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Different endothelins stimulate cytokine production by peritoneal macrophages and microglial cell line

L. SPECIALE, K. RODA, M. SARESELLA, D. TARAMELLI* & P. FERRANTE* *Laboratory of Biology, Don C. Gnocchi Foundation, IRCCS, Milan, and *Institute of Medical Microbiology, University of Milan, Italy*

SUMMARY

Endothelins (ETs), potent vasoconstricting peptides, are produced by macrophages upon stimulation and may participate in the amplification or regulation of the inflammatory response. However, it is not clear whether ETs can act in an autocrine manner on macrophages and which role they play in relationship with other cytokines. To address these issues, we studied the effects of ETs on the production of inflammatory cytokines by mouse peritoneal macrophages or by a retrovirus-transformed microglial cell line. Here, we report that ET-2, but not ET-1 or ET-3, is able to stimulate the production of interleukin-1 (IL-1) and interleukin-6 (IL-6) by peptone-elicited mouse macrophages (pMO). In contrast, ET-3 and ET-1, but not ET-2, are active on microglial cells. No tumour necrosis factor-α (TNF-α) or nitric oxide (NO) were detected in the supernatants of ET-stimulated cultures. The activity of ET-2 on pMO was time and dose dependent and was inhibited by the addition of ET_A and ET_B receptor antagonists, BQ123 and IRL1038, respectively. In addition, when pMO were stimulated by interferon-γ (IFN-γ) in the presence of ET-2, a significant inhibition of IL-6 and IL-1 production was observed compared with the effects of the same doses of IFN-γ or ET-2 used separately. The inhibition was specifically due to the activity of ET-2, since it was reversed by the addition of BQ123 or IRL1038. Similar results were seen when the content of NO in the supernatants of pMO stimulated by IFN-γ plus ET-2 was evaluated. These results suggest that ETs may possess both a pro-inflammatory action on macrophages from different tissues and a regulatory activity on IFN-γ.

INTRODUCTION

Endothelins (ETs) are 21-amino acid peptides known as potent and long-lasting vasoconstricting agents in humans.[1] The three isoforms, derived from a 38-amino acid precursor named big ET, are named ET-1, ET-2 and ET-3. ETs possess a wide range of biological activities within the cardiovascular system and in other organs, including lungs,[2] kidney[3] and brain.[4,5] These peptides act mostly as local paracrine or autocrine hormones. However, there are substantial elevations of the circulating and/or tissue levels of ET-1, in some pathological states, such as coronary vasospasm,[6] ischaemia reperfusion injury[7] and septic shock.[8] The gene expression of ETs is tissue-restricted,[9] since endothelial cells and cardiac tissue express exclusively the ET-1 gene,[10] whereas both ET-1 and ET-3 are present in fetal lung,[11] and in spleen and central nervous system.[12] Two types of cellular receptors have been identified so far and are currently named ET_A and ET_B. ET_A binds ET-1 and ET-2 more efficiently than ET-3,[13] while ET_B is considered a promiscuous receptor since it is capable of binding all three ETs. It has been shown that ET_A and ET_B show a different tissue distribution.[14]

Several lines of evidence suggest an additional important role for ETs in the regulation of the immune response. Recently, it has been reported that ET-1 can induce and modulate interleukin-6 (IL-6) production in human monocytes,[15] stromal cells[16] and human umbilical vein endothelial cells.[17] In addition, ET-1 induces the release of histamine from mast cells,[18] stimulates the chemotaxis of neutrophils[19] and regulates DNA synthesis and migration of endothelial cells.[20] The production of ETs is not limited to endothelial cells since in the presence of appropriate stimuli, such as bacterial lipopolysaccharide (LPS) or human immunodeficiency virus glycoprotein 120,[21] cells of monocyte/macrophage origin are able to synthesize ETs.[22] The observation that murine macrophages[23] and human monocytes[24] express ET_B receptors, suggests that ETs may contribute to macrophage activation in an autocrine manner.

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Abbreviations: Con A, concanavalin A; ETs, endothelins; FCS, fetal calf serum; IFN-γ, interferon-γ; IL-1, interleukin-1; IL-6, interleukin-6; LPS, lipopolysaccharide; MTT, (3-4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; NO, nitric oxide; PBS, phosphate buffered saline; pMO, peptone-elicited peritoneal macrophages; TNF-α, tumour necrosis factor-α.

Correspondence: Dr P. Ferrante, Laboratory of Biology, Don C. Gnocchi Foundation, IRCCS, Via Capecelatro, 66, I-20148, Milan, Italy.

To verify this hypothesis, we studied the effect of ETs on the production of inflammatory cytokines and nitric oxide (NO) by mouse macrophages and by a microglial mouse cell line.

MATERIALS AND METHODS

Mice

Specific pathogen-free female CD1 mice, (6–8 weeks old, 23–25 g) were obtained from Charles River (Calco, Italy). Mice were maintained under conventional conditions and fed with mouse pellet and water *ad libitum*.

Culture medium and reagents

The complete medium (CM) used in these studies was RPMI-1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with 100 U/ml of penicillin, 2 mM glutamine, 100 mg/ml streptomycin, 20 mM HEPES buffer (Whittaker Bioproducts) and 5% fetal calf serum (FCS) (Imperial Products, Andover, UK). All reagents were tested for endotoxin contamination by the LAL assay (Whittaker Bioproducts). LPS from *Escherichia coli* 0111:B4 and Polymyxin B were purchased from Sigma (Milan, Italy), diluted in sterile phosphate-buffered saline (PBS) and stored at −20° in aliquots. ET-1, ET-2, ET-3 and big ET-1 were obtained from Calbiochem Corp. (San Diego, CA). Interferon-γ (IFN-γ) from Genzyme (Cambridge, MA) was dissolved in CM and used at 5–50 U/ml. BQ123 and IRL1038, antagonists of ET_A and ET_B receptors, respectively, were obtained from Inalco (Milan, Italy) and used at 0·1 μM.

Isolation of cells and production of culture supernatants

Macrophages were obtained by harvesting peritoneal exudate cells from mice injected intraperitoneally 2 days earlier with 10% proteose peptone (Difco, Detroit, MI). Cells were washed twice and resuspended in CM. Differential cell counts of macrophage population were made on Wright–Giemsa-stained smears (Hemacolor, Merck, Darmstadt, Germany) made by cytocentrifugation (Labofuge, Hereus, Milan, Italy) and the cell suspension was then adjusted to the desired macrophage concentration. Macrophages were purified by adherence to plastic: briefly, 0·1 ml of a suspension of 2×10^6 cells/ml in CM were distributed in 96-well plates (Costar N.3596, Cambridge, MA) and incubated for 2 hr at 37° in 5% CO_2. Hereafter, we will refer to this population as pMO. After serial washing, pMO monolayers were treated with different concentrations of ETs and incubated for 24 or 48 hr at 37° in 5% CO_2. Plates were then centrifuged and the supernatants were collected and assayed for cytokine activity or kept frozen. For some experiments, we used resident macrophages obtained from CD1 mice without injection with peptone.

An immortalized retrovirus-transformed mouse microglial cell line of CD1 origin was kindly provided by Prof. P. Ricciardi Castagnoli (CNR University of Milan).[25] The cells were maintained in CM with 5% FCS and routinely split. The supernatants from an ET-stimulated microglial cell line were obtained after seeding 10^5 cells/well for 48 hr and collection as described above. Doubling time for this microglial cell line was 18 hr, therefore the number of the cells at end of treatment would be approximately $2{\cdot}6 \times 10^6$/well.

Assays for cytokines

IL-6 was measured in terms of hybridoma growth factor (HGF) activity, using the 7TD1 cell line as already described.[26] Briefly, supernatants in quadruplicate were serially diluted in microplates before the addition of 2×10^3 7TD1 cells/well. Cultures were incubated for 72 hr at 37° in 5% CO_2. Then, 20 μl/well of a 5 mg/ml solution of the (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) in PBS were added and further incubation for 3 hr at 37° was performed. Plates were then centrifuged, the supernatants were discarded and the pellets were dissolved using 100 μl/well of lysing buffer consisting of a solution of 20% (w/v) sodium dodecyl sulphate (Sigma), 40% *N*,*N*-dimethyl formamide (Merck, Darmstadt, Germany) in H_2O, pH 4·7. After dissolving the dark blue formazan crystals, the plates were read in a microplate reader (Molecular Devices Co., Manlo Park, CA) using a test wavelength of 550 nm and a reference wavelength of 650 nm. IL-6 activity resulting in half maximal stimulation of target cell growth was arbitrarily defined as 1 U. The reference standard used in these experiments consisted of recombinant IL-6 purchased from Prepotec (Inalco). Some supernatants were assayed using murine IL-6 kit from Endogen (Woburn, MA) or Genzyme (Cambridge, MA) and the results were expressed as pg/ml.

IL-1 activity was measured by using the standard D10 G4.1 proliferation bioassay with slight modifications.[27] Briefly, 10^4 cells/well were grown overnight in 96-well microtitre plates (Costar 3799) in CM containing 10% FCS, 2-mercaptoethanol (5×10^{-5} M) and concanavalin A (Con A; 3 mg/ml). Serial dilutions of supernatants were then added and incubation was prolonged for 48 hr. For the last 3 hr, MTT was added and the assay was processed as reported above. The results are expressed as arbitrary U/ml of IL-1 calculated from a standard curve with recombinant IL-1 (Prepotec) run in parallel in every test.

Tumour necrosis factor-α (TNF-α) activity in the supernatants was assayed using the Wehi 164 Clone 13 cell line in a MTT tetrazolium cytotoxicity assay, as described.[27] Cells were seeded in quadruplicate at 4×10^3/well with dilution of supernatants or with known amount of rTNF-α (Boehringer-Mannheim, Mannheim, Germany) in CM in 200 μl. The plates were then incubated for 72 hr at 37° in 5% of CO_2 and for the last 3 hr, 20 μl of a 5 mg/ml solution of MTT were added to each well, as above. The percentage of cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = 100 - \left(\frac{\text{OD test} - \text{OD control}}{\text{OD control}}\right) \times 100$$

The results are expressed U/ml of TNF-α calculated from a standard curve with recombinant TNF-α (Boehringer-Mannheim) run in parallel in every test.

Nitrite determination

The accumulation of nitrite, which is the measurable form of NO in aqueous solution, was measured by mixing 100 μl of supernatants with an equal volume of Griess reagent (0·1% naphthyl-ethylenediamine dihydrochloride, 1% sulphanilamide in 5% concentrated H_3PO_4), in triplicate.[28] Plates were incubated at room temperature for 10 min, and then the absorbance was measured in a microtitre plate reader (Molecular Devices) at 550 nm against a standard curve of sodium nitrite in CM.

Statistical analysis

All the tests were performed at least three times in triplicate or quadruplicate, data are given as means ± SEM and the statistical analysis was performed by Student's *t*-test. Differences with $P < 0{\cdot}05$ were considered significant.

RESULTS

Production of IL-6 and IL-1 by peritoneal macrophages treated with ETs

When pMO were stimulated with ETs, only ET-2 was able to induce significant IL-6 and IL-1 production. In contrast, ET-1, big-ET-1 and ET-3 were ineffective at all the doses employed. The production of IL-6 and of IL-1 was dose-dependent in the range between 1 and 20 nM ET-2; it augmented progressively to a value of 10^4 U/ml of IL-6 and 25·5 U/ml of IL-1 at 20 nM ET-2 (Fig. 1). No TNF-α or NO was detectable in the same supernatants. LPS was used as positive control at 10 μg/ml and strong production of IL-6 (Fig. 1a), IL-1 and TNF-α (data not shown) was seen in LPS-stimulated pMO. When the same experiments were performed using resident macrophages or resting macrophages, which were cultured for 24 hr before stimulation, the effect of ETs and LPS on IL-6 and IL-1 production was similar (data not shown).

Table 1. Effect of polymyxin B (pmx B), BQ123 and IRL1038 receptor antagonists on IL-6 production by pMO stimulated by ET-2

Stimulus	IL-6 (pg/ml)		
Exp. 1	−pmx B	+pmx B	
Medium	394 ± 21·2	434·4 ± 19·2	
ET-2 20 nM	1905 ± 142·4	1800·0 ± 80·0	
LPS 10 μg/ml	3000 ± 120·2	454·5 ± 22·4	
Exp. 2	None	+BQ123	+IRL1038
Medum	378 ± 12·4	400 ± 22·1	497 ± 18·2
ET-2 20 nM	1020 ± 115	495 ± 23·4	556 ± 20·5

pMO were treated with ET-2 for 48 hr in the presence of polymyxin B (10 mg/ml) or BQ123 (0·1 μM) or IRL1038 (0·1 μM). The supernatants were assayed by enzyme-linked immunosorbent assay and the results were expressed as pg/ml of IL-6. Each result represents mean ± SEM of four experiments performed in triplicate. Statistical analysis was performed by Student's *t*-test ($P < 0{\cdot}05$).

The effects of ET-2 were not due to contamination by LPS since polymyxin B added during the stimulation had no effect (Table 1, Exp. 1). Consistent, although unexpected, results were obtained when ET_A or ET_B receptor antagonists were used. As shown in Table 1, Exp. 2, both ET_A-receptor antagonist, BQ123, and ET_B-receptor antagonist, IRL1038, completely inhibited ET-2-induced IL-6 production by pMO.

The stimulation of IL-1 and IL-6 release by ET-2 was time-dependent. IL-1 was already evident 2–4 hr after stimulation and it reached a plateau after 18 hr, whereas IL-6 appeared to rise progressively to a value of 9×10^3 U/ml at 48 hr (Fig. 2).

Production of IL-6 by microglial cell line treated with ETs

A similar set of experiments was conducted using a retrovirus-transformed microglial cell line. In contrast with the results obtained with pMO, microglial cells were activated by ET-1 and ET-3, but not by ET-2. The production of IL-6 was dose-dependent and it reached 60 U/ml or 80 U/ml at 40 nM of

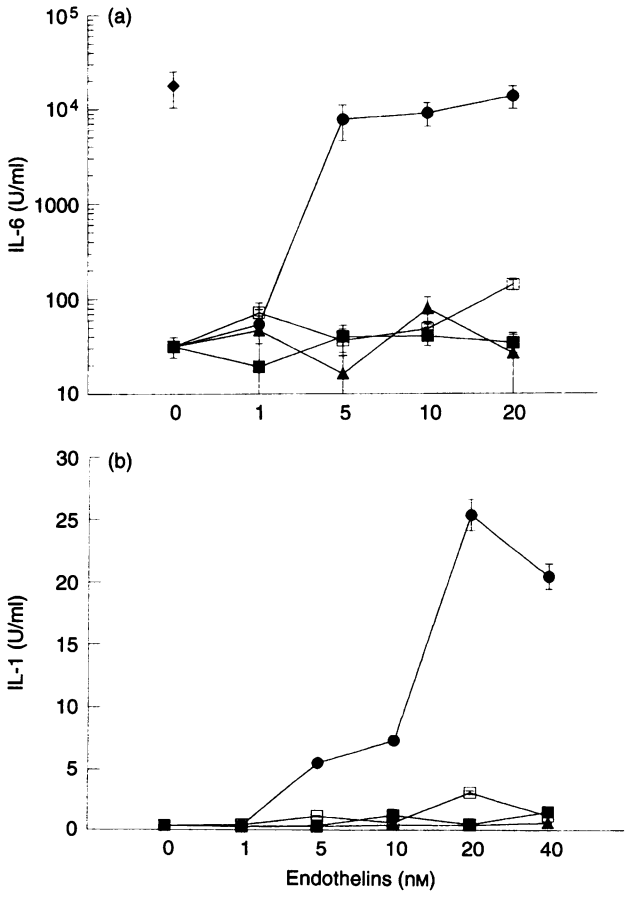

Figure 1. IL-6 (a) and IL-1 (b) production by pMO stimulated for 24 hr with different doses of ET-1 (■), ET-2 (●), ET-3 (□) and big ET-1 (▲). LPS 10 μg/ml (◆) was used as positive control. Each point is expressed in U/ml and represents the mean ± SEM of three experiments performed in quadruplicate.

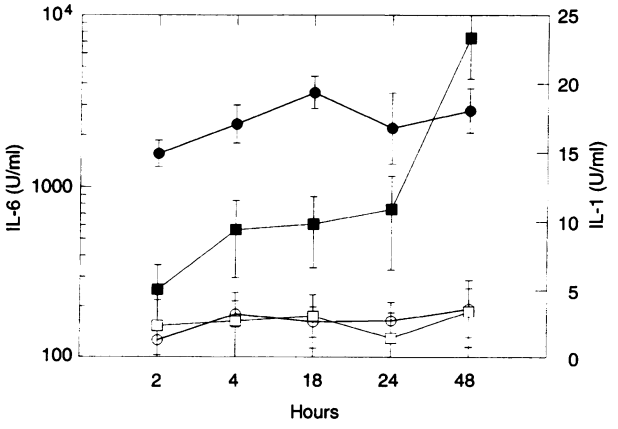

Figure 2. Kinetics of IL-6 (■) and IL-1 (●) production by pMO treated with 20 nM ET-2. Open symbols represent the spontaneous release of cytokine at different time-points in the absence of ETs. Results are expressed as U/ml and each point represents the mean ± SEM of three experiments performed in quadruplicate.

ET-1 or ET-3, respectively (Fig. 3). No IL-1 or TNF-α was detectable in the supernatants of the microglial cell line although significant production of IL-1, IL-6 and TNF-α was observed with LPS stimulation (data not shown).

Effect of ET-2 on IFN-γ stimulation of pMO

We then investigated the effect of ET-2 on pMO stimulated by IFN-γ. The results, shown in Table 2, indicate that IFN-γ (50 U/ml) alone induced significant levels of IL-6. However, when pMO were stimulated with IFN-γ in the presence of different doses of ET-2, a significant decrease of IL-6 production was observed compared to the effects of IFN-γ or ET-2 used alone. The inhibition was observed only when IFN-γ and ET-2 were added at same time and not when ET-2 was added before or after IFN-γ. Similar results were seen when the same supernatants were assayed for NO except that ET-2 was not able by itself to induce NO (Table 2). The addition of IRL1038, on ET_B receptor antagonist, or BQ123, an ET_A receptor antagonist, restored normal levels of IL-6, suggesting that the inhibition was mediated by ET-2 and that both receptors were involved. The same receptor antagonists did not affect the response of pMO to IFN-γ alone (Table 2, Exp. 2). Moreover, we excluded the possibility that the inhibition was due to a direct toxicity of the compounds on pMO since the metabolic activity of the cells, measured by reduction of MTT, was not affected. Comparable results were obtained when the supernatants were assayed for IL-1 production (data not shown).

Table 2. Effect of ET-2 on IL-6 and NO production by pMO stimulated with IFN-γ

Treatments	Receptor antagonists	IL-6 (pg/ml)	NO_2^- (μM)
Exp.1			
Medium		215±15	9·8±0·5
IFN-γ		540±12	60·2±0·2
IFN-γ+ET-2 0·6 nM		420±14*	25·2±0·2†
INF-γ+ET-2 2·5 nM		220±11†	19·4±0·8†
IFN-γ+ET-2 10 nM		78±8†	16·6±0·8†
ET-2 10 nM		580±18	8·5±0·1
Exp.2			
Medium		380±16	8·5±0·7
IFN-γ		800±18	56·5±0·4
IFN-γ	BQ123	821±16	54·7±0·6
IFN-γ	IRL1038	778±18	55·1±0·4
ET-2 10 nM		890±19	9·2±0·7
IFN-γ+ET-2 10 nM		440±22†	15·5±0·3†
IFN-γ+ET-2 10 nM	BQ123	790±26	52·4±0·4
IFN-γ+ET-2 10 nM	IRL1038	780±15	50·1±0·5

pMO were treated with IFN-γ (50 U/ml) or different doses of ET-2, alone or in combination for 24 hr. BQ123 or IRL1038 at 0·1 μM were added at beginning of the stimulation. The supernatants were assayed for IL-6 and NO_2^- as described in the Materials and Methods. The results were expressed as pg/ml of IL-6 and μM for NO_2^- respectively. Each result represents mean±SEM from a representative experiment run in triplicate. Statistical analysis was performed by Student's *t*-test *$P<0·01$; †$<0·001$.

DISCUSSION

Endothelins are multifunctional vasoconstricting peptides produced by different cell types and acting in various tissues. In this report, we have shown that ETs may represent an autocrine, immunoregulatory signal for macrophages. ET-2, but not ET-1 or ET-3, is able to induce IL-1 and IL-6 production by peptone-induced or resident mouse peritoneal macrophages, whereas ET-3 and ET-1, but not ET-2, are active on microglial cells. For each ET, the effects are strictly dependent on the dosage used. In our case, the stimulation of cytokine production by pMO occurred in the range of 5–40 nM ET-2. In addition, the optimal doses of ET-2 appeared to be different depending on the type of cytokine assayed: IL-1 production required between 20 and 40 nM ET-2, whereas only 5–20 nM were necessary for optimal stimulation of IL-6. In this range of doses, only ET-2 was active on pMO, whereas ET-1, big ET-1 and ET-3 were ineffective. These results confirm previous observations that ETs possess a narrow and peculiar dose range. Elferink & de Koster[19] reported that low doses of ET-3 are stimulatory for neutrophil chemotaxis, but high doses are inhibitory. Data from our group indicate that ET-1 is also able to stimulate pMO but only at picomolar doses (data not shown). A similar dose–response was described when the effects of ET-1 and ET-3 on endothelial cell proliferation were analysed.[20] Furthermore, other authors have hypothesized complex interactions among ETs, receptors and intracellular signal transduction pathways to explain such atypical behaviour without conclusive results.[29]

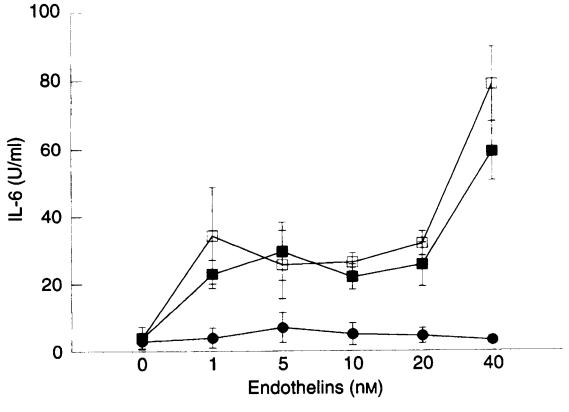

Figure 3. IL-6 production by microglial cells stimulated for 48 hr with different doses of ET-1 (■), ET-2 (●) or ET-3 (□). Results are expressed as U/ml and each point represents mean±SEM of three experiments performed in quadruplicate.

The production of IL-1 and IL-6 by ET-2-stimulated pMO was time-dependent. The release of IL-1 preceded that of IL-6 and reached a plateau at 18 hr. IL-6 became evident at 18 hr and showed its maximum release after 48 hr. It is possible that IL-6 production was a consequence of the IL-1 induced by ET-2 and not a direct effect of ET-2 on pMO. In any case, the results indicate that ET-2 can activate the cytokine cascade and participate in the amplification of the inflammatory response.

The specificity of ET-2 action was confirmed in different ways. First, by using polymyxin B, we ruled out that LPS contamination was responsible for ET-2-mediated effects.

Second, BQ123 and IRL1038, two antagonists of ET_A and ET_B receptors, respectively, completely inhibited ET-2 stimulation. The effect of these receptor antagonists was somewhat unexpected and made difficult the overall interpretation of the results. The fact that antagonists for both ET_A and ET_B receptors completely blocked ET-2 stimulation suggests that both receptors need to be engaged by ET-2 in order to activate fully pMO. It is also true, however, that most of the published work has been done with ET-1 and not with ET-2 and it cannot be excluded that the two ETs act differently in different model systems. Alternatively, we have to admit that the specificity of the receptor antagonists used in our assay is not what we expected. Whereas no doubt exists in the literature about BQ123, which is considered a selective ET_A receptor antagonist, some questions have recently been raised concerning the selectivity of IRL1038.[30] If this is true, our data seem to confirm the absence of specificity of IRL1038 for ET_B receptors and suggest a cross-reaction with ET_A receptors instead. We can only confirm that the data were quite reproducible, at least in the conditions described in our assay. At present it is beyond the aim of the present work to investigate the molecular basis of the ET-2 interaction with ET_A or ET_B receptor on macrophages, or the specificity of the antagonists. We would like only to outline that our report is the first one showing that the ET_A receptor antagonist, BQ123, inhibits ET-2 action on pMO, suggesting the presence of ET_A receptors on these cells. This is at variance with the results of Kishino *et al.*,[23] which demonstrated the presence of only ET_B receptors on peritoneal macrophages. This discrepancy may be due to the different strain of origin of the macrophages used in the two studies: CD1 versus C3H/HeN mice, respectively. Binding studies will help to clarify this point. These data suggest a role for ETs as autocrine factors for macrophages. A similar feedback action has been reported for ET-1, which is induced by transforming growth factor-β[31] and by IL-1 on endothelial cells,[32] and acts back on the same cells.[20] An autocrine effect has also been described for ET-1 on keratinocytes.[33] In our situation, ETs released by macrophages may be part of an amplification loop during the early phase of an inflammatory response.

The response of microglial cells was different from that of pMO. In the same dose range, ET-3 and ET-1, but not ET-2, induced IL-6 production. In any case, however, we did not observe production of IL-1, although the cell line was responsive when LPS was used as stimulus. Several reports indicate that ET-1 and ET-3, but not ET-2, are widely produced in the central nervous system, especially by astrocytes, and act as neuropeptides[34–36] on microglial cells. In damaged rat brain and in the pathogenesis of human cerebrovasospasm, there is an increase of ET-1 and ET-3 production by astrocytes[37–39] and glial cells and the expression of ET receptors is also augmented.[34,35] Our data further support the observation that ET-1 and ET-3 act preferentially at the central level, and not in periphery. Conversely, ET-2 stimulates peripheral pMO, but not microglial cells. These results extend previous observations on the different tissue distribution of ETs. ET-2, in fact, is a potent constrictor of intestinal smooth muscle cells and it is produced by several cell types of the gastrointestinal tract.[41] It can be hypothesized that ET-2, produced *in situ* may act as paracrine factor on macrophages from the same site of origin. In contrast, ET-1 and ET-3 probably act as paracrine factors on microglial cells.

Our study of the response of pMO to IFN-γ in the presence of ET-2 demonstrates that ET-2 possesses an inhibitory effect. Both IFN-γ and ET-2 induce IL-6 and IL-1 production by pMO, however, the presence of both stimuli, at the same time, resulted in inhibition of IL-6 and IL-1 production. Similar results were obtained when we measured NO on the same supernatants. Again, IRL1038 and BQ123, ET_A and ET_B receptor antagonists respectively, blocked the inhibitory effect of ET-2. Similar data were reported by Beck *et al.*[42] who showed an inhibitory effect of ET-1 on cytokine-induced NO production in glomerular mesangial cells. While the precise mechanism(s) and biological relevance of these ET-2 effects are presently unclear, it is likely that down-regulation of IL-6, IL-1 and NO by ET-2 may help to prevent massive NO or inflammatory cytokine build-up when IFN-γ is produced in the development of the immune response. Furthermore, these appear to be the first data showing inhibition of IFN-γ activity by ETs. In conclusion our data seem to add further complexity to the range of activities ascribed to ETs, either as pro-inflammatory or anti-inflammatory molecules.

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