Endothelin-2 synthesis is stimulated by the type-1 tumour necrosis factor receptor and cAMP: comparison with endothelin-converting enzyme-1 expression

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ABSTRACT

The synthesis of the vasoconstrictor peptide endothelin-2 (ET-2) is dependent on hydrolysis of the biologically inactive intermediate big ET-2 by an endothelin-converting enzyme (ECE). Here, mechanisms inducing ET-2 synthesis have been investigated using the human renal adenocarcinoma cell line (ACHN). Synthesis of ET-2 by ACHN cells was inhibited by phosphoramidon ($IC_{50} \approx 11 \mu M$). To determine whether ET-2 synthesis occurs in parallel with the metallo-peptidase ECE-1, a putative processing peptidase for big ET-2, changes in the levels of their mRNAs were compared by semi-quantitative RT-PCR under conditions causing the upregulation of ET-2 synthesis. Tumour necrosis factor- α (TNF α), forskolin and a cell-permeable cAMP analogue (dibutyryl cAMP) caused concentration-dependent increases in ET-2 synthesis. Combination of forskolin or dibutyryl cAMP with TNF α produced a significantly greater increase in ET-2 production

than these agents alone, indicating that adenylate cyclase and TNF α induce ET-2 synthesis by separate signalling pathways. Studies using receptor selective TNF α mutants, ^{125}I -TNF α binding and TNF receptor mRNA showed that type-1 TNF receptors mediate the ET-2 response to TNF α . PreproET-2 mRNA levels were increased by TNF α at 1 h and 2 h, but returned to control levels at 4 h. Treatment with forskolin significantly increased preproET-2 mRNA levels after 1 h and 4 h. ACHN cells expressed ECE-1b and ECE-1c, but not the ECE-1a isoform of this peptidase. RT-PCR for the combined isoforms ECE-1b/c/d showed TNF α to increase mRNA levels at 2 h and 4 h. Forskolin had no effect on ECE-1b/c/d mRNA levels. Thus, expression of ET-2 and ECE-1b/c/d mRNAs in ACHN cells do not display the co-ordinated regulation observed with typical peptide prohormone processing enzymes and their substrates. *Journal of Molecular Endocrinology* (2000) **24**, 273–283

INTRODUCTION

The endothelins (ET-1, ET-2 and ET-3) are a family of 21 amino acid vasoconstrictor peptides first identified in 1988 (Yanagisawa *et al.* 1988, Inoue *et al.* 1989). All three endothelins follow a similar pattern of biosynthesis with initial synthesis as precursor proteins (≈ 200 amino acid residues) which undergo selective proteolysis to yield the biologically inactive intermediates called big endothelins (Yanagisawa *et al.* 1988, Bloch *et al.* 1989, Ohkubo *et al.* 1990). The active endothelins are generated by hydrolysis of the Trp²¹-Val²² bond in big ET-1 and big ET-2, or Trp²¹-Ile²² in big ET-3 (Brooks & Ergul 1998). This cleavage is

unique to the endothelins and hence the role of a specific endothelin-converting enzyme (ECE) was proposed (Yanagisawa *et al.* 1988). Whether the hydrolysis of each of the three big ET molecules is dependent on a distinct ECE has yet to be elucidated.

Purification and molecular cloning of a phosphoramidon-sensitive ECE, named ECE-1, has been described (Schmidt *et al.* 1994, Shimada *et al.* 1994, Xu *et al.* 1994, Turner *et al.* 1998). There are at least four distinct isoforms of this enzyme encoded by the same gene but regulated by different promoters. Termed ECE-1a, ECE-1b, ECE-1c and ECE-1d, the four isoforms differ only in their N-terminal cytoplasmic regions (Valdenaire *et al.*

1995, Schweizer *et al.* 1997, Valdenaire *et al.* 1999). ECE-1 is a highly glycosylated type II transmembrane protease with a neutral pH optimum. It has structural and sequence homology to neutral endopeptidase (E.C.24.11) and to Kell blood group protein (Turner & Tanzawa 1997). ECE-1 mRNA has been demonstrated in ovary, heart, placenta, pancreas, adrenal and lung, and has been localised to vascular endothelial cells and some secretory cells (Schmidt *et al.* 1994, Shimada *et al.* 1994, Xu *et al.* 1994, Takahashi *et al.* 1995). Previous studies have shown that the human renal adenocarcinoma cell line (ACHN) synthesises ET-2 but not ET-1 or ET-3 (Ohkubo *et al.* 1990, Yorimitsu *et al.* 1992). ECE-1b was cloned from ACHN cells (Yorimitsu *et al.* 1995), but a number of studies have reported big ET-2 to be hydrolysed inefficiently by isoforms of ECE-1 (Schmidt *et al.* 1994, Shimada *et al.* 1994, Xu *et al.* 1994), hence casting doubts on its role in ET-2 synthesis.

Cytokines, such as tumour necrosis factor- α (TNF α), cause a marked induction of ET-1 synthesis in endothelial cells by increasing the steady-state levels of ET-1 mRNA (Marsden & Brenner 1992). In contrast to ET-1, the intracellular signalling mechanisms inducing the expression and synthesis of ET-2 have not been extensively investigated. Here, we show that ET-2 synthesis in ACHN cells is regulated by TNF α and cAMP. To determine the relationship between the expression of ET-2 and ECE-1 in ACHN cells, the levels of their mRNAs were compared by semi-quantitative RT-PCR after treatment with forskolin and TNF α .

MATERIALS AND METHODS

Cell culture

The human cell lines ACHN (ECACC No. 88100508), A549 (ECACC No. 86012804) and U937 (ECACC No. 85011440) were obtained from the European Collection of Animal Cell Cultures (ECACC; Salisbury, Wilts, UK). The human endothelial cell line EA.hy 926 was provided by Dr C-J S Edgell (Edgell *et al.* 1983). ACHN, A549 and EA.hy 926 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, penicillin (100 U/ml), streptomycin (100 μ g/ml) and amphotericin (2.5 μ g/ml) at 37 °C in a humidified CO₂ incubator (95% air, 5% CO₂). U937 were grown in RPMI 1640 medium with the same supplements and conditions. Medium for EA.hy 926 cells also included the HAT supplements (100 μ M hypoxanthine, 0.4 μ M aminopterin, 16 μ M thymidine; Sigma, St Louis, MO, USA).

ET-2 release experiments

ACHN cells were grown to confluence on 6 $\times$ 9.5 cm² well plates. Cells were incubated for 6 h with 2 ml serum-free DMEM containing the agents to be tested. Forskolin and dibutyryl cAMP were from Sigma. Human TNF α was from R & D Systems (Minneapolis, MN, USA). Initial experiments studied the effect on ET-2 synthesis of phosphoramidon (0.03–300 μ M; Peptide Institute, Osaka, Japan). Because low concentrations of phosphoramidon increased the stability of the released ET-2 without affecting synthesis, 0.3 μ M was included in the medium for all subsequent experiments. To demonstrate that the test agents were free of cytotoxic effects, mitochondrial dehydrogenase activity was measured at the end of the treatment period by conversion of MTT (Sigma) to formazan. In initial time-course experiments, ACHN cells grown on 9.5 cm² plates were treated with TNF α (3 ng/ml), forskolin (30 μ M) or the combination, for periods of 0.5–4 h. Conditioned media were collected for ET-2 measurement and the cells were harvested in RNazol B (Biogenesis, Poole, Dorset, UK) for subsequent extraction of RNA. However, as levels of secreted ET-2 during short time-periods were low, later time-course studies were performed using ACHN cells grown on 75 cm² flasks.

Measurement of secreted ET-2

Conditioned media were acidified with glacial acetic acid and extracted using disposable Extra-Sep C₁₈ columns (Lida, Kenosha, WI, USA), eluted with 80% acetonitrile containing 0.1% trifluoroacetic acid, and dried down at 50 °C under a stream of nitrogen (Corder *et al.* 1995). ET-2 was measured either by RIA using a rabbit antiserum raised against the common C-terminal sequence of the endothelins, His-Leu-Asp-Ile-Ile-Trp (Corder *et al.* 1995), with ¹²⁵I-ET-1 as tracer and ET-2 as standard, or by using an ET-1 ELISA (R & D Systems). The cross-reactivity of ET-2 relative to ET-1 was 100% in both assays.

RT-PCR

Total cellular RNA was extracted using RNazol B. RT-PCR was performed using Access RT-PCR reagents (Promega, Madison, WI, USA) according to the instructions supplied. PCR primers were initially designed for preproET-2, ECE-1a, ECE-1b, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), type-1 TNF receptors (TNFR-1) and TNFR-2 by reference to the respective human cDNA sequences (Table 1) (Ercolani

TABLE 1. Oligonucleotide primers used for RT-PCR of preproET-2 (Ohkubo *et al.* 1990), ECE-1a, ECE-1b/c/d, ECE-1b, ECE-1c (Valdenaire *et al.* 1995, Schweizer *et al.* 1997), GAPDH (Ercolani *et al.* 1988), TNFR-1 (Loetscher *et al.* 1990) and TNFR-2 (Beltinger *et al.* 1996)

Gene	Predicted PCR product size	Sequence	Sense/antisense	Annealing time/temp.	Extension time/temp.	Cycles
preproET-2	309	5'-AGCCAGCGTCCTCATCTCAT-3' 5'-TGCCAGTCTGGAACACGTCT-3'	S A	0.5/60	1/72	25
ECE-1a	734	5'-CTGAGACAGGAGGCAGC-3'	S	1/65	2/72	40
ECE-1b/c/d	699	5'-GATGTGCGACGTACAAGC-3'	S	1/60	2/68	25
ECE-1b specific	715	5'-TGCTGTCGGCGCTGGGGATG-3'	S	1/62	2/68	24
ECE-1c specific	715	5'-GGAGCACGCGAGCTATGATG-3'	S	1/62	2/68	24
ECE-1 common		5'-CTGTTGGAGTTCTTGGAAATC-3'	A			
GAPDH	859	5'-TGAAGGTCGGAGTCAACGGA-3' 5'-GTGTGCGCTGTTGAAGTCAGA-3'	S A	1/65	2/72	20
TNFR-1	495	5'-ACCTCCAGCTCCACCTATAC-3' 5'-GCAGCCTCATCTGAGAAGAC-3'	S A	1/62	2/72	40
TNFR-2	473	5'-GACTGAGTCCTCGTAGCCAT-3' 5'-CAAGCTCAAGTCCAGTGGAA-3'	S A	1/62	2/72	40

Each PCR reaction used a 94 °C denaturation for 0.5 min at the start of each cycle followed by the indicated annealing and extension times (min) and temperatures (temp.; °C). At the completion of the indicated number of cycles a final extension for 7 min at 72 °C was performed for each reaction.

et al. 1988, Loetscher *et al.* 1990, Ohkubo *et al.* 1990, Valdenaire *et al.* 1995, Beltinger *et al.* 1996). Primers were synthesised by Eurogentec (Abingdon, Oxon, UK). Following publication of cDNA sequences for ECE-1c, and more recently ECE-1d, it became clear that the sense primer used for comparative measurements of ECE-1b and preproET-2 mRNA levels was homologous with the corresponding sequences of ECE-1c and ECE-1d mRNAs (Schweizer *et al.* 1997, Valdenaire *et al.* 1999). Therefore, this primer is referred to as ECE-1b/c/d. Additional sense primers were designed based on the specific gene sequences of human ECE-1b and ECE-1c (Schweizer *et al.* 1997). For the design of primers for a human preproET-2 PCR product, reference was made to the sequence of the rat preproET-2 gene (accession numbers U59510, U64946–49) to obtain primers that should be intron spanning for the human sequence. DNA contamination did not contribute to the PCR products obtained. This was confirmed by performing PCR, with and without RT, and by using a sample contaminated with DNA (data not shown). The preproET-2 primers were not homologous with human preproET-1 or preproET-3 cDNA sequences. Except for TNFR-2, all other primer sequences were intron-spanning.

Oligo-dT₁₅ (1 μ M final concentration) was added to all ECE-1b/c/d, TNFR-1 and TNFR-2 PCR reactions to prime the RT reaction. For each reaction total RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase at 48 °C for 45 min. Preliminary experiments were conducted for preproET-2, ECE-1, and GAPDH to

define semi-quantitative PCR conditions. This was done by using 50–200 ng total RNA to determine the PCR amplification conditions required to give a yield of PCR product which was proportional to the quantity of RNA template. Subsequent studies used 100 ng total RNA per reaction. Optimised cycling conditions for each gene in a total volume of 50 μ l are shown in Table 1. PCR products (10 μ l per lane) were electrophoresed in 1% agarose gels at 120 V, and stained with ethidium bromide. Band intensities were quantified with a Gel Doc 1000 system using Molecular Analyst software (Bio-Rad, Hercules, CA, USA). For each sample the yield of PCR products for preproET-2 or ECE-1b/c/d mRNA was expressed relative to that obtained for GAPDH mRNA.

Characterisation of TNF receptors in ACHN cells

To complement RT-PCR identification of TNF receptor mRNA in ACHN cells, the roles of specific TNF receptors in the induction of ET-2 synthesis were investigated by using receptor-selective mutants of human TNF α . Studies of ¹²⁵I-TNF α binding to ACHN cells (24 $\times$ 2 cm² well plates) were also performed for comparison. Binding of ¹²⁵I-TNF α was determined in the presence of receptor selective TNF α mutants or blocking antibodies. The effects of receptor selective TNF α mutants on ¹²⁵I-TNF α binding were studied using binding medium (DMEM with 2.5 mM Hepes and 2.5% bovine serum albumin (BSA)) containing human TNF α (R & D Systems), the TNFR-1

selective mutant, R32W-S86T, or the TNFR-2 selective mutant, D143N-A145R (0.5–2000 ng/ml) (Hoffman-La Roche Ltd, Basel, Switzerland) (Loetscher *et al.* 1993). For studies of blocking antibodies, binding medium containing anti-human TNFR-1 or TNFR-2 neutralising monoclonal antibodies (0.5–100 ng/ml) (R & D Systems) was incubated for 50 min at 37 °C. After pretreatment with the neutralising antibodies, or immediately after the addition of TNF α or TNF α mutants to cells, 125 I-TNF α was added to each well to give a total incubation volume of 0.2 ml. 125 I-TNF α was trimeric TNF α labelled with 125 I-Bolton and Hunter reagent and chromatographically purified by gel filtration to ensure intact TNF α trimer (Amersham International plc, Amersham, Bucks, UK). The final concentration of 125 I-TNF α was $\approx$ 300 pM expressed as 17.5 kDa TNF α monomer, with a specific activity of 10.4 MBq/nmol TNF α monomer. After incubation of 125 I-TNF α at 37 °C for 2.5 h, cells were washed three times with phosphate-buffered saline containing 0.1% BSA (1 ml per well), and then lysed with 0.1 M NaOH, 0.5% SDS (200 μ l per well) for measurement of bound radioactivity.

Analysis of results

All results are expressed as means $\pm$ s.e.m. and statistical comparisons were made by analysis of variance with Fisher's LSD test.

RESULTS

Effect of phosphoramidon on ET-2 synthesis

Phosphoramidon (0.03–3 μ M) caused a 40–55% increase in the amount of ET-2 accumulating in the conditioned media over 24 h ($P < 0.01$, Fig. 1). Higher concentrations resulted in a concentration-dependent reduction in ET-2 synthesis. Based on the maximum level of ET-2 release, the IC₅₀ for inhibition of ET-2 synthesis by phosphoramidon was $\approx$ 11 μ M, which is 17-fold less than that for inhibition of ET-1 synthesis by A549 cells (IC₅₀ $\approx$ 190 μ M; Fig. 1) (Corder *et al.* 1995).

Stimulatory effects of TNF α , forskolin and dibutyryl cAMP on ET-2 release

Treatment of ACHN cells for 6 h with TNF α (0.1–100 ng/ml), forskolin (0.1–30 μ M), or dibutyryl cAMP (10–1000 μ M) caused concentration-dependent increases in ET-2 release (Fig. 2). The threshold concentration for TNF α was 0.3 ng/ml

FIGURE 1. Effect of phosphoramidon on ET-1 and ET-2 secretion from A549 and ACHN cells respectively. Results are from three to five separate experiments. Basal release over 24 h was 41 ± 4 fmol ET-1/cm² for A549 and 14 ± 0.4 fmol ET-2/cm² for ACHN. †† $P < 0.01$, ** $P < 0.01$ compared with basal release of ET-1 and ET-2 respectively.

and the maximum response was obtained at 3 ng/ml, which represented an almost twofold increase compared with basal release ($P < 0.001$; Fig. 2). Forskolin (30 μ M) increased ET-2 secretion by $316 \pm 56\%$ (Fig. 2). Higher concentrations showed cytotoxicity. The effect of dibutyryl cAMP confirmed that forskolin induced ET-2 synthesis via a cAMP-dependent mechanism.

Combination of forskolin (30 μ M) with an excess of TNF α (100 ng/ml) produced a significantly greater increase in ET-2 secretion ($297 \pm 18\%$ higher than basal release, $P < 0.01$) than either agent alone (increases for forskolin and TNF α were $202 \pm 11\%$ and $80 \pm 12\%$ above basal respectively). Dibutyryl cAMP (500 μ M) combined with TNF α (3 ng/ml) also produced a significantly greater increase in ET-2 secretion ($326 \pm 26\%$ greater than basal release, $P < 0.01$) than treatment with dibutyryl cAMP alone ($226 \pm 20\%$ above basal release).

Comparison of ET-2 synthesis with levels of preproET-2 mRNA

Basal release of ET-2 increased in a time-dependent manner over the 4-h study period (Fig. 3a). After 1 h, ET-2 secretion was increased by 50% by forskolin (30 μ M) ($P < 0.05$). However, TNF α (3 ng/ml) only induced a significant increase in ET-2 secretion after 4-h stimulation ($P < 0.01$; Fig. 3a). Combination of the two agents produced

FIGURE 2. Effect of (a) TNF α , (b) forskolin and (c) dibutyryl cAMP on ET-2 release from ACHN cells. ACHN cells were treated with (a) TNF α , (b) forskolin, or (c) dibutyryl cAMP for 6 h. Results are triplicate evaluations from three to five separate experiments. Mean basal release of ET-2 was (a) 22.5 ± 2.8 , (b) 28.9 ± 1.3 and (c) 48.0 ± 4.3 fmol/9.5 cm² well/6 h. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with basal release.

significantly higher levels of ET-2 in the medium at 0.5 h, 2 h and 4 h compared with the corresponding basal secretion. At 4 h, ET-2 levels with the combined treatments were greater than with either forskolin or TNF α alone ($P < 0.001$).

Time-dependent effects of TNF α (3 ng/ml) and forskolin (30 μ M) on levels of mRNA for preproET-2 were investigated using semi-quantitative RT-PCR. A single PCR product of the correct size was obtained for preproET-2. Basal levels of preproET-2 mRNA did not change over the 4-h study period (Fig. 3b). Treatment with TNF α , forskolin or the combination for 1 h caused increases of approximately 80% in the levels of preproET-2 mRNA (expressed as preproET-2/GAPDH ratios) (Figs 3b and 4c; $P < 0.05$). This paralleled small changes in ET-2 synthesis over this period. The largest increase in ET-2 release was observed at 4 h, which therefore reflects the increased biosynthesis over the preceding 2 h. In agreement with this, the levels of preproET-2 mRNA at 2 h with TNF α alone were approximately twofold higher than control ($P < 0.05$), and in combination with forskolin were elevated more than threefold ($P < 0.001$; Fig. 3b). By 4 h, levels of preproET-2 mRNA in the presence of TNF α had declined to those of untreated control cells. With forskolin treatment, levels of preproET-2 mRNA at 2 h were not different from control, but were raised by 4 h, supporting an increase in ET-2 synthesis between 2 and 4 h ($P < 0.001$).

Expression of ECE-1 mRNAs

Initial experiments investigated which isoforms of ECE-1 could be detected in ACHN cells by RT-PCR. ECE-1a was undetectable after 40 cycles (over-amplified conditions) using a specific ECE-1a sense primer and a common ECE-1 antisense primer (Fig. 4a). The same cycling conditions yielded a PCR product of the correct size from human endothelial cell line EA.hy 926 (Fig. 4a). By comparison, ECE-1b/c/d was readily detected in both cell lines using 25 cycles (Fig. 4b).

In contrast to preproET-2 mRNA levels, levels of ECE-1b/c/d mRNA did not display the same large increases at 2 h when the phase of maximum ET-2 synthesis appears to be initiated (Fig. 3c). At 4 h, TNF α -stimulated cells showed increased levels of ECE-1b/c/d mRNA both alone and combined with forskolin ($P < 0.05$; Fig. 3c). Treatment with forskolin did not alter ECE-1b/c/d mRNA expression during the 4-h study period (Fig. 3c).

To determine whether ACHN cells express both ECE-1b and ECE-1c mRNAs, RT-PCR was performed using isoform specific primers. While

approximately equal amounts of ECE-1b and ECE-1c mRNA were present in ACHN cells (Fig. 4b), EA.hy 926 cells showed similar amounts of ECE-1c mRNA, but ECE-1b mRNA was virtually undetectable (Fig. 4b). As preproET-2 mRNA levels were increased by TNF α and forskolin at 1 h (Fig. 3b), the effects of TNF α and forskolin on ECE-1b and ECE-1c mRNA levels were re-evaluated at 1 h using the isoform specific primers. Consistent with earlier experiments, TNF α or forskolin increased preproET-2 mRNA levels by approximately 75% ($P < 0.01$, $n = 3$, data not shown), but neither ECE-1b nor ECE-1c mRNA levels were altered by these treatments (data not shown, $n = 3$).

Characterisation of TNF receptors regulating ET-2 release

To investigate the expression of TNFR-1 and TNFR-2 mRNA in ACHN cells, total RNA was subjected to RT-PCR using primers specific for TNFR-1 or TNFR-2. The human A549 and U937 cell lines express TNFR-1 mRNA and TNFR-2 mRNA respectively (Iwamoto *et al.* 1994, Shimomoto *et al.* 1995). Total RNA extracted from these cells was subjected to RT-PCR in parallel with ACHN samples. A PCR product of the correct size was obtained in each positive control (Fig. 4d and e). However, in ACHN cells, only TNFR-1 mRNA was expressed and TNFR-2 mRNA was undetectable (Fig. 4d and e).

To determine the roles of the TNFR-1 and TNFR-2 receptors in TNF α -mediated ET-2 release, ACHN cells were stimulated with the TNFR-1 selective mutant, R32W-S86T (0.2–20 ng/ml), the TNFR-2 selective mutant D143N-A145R (0.6–200 ng/ml) or both (0.2–20 ng/ml) for 6 h. The TNFR-1 selective mutant, R32W-S86T, increased ET-2 secretion in a concentration-dependent manner (Fig. 5a). The maximal increase in release ($89 \pm 14\%$ above basal levels; $P < 0.001$)

FIGURE 3. Time-dependent changes in (a) ET-2 secretion, (b) preproET-2 and (c) ECE-1b/c/d mRNA levels after treatment of ACHN cells with forskolin, TNF α or the combination. ACHN cells on 75 cm² flasks or 6 $\times$ 9.5 cm² well plates were treated with 30 μ M forskolin (F), 3 ng/ml TNF α (T) or the combination (T+F) for 0.5–4 h. (b) PreproET-2 and (c) ECE-1b/c/d mRNA levels were measured by RT-PCR and are expressed relative to GAPDH values. Data are from four separate experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with corresponding control (C) levels, † $P < 0.001$ compared with 4-h treatment with TNF α or forskolin alone.

FIGURE 4. Evaluation of mRNA expression in ACHN cells for (a) ECE-1a, (b) ECE-1b/c/d, ECE-1b and ECE-1c, (c) preproET-2, ECE-1b/c/d and GAPDH, (d) TNFR-1 and (e) TNFR-2. RNA samples from ACHN cells were subjected to RT-PCR using sense primers for (a) ECE-1a or (b) ECE-1b/c/d, ECE-1b and ECE-1c with a common ECE-1 antisense primer, and compared with EA.hy 926 cells. (c) This shows representative PCR products for preproET-2, ECE-1b/c/d and GAPDH mRNA after 1-h treatment of ACHN with TNF α (T), forskolin (F) or the combination (T+F) compared with control (C). RT-PCR for TNF receptor mRNA using primers for TNFR-1 (d) and TNFR-2 (e) – comparison of ACHN with samples from A549 and U937 cell lines as sources of TNFR-1 and TNFR-2 mRNA. RT-PCR conditions were semi-quantitative in (b) and (c), and over-amplified (40 cycles) in (a), (d) and (e). M; molecular size markers (ϕ X174 RF DNA HaeIII fragments; Gibco (Paisley, UK)).

was comparable to native TNF α (Figs 2a and 5a). Combination of the two mutants did not produce a greater effect than the TNFR-1 selective mutant alone (Fig. 5a). The TNFR-2 selective mutant D143N-A145R alone had no significant effect on ET-2 secretion at any concentration tested (Fig. 5a).

Studies of 125 I-TNF α binding to ACHN cells showed wild-type TNF α and the TNFR-1 selective mutant, R32W-S86T, caused a concentration-dependent inhibition of binding (Fig. 5b; IC₅₀ values $\approx$ 12 and 29 ng/ml respectively). The higher IC₅₀ value obtained for R32W-S86T is consistent with the twofold lower binding affinity of this mutant for the TNFR-1 receptor (Loetscher *et al.* 1993). The

TNFR-2 selective mutant, D143N-A145R, did not inhibit binding of 125 I-TNF α . The role of the TNFR-1 receptor in binding 125 I-TNF α was also confirmed using receptor selective neutralising monoclonal antibodies (Table 2).

DISCUSSION

These results from ACHN cells have identified two distinct signalling pathways regulating preproET-2 synthesis – namely, a TNFR-1 receptor-linked induction and a cAMP-dependent mechanism. The increased levels of preproET-2 mRNA with TNF α

FIGURE 5. Effect of receptor selective TNFα mutants on ACHN cells (a) ET-2 release and (b) ¹²⁵I-TNFα binding. (a) ACHN cells were incubated with TNFR-1 selective TNFα mutant, R32W-S86T, TNFR-2 selective TNFα mutant, D143N-A145R (Loetscher *et al.* 1993), or both for 6 h. Results are triplicate evaluations from four separate experiments. Mean basal release of ET-2 was 28.9 ± 1.3 fmol/9.5 cm² well per 6 h. ****P* < 0.001 compared with basal release. (b) ACHN cells were treated with TNFα (wt, wild type), or the receptor selective TNFα mutants with ¹²⁵I-TNFα (≈ 300 pM) for 2.5 h at 37 °C. Results are quadruplicate evaluations from three separate experiments. Mean maximum binding was 2671 ± 176 c.p.m./2 cm² well.

and forskolin suggested that increased gene transcription underlies the induction of ET-2 synthesis by these stimuli. The synthesis of ET-2 in response to TNFα was significantly increased by combination with forskolin or dibutyryl cAMP, even though TNFα was used at concentrations eliciting a maximal response. In addition, the time-course for changes in the levels of preproET-2 mRNA were different for TNFα and forskolin. Thus, it seems likely that TNFα and cAMP activate gene

TABLE 2. Inhibition of ¹²⁵I-TNFα binding by TNF receptor selective neutralising antibodies

Antibody concentration (ng/ml)	% Inhibition of ¹²⁵ I-TNFα binding	
	TNFR-1 neutralising antibody	TNFR-2 neutralising antibody
0.5	43.3 ± 4.6	3.0 ± 8.0
5	69.0 ± 3.0	−2.4 ± 10.2
10	75.9 ± 2.8	−1.4 ± 7.0
100	82.9 ± 1.2	4.7 ± 12.5

Results are quadruplicate evaluations from three separate experiments. Mean maximum binding was 2671 ± 176 c.p.m./2 cm² well.

transcription through distinct mechanisms. Indeed, analysis of the promoter region of the human ET-2 gene (accession numbers E07665 and E07666) identifies a number of putative DNA-binding sequences for cAMP response element-binding protein and a variety of regulatory elements associated with TNF signalling, including NF-κB. These results indicate some similarities with the regulation of the ET-1 gene as its synthesis is also induced by TNFα (Marsden & Brenner 1992), but in contrast to preproET-2, forskolin suppresses preproET-1 expression (Malek *et al.* 1993).

The TNFR-1 receptor mediates many of the actions of TNFα (Heller & Krönke 1994). In the studies described here, the effect of TNFα was mimicked by a TNFR-1 selective TNFα mutant, but a TNFR-2 selective TNFα mutant was without effect. Similarly, studies with receptor selective TNFα mutants and neutralising antibodies showed that binding of ¹²⁵I-TNFα to ACHN cells could be accounted for by the TNFR-1 receptor. Furthermore, TNFR-1 mRNA but not TNFR-2 mRNA was identified in ACHN cells, confirming that the TNFR-2 receptor is not involved in the induction of ET-2 synthesis by TNFα in these cells.

Studies of ET-1 biosynthesis have shown that processing of its precursor proendothelin-1, including the hydrolysis of big ET-1 by ECE to ET-1, occurs intracellularly prior to release (Harrison *et al.* 1993, 1995, Xu *et al.* 1994, Corder *et al.* 1995, Russell & Davenport 1999). This bears a considerable similarity to the processing of many endocrine peptides. In agreement with studies of ET-1 synthesis (Corder *et al.* 1995), phosphoramidon inhibited ET-2 synthesis in a concentration-dependent manner. This effect was consistent with inhibition of the physiologically relevant ECE, as HPLC analysis of the conditioned medium followed

by big ET-2 immunoassay of the HPLC fractions showed big ET-2_[1-38] to accumulate in the conditioned media (data not shown). However, inhibition of ET-2 synthesis in ACHN cells displayed a greater than tenfold higher sensitivity to phosphoramidon compared with ET-1 synthesis by A549 cells. This suggests that different ECE isoforms are responsible for hydrolysis of big ET-1 and big ET-2.

Because efficient processing in secretory vesicles of endocrine peptide prohormones into bioactive peptides is dependent on the co-ordinated expression of the prohormone mRNA with the relevant processing enzymes (prohormone convertases, carboxypeptidase H) (Bloomquist *et al.* 1991, 1994, Schuppin & Rhodes 1996), changes in levels of preproET-2 mRNA induced by TNF α or forskolin were compared with those of ECE-1b/c/d mRNA. This was performed to determine whether synthesis of ECE-1 correlated with that of preproET-2 in the manner of a typical processing enzyme to substrate relationship, which might be expected if ECE-1 has a role in big ET-2 cleavage in constitutive secretory vesicles of ACHN cells.

The time-course experiments with TNF α and forskolin were performed before the distinction between the ECE-1b, ECE-1c and ECE-1d isoforms was established. The PCR product referred to as ECE-1b/c/d provided a measure of the combined levels of mRNA for these three ECE-1 isoforms as the DNA sequence corresponding to the sense primer is identical in ECE-1b, ECE-1c and ECE-1d (Valdenaire *et al.* 1999). Although the relative levels of each of these ECE-1 isoforms may have fluctuated, the amounts of the combined ECE-1b/c/d PCR product did not increase in parallel with those of preproET-2 mRNA during stimulation with either TNF α or forskolin. After the description of the cDNA sequence for ECE-1c, the relationship between levels of preproET-2 mRNA and ECE-1b or ECE-1c was re-examined using specific primers, but again no parallels were observed, indicating that the regulation of these ECE-1 isoforms is not closely linked to the ET-2 gene. Comparisons of levels of ECE-1d mRNA with those of preproET-2 mRNA were not undertaken in these investigations.

The four isoforms of ECE-1 have been reported to have different locations when expressed in CHO cells or Madin-Darby canine kidney (MDCK) cells (Schweizer *et al.* 1997, Azarani *et al.* 1998, Brown *et al.* 1998, Schweizer *et al.* 1999, Valdenaire *et al.* 1999). ECE-1a is localised to the cell surface, ECE-1b is predominantly intracellular with a pattern of distribution comparable to the trans Golgi network (TGN), ECE-1c is found

mainly on the cell surface with some associated with the TGN, and ECE-1d has been identified at intracellular sites and on the cell surface. Consistent with a cell surface localisation, both ECE-1a and ECE-1c isoforms expressed in CHO cells function as ectoenzymes with big ET-1 as the substrate (Xu *et al.* 1994, Takahashi *et al.* 1995). Based on available results describing the cellular localisation of the four isoforms in CHO and MDCK cells, it is not possible to determine which ECE-1 isoform, if any, is likely to be most important in the intracellular processing of the big ETs.

In endothelial cells, human ECE-1a (ECE-1 β), and rat and human ECE-1c (ECE-1 α) are localised, at least in part, to the plasma membrane (Takahashi *et al.* 1995, Barnes *et al.* 1998, Brown *et al.* 1998, Russell *et al.* 1998). However, intracellular co-localisation of big ET-1 with ECE-1, including the ECE-1a and ECE-1c isoforms, has also been described (Barnes *et al.* 1998, Russell *et al.* 1998), with ECE-1 cycling between the cell surface and the TGN (Barnes *et al.* 1998). This suggests that ECE-1 processing of big ETs could occur during vesicle translocation from TGN to the plasma membrane, with the ECE-1 then undergoing recycling. However, because ET-1 synthesis still occurs in non-viable fetuses obtained on ECE-1 gene knockout (Yanagisawa *et al.* 1998), conclusive evidence for ECE-1 playing a role in ET-1 biosynthesis has yet to be described.

Although we cannot exclude the possibility that synthesis of ECE-1 protein may be sufficient to accommodate changes in the rate of ET-2 synthesis, based on the precedents of endocrine peptide processing, the absence of any relationship between ECE-1 expression and ET-2 synthesis casts doubts on its potential role as an intravesicle ECE. Moreover, when the many studies showing poor efficiency of big ET-2 hydrolysis by ECE-1 (Schmidt *et al.* 1994, Shimada *et al.* 1994, Xu *et al.* 1994) and the higher sensitivity to phosphoramidon of ET-2 synthesis compared with ET-1 are taken into account, it does not seem likely that ECE-1 plays a role in big ET-2 processing in ACHN cells.

ACKNOWLEDGEMENTS

We are indebted to Liz Wood for providing the cultured cells, Amersham International plc for the supply of ¹²⁵I-TNF α , and Dr W Lesslauer and Dr H Loetscher of Hoffmann-La Roche Ltd for the TNF α receptor selective mutants R32W-S86T and

D143N-A145R. We are grateful to the Institut de Recherches Internationales Servier for the financial support provided for this work. D M L was supported by a European Commission Marie Curie Research Grant (Training and Mobility of Researchers Programme).

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RECEIVED 24 August 1999