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ORIGINAL CONTRIBUTION

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Endothelin-1 induces interleukin-6 release via activation of the transcription factor NF-κB in human vascular smooth muscle cells

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Abstract The potent vasoconstrictor peptide endothelin-1 (ET-1) has been implicated in the pathophysiology of atherosclerosis and its complications. Since inflammation of the vessel wall is a hallmark of atherosclerosis, the purpose of the present study was to investigate the influence of ET-1 on cytokine production in human vascular smooth muscle cells (SMC) as a marker of inflammatory cell activation. ET-1 (100 pM – 1 μM) stimulated interleukin-6 (IL-6) secretion from human vascular SMC in a concentration-dependent manner. The ET-A-receptor antagonist BQ-123 (10 μM), but not the ET-B-receptor antagonist BQ-788, inhibited IL-6 release. ET-1 also transiently increased IL-6 mRNA compatible with regulation of IL-6 release at the pretranslational level. Electrophoretic mobility shift assays demonstrated time- and concentration-dependent activation of the proinflammatory transcription factor nuclear factor-κB (NF-κB) in ET-1-stimulated human vascular SMC. A decoy oligodeoxynucleotide bearing the NF-κB binding site inhibited ET-1-stimulated IL-6 release to a great extent suggesting that this transcription factor plays a key role for cytokine production elicited by ET-1. Moreover, the antioxidant pyrrolidine dithiocarbamate (10 μM) inhibited ET-1-induced IL-6 release indicating involvement of reactive oxygen species in ET-1 signaling. ET-1-stimulated IL-6 secretion was also suppressed by diphenylene iodonium (40 μM), an inhibitor of flavon-containing enzymes such as NADH/NADPH oxidase. The results demonstrate the ability of ET-1 to induce an inflammatory response in human vascular SMC. These observations may contribute to a better understanding of the role of ET-1 in inflammatory activation of the vessel wall during atherogenesis.

Key words Atherosclerosis – cytokines – endothelins – inflammation – free radicals

Introduction

Several lines of evidence suggest a role of endothelin in the pathophysiology of cardiovascular diseases including hypertension, atherosclerosis, and restenosis following angioplasty (26, 28, 33). Besides its vasoactive effects, endothelin-1 (ET-1) is a strong mitogen promoting smooth muscle cell (SMC) proliferation via induction of protooncogenes and stimulation of the mitogen activated protein kinase cascade (11, 12, 39). Moreover, increased ET levels were found in the coronary and systemic circulation of humans with coronary endothelial dysfunction, suggesting involvement of this peptide in early atherosclerosis (18), as well as in advanced 'unstable' atherosclerotic plaques ultimately leading to acute coronary syndromes (17, 40, 41). The concept of ET as an important mediator in cardiovascular disease is further supported by animal studies showing reduced neointimal hyperplasia in a porcine coronary stent model and suppressed devel-

opment of graft arteriosclerosis in rat cardiac allografts by ET receptor antagonists (26, 28).

Chronic inflammation of the vessel wall is a major feature of atherosclerosis during all stages of the disease (1, 31) and is characteristic for complications leading to organ damage (20, 38). The present study was therefore designed to test the hypothesis that ET-1 can induce an inflammatory response in human vascular SMC, the most abundant cell type in all stages of atherosclerotic lesion formation. The inflammatory potential of ET-1 was assessed by measuring production of the multifunctional cytokine interleukin-6 (IL-6) and by determination of activation of the proinflammatory transcription factor nuclear factor-κB (NF-κB).

Methods

Materials

Endothelin-1, pyrrolidine dithiocarbamate (PDTC) and diphenylene iodonium (DPI) were from Sigma (St. Louis, MO, USA). BQ-123 and BQ-788 were from Calbiochem-Novabiochem Corporation (San Diego, CA, USA). Losartan was a gift from Merck (Whitehouse Station, NJ, USA). Recombinant human TNFα was purchased from Endogen (Woburn, MA, USA). Testing for bacterial endotoxin with the Limulus amoebocyte lysate assay (BioWhittaker, Wakersville, MD, USA) revealed levels ≤ 0.25 EU/ml for all agents. Monoclonal antibody HHF-35 (mouse IgG1) was from Enzo Diagnostics (Sysossat, NY, USA).

Cell preparation and culture

Vascular SMC were isolated from unused portions of human saphenous veins harvested for coronary bypass surgery and were grown in Dulbecco's modified Eagle's medium (Eurobio, Raunheim, FRG) supplemented with 10 % (vol/vol) fetal calf serum (Eurobio), 100 U/ml penicillin, 100 µg/ml streptomycin, 1.25 µg/ml amphotericin B, and 2 mM L-glutamine in a humidified atmosphere of 5 % CO_2 and 95 % air. The cells showed the typical „hill and valley" growth morphology of SMC and many reacted with the monoclonal antibody HHF-35 recognizing muscle-specific actin (37). Cells were passaged by brief trypsinization and were used between passages 2 to 5 for the experiments after preincubation for 1–2 d in serum-free insulin-transferrin (IT-) medium to achieve growth arrest and to prevent exposure to serum components before addition of stimuli. IT-medium consisted of DMEM and Ham's F12 (Eurobio, 1:1, vol/vol) supplemented with 1 µM insulin and 5 µg/ml transferrin (21).

Determination of IL-6 release

Human vascular SMC were plated on gelatin-coated 96-well tissue culture dishes (CoStar, Cambridge, MA, USA) and incubated at 37 °C until confluency. They were kept in IT-medium for 2 d before the experiment. For some experiments cells were preincubated with BQ-123, BQ-788, PDTC, or DPI, respectively, for 1 h. After addition of the stimuli, cells were incubated for 12 or 24 h, respectively, then the supernatants were collected and frozen. TNFα served as positive control stimulus for IL-6 release. Assays for IL-6 were performed with an ELISA kit (Endogen) according to the manufacturer's instructions. The assay selectively recognizes IL-6, with a limit of detection of < 1 pg/ml.

RNA isolation and RT-PCR

Total RNA was isolated from confluent human vascular SMC grown in 10 cm Petri dishes using RNAzol B (Wak-Chemie, Bad Homburg, FRG) according to the manufacturer's instructions. Single-stranded complementary DNA (cDNA) was synthesized from 1 µl samples of total RNA (1 µg) using Moloney murine leukemia virus reverse transcriptase (Fermentas, Vilnius, Lithuania). Specific cDNA from the reverse transcriptase reaction product was amplified using human IL-6 specific primers (sense: 5'-ATGAACTCCTTCTCCACAAGCGC-3', antisense: 5'-GAAGAGCCCTCAGGCTGGACTG-3') and GAPDH specific primers (sense: 5'-CCACCCATGGCAAATTCCATGGCA-'3, antisense: 5'-TGCTAAGCAGTTGGTGGTGCAGGAG-3'). Amplification was performed with Taq DNA polymerase (Fermentas) in a DNA thermal cycler (Stratagene, La Jolla, CA, USA) with 34 cycles consisting of 45 s at 94 °C, 45 s at 65 °C, and 1.5 min at 72 °C. The amplification products were analyzed by electrophoresis on 1 % agarose gels and visualized by ethidium bromide staining. The predicted size of the products was 622 bp for the interleukin-6 gene and 333 bp for the GAPDH gene.

Electrophoretic mobility shift assay (EMSA)

Protein extracts from SMC were prepared as follows: after washing in ice-cold PBS three times, cells were harvested by scraping, resuspended and sedimented by centrifugation. The cell pellet was lysed in a buffer composed of 20 mM HEPES-KOH (pH 7.9), 0.35 M NaCl, 20 % glycerol, 1 % NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 10 µg/ml leupeptin, 0.5 mM DTT and 0.2 mM PMSF by incubation on ice for 30 min. After centrifugation, the supernatant containing the protein fraction was frozen at –80 °C. For electrophoretic mobility shift assays, a double-stranded oligonucleotide (Promega, Madison, WI, USA) representing the consensus

sequence for NF-κB binding was labeled with γ-^{32}P-ATP (NEN, Boston, MA, USA) using T-4 polynucleotide kinase (Promega). Cell proteins (10 µg) and labeled oligonucleotides (50 000–70 000 cpm) were incubated for binding of active NF-κB for 20 min at room temperature in a buffer containing 20 µg poly (dI:dC), 8 % Ficoll 400, 44 mM HEPES-KOH (pH 7.9), 140 mM KCl, 4 % glycerol, 0.05 % NP-40, 0.1 mM EDTA, 4.4 mM DTT, and 0.06 mM PMSF. Immediately after binding, the protein/DNA complexes were separated from unbound oligonucleotides by electrophoresis on a native 5 % polyacrylamide gel in TBE. Autoradiography was performed with the dried gels using Hyperfilm (Amersham, Buckinghamshire, UK). For NF-κB/DNA binding specificity testing, antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against the p65 subunits of NF-κB were added to the proteins in some experiments, resulting in further retardation of electrophoretic mobility ('supershift'), or a 160-fold molar excess of unlabeled 'cold' competitor oligonucleotide was added to the binding reaction, leading to a decrease in NF-κB-bound radioactivity.

Decoy oligodeoxynucleotide technique

Double-stranded decoy oligodeoxynucleotides with the consensus binding sequence for NF-κB were obtained from complementary single-stranded oligodeoxynucleotides with phosphorothioate modifications (MWG Biotech, Ebersberg, FRG) by melting at 95 °C and cooling-down for 3 h at room temperature (15). SMC grown in 96-well plates were preincubated with 10 µM double-stranded oligodeoxynucleotide in IT-medium for 4 h. Thereafter the medium was replaced with fresh oligodeoxynucleotide-containing IT-medium with the appropriate stimulus for 12 h. The medium was collected and assayed for IL-6 concentration. The single-strand sequence of the oligodeoxynucleotide was 5'-AGTTGAGGGGACTTTC-CCAGGC-3' with underlined letters denoting phosphorothioate-modified bases.

Statistical analysis

Data are presented as arithmetic mean ± SEM. Statistical analysis was performed with ANOVA followed by Fisher's exact test. Differences were considered statistically significant at a p-value of < 0.05.

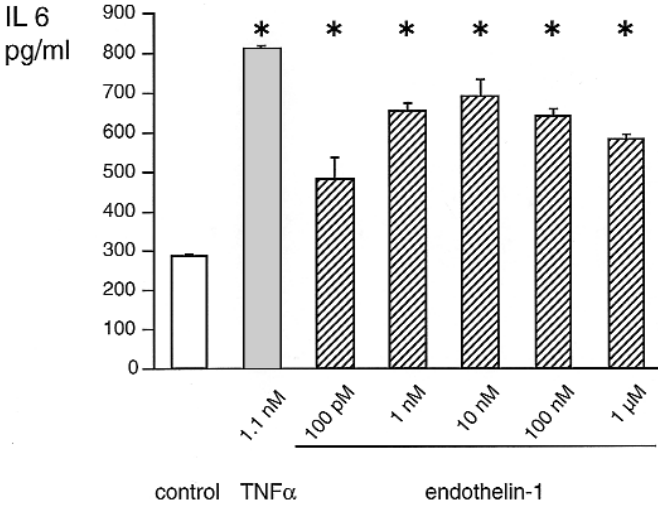

Fig. 1 Endothelin-1 stimulates IL-6 release from human vascular SMC. SMC grown to confluency were growth-arrested in insulin-transferrin medium for 2 d in gelatin-coated 96-well plates. For stimulation experiments ET-1 and the positive control TNFα (1.1 nM) were added together with fresh IT-medium. Supernatants were collected after 24 h and assayed for IL-6 concentration by ELISA. Endothelin-1 concentrations are indicated at the bottom of the bars. A representative experiment of 4 independent experiments performed is illustrated. Results are expressed as mean ± SEM. * $p < 0.05$ vs. respective control value.

Results

ET-1 induces IL-6 release from human vascular smooth muscle cells

IL-6 release into the tissue culture medium was measured to gauge the ability of ET-1 to induce cytokine production, since this member of the cytokine family is rapidly secreted upon its induction (24). Unstimulated human vascular SMC exhibited low basal IL-6 secretion. Maximal levels of IL-6 release were detected after stimulation with TNFα (1.1 nM). ET-1 caused concentration-dependent IL-6 release from human vascular SMC from 100 pM up to 1 µM (Fig. 1) with maximal IL-6 secretion at 10 nM. Four experiments with cells from different donors showed similar results. Basal IL-6 secretion varied between cells from different donors (mean 288 pg/ml, range 118–456 pg/ml), reflecting heterogeneiety of human cells. The different cell lines, however, responded with a similar relative increase in IL-6 release upon stimulation with TNFα and ET-1.

ET-1-induced IL-6 release from human vascular smooth muscle cells depends on the ET-A-receptor

Endothelin-1 binds to the ET-A-receptor with high affinity (19, 36). BQ-123 (10 µM), a highly potent and selective ET-A-receptor antagonist, reduced ET-1-stimulated IL-6 release from human vascular SMC to basal levels (Fig. 2). This find-

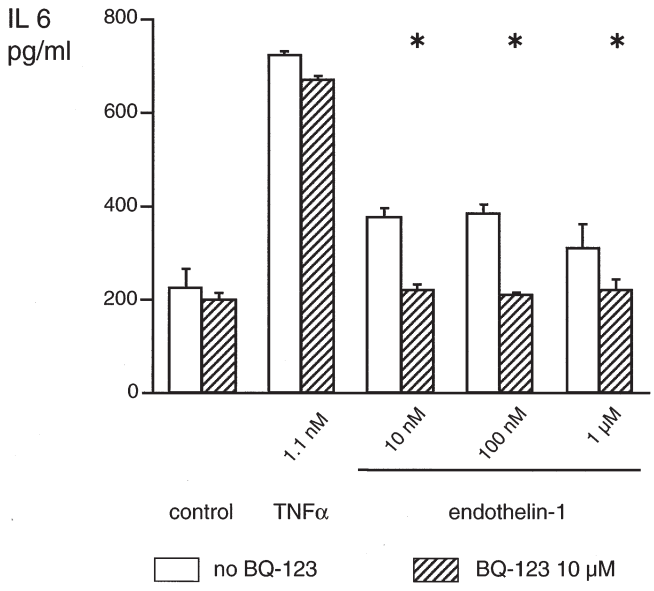

Fig. 2 The stimulatory effect of ET-1 on IL-6 release from human vascular SMC is inhibited by the selective ET-A-receptor antagonist BQ-123. Human vascular SMC were treated as described in Fig.1. Furthermore, cells were preincubated with BQ-123 (10 µM) starting 1 h before stimulation with ET-1 or the positive control stimulus TNFα (1.1 nM). The supernatants were collected after 24 h and assayed for IL-6 concentration by ELISA. A representative experiment of three independent experiments performed is illustrated. Results are expressed as mean ± SEM, * $p < 0.05$ vs. respective value without BQ-123.

ing demonstrates both specificity of the ET-1 effect and involvement of the ET-A-receptor. Three independent experiments with cells from different donors showed similar results. The ET-B-receptor antagonist BQ-788 (10 µM) did not inhibit ET-1-induced IL-6 release (data not shown). Since angiotensin II is present inside vascular SMC (10) and stimulates IL-6 release from human vascular SMC (14), ET-1-induced IL-6 release could be due to auto-/paracrine release of angiotensin II. We therefore investigated ET-1-induced IL-6 release in the presence of the angiotensin II-AT1 receptor antagonist losartan (10 µM). Losartan did not inhibit ET-1-stimulated IL-6 release (data not shown).

ET-1 stimulates accumulation of IL-6 mRNA

The effect of ET-1 on IL-6 mRNA accumulation in vascular SMC was assessed by RT-PCR analysis. Both TNFα (1.1 nM) and ET-1 (1 µM) transiently increased IL-6 mRNA levels compared to control conditions with a maximal response at 2 h, whereas levels of the constitutively expressed GAPDH mRNA remained stable over the experimental period of 6 h (Fig. 3). Four experiments with cells from different donors showed similar results.

ET-1 activates the proinflammatory transcription factor NF-κB

Activation of NF-κB was investigated by EMSA. Both TNFα (1.1 nM) and ET-1 (10 nM/1 µM) activated NF-κB in human vascular SMC. Active NF-κB was already present after 30 min stimulation. Maximal NF-κB activation was found after 1 h stimulation (Fig. 4a). The specificity of the shifted autoradiographic bands was ascertained in two ways (Fig. 4b): a) an excess of unlabeled 'cold' competitor oligonucleotide reduced the signal intensity of the band associated with active NF-κB and b) addition of antibodies against the p65 subunit of NF-κB resulted in a further retardation of the mobility of the NF-κB/oligonucleotide complex ('supershift'). Three independent experiments showed comparable results.

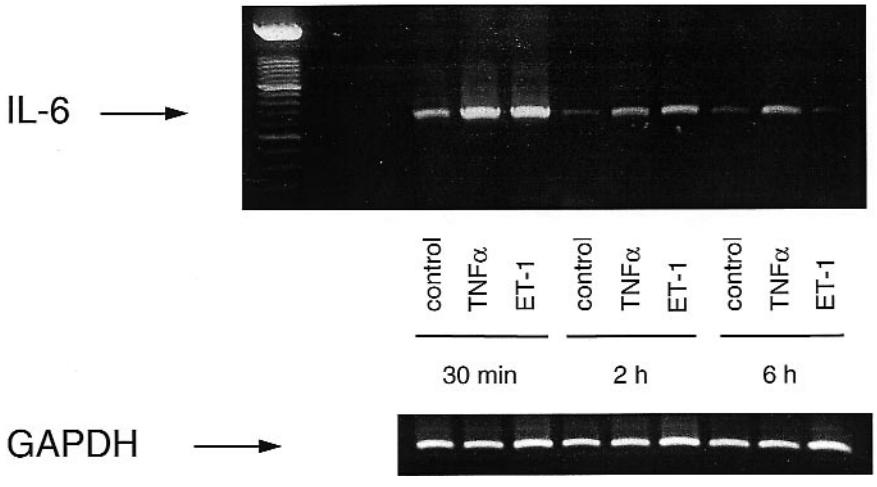

Fig. 3 Endothelin-1-induced IL-6 mRNA accumulation in human vascular SMC. Confluent and growth arrested cells were treated with TNFα (1.1 nM) or ET-1 (1 µM) for the indicated time periods. One microgram of total RNA was used for RT-PCR analysis, yielding PCR products of 622 bp for the IL-6 gene and 333 bp for the GAPDH gene, respectively. Ethidium bromide-stained agarose gels of RT-PCR products for IL-6 mRNA (upper panel) and GAPDH (lower panel) are shown. Results are representative for four independent experiments with cells from different donors.

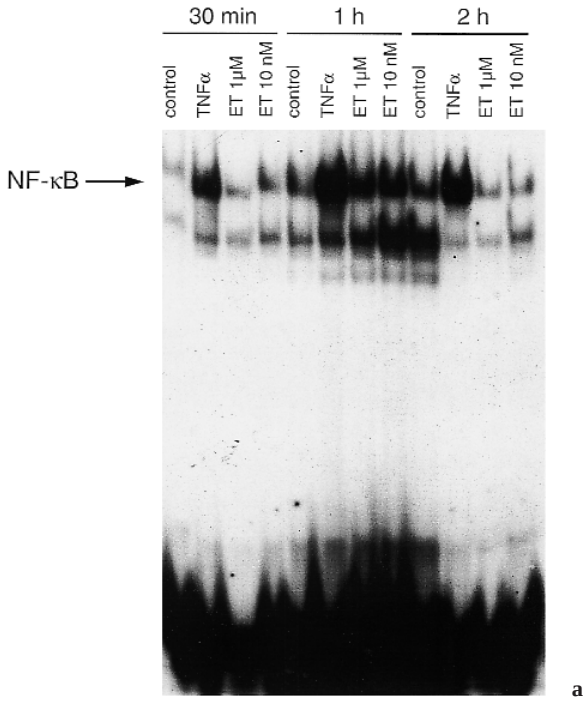

Fig. 4 Endothelin-1 activates NF-κB. Confluent and growth arrested cells were stimulated with insulin-transferrin medium alone (control), TNFα (1.1 nM) or ET-1 (1 μM, 10 nM). Cell protein extracts were prepared and incubated with ^{32}P-ATP-labeled oligonucleotides corresponding to the NF-κB consensus sequence. Binding of activated NF-κB to the oligonucleotide was visualized by autoradiography after separation by non-denaturing polyacrylamide gel electrophoresis. Arrows denote oligonucleotide/NF-κB protein complex. **a)** Time-dependent activation of NF-κB by endothelin-1. **b)** To demonstrate specificity of NF-κB detection, protein extracts (control, TNFα 1.1 nM, ET-1 10 nM) were subjected to EMSA in the presence of excess unlabeled competitor oligonucleotide or of an antibody against the p65 subunit ('supershift'). Three independent experiments showed comparable results.

To test whether ET-1-induced IL-6 release depends on NF-κB we used the decoy oligonucleotide method to inhibit NF-κB binding to promoter regions of NF-κB-inducible genes. The decoy oligonucleotide used for these experiments has been shown to inhibit specifically activation of NF-κB but not of other transcription factors such as Stat-1 (15). Preincubation of SMC with decoy oligonucleotides reduced ET-1-stimulated IL-6 release by 84 %. This finding suggests that NF-κB is a key transcription factor for ET-1-induced cytokine release from SMC (Fig. 5).

The antioxidant PDTC and DPI, an inhibitor of flavon-containing enzymes, suppress ET-1-induced IL-6 release

Since reactive oxygen species activate NF-κB and induce subsequent production of cytokines (32, 34), the effect of the antioxidant PDTC on ET-1-induced IL-6 release by vascular SMC was examined. PDTC (10 μM) inhibited basal and ET-1-induced IL-6 release suggesting involvement of reactive oxygen intermediates in the regulation of both basal and ET-1-stimulated IL-6 secretion (Fig. 6a). In vascular SMC,

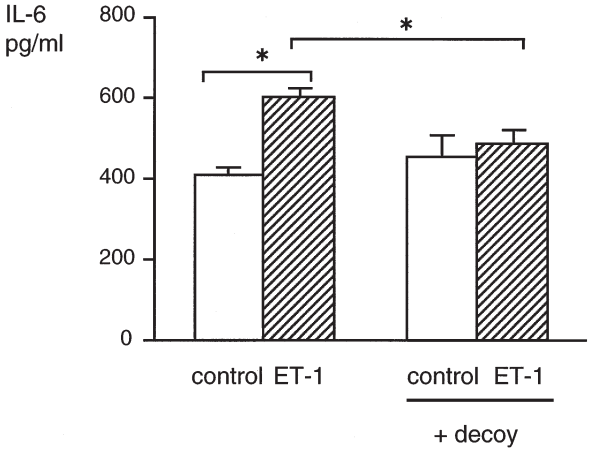

Fig. 5 Decoy oligonucleotides for NF-κB inhibit ET-1-induced IL-6 release. Confluent and growth arrested cells were preincubated for 4 h with 10 μM double-stranded, phosphorothioate-modified oligodeoxynucleotides which represent the consensus binding sequence for activated NF-κB. Then the cells were stimulated with ET-1 for 12 h and the medium was assayed for IL-6 concentration. A representative experiment of 2 independent experiments is illustrated. Results are expressed as mean ± SEM. * $p < 0.05$.

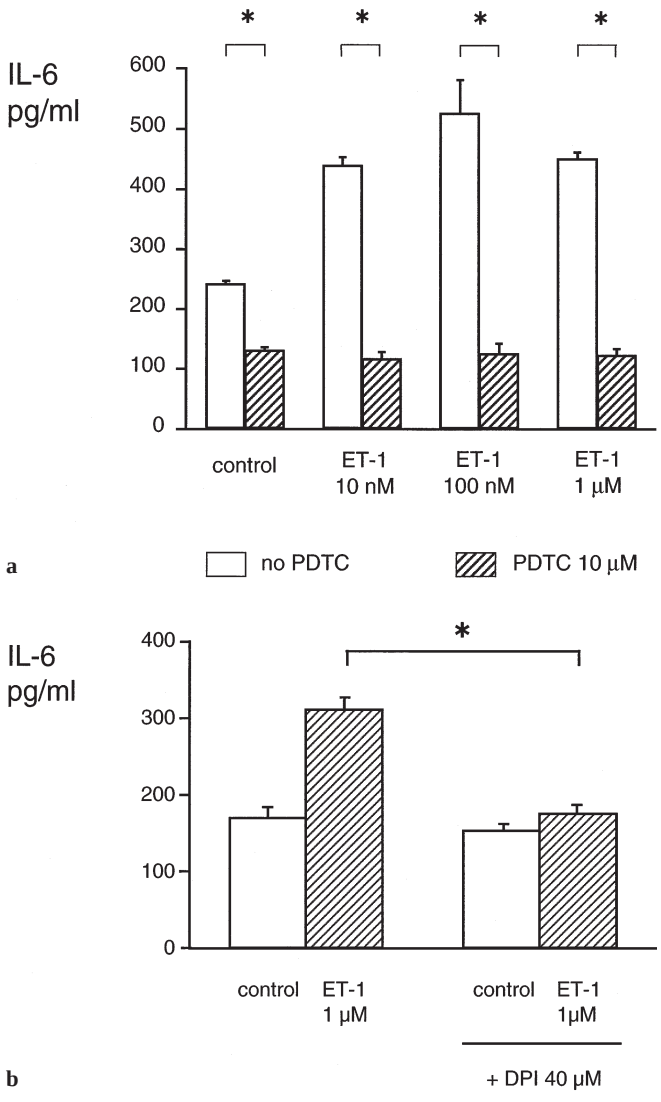

Fig. 6 Endothelin-1-induced IL-6 release by human SMC is suppressed by the antioxidant PDTC and by DPI, an inhibitor of flavon-containing enzymes. Confluent cells were growth-arrested in insulin-transferrin (IT-) medium for 2 d. For the experiment, the medium was replaced by fresh IT-medium with or without ET-1 in the absence or presence of the antioxidant agent pyrrolidine dithiocarbamate (PDTC, 10 µM; Fig. 6a) or of diphenylene iodonium (DPI, 40 µM, Fig. 6b), an inhibitor of flavon-containing enzymes such as NADH/NADPH oxidase. After 24 h the medium was collected and assayed for IL-6 concentration by ELISA. Results are expressed as mean ± SEM, * $p < 0.05$ vs. respective value without PDTC or DPI. Two independent experiments gave similar results.

reactive oxygen species can be produced by NADH/NADPH oxidase, a membrane bound enzyme system (9). DPI, an inhibitor of flavon-containing enzymes, including NADH/NADPH oxidase, suppressed IL-6 release stimulated by ET-1 (Fig. 6b). Since activation of protein kinase C has been implicated in mouse osteoblast-like cells (25), we tested the effect of the protein kinase C inhibitor staurosporin on IL-6 release from SMC. Staurosporin (10 nM) did not inhibit ET-1-stimulated IL-6 release (data not shown).

Discussion

Since inflammation of the vessel wall is a hallmark in the complex process of atherogenesis the present study was designed to investigate the effect of ET-1 on inflammatory activation of human vascular SMC. Particularly acute coronary syndromes are characterized by inflammation of the culprit lesion (38). The observation that tissue ET-1-like immunoreactivity is significantly increased in atherosclerotic lesions of patients with acute coronary syndromes (40, 41) lead us to the hypothesis that ET-1 might be able to induce inflammatory activation of crucial cellular components of atherosclerotic plaque tissue via stimulation of cytokine pathways. Besides the vascular endothelium, macrophages and vascular SMC have been identified as potent sources of ET production making multiple interactions between the involved cellular components very likely (7, 30). Recently published studies demonstrated increased IL-6 release from murine osteoblasts, murine peritoneal macrophages and a microglial cell line stimulated with endothelin (29, 35). To our knowledge our study demonstrates for the first time that ET-1 can induce cytokine production in human vascular SMC. ET-1 induced IL-6 release from SMC in a concentration-dependent manner. IL-6, a multifunctional cytokine, is the principal stimulus of the acute phase reaction, e.g., synthesis of C-reactive protein (CRP) (16). Both elevated plasma levels of IL-6 and CRP correlate with an adverse outcome of patients with acute coronary syndromes (4, 23). Previous work has identified SMC as major source of IL-6 in the vessel wall (24). Known stimuli of IL-6 production in SMC are other cytokines, thrombin, and angiotensin II (13, 14, 24). All these agents are involved in the pathogenesis of atherosclerosis. Two types of endothelin receptors, ET-A and ET-B, have been described. Almost all ET receptors in the vasculature, both in normal and in atherosclerotic vessels, are of the ET-A subtype (2, 6). ET-1 binds to the ET-A-receptor with high affinity and it has been shown that selective ET-A-receptor antagonism can prevent ET-1-induced mitogenic effects (27). Our results suggest that ET-1-stimulated IL-6 release is mediated via ET-A-receptors since the highly selective ET-A-receptor antagonist BQ-123, but not the ET-B-receptor antagonist BQ-788, reduced IL-6 secretion caused by ET-1.

Stimulation of human vascular SMC by ET-1 resulted in transiently increased accumulation of IL-6 mRNA, suggesting interaction of ET-1 with IL-6 release at the pretranslational, probably transcriptional level. Therefore the intracellular signaling pathway by which ET-1 leads to IL-6-mRNA accumulation is of interest. Transcriptional activation of cytokine

genes commonly requires induction of NF-κB (3) and activation of IL-6 gene expression also depends on NF-κB (22). In this study, we showed that ET-1 is a potent stimulus for activation of NF-κB in human vascular SMC, extending recent observations of activation of NF-κB by ET-1 in hepatic stellate cells (8). Moreover, application of a decoy oligodeoxynucleotide against NF-κB prior to stimulation inhibited ET-1-induced IL-6 release from SMC to a great extent. This finding supports a major role for NF-κB in IL-6 generation stimulated by ET-1. Oxygen free radicals can serve as second messengers for the activation of NF-κB (34) and the expression of cytokines (32). Other vasoactive peptides like angiotensin II may signal NF-κB activation through generation of O_2^-. Results from our laboratory and others support the hypothesis that reactive oxygen species participate in angiotensin II-induced cytokine production by vascular SMC (5, 14). A major source of angiotensin II-stimulated O_2^- production is NADH/NADPH oxidase, a membrane-bound enzyme system (9). The results demonstrated here are compatible with an involvement of redox-sensitive mechanisms in ET-1 signaling since the antioxidant PDTC strongly inhibited ET-1-induced cytokine production by vascular SMC. Moreover, DPI, an inhibitor of flavon-containing enzymes such as NADH/NADPH oxidase, suppressed IL-6 release stimulated by ET-1. The latter data are in keeping with a role for reactive oxygen species derived from NADH/NADPH oxidase in ET-1-induced cytokine production by vascular SMC, but must be interpreted with caution, since DPI is an inhibitor of a broad variety of other enzymes. The lack of inhibition of IL-6 release by staurosporin argues against a role of protein kinase C for IL-6 release elicited by ET-1.

In conclusion, the present data show the ability of ET-1 to initiate an inflammatory response in human vascular SMC by activation of the proinflammatory transcription factor NF-κB and stimulation of cytokine production. This occurs probably through involvement of reactive oxygen intermediates which are possibly generated by activation of NADH/NADPH oxidase. ET-1 may therefore act not only as local mediator of vascular tone but also as a trigger of inflammatory pathways participating in vascular disease. In this way vasoactive peptides like ET-1 may contribute to cardiovascular pathophysiology beyond their vasoconstrictor and growth promoting properties. Pharmacological intervention interfering with ET-induced activation of vascular cells, e.g., ET-receptor blockade, may be beneficial in the prevention and treatment of acute coronary syndromes.

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