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The p55 tumor necrosis factor receptor (CD120a) induces endothelin-1 synthesis in endothelial and epithelial cells

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Abstract

Synthesis of the vasoconstrictor peptide endothelin-1 by endothelial and epithelial cells is strongly induced by tumor necrosis factor α (TNF-α). The actions of TNF-α are mediated by two transmembrane receptors of approximately 55 (p55, CD120a) and 75 kDa (p75, CD120b). Reagents activating selectively these receptor subtypes have been used to identify which TNF receptor mediates the induction of endothelin-1 synthesis. Stimulation of bovine aortic endothelial cells or human HEp-2 epithelial cells with a p55-selective mutant of human TNF-α (R32W-S86T) induced significant and concentration-dependent increases in endothelin-1 release. A p75 receptor-selective TNF-α mutant (D143N-A145R) was ineffective alone or in combination with the p55-selective mutant. Competitive binding experiments with [^{125}I]TNF-α showed the p55-selective mutant, but not the p75-selective mutant, to inhibit the binding of [^{125}I]TNF-α to endothelial and HEp-2 cells. Similar results were obtained with the p55 agonist antibody htr1 in both cell lines. These results establish the p55 TNF receptor as the main receptor involved in the induction of endothelin-1 synthesis by TNF-α.

Keywords: Endothelin; TNF (tumor necrosis factor) receptor; Endothelial cell; Epithelial cell; Inflammation; Atherosclerosis

1. Introduction

Endothelin-1 is a 21 amino acid vasoconstrictor peptide released mainly by endothelial and epithelial cells (Yanagisawa et al., 1988; Rubanyi and Polokoff, 1994). A great number of agents increase the transcription of the preproendothelin-1 gene, but the precise mechanisms controlling basal and stimulated endothelin-1 synthesis remain poorly understood. A pathophysiological role for endothelin-1 has been postulated in a large number of diseases on the basis of its potent vasoconstrictor and mitogenic properties and the observation that its expression is frequently increased (Rubanyi and Polokoff, 1994; Haynes and Webb, 1998). The proinflammatory cytokine tumor necrosis factor α (TNF-α) has been shown to increase endothelin-1 synthesis in endothelial and epithelial cells in vitro, by increasing the transcriptional rate of the preproendothelin-1 gene (Marsden and Brenner, 1992; Nakano et al., 1994; Corder et al., 1995). In vivo, both exogenously administrated TNF-α and endogenously synthesised TNF-α induce the synthesis of sufficient endothelin-1 to cause coronary vasoconstriction (Klemm et al., 1995). This has implications for a number of disease conditions. Indeed, an abnormal expression of TNF-α occurs in two widespread diseases, atherosclerosis and asthma, where endothelin-1 production from endothelial and epithelial cells, respectively, is also increased (Lerman et al., 1991; Mattoli et al., 1991; Rus et al., 1991; Cembrzynska-Nowak et al., 1993). Atherosclerotic plaques in particular contain both macrophages and smooth muscle cells, which are able to produce TNF-α (Beutler and Cerami, 1989; Rus et al., 1991) and endothelin-1-producing cells (endothelial and smooth muscle cells), and a high endothelin-1 expression is detected in macrophage-rich regions (Lerman et al., 1991). It is thus of critical importance to reach a better understanding of the mechanisms linking TNF-α stimulation to endothelin-1 synthesis.

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TNF-α binds to two transmembrane receptors of approximately 55 (p55, TNFR1, CD120a), and 75 kDa (p75, TNFR2, CD120b) (Aggarwal and Natarajan, 1996). While the p55 TNF receptor is ubiquitously expressed, the p75 TNF receptor is predominantly expressed by haematopoietic and endothelial cells. These receptors do not possess any intrinsic kinase activity or previously described consensus sequence involved in signal transduction and show no homology in their intracellular domains, which suggests they activate distinct signalling pathways. Upon TNF-α stimulation, the signal is transduced by association of the cytoplasmic domain of the TNF receptor with proteins belonging mainly to two novel protein families, the TNF receptors-associated factors (TRAFs) and death domain containing proteins (Darnay and Aggarwal, 1997). Although some of the proteins interacting with the p75 TNF receptor are able to associate indirectly with the p55 receptor, the majority of proteins recruited to the p55 receptor are unable to interact with the p75 receptor (Darnay and Aggarwal, 1997), further suggesting that these two receptors trigger different intracellular signalling pathways and mediate distinct cellular responses. A number of studies have focused on the identification of the TNF receptor involved in different specific actions of TNF-α and, so far, the great majority have been attributed to the p55 receptor (Aggarwal and Natarajan, 1996).

Treatment of endothelial cells with TNF-α induces a process of cell activation, characterised by the increased expression of a number of genes including leukocyte cell adhesion molecules (such as intercellular adhesion molecule type 1, vascular adhesion molecule type 1 or E-selectin) and tissue factor. These responses have been shown to be mediated primarily by the p55 TNF receptor (Mackay et al., 1993; Barbara et al., 1994; Schmid et al., 1995; Clauss et al., 1996). The aim of the work described here was therefore to determine whether endothelin-1 induction was also mediated by the p55 TNF receptor as part of a coordinated response to cytokines in endothelial cells, and for comparison, parallel investigations were made in the epithelial cell line HEp-2.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), foetal calf serum, HEPES, L-glutamine, bovine serum albumin, MTT and sodium hydroxide were purchased from Sigma (Poole, England). Collagenase was from Worthington (Lakewood, USA) and trypsin was from Gibco-BRL (Paisley, UK). Amphotericin B, penicillin, and streptomycin were obtained from ICN Biomedicals (Costa Mesa, USA). Sodium dodecyl sulphate (SDS) was from Bio-Rad (Hercules, USA). Human recombinant TNF-α was from R&D Systems (Minneapolis, USA). The two receptor-selective TNF-α mutants R32W-S86T and D143N-A145R have been described previously and were generated by site-directed mutagenesis (Loetscher et al., 1993). Iodinated human TNF-α ([^{125}I]hTNF-α) was prepared by the lactoperoxidase method and purified by gel filtration to ensure that it was composed of trimeric TNF-α (specific activity 23 TBq/mmol). Hybridoma supernatant of the mouse monoclonal antibody htr1, raised against TNF-α-binding proteins isolated from HL-60 cells (Brockhaus et al., 1990) was kindly provided by Drs. Lesslauer and Loetscher.

2.2. Cell culture

Experiments were performed on confluent cultures of clonal bovine aortic endothelial cells prepared from single cells isolated from primary cultures, and on cells from the human epidermoid carcinoma line HEp-2 (European Collection of Animal Cell Cultures, Salisbury, England). Endothelial cells were isolated from bovine aorta by scraping the intimal surface and were then treated with class 1 collagenase. Endothelial and HEp-2 cells were grown in DMEM containing 25 mM HEPES, 4 mM L-glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 2.5 μg/ml amphotericin B (complete DMEM) and supplemented with 10% foetal calf serum. Subcultures were prepared by treating confluent cultures with trypsin (0.05%) and seeding into 24-well plates for use when confluent.

2.3. Endothelin-1 release

Cells were rinsed once with complete DMEM before stimulation. Cells were stimulated in triplicate with increasing concentrations of wild-type human TNF-α, the receptor-selective mutants (Loetscher et al., 1993), or the agonist antibody htr1 (Brockhaus et al., 1990) in complete DMEM without foetal calf serum for 6 h. Conditioned media were collected and the amounts of endothelin-1 release were measured by a specific radioimmunoassay (cross-reactivity with big-endothelin-1 $< 0.015\%$; Corder et al., 1993). Results are expressed as percent change from basal release. Cell viability was assessed at the end of each experiment using a modification of the MTT assay (Mosmann 1983), by incubating cells for 1 h at 37°C in 0.4 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) in complete DMEM. The medium was then aspirated and the amount of formazan precipitate formed as a result of mitochondrial dehydrogenase activity was determined. None of the reagents used displayed any cytotoxicity under the conditions being used.

2.4. [^{125}I]hTNF-α binding

Cells were rinsed once with complete DMEM containing 0.1% bovine serum albumin. They were incubated for

2.5 h at 37°C in 200 μl complete DMEM containing 2% bovine serum albumin, various concentrations of wild-type TNF-α or the receptor-selective TNF-α mutants and 2.6 ng/ml [^{125}I]hTNF-α (specific activity 23 TBq/mmol). For experiments using the antibody htr1, cells were preincubated for 45 min at 37°C with the antibody before addition of 2.6 ng/ml [^{125}I]hTNF-α and further incubated for 2.5 h at 37°C. Cells were washed three times with phosphate-buffered saline containing 0.1% bovine serum albumin and lysed in 250 μl of 0.5% SDS in 0.1 M NaOH. The bound radioactivity in the lysates was counted and expressed as percent of maximum binding.

2.5. Statistical analysis

ANOVA (analysis of variance) with Tukey's post-test was used for statistical analysis.

3. Results

3.1. Effect of TNF-α mutants on endothelin-1 secretion and [^{125}I]hTNF-α binding on endothelial and epithelial cells

These studies have used previously described mutants of human TNF-α to activate selectively either TNF receptor 1 (p55) or TNF receptor 2 (p75) (Loetscher et al., 1993). Each mutant carries a double amino acid substitution abolishing its ability to bind one of the two TNF receptors. In the p55-selective mutant, the amino acids in positions 32 and 86, arginine and serine in wild-type TNF-α, are replaced by tryptophan and threonine, respectively (R32W-S86T). In the p75-selective mutant, the aspartic acid residue in position 143 and the alanine in position 145 of wild-type TNF-α are substituted by asparagine and arginine (D143N-A145R).

The effects of human wild-type TNF-α and mutants on endothelin-1 secretion from bovine aortic endothelial cells are shown in Fig. 1A. TNF-α stimulated endothelin-1 release in a concentration-dependent manner. A concentration as low as 1 ng/ml TNF-α was sufficient to increase significantly endothelin-1 production above basal levels. The p55-selective mutant was also able to induce endothelin-1 secretion. The threshold concentration for this effect was about 50 times greater than native TNF-α. The amount of endothelin-1 secreted in the medium over the 6-h incubation period was significantly less than that obtained with the corresponding concentration of wild-type TNF-α for all the concentrations tested ($P < 0.05$). The p75-selective mutant had no effect, either alone or in combination with the p55-selective mutant. Human wild-type TNF-α and mutants were also used in competitive binding experiments to determine the type of TNF receptor molecules

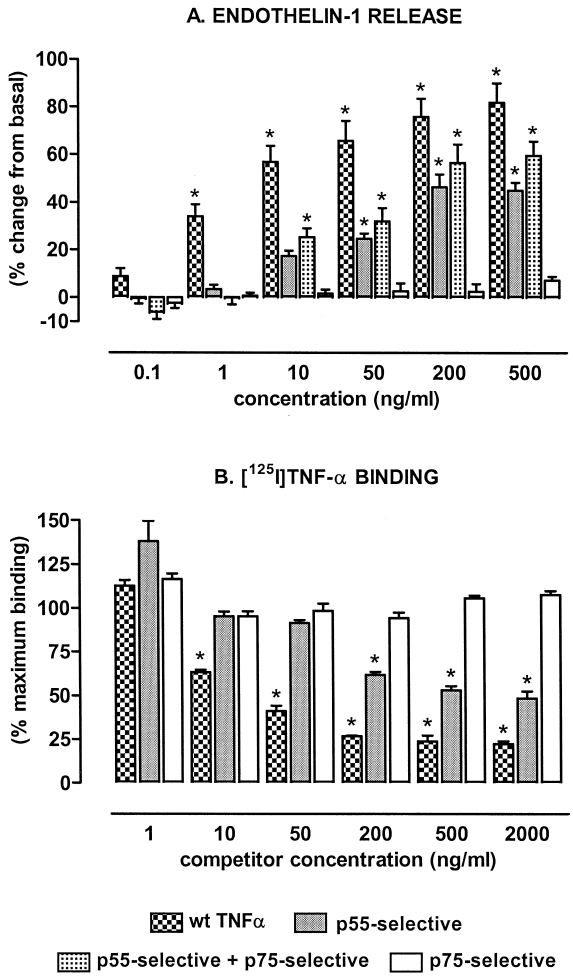

Fig. 1. Endothelin-1 release and competitive binding of wild-type TNF-α and receptor-selective TNF-α mutants to bovine aortic endothelial cells. (A) Cells were treated for 6 h in serum-free medium with increasing concentrations of wild-type or mutant TNF-α. Basal release was 35 ± 2 fmol/cm^2, $n = 6$–12. (B) Cells were incubated for 2.5 h with increasing concentrations of wild-type or mutants TNF-α in the presence of 2.6 ng/ml [^{125}I]hTNF-α. Maximum binding was 2.3 ± 0.2 pg/cm^2, $n = 4$–8. * $P < 0.001$ versus basal release (A) or maximum binding (B), ANOVA with Tukey's post-test.

expressed at the surface of bovine aortic endothelial cells (Fig. 1B). Both the wild-type and p55-selective mutant TNF-α were able to inhibit in a concentration-dependent manner the binding of [^{125}I]hTNF-α to these cells. The p55-selective mutant showed a decreased affinity compared to TNF-α, the inhibition being significant for concentrations greater or equal to 200 ng/ml R32W-S86T, compared to 10 ng/ml for TNF-α. The maximal inhibition achieved using 2000 ng/ml competitor was also significantly less with the p55-selective mutant than with TNF-α ($P < 0.05$). The p75-selective mutant did not significantly inhibit binding at the concentrations tested.

The cell line HEp-2 was used for comparison. These cells are of human origin which eliminates potential problems of species specificity, and they express only the p55

TNF receptor and thus offer a simplified system (Brockhaus et al., 1990; Barbara et al., 1994). The effect of wild-type TNF-α and mutants on endothelin-1 secretion from HEp-2 is shown in Fig. 2A. TNF-α and the p55-selective mutant induced concentration-dependent increases in endothelin-1 release with a threshold concentration of 10 ng/ml. The efficiency of the p55-selective mutant was not different from that of wild-type TNF-α. The p75-selective mutant had no effect on endothelin-1 production, alone or in combination with the p55-selective mutant.

Competitive binding experiments (Fig. 2B) showed both TNF-α and the p55-selective mutant to inhibit the binding of [^{125}I]hTNF-α to HEp-2. Although the maximal inhibition obtained with TNF-α or the p55-selective mutant was similar, the p55-selective mutant displayed a significantly reduced affinity compared to TNF-α. The p75-selective mutant had no effect on [^{125}I]hTNF-α binding.

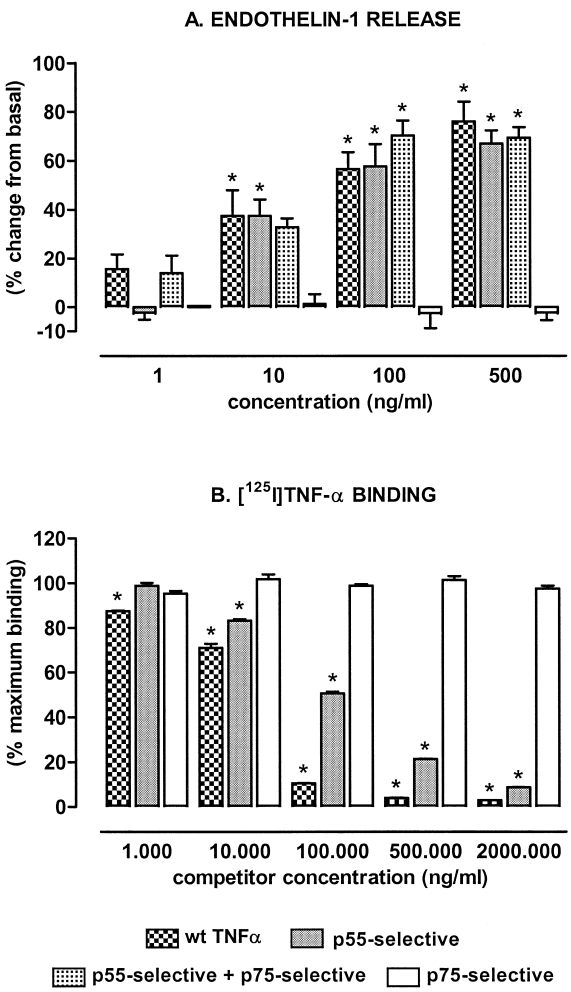

Fig. 2. Endothelin-1 release and competitive binding of wild-type TNF-α and mutants to HEp-2 cells. (A) HEp-2 cells were treated for 6 h in serum-free medium with increasing concentrations of wild-type or mutant TNF-α. Basal release was 3.5 ± 0.3 fmol/cm^2, $n = 6$–12. (B) HEp-2 were incubated for 2.5 h with increasing concentrations of wild-type or mutant TNF-α in the presence of 2.6 ng/ml [^{125}I]hTNF-α. Maximum binding was 32.68 ± 0.55 pg/cm^2, $n = 6$. $^*P < 0.001$ versus basal release (A) or maximum binding (B), ANOVA with Tukey's post-test.

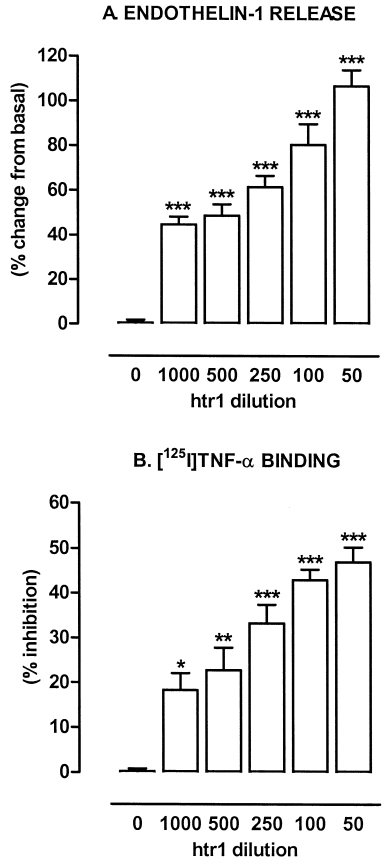

Fig. 3. Effect of the p55 TNF receptor agonist antibody htr1 on endothelin-1 release and [^{125}I]hTNF-α binding to bovine aortic endothelial cells. (A) Cells were treated for 6 h in serum-free medium with dilutions of htr1. Basal release was 20 ± 1 fmol/cm^2, $n = 12$. (B) Cells were preincubated for 45 min at 37°C with dilutions of htr1 before addition of 2.6 ng/ml [^{125}I]hTNF-α and further incubated for 2.5 h at 37°C. Maximum binding was 2.2 ± 0.03 pg/cm^2, $n = 12$. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus basal release (A) or maximum binding (B), ANOVA with Tukey's post-test.

3.2. Effect of the p55 TNF receptor agonist antibody htr1 on endothelin-1 production and [^{125}I]hTNF-α binding on endothelial and epithelial cells

The antibody htr1 is an agonist of the p55 TNF receptor. It has been shown to mimic several of the actions of TNF-α on endothelial cells such as the expression of leukocyte adhesion molecules (intercellular adhesion molecule type 1, vascular cell adhesion molecule type 1 or E-selectin) or tissue factor (Mackay et al., 1993; Schmid et al., 1995).

Htr1 induced dose-dependent and significant increases in endothelin-1 release from bovine aortic endothelial cells (Fig. 3A). Pretreatment with the same dilutions of htr1 significantly inhibited the binding of [^{125}I]hTNF-α to these cells (Fig. 3B). Both these effects were significant even at the highest dilution tested of 1/1000.

Similar results were obtained with HEp-2 (Fig. 4). These cells were however less sensitive to htr1 than en-

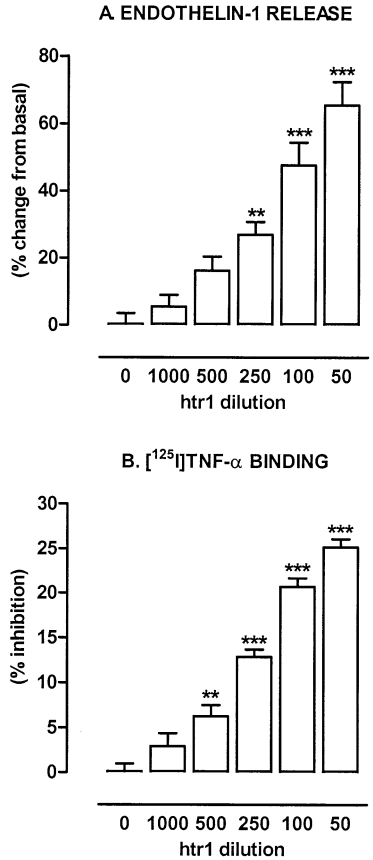

Fig. 4. Effect of the p55 TNF receptor agonist antibody htr1 on endothelin-1 release and [^{125}I]hTNF-α binding to HEp-2. (A) HEp-2 were treated for 6 h in serum-free medium with dilutions of htr1. Basal release was 3.5 ± 0.8 fmol/cm^2, $n = 12$. (B) HEp-2 were preincubated for 45 min at 37°C with dilutions of htr1 before addition of 2.6 ng/ml [^{125}I]hTNF-α and further incubated for 2.5 h at 37°C. Maximum binding was 26.1 ± 0.7 pg/cm^2, $n = 12$. $^{**}P < 0.01$ and $^{**}P < 0.001$ versus basal release (A) or maximum binding (B), ANOVA with Tukey's post-test.

dothelial cells, since endothelin-1 increases were not significantly different from basal for dilutions of htr1 greater than 1/250 (Fig. 4A) and the binding was only significantly inhibited when dilutions of 1/500 or less were used (Fig. 4B).

4. Discussion

This study has shown that selective stimulation of the p55 TNF receptor using mutant TNF-α or an agonist antibody was sufficient for induction of endothelin-1 synthesis in two independent systems, endothelial and epithelial cells.

The p55-selective mutant stimulated endothelin-1 release from bovine aortic endothelial cells and inhibited the binding of [^{125}I]hTNF-α to these cells, but both effects of this mutant were less than those of native human TNF-α. In agreement with our results, the double mutation introduced in the p55-selective mutant R32W-S86T has been shown to abolish its binding to the p75 TNF receptor but also to decrease slightly its affinity for the p55 receptor (Loetscher et al., 1993). The reduced maximal response with the p55-selective mutant may be due simply to a reduction in intrinsic activity on bovine p55 TNF receptor. Indeed, the endothelin-1-stimulating activity of the p55-selective mutant on HEp-2 cells was very similar to that of TNF-α, even though it displayed a reduced ability to inhibit [^{125}I]hTNF-α binding. However, a comparison of the effect of TNF-α on endothelin-1 synthesis in these two cell types showed that bovine endothelial cells were about 10 times more sensitive to TNF-α than HEp-2. This suggests that endothelin-1 synthesis induced by TNF-α, even though mediated mainly by the p55 TNF receptor, may also be facilitated by p75 TNF receptor stimulation in endothelial cells. Similar conclusions were reached by Barbara et al. (1994) for the induction of proinflammatory effects using TNF receptor-selective TNF-α mutants, including the mutant R32W-S86T. This showed that although the p55 TNF receptor was the major receptor involved in endothelial cell activation, the response was significantly reduced compared to native TNF-α, while the cytotoxicity of wild-type TNF-α and the mutant R32W-S86T towards cells from the HEp-2 line were not significantly different (Barbara et al., 1994).

cDNA cloning of the TNF receptors showed that, at least in the case of the murine receptors, the p75 TNF receptor is species specific (Lewis et al., 1991) and is not activated by human TNF-α. The double mutation introduced into the p75-selective mutant may thus be sufficient to abolish its affinity or intrinsic activity for bovine p75 TNF receptor. The p75 TNF receptor may also increase the magnitude of the response in endothelial cells by ligand-passing or formation of ligand-binding heterocomplexes with the p55 receptor (Tartaglia et al., 1993; Pinckard et al., 1997), and this will not occur when receptor-selective TNF-α mutants are used. Alternatively, the lack of response to p75-selective mutant may be due to the requirement for the transmembrane form of TNF-α for activation of this receptor subtype (Grell et al., 1995). Nevertheless, the fact that endothelin-1 synthesis was strongly stimulated by wild-type human TNF-α and the p55-selective mutant showed that activation of the p75 TNF receptor is not required for the endothelin response to occur. Moreover, our data confirm that cells from the HEp-2 line do not express the p75 TNF receptor and yet TNF-α up-regulates endothelin-1 production from these cells.

Another p55-selective stimulus, the monoclonal antibody htr1 was used to confirm the role of the p55 TNF receptor in endothelin-1 induction. In endothelial cells as well as in HEp-2 cells, htr1 induced significant increases in endothelin-1 release above basal levels and prevented the binding of [^{125}I]hTNF-α to the cell surface. Interestingly, the magnitude of the maximal endothelin-1 release was very similar to that obtained after stimulation with wild-type TNF-α on both endothelial and epithelial cells,

further establishing the p55 receptor as the main TNF receptor involved in endothelin-1 synthesis.

In conclusion, these results establish endothelin-1 as one of the numerous genes up-regulated during TNF-α induced endothelial cell activation, which also includes cell adhesion molecules and tissue factor. The fact that all these events are mediated by the p55 TNF receptor emphasises the need for new therapeutic agents which target the p55 TNF receptor in order to block the deleterious effects of TNF-α in states of vascular inflammation.

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