Investigation into the Potential Anti-Inflammatory Effects of Endothelin Antagonists in a Murine Model of Experimental Monosodium Urate Peritonitis

Stephen J. Getting, Clara Di Filippo, Connie W. Lam, Francesco Rossi, and Michele D’Amico

The William Harvey Research Institute, Charterhouse Square, London, United Kingdom (S.J.G., C.W.L.); and Department of Experimental Medicine, Second University of Naples, Naples, Italy (S.J.G., C.D.F., F.R., M.D.A.)

Received January 16, 2004; accepted March 2, 2004

ABSTRACT

Endothelin (ET)-1 has been detected in many inflammatory pathologies, including rheumatoid arthritic patients, asthma, and ischemia-reperfusion injury. In this study, we have investigated the effect of a panel of different ET-1 antagonists displaying different selectivities for the receptors in a murine model of experimental inflammatory peritonitis. Systemic treatment of mice with the ETA antagonist C\(_{33}\)H\(_{42}\)N\(_{5}\)O\(_{7}\), N\(\text{cis}\)-[N\(\text{cis}\)-[N(hexahydro-1\(H\)-azepin-1-yl)carbonyl]-l-leucyl]-1-methyl-o-tryptophyl]-3-(2-pyridinyl)-o-alanine (FR139317) inhibited neutrophil accumulation. However, a greater degree of inhibition was observed with the ETB antagonist C\(_{34}\)H\(_{51}\)N\(_{5}\)O\(_{7}\), [N\(\text{cis}\)-[N(hexahydro-1\(H\)-azepin-1-yl)carbonyl]-l-leucyl]-1-methyl-o-tryptophyl]-3-(2-pyridinyl)-o-alanine (BQ-788) and the ET(A and B) antagonist C\(_{52}\)H\(_{65}\)N\(_{7}\)O\(_{10}\), N-acetyl-[10,11-dihydro-5\(H\)-dibenz[a,d]cycloheptadien-5-yl]-o-Gly-Leu-Asp-Ile-Ile-Trp (PD145065); all these effects occurred without altering peripheral blood cell counts. Release of the CXC chemokine KC was significantly reduced by the FR139317 and PD145065 but not by BQ-788. Evaluation of the therapeutic potential of these antagonists showed that PD145065 inhibited neutrophil migration and KC release, whereas the others caused a nonsignificant reduction in these parameters. Parameters of endothelial cell activation showed that urate-stimulated interleukin-1\(\beta\) release was inhibited by BQ-788 and PD145065 but not by FR139317, whereas ET-1 was only inhibited by the mixed antagonist. A different scenario was observed with respect to release of the CXC chemokine KC with FR139317 and PD145065 being effective, whereas with a marker of polymorphonuclear activation the ETA and mixed antagonist inhibited adhesion molecule expression. These data show that ET-1 antagonists elicit different mechanisms of actions in the way they display their antimigratory effects in a murine model of monosodium urate crystal peritonitis.
Anti-Inflammatory Effects of Endothelin Antagonists

Murray IL-1β and KC levels in the lavage fluids and plasma were quantified with a commercially available Quantikine ELISA purchased from R&D Systems (Oxfordshire, UK). In brief, lavage fluids (50 µl) were assayed for IL-1β and compared with a standard curve ranging from 0 to 0.5 ng/ml standard cytokine or for KC and compared with a standard curve ranging from 0 to 1 ng/ml standard chemokine. The ELISAs showed negligible (<1%) cross-reactivity with several murine cytokines and chemokines (data as furnished by the manufacturer).

Cytokine and Chemokine Quantification by ELISA

Local IL-1β levels in the cell culture supernatants were quantified with a commercially available ELISA purchased from Cayman Chemical (Ann Arbor, MI). In brief, cell culture supernatants (100 µl) were assayed for ET-1 and compared with a standard curve ranging from 0 to 1 ng/ml standard ET-1.

Drug Treatment

The ETα antagonist FR139317 (100–1000 pmol) (Sogabe et al., 1993; Filep et al., 1995), ETβ antagonist BQ-788 (100–1000 pmol) (Karaki et al., 1994; Hayasaki et al., 1996), mixed ETα and ETβ antagonist PD145065 (100–1000 pmol) (McMurdo et al., 1993; Battistini et al., 1994), or PBS (100 µl) was administered i.v. either 30 min before 2 h after MSU crystals (3 mg in 0.5 ml of sterile PBS i.p.). FR139317 was purchased from Tocris Cookson Inc. (Avonmouth, Bristol, UK), BQ-788 was purchased from Bachem Ltd. (Saffron Walden, Essex, UK), and PD145065 was purchased from Sigma Chemical (Poole, Dorset, UK). All peptides were stored according to the manufacturers’ instructions.

Assays of Cell Activation

Endothelial Cell Culture. Murine endothelial cells (300 × 10^6/well) were obtained as described previously (Lim et al., 2003) and maintained and cultured in Dulbecco’s modified Eagle’s medium with glutamax, sodium pyruvate, glucose, pyrrodone, 10% fetal calf serum, 100 µM HEPES, 1% nonessential amino acids, and 0.05 mg/ml gentamicin and grown on gelatin-coated flasks (0.5%) at 37°C under humidified conditions with 5% CO2, 95% air. Nonadherent cells were then washed off and adherent cells were incubated with the ET-1 antagonists FR139317, BQ-788, and PD145065 (100–1000 nM) for 30 min in Dulbecco’s modified Eagle’s medium. Cells were then stimulated with 1 mg/ml MSU crystals (a concentration chosen from preliminary experiments) (Getting et al., 1999), and the cell-free supernatants were collected 30 min later. KC and IL-1β levels were measured by ELISA as described above.

Primary Culture of MØ. An enriched population of peritoneal MØ (>95% pure) was prepared by 2-h adherence at 37°C under humidified conditions with 5% CO2, 95% air in RPMI 1640 medium supplemented with 10% fetal calf serum, by culturing 1 × 10^6 MØ in 24-well plates. Nonadherent cells were washed off using warm media, and adherent cells (>95% MØ) were then incubated with either PBS or 1000 nM FR139317, BQ-788, and PD145065 for 30 min in RPMI 1640 medium. Cells were then stimulated with 1 mg/ml MSU crystals (a concentration chosen from previous experiments) (Getting et al., 1999), and cell-free supernatants were collected 2 h later. KC levels were measured by ELISA as described above.

PMN CD11b Up-Regulation. A whole blood protocol was used to quantify CD11b expression in basal conditions and after cell stimulation (Tailer et al., 1997). Briefly, 1 ml of mouse blood was incubated
with the different antagonists (1000 nM) for 15 min at 37° C in a
shaker water bath before addition of 0.1 μM platelet-activating fac-
tor (PAF) and a further 15-min incubation. Control tubes were incu-
bated without PAF. At the end, blood aliquots (200 μl) were incu-
bated with rat IgG (10 μg/ml) or rat anti-mouse CD11b (clone 5C6;
Serotec, Oxford, UK). After 1 h at 4°C, fluorescein isothiocyanate-
conjugated rabbit anti-mouse IgG was added for a further 45 min,
and fluorescence-activated cell sorting analysis performed using a
FACScan II analyser (BD Biosciences, Mountain View, CA) with
air-cooled 100-mV argon ion laser tuned to 548 nm and a Consort 32
computer running Lysis II software. CD11b expression was mea-
sured in the FL1 (green) channel and quantified as computed units
(channel numbers) and expressed as median fluorescence intensity.

Statistics

Data are reported as mean ± S.E. of n distinct observations.
Statistical differences were calculated on original data by analysis of
variance followed by Bonferroni’s test for intergroup comparisons
(Berry and Lindgren, 1990), or by unpaired Student’s t test (two-
tailed) when only two groups were compared. A threshold value of
P < 0.05 was taken as significant.

Results

Effect of ET-1 Antagonists on PMN Accumulation
and KC Levels in a Model of MSU Crystal Peritonitis.
As shown in Table 1, the in vivo administration of FR139317,
BQ-788, and PD145065 did not alter circulating peripheral
blood leukocyte counts or plasma levels of the CXC chemo-
kine KC as measured at 2 h after collection of blood by
cardiac puncture.

We then evaluated the effect of selective and mixed antag-
onists on PMN migration and as a marker of inflammation,
the CXC chemokine KC in cell-free lavage fluids. The selec-
tive ETA antagonist FR139317 caused a dose-dependent at-
tenuation of PMN migration into the peritoneal cavity. The
highest dose tested (1000 pmol) caused a 32% reduction, 300
pmol caused a more modest 15% reduction, whereas the
lowest dose was essentially inactive (Fig. 1A). At the anti-
migratory dose of 1000 pmol, FR139317 caused a significant
attenuation of KC release into the peritoneal cavity, with a
28% reduction being observed. Surprisingly, the lower dose of
300 pmol caused a 40% reduction in KC release (Fig. 1B). The
selective ETB antagonist BQ-788 showed a similar degree of
inhibition at 300 and 1000 pmol with a 25 and 35% reduction
being observed. Surprisingly, the lower dose of
300 pmol caused a more modest 15% reduction, whereas the
highest dose tested (1000 pmol) caused a significant
 attenuation of PMN migration into the peritoneal cavity. The

![Fig. 1. Anti-inflammatory effects of the ETA antagonist FR139317 in
MSU crystal-induced peritonitis. Mice were treated i.v. with FR139317
(100–1000 pmol) or PBS (100 μl) 30 min before an i.p. injection of MSU
crystals (3 mg in 0.5 ml of sterile PBS) on PMN migration (A) and KC
release (B) as assessed at 6-h time point. Data are mean ± S.E. of n = 8
mice per group. * P < 0.05 versus control group.](image)

![Fig. 2. Anti-inflammatory effects of ETB antagonist BQ-788 in MSU
crystal-induced peritonitis. Mice were treated i.v. with BQ-788 (100–
1000 pmol) or PBS (100 μl) 30 min before an i.p. injection of MSU
crystals (3 mg in 0.5 ml of sterile PBS) on PMN migration (A) and KC
release (B) as assessed at 6-h time point. Data are mean ± S.E. of n = 8
mice per group. * P < 0.05 versus control group.](image)

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Blood Cells</th>
<th>Plasma KC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMN</td>
<td>Monocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>6.3 ± 1.1</td>
<td>7.2 ± 0.9</td>
</tr>
<tr>
<td>FR139317</td>
<td>5.8 ± 1.6</td>
<td>6.8 ± 1.3</td>
</tr>
<tr>
<td>BQ-788</td>
<td>6.4 ± 0.9</td>
<td>7.5 ± 1.5</td>
</tr>
<tr>
<td>PD145065</td>
<td>6.0 ± 0.8</td>
<td>7.0 ± 0.5</td>
</tr>
</tbody>
</table>

**Therapeutic Administration of the Selective ET-1 Antagonists on PMN Accumulation in Experimental**
Gout. In these experiments, the therapeutic potential of the antagonists was evaluated at the dose of 1000 pmol per mouse because this was found to be the most active when given prophylactically. The selective ETA antagonist FR139317 caused a nonsignificant 25% reduction in the neutrophil migration into the peritoneal cavity when given 2 h after the initiation of the inflammatory response (Fig. 4A), and this inhibition was associated with a 32% reduction in the CXC chemokine KC (Fig. 4B) and 23% reduction in IL-1β release (Fig. 4C). A different scenario was observed for the selective ETB antagonist BQ-788; this failed to significantly attenuate the neutrophil migration (Fig. 4A), and KC or IL-1β levels (Fig. 4, B and C). To ascertain whether inhibition of both receptors led to an enhanced therapeutic anti-inflammatory effect, the mixed ET(A and B) antagonist PD145065 was evaluated. PMN migration was inhibited by 51% (Fig. 4A), whereas KC (Fig. 4B) and IL-1β (Fig. 4C) were both inhibited by a significant 32%.

Effect of Antimigratory Dose of Endothelin Antagonists over a Time Course. We next decided to see whether the antagonists were able to modulate the inflammatory response elicited by MSU crystals over a time course. Mice were treated with 1000 pmol of PD145065, FR139317, or BQ-788, and their effects on PMN migration and release of KC were investigated at different time points. MSU crystal injection caused a peak release of the chemokine at 2 h (3272 ± 190 pg/ml); it then fell dramatically to 882 ± 125 pg/ml at 6 h and back to a near basal level between 24 and 48 h. The maximal detection of PMN migration was observed 6 h after MSU crystal injection and remained high up to 24 h. Investigation of the effect of the mixed antagonist PD145065 showed a significant reduction of 38% at 2 h, reducing PMN migration from 1.3 ± 0.3 to 0.8 ± 0.1 × 10⁶/mouse (P < 0.05 versus PBS; n = 4) and a 33% reduction in the release of the CXC chemokine KC, reducing it from 3272 ± 190 to 2192 ± 75 pg/ml (P < 0.05 versus PBS; n = 4). At the later time point of 24 h, an anti-migratory effect was observed, although no effect was seen at 48 h. With respect to the chemokine, an inhibitory effect was seen at 6 h, although at later time points no effect was observed. We next evaluated the ETA antagonist FR139317 that was inactive at 2, 24, and 48 h post-MSU crystal stimulation although no effect was observed on KC release (Table 2).

In Vitro Effect of ET-1 Antagonists on IL-1β and ET-1 Release from Cultured Endothelial Cells. The effect of ET antagonists on IL-1β and ET-1 release in vitro was evaluated as a marker of endothelial cell activation after stimulation with urate crystals using the most effective dose observed in vivo. FR139317 at 100 to 1000 nM was inactive and failed to inhibit IL-1β release, whereas BQ-788 caused a nonsignificant inhibition of 36% at 300 nM and a significant 60% inhibition at 1000 nM, respectively. PD145065 caused a 28% reduction at 300 nM, similar to that observed for BQ-
788, whereas at 1000 nM there was a significant inhibition of 52% (Fig. 5A). A different scenario was observed with respect to the effect of the antagonists on ET-1 release. FR139317 and BQ-788 were inactive and failed to inhibit ET-1 release. However, antagonism of both receptors using PD145065 caused a near complete abrogation of ET-1 release at 1000 nM with an approximate 90% reduction being observed (Fig. 5B).

**Effect of ET-1 Antagonists on KC Release in Vitro from Cultured Peritoneal MØ.** The effect of ET-1 antagonists on release of the CXC chemokine KC in vitro was evaluated as a marker of MØ activation. The ETA antagonist FR139317 at 1000 nM caused a 57% reduction in KC levels (Fig. 6A). FR139317, BQ-788, and PD145065 (1000 nM) were added to adherent cultured MØ (300 × 10⁶), 30 min before stimulation with 1 mg/ml MSU crystals. Supernatants were removed 30 min later, and cell-free aliquots were analyzed for KC (by ELISA). Data are mean ± S.E.M. of n = 4 determinations in triplicate. *P < 0.05 versus relevant PBS control.
FR139317 and PD145065 at 1000 nM caused a 28 and 31% reduction, respectively, in CD11b expression (P < 0.05 versus PBS; n = 4), whereas no effect was observed with BQ-788 (Fig. 6B).

Discussion

In this study, we report the novel discoveries that ET-1 antagonists are anti-inflammatory in a model of experimental MSU peritonitis. They exert their effects in part by suppressing the release of chemotactic agents and reducing adhesion molecule expression, ultimately leading to inhibition of neutrophil accumulation. This study has been prompted by the observation that ET-1 led to the migration of rabbit neutrophils (Elferink and de Koster, 1994). However, we now report here for the first time that ET-1 antagonists are efficacious in a murine model of gouty peritonitis. These data suggest that antagonism of ET receptors could be of benefit in neutrophil-driven pathologies.

MSU crystals injected into the peritoneal cavity have been shown to produce an intense and long-lasting accumulation of PMN into the mouse peritoneal cavity, recently characterized in terms of role of resident cells and requirements for adhesion molecules (Getting et al., 1997). Before the accumulation of PMN, there is a release of chemotactic cytokines and chemokines during this inflammatory reaction (Getting et al., 1999). In this study, we decided to monitor the CXC chemokine KC and the proinflammatory cytokine IL-1β, because these have been shown to be produced in other models of experimental (Matsukawa et al., 1998; Terkeltaub et al., 1998) and human (di Giovinne et al., 1991; Hachicha et al., 1995) gouty arthritis.

Systemic administration of the ETA antagonist FR139317 inhibited MSU crystal-induced PMN accumulation into the mouse peritoneal cavity, and this was associated with a reduction in KC release in the inflammatory exudates. These findings suggest that ETA antagonists can inhibit neutrophil migration, a finding observed previously with BQ-123 and migration of rabbit neutrophils (Elferink and de Koster, 1994) and in neutrophil-driven ischemia in hearts with the selective antagonist LU135252 attenuating the neutrophil-mediated injury (Andrasi et al., 2002). The importance of ETA antagonists in inhibiting inflammation has also been demonstrated in models of asthma with FR139317 inhibiting airway hyperresponsiveness (D’Agostino et al., 1999), lung eosinophilia (Fujitani et al., 1997), and paw edema (Sampaio et al., 1995). The ETB antagonist BQ-788 also inhibited PMN migration but failed to modulate KC levels, whereas the ETA (A and B) antagonist PD145065 inhibited both parameters. A potential reason for the lack of efficacy on KC release with BQ-788 is that both FR139317 and PD145065 are inhibiting KC release from mast cells and MØ. MSU crystals have long been known to release KC from MØ and mast cells (Getting and Perretti, 2000) and cause PMN migration (Schramm et al., 2002). Because the ETA antagonist BQ-610 but not the ETB antagonist IRL-1628 can inhibit mast cell degranulation, it is possible that mast cells express ETA receptors and antagonism of the receptor will prevent the release of this chemokine (Boros et al., 2002). This antiinflammatory effect is in agreement with other studies, which have shown a protective effect in other neutrophil-driven pathologies such as ischemic reperfusion injury (Pernow and Wang, 1997) and in coronary vasoconstriction with the nonselective antagonist SB209670 (Klemm et al., 1995). We believe this is the first time that an anti-inflammatory activity has been described in a model of MSU crystal peritonitis.

We then investigated whether these antagonists possessed any therapeutic potential, so they were administered after the onset of inflammation. Antagonists were administered 2 h after MSU crystal injection, the time point chosen due to the fact that this is where the peak release of the CXC chemokine KC was observed (Getting et al., 1999). Both FR139317 and BQ-788 caused a modest inhibition, although this was not significant and little effect on the levels of detectable KC and IL-1β were observed. However, a significant inhibition was observed for the mixed antagonist PD145065 and its antiinflammatory effect seemed to be due to inhibition of KC and IL-1β. These findings are not surprising given the fact that this antagonist when dosed prophylactically inhibited the release of this chemokine. Therefore, antagonism of both receptors seems to be required once the inflammatory response has been initiated for an antimigratory effect to occur. The requirement of switching off both receptors for an enhanced anti-inflammatory profile is not altogether surprising given the fact that ETA receptors are expressed on neutrophils and FR139317 can inhibit ET-1-induced up-regulation of CD11b/CD18 expression, whereas BQ-788 had no effect. However, BQ-788 but not FR139317 can inhibit intercellular adhesion molecule expression on endothelial cells (Zouki et al., 1999).

Given the antiinflammatory effects of these antagonists, their effect over a time course was evaluated as to allow determination at which time point they exerted their effects. MSU crystal injection caused a peak of release of the chemokine KC at 2 h, and this was followed by a maximal accumulation of PMN at 6 h and is in agreement with previous studies (Getting et al., 1997). At the 2-h time point, only PD145065 inhibited PMN migration, whereas at 6 h all antagonists were antimigratory. At 24 h, only the mixed and ETB antagonist displayed any antimigratory effect. These data could suggest that antagonism of either A or B receptors at early time points will lead to an inhibition in PMN migration, whereas at later time points antagonism of the B receptor will prevent cell migration.

We then evaluated the effect of the ET-1 antagonists on MSU crystal stimulated endothelial cells by measuring IL-1β and ET-1 because urate crystals have previously been shown to release IL-1β in a murine model of peritonitis (Getting et al., 2001). We found that BQ-788 and PD145065 exhibited a dose-dependent inhibition in IL-1β release, whereas FR139317 was essentially inactive compared with the control. A different profile was observed on ET-1 release with antagonism of both receptors being required to see an inhibitory effect. Therefore, antagonism of ETB receptors leads to a decrease in the production of IL-1β and ET-1 from endothelial cells and ETA receptors seem not to play a role. An observation that is in agreement with the fact that endothelial cells do not express ETA receptors (Zouki et al., 1999) and that inhibition of IL-1β will prevent the induction of ET-1 (Yoshizumi et al., 1990).

Previous studies have highlighted that MSU crystals can stimulate resident peritoneal MØ to release the proinflammatory chemokine KC (Getting et al., 1999). FR139317 and PD145065 inhibited KC release from cultured MØ at 1000
Fig. 7. Schematic model for the involvement of ET-1 antagonists in modulating the host inflammatory response elicited by MSU crystals. Antagonists that block the ETA receptor (filled squares) lead to an inhibition of PAF-induced CD11b expression in the PMN. MSU crystal stimulation of MØ leads to release of KC release, which is inhibited by antagonists that block the ETA receptor, whereas activation of ETB (filled circles) receptors on endothelial cells (EC) leads to IL-1β release and ET-1, which is abrogated by antagonists of this receptor. Therefore, inhibition of adhesion molecule expression and proinflammatory chemokine/cytokines ultimately lead to a reduction in PMN migration to the inflamed site.

nM, whereas BQ-788 was inactive. This inhibition in KC from cultured MØ could explain the anti-inflammatory effects observed in vivo, because FR139317 and PD145065 inhibited KC as detected in lavage fluids from the peritoneal cavity. An effect of an ETA receptor antagonist on mouse peritoneal MØ function is in agreement with a previous study showing that BQ-123 could inhibit ET-1-induced production of prostaglandin E₂ and the expression of cyclooxygenase 2 protein. However, in this study they also found an effect with BQ-788, something that we were unable to do. This could be due to the different strain of mice used (C57 compared with Swiss albino).

Finally, given the fact that ET-1 receptors can be activated by PAF (Yoshizumi et al., 1990), we investigated the effect of the antagonists on PAF-induced up-regulation of CD11b expression on murine PMN. FR139317 and PD145065 inhibited CD11b expression, whereas BQ-788 was inactive. Given that ET-1 has been shown to down-regulate the expression of L-selectin and increase CD11b expression (Zouki et al., 1999), a scenario that will lead to an increase in leukocyte migration. Our findings showing an effect of the ETA and ET(A and B) antagonist but no effect with an ETB antagonist are in agreement with this study.

In conclusion, the ET antagonists display novel antimigratory effects in a murine model of MSU crystal-induced inflammation. Different profiles were observed for each antagonist and their effect was altered depending on whether given prophylactically or therapeutically. The data also suggest that inhibition of both receptors leads to an enhanced anti-inflammatory effect and this effect is partially due to the fact that the antagonists target the release and action of cytokines and chemokines, and adhesion molecule expression either on the surface of the emigrating PMN or on the endothelium. These findings suggest that ET antagonists could be beneficial in treating certain inflammatory conditions. In the future, further studies could be used in the rabbit because both CXCR1 and CXCR2 are expressed as in humans. Previously, an anti-IL-8 antibody has been shown to attenuate neutrophil infiltration and joint swelling associated with MSU crystal injection (Nishimura et al., 1997). Figure 7 shows a schematic model for the involvement of ET-1 antagonists in modulating the host inflammatory response elicited by MSU crystals.

Acknowledgments

We thank Drs. R. de Médicis and A. Lussier (University of Sherbrooke, Sherbrooke, Canada) for the supply of MSU crystals.

References


