulates IL-1α release from LPS stimulated human EC. © 2000 Academic Press

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Interleukin-1 (IL-1) is an important mediator of the host defense responses and acute inflammatory mechanisms that develop during bacterial sepsis and infection with gram negative organisms (1). Disordered regulation of IL-1 secretion/release may also be associated with chronic inflammatory states (2). IL-1 has multiple biological activities and is produced by activated monocyte-macrophages (Mϕ) and endothelial cells (EC) (1–3).

The biological activity of IL-1 is derived from two distinct but related gene products, IL-1α and IL-1β that are approximately 27% homologous (4). The primary translation products of the two forms are synthesized as precursor molecules of 31 to 35 kDa (5, 6). The precursor form of IL-1α, pro-IL-1α, has biological activity while pro-IL-1β does not and must undergo post-translational modification, prior to interaction with the receptor (7). IL-1β is typically cleaved to the biologically active 17 kDa form by the action of IL-1β converting enzyme, a cysteine protease or caspase (8, 9). Cleavage of IL-1α to the 17 kDa derivative is not required for biologic activity and active forms were originally isolated in a variety of 10 to 20 kDa proteins (10, 11). Subsequent data has suggested that pro-IL-1α may be also cleaved by a calpain-like enzyme (12, 13). In addition, calcium ionophores A23187 and ionomycin (14), as well as ATP, have been reported to induce processing of IL-1α (15).

IL-1α is an important cytokine mediating multiple facets of EC activation. Tissue factor and vascular endothelial growth factor-C can be induced in response to stimulation with IL-1α (16, 17). Supernatants of IL-1α stimulated EC potentiate the effects of cytokines on proliferation of hematopoietic progenitors (18). CD44 is induced by IL-1α exposure in EC (19, 20). Spontaneous release of IL-1α has the potential to activate EC in culture (21) and may in addition regulate human EC migration *in vitro* (22).

The manner of IL-1 secretion from cells is unclear as neither of the IL-1 isoforms contain signal sequences for targeting to the secretory apparatus of the cell (4). Thus IL-1 has been reported to be synthesized on free ribosomes and to exist as a cytosolic protein (23, 24). One hypothesis proposes that IL-1 can be only released in response to cell injury or pericellular proteolysis (25,

Abbreviations used: EC, endothelial cells; Mϕ, monocyte–macrophages; LPS, lipopolysaccharide; IL-1, interleukin-1; NTPDase, nucleoside triphosphate diphosphohydrolase; AdCD39, recombinant CD39 adenovirus; Adβ-gal, recombinant β-galactosidase adenovirus; HUVEC, human umbilical vein endothelial cells.

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26). Such efficient proteolytic processing and release of mature, activated IL-1 requires a secondary signal, in addition to the primary stimulus (e.g., LPS), that promotes transcription and translation (15, 27–29). This requirement for a second stimulus provides an additional checkpoint by which cells may regulate biogenesis of this important cytokine. For example, peritoneal macrophages activated with LPS *in vivo* require a second stimulus to initiate efficient IL-1β maturation/release. Purinergic stimulation with extracellular ATP could provide this signal (30).

Both IL-1α and IL-1β are released from LPS activated peritoneal macrophages following ATP stimulation *in vitro* (15, 29) and *in vivo* (30). Cytolytic T cells (15), and freshly isolated human monocytes in response to LPS activation also release IL-1 (31). ATP appears to trigger IL-1β release by stimulating macrophages and microglial cells via the P2X7 receptor and this process precedes the leakage of cytoplasmic markers by cell death; this pathway is presumably based on an autocrine loop involving LPS-triggered ATP secretion (32, 33).

EC express high levels of the membrane associated CD39/nucleoside triphosphate diphosphohydrolase (NTPDase; ENTPD I) (34, 35). Although monocytes elaborate less of this specific NTPDase, they also secrete a soluble ectonucleotidase CD39L4 that has preferential ADPase activity. Therefore, CD39 expressed by EC could modulate IL-1 release. Few studies have examined IL-1α export, but mechanisms of release appears to be comparable; albeit there may be a greater tendency for cell-association of this IL-1 isoform (36).

In this study, we demonstrate that ATP and IL-1α are released from human EC following LPS stimulation. The secretion of ATP precedes IL-1α release and exposure to NTPDases abrogates this ATP paracrine loop, and decreases IL-1α release. Our data therefore indicate that CD39 modulates IL-1α release from LPS stimulated EC.

MATERIALS AND METHODS

Adenoviral vectors. A replication defective adenovirus vector, type 5, encoding for CD39 cDNA (37) and the control recombinant β-galactosidase adenovirus (Adβ-gal) contained the *Escherichia coli lac Z* gene were generated as described (38). Viral titers were determined by an average of two plaque assays and expressed as plaque forming units per ml (pfu/ml), by standard convention (39). Virus stocks were expanded as previously described (40), aliquoted in small volumes, and stored in PBS with 10% glycerol at −80°C and thawed on only one occasion. The viral titers used for these experiments ranged from 10^{10} to 10^{13} pfu/ml.

Cell cultures. Human umbilical vein endothelial cells (HUVEC) were cultured in medium 199 (BIO WHITTAKER, Walkersville, MD) supplemented with 20% fetal bovine serum (ATRANTA Biologicals, Norcross, GA), 50 μg/ml epidermal mitogen (Biomedical Technologies Inc., Stoughton, MA), 25 units/ml heparin (Sigma), 50 units/ml Penicillin G sodium (GIBCO BRL; Life Technologies, Inc., Grand Island, NY), 50 μg/ml streptomycin sulfate (GIBCO BRL), and 2 mM L-glutamine (GIBCO BRL) (41). One day prior to stimulation, HUVEC were incubated with Dulbecco's modified Eagle medium (GIBCO BRL) supplemented with 15% fetal bovine serum, 50 units/ml penicillin G sodium, 50 μg/ml streptomycin sulfate, 2 mM L-glutamine, and 100 μM adenosine (Adenocard; Fujisawa, Japan) to ensure adequate cellular nucleotide salvage.

The U937 cell (monocyte cell line) line (ATCC No. CRL-1593.2) was cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 50 μg/ml epidermal mitogen, 25 units/ml heparin, 50 units/ml Penicillin G sodium, 50 μg/ml streptomycin sulfate, and 2 mM L-glutamine.

In selected experiments, HUVEC were infected with recombinant adenoviruses in serum-free medium at 100 pfu/cell for 1 h. This was followed by washing and replacement with the original medium containing 20% serum. Two days later, cells were exposed to LPS (1 or 10 μg/ml).

Under certain conditions, HUVEC culture media were also supplemented with 0.8 U/ml of potato apyrase (Sigma; potato ATP diphosphohydrolase) to hydrolyze extracellular ATP right prior to LPS stimulation.

LPS or ATP-stimulation. HUVEC were stimulated with 1 or 10 μg/ml of LPS (Sigma: *Escherichia coli* Serotype 055:B5), with or without 1 μM, 10 μM, 100 μM or 1 mM of ATP (Sigma). Selected cultures of HUVEC were exposed to 1 mM of ATP, 2 h post LPS (1 μg/ml) stimulation. U973 cells were treated in the same manner.

IL-1 assays. IL-1α and IL-1β concentrations in cell culture medium and cell lysates post-stimulation were measured by ELISA using Quantikine Human IL-1 Immunoassay (R&D Systems, Minneapolis, MN). HUVEC lysates were prepared for assay by freeze-thaw cycles.

Measurement of extracellular ATP. ATP concentrations were determined using the specific enzymatic reaction of firefly luciferase with luciferin, as previously described (41).

Measurement of ATPDase activity assay. Membrane-bound NTPDase activity was determined by measurement of the amount of liberated inorganic phosphate, hydrolyzed from exogenous, extracellular ATP. Adenoviral-infected HUVEC were incubated in 20 mM Tris pH 8.0 containing 5 mM $CaCl_2$, and either 200 μM ADP or 200 μM ATP for 15 min at 37°C. Release of free phosphate was then determined using published methodology, using the Malachite green technique (42, 43).

Statistical analysis. The statistical analysis was performed by a two-tailed paired *t* test for comparison with the control group (Microsoft Excel 5; Microsoft). Differences were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Release of IL-1 by HUVEC. IL-1α release followed LPS stimulation in both HUVEC and U937 cells but was a relatively minor product in the latter. Non-stimulated HUVEC released 0.9 ± 0.1 pg/10^6 cells of IL-1α by 24 h and 1.3 ± 0.3 at 48 h (mean ± SD, $n = 5$). Following LPS stimulation (1 μg/ml), the released levels of IL-1α were 11.1 ± 1.4 pg/10^6 HUVEC by 24 h and 15.9 ± 2.9 at 48 h. Higher LPS concentrations (to 10 μg/ml) increased the levels of IL-1α released by 24 h (17.2 ± 6.8 pg/10^6 cells) and also at 48 h (23.9 ± 8.3). U937 cells released 1.6 ± 0.3 pg/10^6 cells of IL-1α by 24 h and 7.9 ± 1.1 at 48 h post-LPS stimulation. Control levels in unstimulated HUVEC

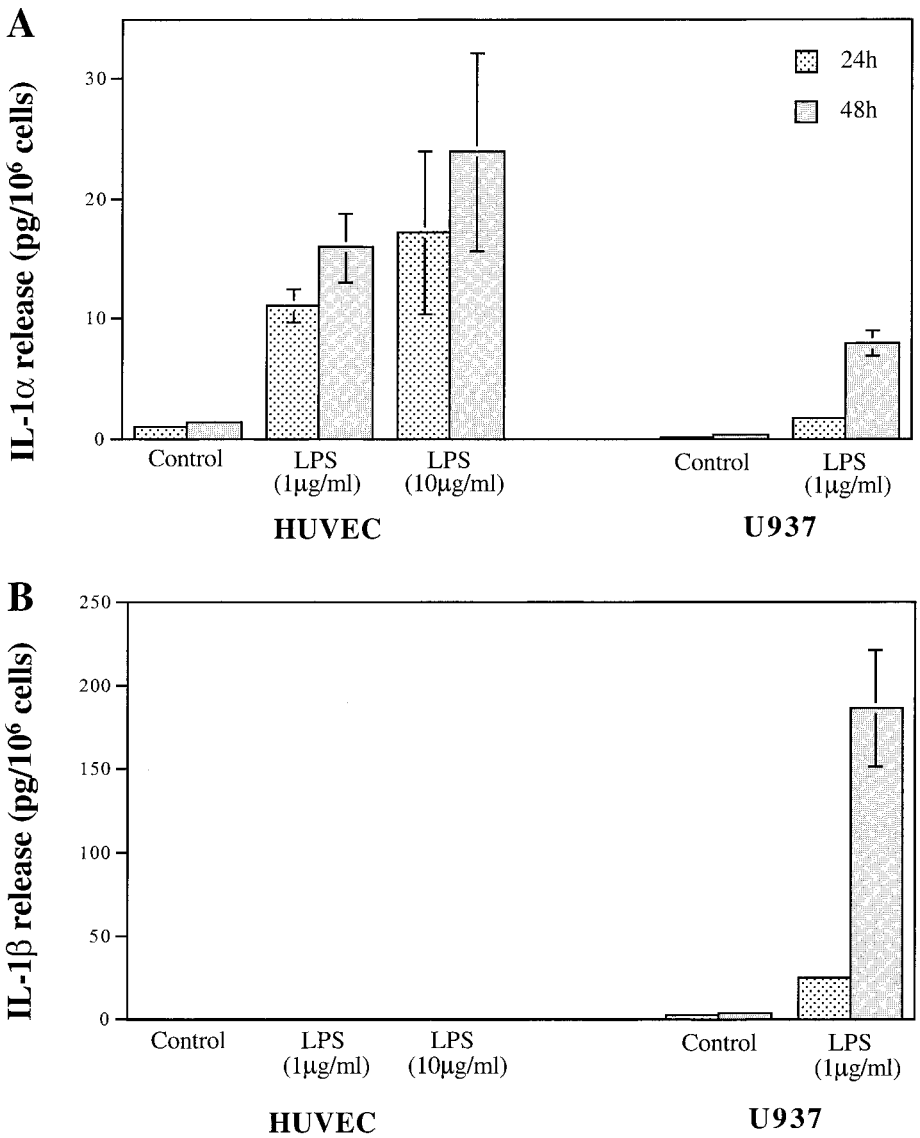

FIG. 1. IL-1 release from LPS-stimulated endothelial cells (HUVEC) and monocyte cell line (U937). HUVEC were stimulated with 1 μg/ml and 10 μg/ml of LPS and the supernatants were collected 24 and 48 h after LPS stimulation. U937 cells were stimulated with 1 μg/ml of LPS and harvest fluids collected free of cells and cytokines measured in same manner. Concentrations of IL-1α (A) and IL-1β (B) in the conditioned medium were measured by ELISA.

were 0.2 $\pm$ 0.1 pg/10^6 cells by 24 h and 0.2 $\pm$ 0.1 at 48 h (mean $\pm$ SD, $n = 3$) (Fig. 1A).

IL-1β was not detected in the medium of HUVEC following LPS stimulation, but was however substantially released from LPS stimulated U937 cells (24.1 $\pm$ 2.5 pg/10^6 cells at 24 h; 185.5 $\pm$ 35.5 pg/10^6 cells (mean $\pm$ SD, $n = 3$) at 48 h) (Fig. 1B). ATP stimulation alone did not result in IL-1β release from U937 (data not shown).

Previous studies have indicated that both IL-1α and IL-1β are expressed in LPS stimulated rabbit aortic tissues (44, 45), hypercholesterolemic monkey aortic segments (46) and tissue homogenates from human abdominal aneurysms (47). These data indicate that IL-1α and IL-1β associated with all components of arterial vasculature including EC, smooth muscle cells and monocytes.

Recent papers have shown that the accumulation and release of endogenous IL-1α may result in spontaneous activation of EC in culture (21). IL-1α may also function as an intracellular regulator of the migratory capacity of the human EC (22) and this isoform of IL-1 may be of importance to EC. In addition, IL-1β deficient mice have normal vascular EC phenotypes (48). Our data suggest that IL-1α is important in initial phase of vascular inflammation prior to leukocyte/monocyte infiltration and IL-1β elaboration.

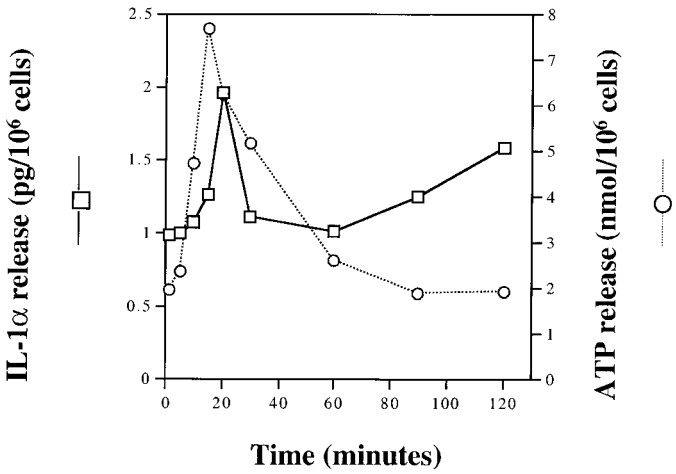

FIG. 2. Early IL-1α release from LPS-stimulated EC. HUVEC were stimulated with 1 μg/ml of LPS and the harvest fluids were collected at predetermined time points (1, 5, 10, 15, 20, 30, 60, 90, 120 min). IL-1α was measured by ELISA and extracellular ATP levels was determined by luciferase–luciferine assay.

IL-1α release in EC treated with ATP following LPS stimulation. Expose of HUVEC to ATP 2 h post LPS (1 μg/ml) stimulation had minimal effects on IL-1α release (LPS + ATP; 14.7 $\pm$ 5.8 pg/10^6 cells at 24 h and 18.8 $\pm$ 6.7 at 48 h; LPS alone; 11.1 $\pm$ 4.1 at 24 h and 16.0 $\pm$ 5.7 at 48 h: mean $\pm$ SD, $n = 3$). ATP stimulation alone (1 μM–1 mM) did not induce IL-1α and IL-1β release from HUVEC (data not shown). These results suggest that the exposure of EC to extracellular ATP at an early phase is necessary to induce IL-1α release from activated EC. Endogenous release of ATP may provide for maximal effects.

Kinetics of IL-1α and ATP release. We next studied rates of IL-1α and ATP release from HUVEC, 2 h after LPS (1 μg/ml) stimulation. Extracellular ATP concentrations increased 15 min after LPS stimulation (2.0 to 7.6 nmol/10^6 cells at 15 min) but then rapidly decreased to baseline levels in keeping with phosphohydrolysis by exposed CD39. Extracellular IL-1α concentrations increased from baseline levels to 2.0 pg/10^6 cells at 20 min post stimulation (Fig. 2). The levels began to climb more substantially at 60 min post-stimulation.

Patterns of IL-1α and ATP release from stimulated HUVEC to 24 h are shown in Fig. 3A. Extracellular ATP concentrations rapidly returned to baseline after the early peak, as described above. However, IL-1α elaboration and consequent release increased constantly to 48 h after stimulation (Fig. 1A).

Overexpression of CD39. Membrane associated ATPDase activities were significantly increased in AdCD39 infected HUVEC (14.5 $\pm$ 3.5 Pi nmol/min: mean $\pm$ SD, $n = 3$) when compared with Adβ-gal infected (1.01 $\pm$ 0.023) ($P < 0.05$) and non-viral-infected control HUVEC (0.95 $\pm$ 0.0025) ($P < 0.05$). Maximum levels of ATP release post-LPS stimulation of AdCD39 infected HUVEC at 15 min were substantially decreased when compared to the control groups (AdCD39; 2.5 nmol/10^6 cells, Adβ-gal; 6.9, control; 7.7)

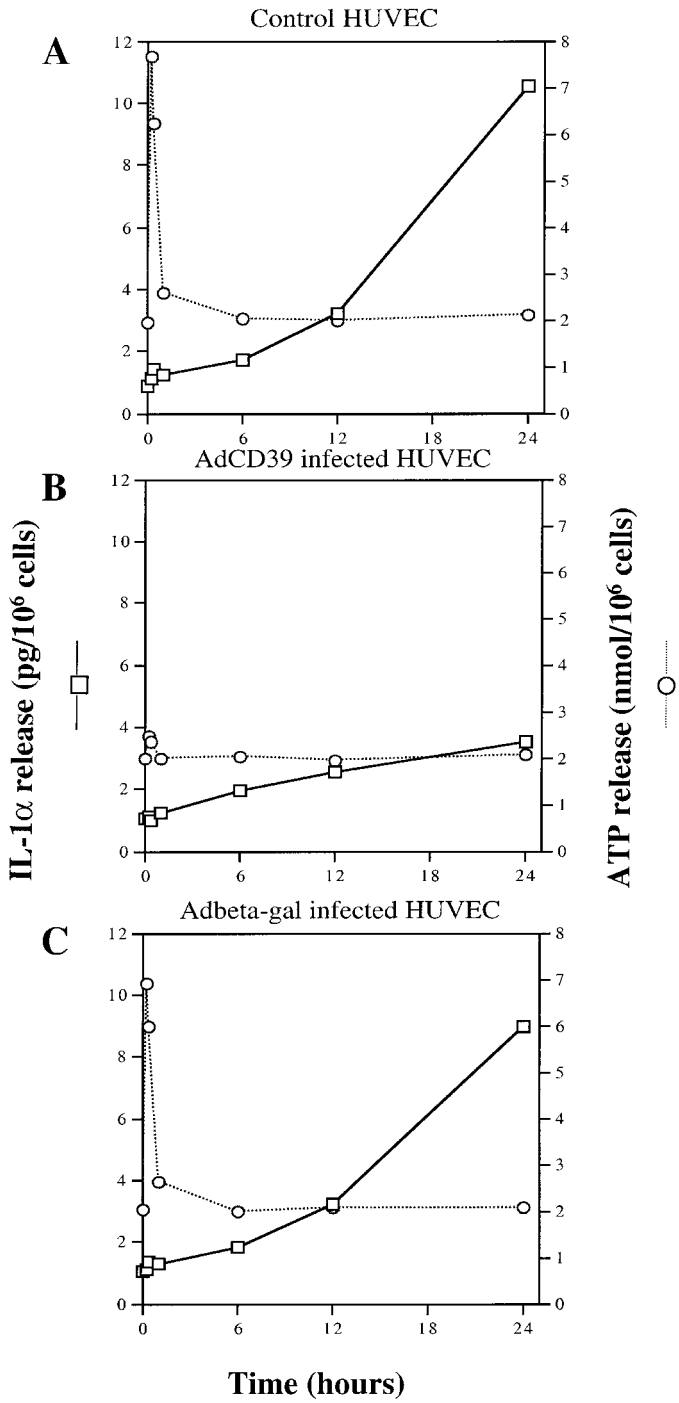

FIG. 3. Delayed IL-1α release from LPS-stimulated adenoviral infected EC. (A) Noninfected. (B) AdCD39-infected and (C) Adβ-gal-infected HUVEC. LPS stimulation as performed before (1 μg/ml). IL-1α and ATP measurements were determined as for Fig. 2.

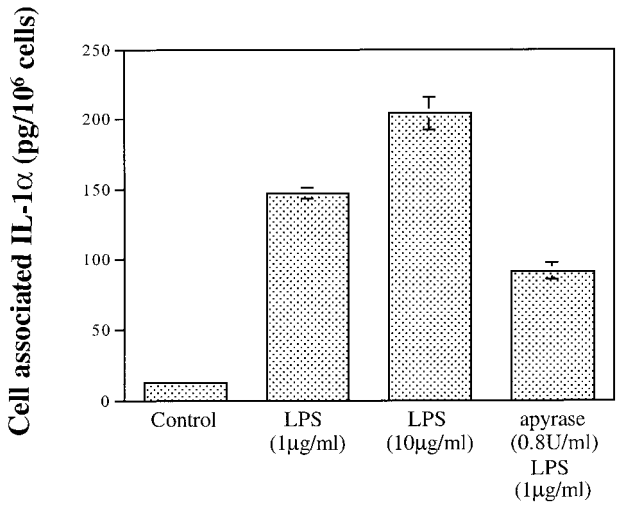

FIG. 4. Intracellular levels of IL-1α in LPS-stimulated endothelial cells. IL-1α concentrations measured in HUVEC cell lysates following 48 h stimulation of LPS (1 μg/ml).

(Fig. 3). IL-1α release from AdCD39 infected HUVEC at 24 h was also much lower than in control groups (AdCD39; 3.49 pg/10^6 cells, Adβ-gal; 8.97, control; 10.51) (Fig. 3). Levels of IL-1α and ATP release from Adβ-gal infected HUVEC were comparable to those seen in non-infected control HUVEC (Figs. 3A and 3C).

Soluble NTPDase supplementation. Levels of IL-1α release from HUVEC treated with apyrase at 24 h post-LPS stimulation (6.4 $\pm$ 0.59 pg/10^6 cells; mean $\pm$ SD, $n = 3$) decreased relative to LPS stimulated HUVEC in the absence of exogenous NTPDase (11.1 $\pm$ 1.37 pg/10^6 cells). The IL-1α concentration in conditioned medium did not differ between apyrase administration and control in the absence of LPS stimulation (data not shown).

IL-1α concentration in cell lysates. IL-1α concentration in HUVEC lysates, 48 h after stimulation with LPS was significantly higher (147 $\pm$ 4.2 pg/10^6 cells 1 μg/ml and 204.5 $\pm$ 12.0 pg/10^6 cells by 10 μg/ml; mean $\pm$ SD, $n = 3$) than in controls (12.9 $\pm$ 0.7 pg/10^6 cells; mean $\pm$ SD, $n = 3$). Apyrase administration significantly decreased IL-1α levels to 91.6 $\pm$ 5.9 pg/10^6 cells ($P < 0.05$) (Fig. 4). These results suggest that LPS induced additional IL-1α protein synthesis in EC and that this increase was ATP-dependent in that it could be inhibited by NTPDase.

Mechanisms of IL-1 release from LPS stimulated human Mϕ are still unclear. Several authors have been shown that extracellular ATP triggers pre-formed IL-1 release via stimulation of purinergic receptor P2Z/K^+ channels (32, 33). ATP stimulates release of both IL-1α and IL-1β from LPS stimulated murine macrophages via a similar mechanism, that is accompanied by cell death (29). We show that ATP release was maximal at 15 min after LPS stimulation, and IL-1α at 20 min (Fig. 2). These results suggest that the mechanism of IL-1α secretion from LPS stimulated EC may be influenced by NTPDase expression via modulation of purinergic mechanisms.

Other authors have indicated that IL-1 release requires a secondary signal in addition to LPS stimulation to promote transcription and translation (15, 27–29), and that extracellular ATP may be a secondary stimulus to initiate IL-1β maturation/release from peritoneal macrophages (30). These data are in keeping with our own observations in EC. However, in addition we have shown that CD39 inhibits IL-1α release from LPS-stimulated EC, further suggesting that ATP is an important mediator of this process.

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