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ENDOTHELIN-1 STIMULATES THE BIOSYNTHESIS OF TUMOUR NECROSIS FACTOR IN MACROPHAGES: ET-RECEPTORS, SIGNAL TRANSDUCTION AND INHIBITION BY DEXAMETHASONE

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Endothelin-1 (ET-1) enhances the biosynthesis of interleukin-6 (IL-6) in endothelial cells and bone marrow-derived stromal cells of the rat. This study investigates (i) whether ET-1 stimulates the formation of tumour necrosis factor α(TNFα) or interferon-γ(IFNγ) in cultured macrophages or in the anaesthetized rat. Incubation of J774.2 macrophages with ET-1 (0.001 — 1 μM) caused a concentration- and time-dependent increase in the concentration of TNFα, but not of IFNγ, in the culture medium. The increase in TNFα caused by stimulation of J774.2 macrophages was abolished by pretreatment of cells with (i) the protein synthesis inhibitor cycloheximide, (ii) with the selective ET_A-receptor antagonists BQ-123 or BQ-485 (but not the selective ET_B-receptor antagonist BQ-788), (iii) the tyrosine kinase inhibitors genistein or tyrphostin AG126, or (iv) with the glucocorticoid, dexamethasone. The inhibition by dexamethasone of the formation of TNFα by cells activated with ET-1 is not due to the formation of lipocortin-1 (LC1), as it was not reduced by a monoclonal antibody against LC1. Systemic administration (*iv*) of ET-1 (1 nmol · kg^{-1}) to anaesthetized rats caused a rapid and sustained (maximum: 45 min; return to baseline: within 180 min) rise in the plasma levels of TNFα. This is the first demonstration that ET-1 can release proinflammatory cytokines *in vitro* and *in vivo*. The generation of TNFα caused by ET-1 involves (in sequence) the (i) activation of ET_A-receptors, (ii) activation of tyrosine kinase resulting in the phosphorylation of intracellular proteins, (iii) the activation of, hitherto, unknown transcription factors, finally resulting in (iv) transcription and translation of the TNFα gene. The generation of TNFα by cells activated with ET-1 points to a pro-inflammatory role of ET-1 in diseases associated with local (e.g. atherosclerosis, heart failure) or systemic inflammation (circulatory shock), which are associated with high ET-1 plasma levels.

Key words: *endothelin-1, tumour necrosis factor α, ET_A-receptor, ET_B-receptor protein tyrosine kinase, dexamethasone, rat*

INTRODUCTION

Tumour necrosis factor α(TNFα) is a potent pro-inflammatory and immunomodulatory cytokine with a molecular weight of 17 kDa (1). TNFα is released by monocyte/macrophages, lymphocytes, granulocytes and vascular

smooth muscle cells after stimulation with lipopolysaccharide (LPS) (2—4). An enhanced formation of TNFα has been implicated in the pathogenesis of conditions which are associated with a local or systemic inflammatory response including rheumatoid arthritis, Crohn's disease, multiple sclerosis and septic shock (5). For instance, infusion of TNFα alone or in combination with low doses of endotoxin mimics several cardiovascular features of circulatory shock, including hypotension, peripheral vasodilatation and organ damage (6). TNFα enhances the recruitment (chemotaxis) and cytotoxicity of macrophges in an autocrine and paracrine fashion (2), and stimulates the expression of adhesion molecules (7). These effects of TNFα are either due to the direct activation of cells through specific TNFα-receptors or secondary to the stimulation by TNFα of the synthesis of other mediators and cytokines such as interleukin (IL-1, IL-6), prostaglandins (due to expression of cyclooxygenase-II) or nitric oxide (NO) (due to expression of the inducible nitric oxide synthase; iNOS) (2, 8, 9).

The endothelins (ET) are a family of peptides which share a common structure of 21 amino acids with four cysteine residues at positions 1, 3, 11 and 15 which link to form two intrachain disulphide bridges between residues 1 and 15, and 3 and 11 (10). Three isoforms of ET have been identified, namely ET-1, ET-2 and ET-3 (10). The complementary DNAs of two human endothelin receptors, ET_A (11) and ET_B (12), have been cloned and expressed. The vasoconstrictor effects of the endothelins are primarily mediated by activation of the ET_A receptor subtype, although ET_B receptors (located like the ET_A receptor on the vascular smooth muscle) also mediate vasoconstriction in certain vascular beds (13, 14). Activation by endothelins of the ET_B-receptor located on the endothelial cells result in the release of NO and prostacyclin and, hence, vasodilatation (15, 16). In addition, ET-1 stimulates the release of autacoids and hormones, decreases glomerular filtration rate and increases cell growth and division (17—20). Interestingly, ET-1 is not only produced by endothelial cells, but also by human macrophages (21) and polymorphonuclear leukocytes (22), suggesting a role for ET-1 in local or systemic inflammation (23). Indeed, enhanced ET-1 serum levels have been documented in experimental models of endotoxaemia (24, 25) as well as in humans with sepsis and septic shock (26). There is increasing evidence that proinflammatory cytokines such as TNFα, IL-1, IL-2 or IL-6 can stimulate the formation of ET-1 *in vitro* and *in vivo* (27—30). Although there are no studies documenting that ET-1 stimulates the formation of TNFα *in vitro* and *in vivo*, there is some evidence that ET-1 enhances the formation of the proinflammatory cytokine IL-6 in aortic endothelial cells (31) and bone marrow-derived stromal cells of the rat (32).

Here we demonstrate that ET-1 stimulates the *de novo* biosynthesis of the pro-inflammatory cytokine, TNFα (but not of IFNγ), in cultured macrophages

(and in the anaesthetized rat). The signal transduction events leading to the generation of TNFα by macrophages activated with ET-1 involve (i) the activation of ET_A-receptors and of protein tyrosine kinase, and (ii) are sensitive to inhibition with dexamethasone.

MATERIALS AND METHODS

Cell culture

The mouse macrophage cell line J774.2 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (3.5 mM) and 10% foetal calf serum (33). Cells were cultured in 96-well plates with 200 µl culture medium until they reached confluence.

Experimental design

(i) Cells were exposed to varying concentrations of ET-1 (1 nM — 1 γM) for 48 h to establish a concentration-response curve to TNFα.

(ii) In order to establish a time-course of the effects of ET-1, cells were incubated in the absence or presence of endothelin-1 (ET-1, 1 γM) for 6, 12, 24, 48 and 72 hours.

(iii) In order to confirm that the rise in TNFα afforded by ET-1 was not due to a contamination with endotoxin, cells were incubated with ET-1 (1 γM) for 48 h in the absence or presence of polymyxin B (0.5 $\gamma g \cdot ml^{-1}$), an agent which binds and inactivates endotoxin. In addition, cells were challenged with ET-1 for 48 h in the absence or presence of a sheep polyclonal antibody (PAb) against human TNFα (100 $\gamma g \cdot ml^{-1}$).

(iv) To investigate whether the formation of TNFα by ET-1 was due to *de novo* protein synthesis, cells were treated with ET-1 (1 γM) for 48 h in the absence or presence of cycloheximide (0.3 $\gamma g \cdot ml^{-1}$).

(v) To investigate whether the increase in TNFα by ET-1 is sensitive to the inhibition with dexamethasone, an anti-inflammatory steroid which prevents the expression of cyclooxygenase-2 (34) and iNOS (35, 36), cells were also challenged with ET-1 for 48 h in the absence or presence of dexamethasone (1 γM at 2 h prior to ET-1).

(vi) To investigate whether the inhibition by dexamethasone of the formation of TNFα by macrophages activated with ET-1 is mediated by lipocortin-1, which mediates many, but not all of the effects of dexamethasone (37), cells were exposed to ET-1 (1 γM) for 48 h and dexamethasone (1 %M at 2 h prior to ET-1) in the absence or presence of a polyclonal sheep antibody raised against human recombinant lipocortin-1 (anti-LC1; 1:60 dilution at 4 h prior to ET-1; (36)).

(vii) To elucidate which ET-receptor subtype mediates the production of TNFα caused by ET-1, cells were challenged with ET-1 (0.1 γM) in the presence or absense of the selective ET_A-receptor antagonists BQ-123 or BQ-485 [1 γM; (38)] or the selective ET_B-receptor antagonist BQ-788 [1 γM; (39)];

(viii) In subsequent experiments designed to evaluate whether any increase in TNFα by ET-1 involves the activation of tyrosine kinase, cells were incubated in the absence or presence of the protein tyrosine kinase inhibitors, genistein (100 γM) or tyrophostin AG126 (30 µM). In all the above studies, the medium was quickly removed after incubation, placed in test tubes, and stored for up to 1 week at −20°C.

Cell viability

To ensure that none of the above drug protocols resulted in a loss of cell viability, mitochondrial respiration (an indicator of cell viability) was assessed by the mitochondria-dependent reduction of MTT [3- (4,5 — dimethylthiazol-2-yl) — 2,5-diphenyltetrazolium bromide] to formazan (40). Cells in 96-well plates were incubated (37°C) with MTT (0.2 mg ml^{-1} for 60 min). Culture medium was removed by aspiration and cells solubilised in dimethylsulphoxide by measurement of OD_{550} using a Molecular Devices microplate reader (Richmond, CA, U.S.A.). Formazan production was expressed as a percentage of the values obtained from untreated cells.

Measurement of tumour necrosis factor α and interferon-γ in the supernatant of J774.2 macrophages or in the serum obtained from rats

The content of TNFα in serum samples (see below) or in the cell supernatant (50 γl) was determined by ELISA (Mouse TNFα ELISA kit, Genzyme, Cambridge, MA, U.S.A.) in 96-well plates (33). Binding was detected by a peroxidase-conjugated polyclonal anti-mouse TNFα antibody using tetramethylbenzidine as a substrate. Following acidification (sulphuric acid, 0.5 M final), the absorbance of each well was measured at 450 nm using a Molecular microplate reader (Anthos Labtec Instruments, Richmond, CA, U.S.A.).

For the determination of IFNγ in serum samples or cell supernatant (100 μl), a Cytoscreen™ rat IFNγ ELISA kit (Biosource International, Camarillo, CA, U.S.A.) was used. Binding was detected by a biotin-conjugated monoclonal anti-rat IFNγ antibody using streptavidin as a substrate. The absorbance of each well (96-well plate) was measured at 450 nm.

Effects of ET-1 on the plasma levels of TNFα and IFNγ in the rat

Male Wistar rats (240—320 g; Glaxo Laboratories Ltd., Greenford, Middx., U.K.) were anaesthetized with thiopentone sodium (Intraval; 120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration and body temperature was maintained at 37°C with a homeothermic blanket (BioSciences, Sheerness, Kent, U.K.). The right carotid artery was cannulated for the withdraw of blood. The femoral vein was cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilise for 15 min. At time 0, animals received a bolus injection of ET-1 (1 nmol $\cdot kg^{-1}$, i.v., $n = 24$). Prior to and at 15 min, 30 min, 45 min, 60 min, 90 min, 120 min, 180 min after injection of ET-1, 200 μl of blood was collected from a catheter placed in the carotid artery. The blood samples were centrifuged (15,000 rpm for 3 min) to prepare serum for the measurement of TNFα and IFNγ by ELISA.

Materials

Polymyxin B, dexamethasone, genistein and cycloheximide were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Tyrphostin AG126 was obtained from Calbiochem Novabiochem (Nottingham, U.K.). Sodium thiopentone (Intraval Sodium) was obtained from Rh—ne M/rieux Ltd. (Harlow, Essex, U.K.). L-Glutamine was obtained from B.D.H (Dagenham, U.K.) and foetal calf serum was obtained from Gibco BRL (U.K.). Endothelin-1 was purchased from Peptide International Inc. (Europe: Scientific Marketing Associates, Barnet, Hardfortshire, U.K.). BQ-788, BQ-485 and BQ-123 were a generous gift from Dr M. Yano (Banyu Pharmaceutical Co., Ltd, Japan). The sheep polyclonal antibody raised against human TNFα was a generous gift from Dr D. Smith (Therapeutic Antibodies Inc., London, U.K.). The polyclonal sheep antibody raised against human recombinant lipocortin-1 was a generous gift from Professor

Rod Flower (Department of Biochemical Pharmacology, William Harvey Research Institute, London, U.K.).

Statistical evaluation

All values in the *Figs* and in the text are expressed as mean ± s.e.mean of n observations, where n represents the number of wells (duplicate determinations) from 3—5 separate experiments or the number of animals studied. A two way analysis of variance (ANOVA) followed, if appropriate, by a Bonferoni's test was used two compare means between groups. A P-value of less than 0.05 was considered as statistically significant.

RESULTS

Effect of endothelin-1 (ET-1) on the production of tumour necrosis factor α (TNFα) or interferon-γ (IFN) in J774.2 macrophages

ET-1 (0.001 — 1 μM) caused within 48 h a concentration-dependent increase in the concentration of TNFα in the culture medium of J774.2 macrophages from 11 ± 6 (baseline) to 351 ± 46 pg · ml^{-1} ($n = 6$, *Fig. 1a*). The increase in the formation of TNFα (measured as accumulation over time) elicited by ET-1 (1 μM) was maximal at 48 h ($n = 6$) and there was no further significant increase at 72 h (*Fig. 1b*). In contrast, incubation of J774.2 macrophages with ET-1 (up to 1 μM) had no effect on the production of IFNγ in J774.2 macrophages ($n = 6$, detection limit: 21.8 pg · ml^{-1}, data not shown). Therefore, in subsequent experiments aimed at investigating the mechanism of the ET-1-induced production of TNFα, cells were incubated with ET-1 (1 μM) for 48 h in the absence and presence of various drugs.

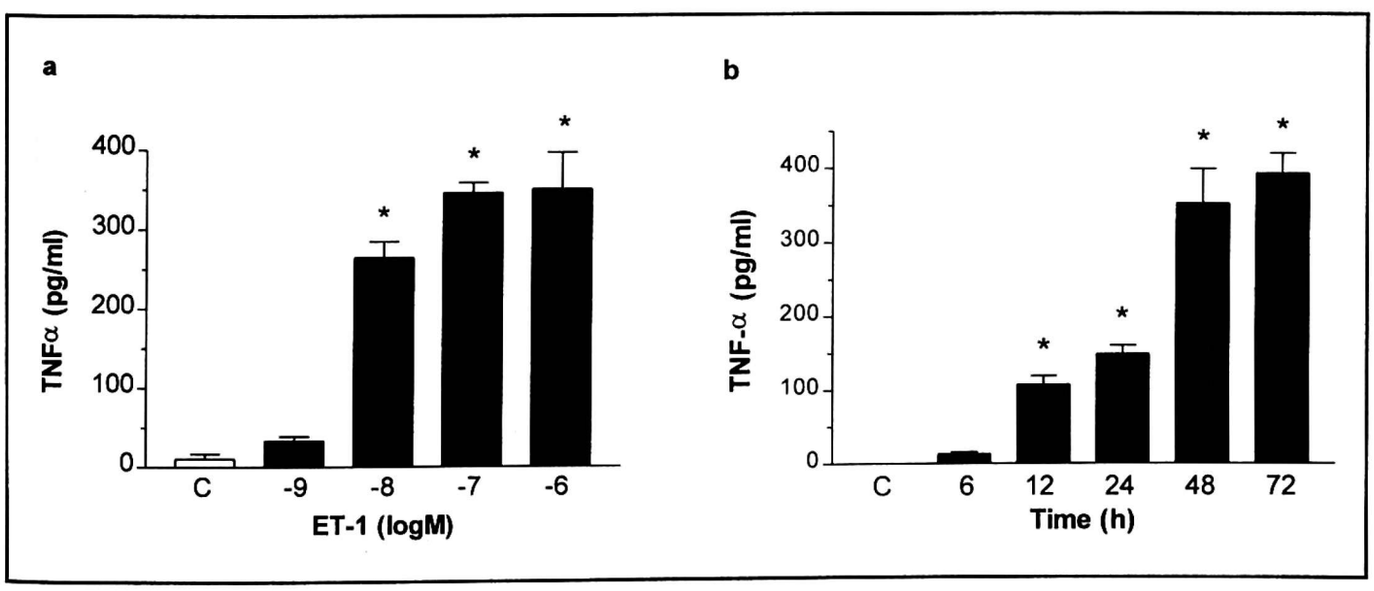

Fig. 1. (a) Endothelin-1 (ET-1) causes a concentration-dependent accumulation of TNFα in the supernatant of J774.2 macrophages. (b) The increase in the accumulation of TNFα by ET-1 (1 μM) is maximal at 48 h. Data are expressed as mean ± s.e.mean from duplicate determinations from three separate experiments ($n = 6$). *$P < 0.05$ represents significant difference compared to the untreated cells.

The effect of exogenous ET-1 on the plasma levels of TNFα in the anaesthetized rat

Bolus injection of ET-1 (1 nmol·kg^{-1}, i.v.) resulted, within 15 min, in a substantial increase in the plasma levels of TNFα, reaching a maximum of 412±41 pg·ml^{-1} ($P<0.05$, $n=8$) at 45 min after injection of ET-1 and returning to baseline within 180 min (*Table 1*). This increase in the plasma levels of TNFα caused by ET-1 was not accompanied by an increase in the plasma levels of IFNγ (*Table 1*).

Table 1. Effects of a bolus injection of saline (C; 1 ml·kg^{-1}, i.v.) or endothelin-1 (ET-1; 1 nmol·kg^{-1}, i.v.) on mean arterial pressure (MAP) and on the release into th plasma of tumour necrosis factor α (TNFα) or interferon-γ (IFNγ) in anaesthetized rats. Data are expressed as mean±s.e. mean of n observations. *$P<0.05$ represents significant difference when compared to control rats.

		Time (min)								
		0	15	30	45	60	90	120	150	180
C ($n=6$)	TNFα (pg/ml)	0	0	20±15	25±19	23±21	18±6	16±13	20±17	22±11
	IFNγ (pg/ml)	0	0	0	0	0	0	0	0	0
	MAP (mmHg)	118±4	116±5	113±5	114±4	113±5	112±4	109±4	110±5	111±4
ET-1 ($n=6-8$)	TNFα (pg/ml)	0	77±13*	152±22*	412±41*	309±45*	262±32*	112±16*	92±11*	31±9
	IFNγ (pg/ml)	0	0	0	0	0	0	0	0	0
	MAP (mmHg)	116±5	133±5*	126±5*	119±6	115±4	114±5	112±4	111±5	109±4

Effects of polymyxin B, a polyclonal antibody against TNFα, dexamethasone or the protein synthesis inhibitor cycloheximide on the production of TNFα caused by ET-1 in J774.2 macrophages

Coincubation of the cells with polymyxin B (0.5 μg·ml^{-1}, $n=6$), an agent which binds and inactivates endotoxin (Kengatharan *et al.*, 1996), had no effect on the production of TNFα elicited by ET-1 (1 μM) (*Fig. 2a*), suggesting that the ET-1 used, was not contaminated with endotoxin. In contrast, addition of a polyclonal antibody against TNFα (100 μg·ml^{-1}, $n=6$) to the cells abolished the detection (ELISA) of the increase in TNFα caused by ET-1 (1 μM) (*Fig. 2a*). Treatment of J774.2 macrophages with cycloheximide (0.3 μg·ml^{-1}, $n=6$), which inhibits the *de novo* biosynthesis of proteins, prevented the increase in

TNFα elicited by ET-1 (1 μM) (*Fig. 2b*). Similarly, dexamethasone (1 μM, $n = 6$) also prevented the increase in TNFα caused by ET-1 (*Fig. 2b*). The inhibition of TNFα formation caused by dexamethasone was not affected by pretreatment of cells with anti-LC1 (1:60 dilution, at 4 h prior to ET-1, $n = 6$) (*Fig. 2b*).

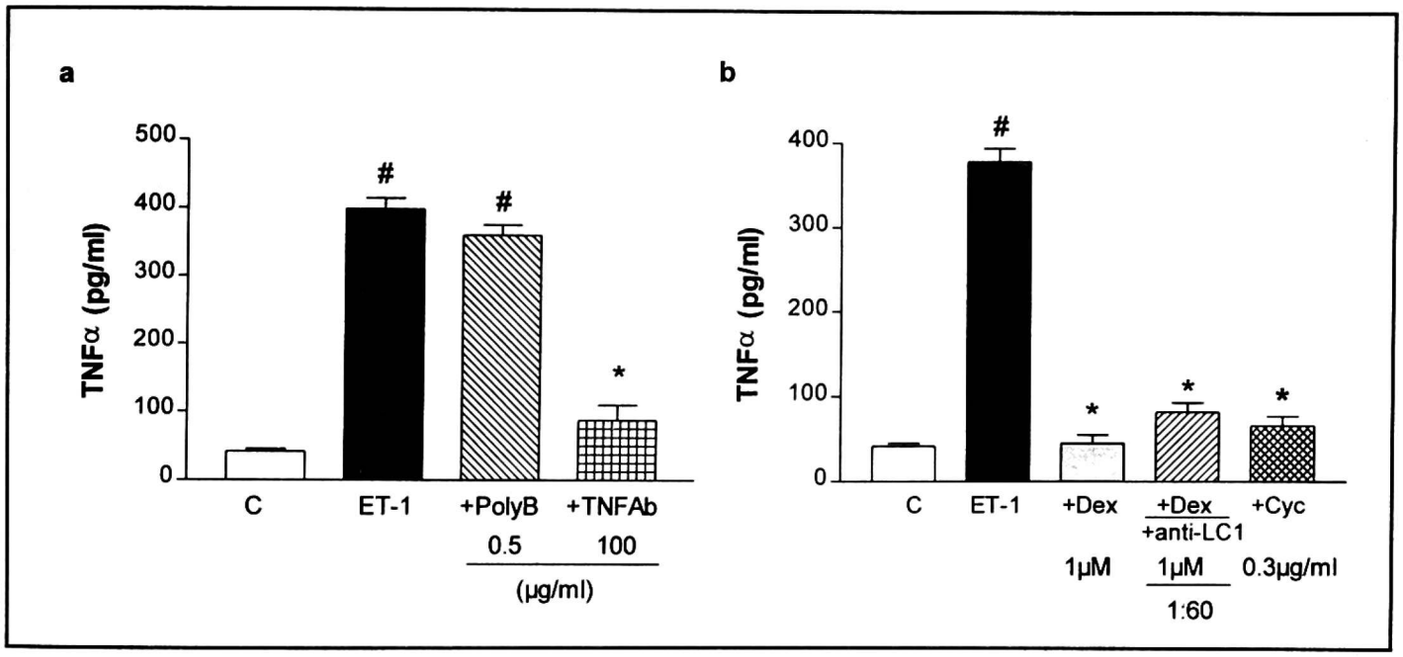

Fig. 2. (a) The detection of the increase in the production of TNFα induced within 48 h by ET-1 (1 μM) in cultured J774.2 macrophages by the ELISA used, is abolished by a polyclonal antibody against human TNFα (TNFAb; 100 $\mu g \cdot ml^{-1}$, squared column), but not by polymyxin B (PolyB; 0.5 $mg \cdot ml^{-1}$, hatched column), an agent which binds and inactivates endotoxin. (b) Treatment of cells with cycloheximide (Cyc; 0.3 $\mu g \cdot ml^{-1}$, crossed column) or dexamethasone (Dex; 1 μM, stippled column) prevented the increase in the production of TNFα by ET-1 (1 μM) in J774.2 macrophages. The inhibition of the formation of TNFα caused by dexamethasone was not affected by pretreatment of cells with a polyclonal sheep antibody raised against human recombinant lipocortin-1 (anti-LC1; 1:60 dilution, at 4 h prior to ET-1, hatched column, $n = 6$). Data are expressed as mean ± s.e.mean from duplicate determinations from three seperate experiments ($n = 6$). #$P < 0.05$ represents significant difference when compared to ET-1. $P < 0.05$ represents significant difference when compared to control.

The effect of the selective ET_A-receptor antagonists, BQ-485 or BQ-123, or the selective ET_B-receptor antagonist, BQ-788, on the production of TNFα caused by ET-1

To determine which ET-receptor suptype (ET_A or ET_B) mediates the increase in the formation of TNFα caused by ET-1 in J774.2 macrophages, cells were incubated for 48 h in the absence or presence of the selective ET_A-receptor-antagonists BQ-485 (1 μM, $n = 6$) or BQ-123 (1 μM; $n = 6$) or the selective ET_B-receptor antagonist BQ-788 (1 μM, $n = 6$). Coincubation of the cells with BQ-485 or BQ-123 significantly inhibited the increase in the formation of TNFα elicited by ET-1 (0.1 μM) ($P < 0.05$, *Fig. 3*). In contrast,

treatment of the cells with BQ-788 had no effect on the formation of TNFα elicited by ET-1 (*Fig. 3*).

Effect of the inhibitors of protein tyrosine kinase on the production of TNFα caused by ET-1

The increase in the biosynthesis of TNFα elicited by ET-1 (1μM) was prevented by coincubation of J774.2 cells with the protein tyrosine kinase inhibitors genistein (100 μM) or tyrphostin AG126 (30 μM) (*Fig. 4*). The vehicle used to dissolve the protein tyrosine kinase inhibitors, DMSO (maximum concentration of 0.05% used), did not affect the production of TNFα induced by ET-1.

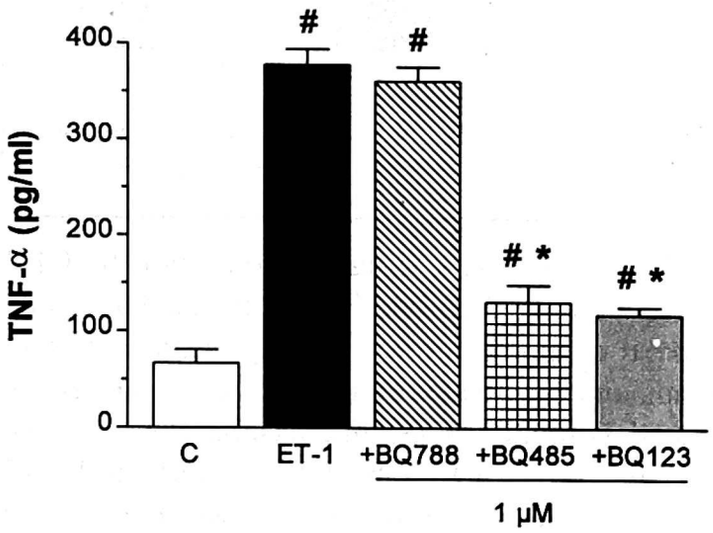

Fig. 3. Effect of the selective ET_A-receptor antagonists, BQ-485 (1 μM, squared column) or BQ-123 (1 μM, stippled column), or the selective ET_B-receptor antagonist, BQ-788 (1 μM, hatched column), on the accumulation of TNFα induced by ET-1 (0.1 μM) in J774.2 macrophages within 48 h. Data are expressed as mean ± s.e.mean from duplicate determinations from three seperate experiments ($n = 6$). *$P < 0.05$ represents significant difference when compared to ET-1. $P < 0.05$ represents significant difference when compared to control.

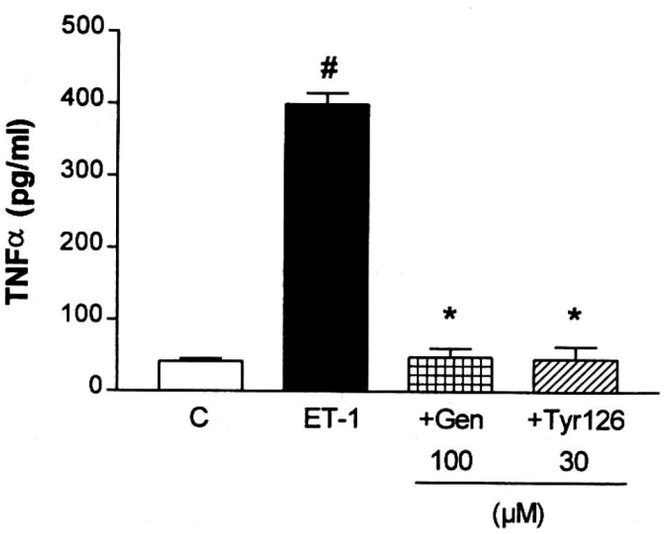

Fig. 4. Treatment of J774.2 macrophages with the tyrosine kinase inhibitors genistein (Gen; 100 μM, squared column) or tyrphostin AG126 (Tyr126; 30 μM, hatched column) prevented the increase in the production of TNFα elicited within 48 h by ET-1 (1 μM). Data are expressed as mean ± s.e.mean from duplicate determinations from three seperate experiments ($n = 6$). *$P < 0.05$ represents significant difference when compared to ET-1. #$P < 0.05$ represents significant difference when compared to control.

The effect of the drugs used on cell viability

None of the drugs used in these studies had any significant effect on cell viability as determined by the MTT-assay.

DISCUSSION

This study demonstrates that ET-1 activates cultured macrophages (J774.2 cell line) to produce TNFα in a concentration- (EC_{50}: approximately 50 nM) and time-dependent fashion. The generation of TNFα by macrophages challenged with ET-1 (1 μM) occured within 12 h and was maximal within 48 h after addition of the peptide to the cell culture medium. In contrast, ET-1 did not stimulate the formation of IFNγ in cultured macrophages.

What, then, is the mechanism by which ET-1 causes the formation of TNFα by cultured macrophages. As endotoxin stimulates macrophages to form TNFα (and other cytokines), it is possible that the observed increase in the formation of TNFα by ET-1 is due to a contamination of either ET-1 itself or of its vehicle with endotoxin. This was, however, not the case, as polymyxin B, an agent which (at the concentration used in this study) binds to endotoxin and, hence, prevents its effects (e.g. the expression of iNOS caused by endotoxin in macrophages, (41)), does not affect the generation of TNFα caused by ET-1. ET-1 selectively stimulated the generation of TNFα, but not of IFNγ, while endotoxin stimulates the generation of both of these cytokines in cultured macrophages (33). The generation of TNFα by macrophages challenged with ET-1 involves the *de novo* synthesis of proteins, as this effect was abolished by the protein synthesis inhibitor cycloheximide. Thus, we propose that ET-1 stimulates the *de novo* synthesis of TNFα in cultured macrophages. Similarly, the enhanced formation of IL-6 by rat aortic endothelial cells (cloned cell line: WAE-1) exposed to ET-1 is associated with an increase in mRNA for IL-6 and, hence, secondary to the transcription of the IL-6 gene (31).

Having demonstrated that ET-1 caused the *de novo* synthesis of TNFα, further studies were designed to elucidate which ET-receptor subtype mediates the activation of J774.2 macrophages by ET-1. There is good evidence that peritoneal macrophages of the rat express both ET_A- and ET_B-receptors (42). The increase in the formation of TNFα caused by ET-1 (10^{-7} M) in J774.2 macrophages was largely attenuated by the selective ET_A-receptor antagonists BQ-485 or BQ-123, but not by the selective ET_B-receptor antagonist BQ-788. Thus, we propose that an activation by ET-1 of ET_A-receptors , but not of ET_B-receptors, on J774.2 macrophages accounts for the enhanced formation of TNFα observed. Although we provide no evidence for the involvement of ET_B-receptors in the enhanced formation of TNFα by macrophages activated with ET-1, there is some evidence that ET_B-receptors mediate the stimulation by ET-1 of the synthesis of IL-6 in rat endothelial cells (cloned cell line: WAE-1) or smooth muscle cells. The hypothesis that the formation of IL-6 by cloned endothelial cells activated with ET-1 is (at least in part) due to activation of the ET_B-receptor, is based on the findings that (i) ET-3 (which primarily activates the ET_B-receptor subtype) also stimulates the generation of

IL-6 in these cells, and (ii) that the generation of IL-6 by ET-3 was abolished by the ET_B-receptor antagonist RES-701-1, but not by the ET_A-receptor antagonist BQ-485 (31).

What, then, are the signal transduction events which link the activation of the ET_A-receptors to the *de novo* synthesis of TNFα in cultured J774.2 macrophages? We demonstrate here that pretreatment of macrophages with the tyrosine kinase inhibitor genistein abolished the formation of TNFα caused by ET-1. This finding strongly indicates that the activation of tyrosine kinase and, hence, the phosphorylation of specific intracellular proteins is a key step in the signal transduction pathway involved in the formation of TNFα by macrophages activated with ET-1. There is, however, some evidence suggesting that some effects of genistein (e.g. the prevention of the expression of cyclooxygenase-2 caused by endotoxin) are independent of the inhibition of tyrosine kinase activity. Our hypothesis that the activation of tyrosine kinase by ET-1 precedes the generation of TNFα in activated macrophages is also supported by our finding that tyrphostin AG126, a potent and selective inhibitor of tyrosine kinase (43), also prevents the formation of TNFα afforded by ET-1 in cultured macrophages. The concept that ET-1 can, in principle, activate tyrosine kinase is not entirely new, as the mitogenic effect of ET-1 in mesangial cells also involves the activation of protein tyrosine kinase (44). For instance in these cells, ET-1 increases tyrosine phosphorylation of cellular proteins and this effect is prevented by the tyrosine kinase inhibitor herbimycin A (44). In addition, herbimycin A and genistein attenuate the mitogenic effects of ET-1 (measured as uptake of [^{3}H]thymidine) and herbimycin attenuated c-fos induction, AP-1 DNA binding, and transcription directed by an AP-1 cis-element in response to ET-1 (44). Thus, we propose that the stimulation by ET-1 of the formation of TNFα in cultured J774.2 macrophages involves the activation of tyrosine kinase leading to the phosphorylation of cellular proteins and, ultimately, to transcription/translation of the TNFα gene.

Further studies were designed to elucidate whether the formation of TNFα by macrophages activated with ET-1 is sensitive to inhibition with the glucocorticoid dexamethasone. Dexamethasone inhibits the release of TNFα from leukocytes in response to various stimuli, including LPS (1, 45). We demonstrate here that dexamethasone also abolishes the synthesis of TNFα by macrophages activated with ET-1. There is good evidence that some, but not all, of the anti-inflammatory effects of dexamethasone are secondary to the formation of lipocortin-1 (37). For instance, the inhibition by dexamethasone of the expression of iNOS, but not of cyclooxygenase-2, protein and activity is mediated by lipocortin-1 (36). Moreover, lipocortin-1 also mediates the prevention by dexamethasone of the release of TNFα from human peripheral mononuclear cells challenged with endotoxin (46). In contrast, this study provides no evidence for the involvement of lipocortin-1 in the inhibition by

dexamethasone of the formation of TNFα in macrophages challenged with ET-1. This conclusion is based on the finding that a monoclonal antibody against lipocortin-1 did not affect the inhibition by dexamethasone of the *de novo* biosynthesis of TNFα caused by ET-1 in murine macrophages. It should, however, be noted that the antibody to lipocortin-1 (in the concentration used) did abolish the reduction in nitrite formation afforded by dexamethasone in J774.2 macrophages challenged with endotoxin (data not shown).

This study also demonstrates that the systemic administration of ET-1 (1 nmol · kg^{-1} i.v.) to anaesthetized rats resulted in an increase in the plasma levels of TNFα, but not of IFNγ, which were maximally elevated after 45 min. Thereafter, the plasma levels of TNFα declined so that they were no longer significantly different from baseline at 180 min after injection of the peptide. The rapid increase in the plasma levels of ET-1 (within 15 min) coincided with a moderate (~17 mm Hg) and transient (for 45 min) increase in blood pressure caused by ET-1. It should, however, be noted that the maximum increase in the plasma levels of ET-1 occured between 45 and 90 min, a period where no significant elevations in blood pressure were detected. Although this result confirms that ET-1 (in a pharmacological concentration) can, in principle, also increase the plasma levels of TNFα, the time-course of the generation of TNFα *in vivo* (maximum within 45 min) and *in vitro* (maximum 48 h) are substantially different. Thus, the mechanism(s) by which ET-1 causes the formation of TNFα in J774.2 macrophages. Comparisons of the kinetics of the release of TNFα *in vivo* and *in vitro* are difficult for the following reasons: (1) The plasma half-life of ET-1 *in vivo* is very short (minutes), as more than 80% of ET-1 are removed by a single passage through the pulmonary circulation (15), and the clearance of ET-1 is blocked by BQ-788 (Fukuroda *et al.*, 1994). (2) In addition to removal of ET-1 involving the binding of ET-1 to ET_B-receptors, ET-1 is also metabolized by polymorphonuclear cells (22). In contrast, there is no evidence that J744.2 macrohages (in culture) either take up or metabolize ET-1. Moreover, systemic administration of ET-1 results in the release of many other mediators and autacoids including prostaglandins, nitric oxide, platelet-activating factor, aldosterone, vasopressin etc (17—20), one or many of which may influence the release and the kinetics of the release of TNFα caused by ET-1 *in vivo*.

In conclusion, this study demonstrates that ET-1 causes the generation of TNFα in cultured J774.2 macrophages as well as in the anaesthetized rat. The formation of TNFα by macrophages exposed to ET-1 is due to the activation of ET_A-receptors which, in turn, leads to the activation of tyrosine kinase. We also provide evidence that the generation of TNFα caused by ET-1 in macrophages is sensitive to the inhibition by the glucocorticoid dexamethasone and involves the *de novo* synthesis of proteins. Thus, we propose that the generation of TNFα caused by ET-1 involves (in sequence) the (i) activation of

ET_A-receptors, (ii) activation of tyrosine kinase resulting in the phosphorylation of intracellular proteins, (iii) the activation of, hitherto, unknown transcription factors, finally resulting in (iv) transcription and translation of the TNFα gene. We propose that the stimulation of the biosynthesis of TNFα mediates some of the effects of ET-1 in conditions associated with local or systemic inflammation.

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