Secretion of Intracellular IL-1 Receptor Antagonist (Type 1) Is Dependent on P2X7 Receptor Activation

Heather L. Wilson, Sheila E. Francis, Steven K. Dower and David C. Crossman

*J Immunol* 2004; 173:1202-1208; doi: 10.4049/jimmunol.173.2.1202

http://www.jimmunol.org/content/173/2/1202
Secretion of Intracellular IL-1 Receptor Antagonist (Type 1) Is Dependent on P2X7 Receptor Activation

Heather L. Wilson,† Sheila E. Francis,* Steven K. Dower,* and David C. Crossman†

Inflammatory mechanisms are critical in the arterial response to injury. Both IL-1 and the naturally occurring inhibitor of IL-1, IL-1R antagonist (IL-1ra), are expressed in the arterial wall, and in particular in the endothelium. Previous studies suggest that endothelial cells only make the intracellular type I isofrom of IL-1ra (icIL-1ra1), an isofrom known to lack a secretory signal peptide. It is unclear how icIL-1ra is released from the endothelial cell to act as an antagonist on cell surface IL-1 type I receptors. IL-1β, which also lacks a secretory signal peptide, may be released by ATP stimulation of the P2X7R. Therefore, we examined whether icIL-1ra1 release occurs in an analogous manner, using both the mouse macrophage cell line RAW264.7 and HUVECs. P2X7R activation caused icIL-1ra1 release from LPS-primed RAW264.7 macrophages and from HUVECs. This release was inhibited in the absence of extracellular calcium, and attenuated by preincubation with oxidized ATP, KN62, and apyrase. Endogenous ATP release, which also facilitated release of icIL-1ra1, was detected during LPS treatment of both RAW264.7 macrophages and HUVECs. Annexin V assays showed that ATP stimulation resulted in a rapid phosphatidylserine (PS) exposure on the cell surface of RAW264.7 macrophages, and that PS-exposed microvesicles contained icIL-1ra1. However, PS flip and microvesicle shedding was not apparent in ATP-treated HUVECs. These data support a general role for the P2X7R in the release of leaderless cytokines into the extracellular medium, and indicate how icIL-1ra1 may act upon its extracellular target, the IL-1R. The Journal of Immunology, 2004, 173: 1202–1208.
to a rapid exposure of phosphatidylserine (PS) upon the cell surface, followed by microvesicle shedding. These small vesicles were found to contain the processed and biologically active mature form of IL-1β (24). These results suggest that microvesicle shedding may represent a general pathway for the release of cytoplasmic proteins that lack leader sequences. Therefore, we sought to determine whether icIL-1ra1 is also released into the extracellular medium via activation of the P2X7-R. This would provide an insight into the cellular mechanisms used to balance IL-1 and IL-1ra levels. The release mechanism was analyzed using both transfected icIL-1ra1 in RAW264.7 cells, as a model macrophage system. In addition, we examined P2X7-R dependence of icIL-1ra1 release from cultured HUVECs. The results indicate that in both cases release of the intracellular form of the IL-1R antagonist can be regulated by P2X7-R activation, however, there are significant differences between the two cell types.

Materials and Methods

Cells and solutions

Murine RAW264.7 macrophages were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 2 mM glutamine, 10% heat-inactivated FCS (BioWhittaker, Walkersville, MD), 100 U/ml penicillin, and 100 μg/ml streptomycin.

HUVECs were isolated by 0.1% type IV collagenase treatment from umbilical cords collected from the Sheffield Hospital Maternity unit. Freshly isolated cells were cultured at 37°C on gelatin-coated flasks in medium 199 (Sigma-Aldrich, St. Louis, MO). From passage 1, cells were grown in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA), to avoid culture in high ATP concentrations. These media were supplemented with 20 μg/ml EC growth supplement (Tatom Biologicals, Northampton, U.K.), 90 μg/ml heparin (Sigma-Aldrich), 1% Fungizone (Invitrogen Life Technologies), 10% FCS, and 10% newborn calf serum (Invitrogen Life Technologies) (25).

RAW264.7 macrophages were transfected using Fugene-6 (Roche, Basel, Switzerland) according to manufacturer’s instructions. HUVECs were transfected at passage 1 using Lipofectin reagent (Invitrogen) (26). To avoid culture in high ATP concentrations, these media were supplemented with 20 μg/ml EC growth supplement (Tatom Biologicals, Northampton, U.K.), 90 μg/ml heparin (Sigma-Aldrich), 1% Fungizone (Invitrogen Life Technologies), 10% FCS, and 10% newborn calf serum (Invitrogen Life Technologies) (25).

Results

P2X7-R-dependent icIL-1ra1 release from the RAW264.7 macrophage cell line

To determine whether icIL-1ra1 is released via a P2X7-R-dependent mechanism, we used RAW264.7 macrophages as a transfected monocyte-derived cell line and model. The only Abs that are available to detect endogenous IL-1ra are unable to distinguish between the secreted and intracellular isoforms of this protein. Therefore, to distinguish the leaderless isoform of IL-1ra released into the extracellular medium following cellular stimulation, we tagged icIL-1ra1 with a myc epitope. Human icIL-1ra1-myc was transfected and expressed in RAW264.7 cells as shown by immunoblotting of cell extracts harvested 24 h posttransfection (Fig. 1A). These cells were cultured under various conditions, and release of icIL-1ra1-myc into the extracellular medium was measured by immunoblotting of the supernatants after gel electrophoresis. Cells that had been pretreated for 3 h with LPS only or with LPS followed by Bz-ATP did release icIL-1ra1-myc into the supernatant; this release being inhibited in the presence of the ATP-degrading enzyme apyrase, inhibited by the absence of extracellular calcium, and partially blocked by the addition of Ox-AKT (Fig. 1, B and C). This activation and inhibition profile indicates that the release of icIL-1ra1-myc is dependent upon activation of the P2X7-R. The ability of LPS pretreatment alone to mediate release of icIL-1ra1-myc is likely due to LPS causing endogenous ATP release, as has been previously demonstrated in RAW264.7 cells (26). We also found that supernatant samples from cells treated with LPS contained detectable levels of ATP (using the luciferase assay; data not shown) compared with supernatants from unstimulated or apyrase-treated cells, where no ATP was present.

P2X7-R-dependent microvesicle shedding by RAW264.7 cells

Release of the leaderless cytokine IL-1β from macrophages and monocytes has been shown to be via a microvesicle-shedding mechanism (24). The microvesicle shedding is preceded by a rapid exposure of PS on the outer surface of the plasma membrane, and also on the surface of the microvesicles containing the IL-1β protein. This may be a general mechanism of release of leaderless proteins, including icIL-1ra1, from the cell. Therefore, we tested, using annexin V conjugated to FITC, whether P2X7-R activation in RAW264.7 macrophages also results in PS flip in the plasma membrane. Fig. 2A shows RAW264.7 macrophages pretreated with LPS, then stimulated with Bz-ATP for various times, in the presence of annexin VFITC. Within 10–15 min, annexin VFITC was clearly seen bound to the cell surface indicating that PS exposure had occurred. Vesicle formation was observed at the cell surface.
from 10 to 15 min after ATP application (Fig. 2A), and live microscopy showed vesicles detaching from the cell surface. Cells that were not stimulated with Bz-ATP did not show any significant annexin V FITC binding when incubated for up to 45 min (data not shown). To determine whether PS-exposed microvesicles containing icIL-1ra1 were released from these macrophages, we isolated microvesicles by pull-down assays using annexin V-coated beads (24). Supernatants from cells stimulated with LPS, or LPS and Bz-ATP, were incubated with annexin biotin linked to streptavidin beads. Fig. 2B shows that the bound fraction in the pull-down assays contained icIL-1ra1-myc, if the protein had been released into the extracellular medium due to activation of the P2X 7 R, but that after LPS stimulation alone the amount was lower and not associated with PS-positive vesicles.

**FIGURE 1.** Release of icIL-1ra1-myc from transfected RAW264.7 macrophages. A, Transfected cell extracts under different treatment conditions were immunoblotted to detect expression of myc-tagged protein corresponding to the molecular mass of the transfected icIL-1ra1-myc. The control (left, first treatment), stimulated with LPS and Bz-ATP, was for EGFP transfection (40 ± 5% transfection efficiency as determined by microscopy). B, Supernatants from these same cell samples were immunoblotted for the presence of icIL-1ra1-myc under these different treatment conditions. A and B, a typical experiment; C, data pooled from a total of five separate determinations of supernatants for mean band density values ± SEM.

**FIGURE 2.** Annexin V binding to stimulated RAW264.7 macrophages following P2X-R activation. A, Bright-field and fluorescent images of RAW264.7 macrophages following Bz-ATP exposure in the presence of FITC-conjugated annexin V (5 μl/100 μl). B, Annexin V biotin pull down of supernatants from RAW264.7 macrophages that were untreated (Con), pretreated with LPS only (LPS), or pretreated with LPS then stimulated with Bz-ATP (LPS + ATP). For each treatment the following samples were analyzed by immunoblotting: supernatant, following concentration using 10-kDa molecular mass cut-off filters; annexin V biotin pull-down fraction (annexin bound); and the annexin V biotin unbound fraction, following concentration with 10-kDa cut-off filters (annexin unbound).

Only icIL-1ra1 has been detected in ECs from human coronary artery and human umbilical vein after treatment with LPS and PMA (4). Therefore, we determined whether icIL-1ra1 is released from ECs by a mechanism similar to that in the RAW264.7 macrophage model. Fig. 3, A and B, shows results from HUVECs.
following various treatments, when supernatants were immunoblotted with IL-1ra Ab. LPS treatment, followed by Bz-ATP stimulation, resulted in a maximal release of IL-1ra into the extracellular medium. Significant release was also observed with LPS treatment alone and even in the absence of exogenous agonists (Fig. 2B), this being inhibited in the absence of extracellular calcium, and attenuated in the presence of apyrase, Ox-ATP, or KN62. This profile of inhibition indicated that the release of IL-1ra was likely dependent upon activation of the P2X$_7$R. The release of IL-1ra from LPS-treated and unstimulated cells was likely the result of the presence of endogenous ATP released from the HUVECs, as these released fractions were found to contain ATP when measured by luciferase assay (Fig. 3C), and no IL-1ra was present in supernatant removed at time zero (Fig. 3, A and B, left bar).

The results in Fig. 3 show release of endogenous IL-1ra from HUVECs. However, use of the IL-1ra Ab meant that we were unable to distinguish what isofrom of IL-1ra had been released into the extracellular medium. Therefore, HUVECs were transfected with an icIL-1ra1-myc construct to determine whether this specific leaderless isofrom could be released from ECs. Detection of icIL-1ra1 was performed by immunoblotting of transfected HUVEC cell extracts (Fig. 4A) and supernatants (Fig. 4, B and C) with anti-myc Ab. Release of icIL-1ra1-myc occurred when cells were pretreated with LPS, then stimulated with Bz-ATP. This was inhibited in the absence of extracellular calcium, or by addition of apyrase or Ox-ATP. LPS treatment alone did lead to release of icIL-1ra1-myc from HUVECs, the levels varying significantly according to cell batch (data not shown).

**P2X$_7$R stimulation does not cause PS translocation in ECs**

We determined whether P2X$_7$R activation of HUVECs resulted in PS exposure, and whether PS-microvesicles contained icIL-1ra1-myc from transfected HUVECs. Fig. 5A shows a typical example of HUVECs pretreated with LPS, then stimulated with Bz-ATP. No annexin V-FITC binding was detected at 15 and 30 min after stimulation, or up to 60 min (data not shown). In contrast, cells treated with staurosporin for 18 h, then washed and incubated with annexin V-FITC, did show binding (Fig. 5B). Annexin biotin pulldown assays showed that icIL-1ra1-myc-containing supernatants from LPS and LPS + Bz-ATP-treated cells did not bind to annexin V-coated beads, being detected in the unbound fraction (Fig. 5C).

**P2X$_7$R activation does not cause cell necrosis/lysis**

To assess whether release of icIL-1ra1 from both RAW264.7 macrophages and HUVECs could have occurred as a result of cell lysis for all treatments, cells were observed for lysis by microscopy when each sample was taken, but no apparent necrotic or apoptotic appearance was noted. Supernatants were also measured for the presence of LDH, which is released upon cell lysis. Fig. 6, A and B, shows LDH levels from RAW264.7 and HUVEC-released supernatants, respectively. None of the fractions contained significant levels of LDH, compared with control samples of cells stimulated with staurosporin, or cells lysed in 1% Triton X-100.

**Discussion**

Our data demonstrate that icIL-1ra1 is released or secreted from cells by an extracellular ATP-dependent mechanism, in both the RAW264.7 macrophage cell model and in HUVECs. Transfection of myc-tagged icIL-1ra1 into both the mouse macrophages and HUVECs enabled us to distinguish between this isofrom and sIL-1ra. The action of extracellular ATP in mediating this release is likely to be attributable to activation of the P2X$_7$R in both cell types. The P2X$_7$R is expressed and functional in RAW264.7 macrophages (27) and also in ECs (28, 29). Release of icIL-1ra1 from RAW264.7 macrophages and HUVECs was inhibited by the removal of extracellular calcium, and was significantly attenuated by incubation with Ox-ATP, KN62, and apyrase. This inhibition profile fits well with an action at the P2X$_7$R. Therefore, we may conclude that ATP-stimulated release of icIL-1ra1 is most likely mediated by activation of the P2X$_7$R.
Stimulation of the P2X 7 R on RAW264.7 macrophages resulted in PS translocation (PSflip), as demonstrated by annexin V FITC binding to the cell surface. By microscopy we observed the formation of microvesicles, which were able to bud off from the cell surface. The PS flip occurred within a relatively rapid time frame, within a few minutes of activation by ATP. As previously demonstrated by MacKenzie et al. (24), PS flip after brief (≤10 min) P2X,R activation in the human monocytic cell line THP-1 is reversible and not associated with cell death. In this study, we did not detect any LDH release from either ATP-stimulated RAW264.7 macrophages or from HUVECs. PS exposure is associated with microvesicle shedding from THP-1 cells (24), the vesicles containing biologically active 17-kDa processed IL-1β. By analogy, we were able to isolate PS-exposed vesicles from Bz-ATP-stimulated RAW264.7 macrophages using annexin-coated beads. The annexin bound fraction contained icIL-1ra1, indicating that, as with IL-1β, PS-exposed microvesicle formation and shedding provides a release mechanism for at least one icIL-1ra. Some icIL-1ra1-myc was detected in the “annexin unbound” fraction for

FIGURE 4. Release of icIL-1ra1-myc from transfected HUVECs. A, Transfected cell extracts under different treatment conditions were immunoblotted to detect expression of myc-tagged protein corresponding to the molecular mass of the transfected icIL-1ra-myc. The control (left, first treatment), stimulated with LPS and Bz-ATP, was for EGFP transfection (25 ± 8% transfection efficiency as determined by microscopy). B, Supernatants from these same cell samples were immunoblotted for the presence of icIL-1ra1-myc under these different treatment conditions. A and B, a typical experiment; C, data pooled from three separate determinations for mean band density values ± SEM.

Stimulation of the P2X,R on RAW264.7 macrophages resulted in PS translocation (PS flip), as demonstrated by annexin VFITC binding to the cell surface. By microscopy we observed the formation of microvesicles, which were able to bud off from the cell

FIGURE 5. Annexin V binding to stimulated HUVECs following P2X,R activation. A, Bright-field (left panel) and fluorescent images of HUVECs following Bz-ATP exposure (at 0, 15, and 30 min) in the presence of FITC-conjugated annexin V (5 μl/100 μl). B, Bright-field (left panel) and fluorescent images of HUVECs following staurosporin treatment (18 h) in the presence of FITC-conjugated annexin V (5 μl/100 μl) at 0, 15, and 30 min after addition of annexin VFITC. C, Annexin V biotin pull down of supernatants from HUVECs that were untreated (Con), pretreated with LPS only (LPS), or pretreated with LPS then stimulated with Bz-ATP (LPS + ATP). For each treatment the following samples were analyzed by immunoblotting: supernatant, following concentration using 10-kDa molecular mass cut-off filters; annexin V biotin pull-down fraction (annexin bound); and the annexin V biotin unbound fraction, following concentration with 10-kDa cut-off filters (annexin unbound).
macrophages treated with LPS alone. Therefore, it is possible that
LPS may stimulate release of icIL-1ra by an independent mecha-
nism, perhaps akin to that observed in HUVECs. However, the
levels of icIL-1ra released due to LPS treatment alone were much
lower than for the ATP-mediated PS-exposed release.

By microscopy we observed the predominant formation of mi-
crovesicles, with fewer large blebbing events, in ATP-activated
RAW264.7 cells using high micromolar concentrations of Bz-ATP
and in a low sodium extracellular solution. Release of icIL-1ra1
occurred in low sodium extracellular solution for both ATP-activ-
ated RAW264.7 murine macrophages and HUVECs. Several
studies indicate that ATP-mediated cytokine-containing mi-
crovesicle shedding and cell surface “tethered” blebbing occur via
separable mechanisms. Microvesicle formation occurs optimally
at submillimolar concentrations of Bz-ATP or ATP in both HEK293
cells and BAC1 murine macrophages, associated with IL-1β re-
lease in the macrophages (30). Whereas P2X, R-mediated, large,
tethered bleb formation requires millimolar ATP concentrations,
and is dependent on the serine/threonine kinase ROCK I (31),
RhoA activation, and Rho-effector kinase activity (30). In addition,
removal of extracellular sodium results in a reduction in P2X, R-
mediated cell blebbing (32), but does not inhibit microvesicle
shedding (24). Hence, the conditions used in this study favored
the microvesicle formation that was observed for RAW264.7 cell
stimulation, resulting in release of icIL-1ra1.

Removal of extracellular calcium prevented the release of icIL-
1ra1 in both RAW264.7 cells and in HUVECs. Release of IL-1β
from THP-1 monocytes is also prevented in the absence of extra-
cellular calcium (24). A recent detailed analysis of the calcium
dependence of IL-1 release from murine peritoneal macrophages
showed that ATP-mediated release IL-1β is independent of cal-
cium influx, but requires release of intracellular calcium, and K+
eflux (33). In this study, removal of extracellular calcium will also
have had the effect of depleting intracellular calcium stores, hence
it is possible that intracellular stores are required for the release of
icIL-1ra1, an area for future investigation.

In HUVECs, we were unable to detect PS flip following activa-
tion of the P2X-R, despite the fact that icIL-1ra1 is released from
these ECs in a P2Xγ-dependent manner. It is possible that any
PS flip in this cell type was at a lower level compared with the
ATP-induced exposure in the monocytes and macrophages, and is
below the threshold of detection in our experimental system.
Alternatively, the P2Xγ-mediated release of icIL-1ra1 from ECs
may occur via a PS flip-independent mechanism, providing an im-
portant area for future investigation. Functional P2Xγ channels
have been measured in bovine aortic endothelium as assessed by
whole cell electrophysiological recording, and by immunocyto-
chemistry (28). However, there was an absence of YO-PRO-1 up-
take in these cells, indicating that the functional regulation of the
P2X-R may differ in this cell type, compared with monocyte-de-

derived cells. This difference in channel function may be attribut-
table to differences in protein-binding interactions due to tissue
specific expression. This may in turn relate to cell-type-dependent differ-
ences in the mechanism of release of leaderless proteins following
activation of the P2X-R. Differences in the expression of other
purinergic receptor subtypes between ECs and macrophages may
also lead to altered responses according to cell type. Alternatively,
differences between the responses of the murine macrophages and
human ECs may be due to the difference between species.

No release of the cytoplasmic protein LDH was observed from
either RAW264.7 macrophages or HUVECs under conditions
where icIL-1ra1 was released, suggesting that the secretory mecha-

nism is selective. Therefore, the release of icIL-1ra1 does not
occur via a general, and nonspecific, export route for any intracel-

lular protein. Nor is it a consequence of breakdown of the plasma
membrane barrier, concomitant, for example, with cell death.

Our investigation has shown that icIL-1ra1 can be released into
the extracellular environment. Keratinocytes, like the endothelium,
predominantly express the intracellular isoform of the receptor an-
tagonist (8). Previously it was shown that icIL-1ra1 is released from
keratinocytes into the supernatant via a leaderless brefeldin A-in-
dependent mechanism, which is not associated with cytotoxicity
by LDH assay (34). The levels of release are altered according to
the maturity of the keratinocytes (34) and are up-regulated in skin
cells from atopic dermatitis patients compared with normal con-
trols (35). icIL-1ra1 is released into the extracellular medium from
airway epithelial cells, and is not associated with LDH-assayed
cytotoxicity (36). In each of these studies it is possible that acti-
vation of the P2X-R, exerted via endogenous ATP, may be re-
quired for icIL-1ra1 release; P2Xγ are expressed in keratino-
cyes (37) and epithelial cells (38).

In the endothelium the only detectable isoform of IL-1ra that
is inducibly expressed is this intracellular leaderless protein.
Increased levels of extracellular ATP at the EC surface are likely
to result in the release of icIL-1ra1. Our results show that LPS-pre-
treated, and even unstimulated, HUVECs were able to release en-
dogenous ATP sufficient to activate the release of icIL-1ra1, ATP
was detectable in the culture supernatants, and secretion of icIL-
1ra1 was blocked by antagonizing the P2X-R or by degradation of
ATP by apyrase. This suggests that an autocrine mechanism con-


crols icIL-1ra1 release, and endogenous ATP concentrations are
sufficient to activate the P2X-R. Other work has shown that during
LPS-mediated acute inflammation or ATP stimulation itself, an
increase in ATP release was measured from ECs (39) (40). The

FIGURE 6. LDH activity of supernatant samples. Supernatant samples
from RAW264.7 macrophages (A) and HUVECs (B). Cells were treated
with staurosporin (stauro), lysed with 1% Triton X-100 (Triton), unstimu-
lated (control), or pretreated with LPS for 3 h (LPS) followed by stimu-
lation with Bz-ATP (LPS + ATP), or in the presence of apyrase (LPS +
Apyr).
endothelium may be exposed to high levels of extracellular ATP under inflammatory conditions, due to release from degranulating platelets, via sympathetic nerve stimulation, from damaged cells in atherosclerosis, and under hypertension, restenosis, or ischemia (41). Hence, activation of the P2X7R resulting in secretion of icIL-1ra1, plays an important role in a number of pathological and immunological processes at the endothelium. The balance between release of proinflammatory IL-1 and the IL-1ra protein is likely to be critical in determining the arterial wall response and resultant pathology (42). It will be important to understand whether there are subtle differences in the mechanisms of release of each of the leaderless proteins, proinflammatory IL-1 and anti-inflammatory icIL-1ra1, from the endothelium, or whether their secretion is under the same control by P2X7R activation.

In summary, we have shown that icIL-1ra1 may be secreted into the extracellular medium, via a P2X7-R-dependent mechanism, from both macrophages and the endothelium. The results suggest that activation of the P2X7R by extracellular ATP may regulate a more general pathway for the release of leaderless cytoplasmic proteins.

Acknowledgments
We are grateful to Gary Shaw for isolation of HUVEC’s, to Ian Palmer for assistance with microscopy, and to Richard Varcoe for discussion.

References