**Abstract:** The vasoactive peptide endothelin-1 (ET-1) may contribute to the pathogenesis of atherosclerosis and its acute complications. Because inflammation of the vessel wall is a characteristic feature of atherosclerosis, this study investigated the effect of ET-1 on the proinflammatory transcription factor NF-κB in monocytes. Monocyte/macrophages are a major source of inflammatory mediators in atheroma and are located in rupture prone plaque areas. In human monocytes ET-1 caused NF-κB activation. Specificity of ET-1-induced NF-κB activation was ascertained by supershift and competition experiments. This ET-1 effect was blocked by the ET-A-receptor antagonist BQ-123 but not by the ET-B-receptor antagonist BQ-788. PI-1, a specific inhibitor of the IκB-α-degrading proteasome complex, also prevented NF-κB activation. ET-1 stimulated expression of the proinflammatory molecule CD40 but not of the cytokine IL-6 in a NF-κB-dependent manner. Conclusion: The data demonstrate the ability of ET-1 to activate inflammatory pathways in human monocytes differentially.

**Key words:** atherosclerosis; endothelin; inflammation; monocytes; NF-κB

**INTRODUCTION**

Endothelial adhesion, transendothelial migration and activation of monocytes in the vessel wall are important steps in initiation and progression of atherosclerosis (Ross 1999). Atherosclerotic plaques show histologic features of chronic inflammation, and particularly acute coronary syndromes result from thrombosis triggered by disruption of an atherosclerotic plaque with high inflammatory activity (Libby 1995). Also, pulmonary arterial hypertension, a disease with growing importance and bad prognosis, is characterised by inflammation involving monocytes (Humbert et al. 2004).

The vasoactive peptide endothelin-1 (ET-1) plays an important role in the pathophysiology of both atherosclerosis and pulmonary arterial hypertension (Schiffrin 2001, Humbert et al. 2004). It exerts potent vasoconstrictory, mitogenic and chemotactic properties especially on vascular smooth muscle cells. Moreover ET-1 activates the proinflammatory transcription factor NF-κB in vascular smooth muscle cells and induces an inflammatory response by increased secretion of IL-6 (Browatzki et al. 2000). We therefore hypothesized that ET-1 may activate NF-κB in human monocytes which subsequently results in enhanced expression of inflammatory mediators such as IL-6 and CD40.

**MATERIALS AND METHODS**

**Materials**

LPS, PDTC and ET-1 were purchased from Sigma (St. Louis, MO, USA). BQ-123, BQ-788, proteasome inhibitor 1 (PI-1, Z-Ile-Glu(OtBu)-Ala-Leu-COH) and N-tocyl-l-phenylalanine chloromethyl ketone (TPCK) were from Calbiochem-Novabiochem (San Diego, CA, USA). Recombinant human TNFα was obtained 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.

**Cell preparation and culture**

Peripheral blood mononuclear cells (PBMC) from healthy volunteers were isolated from whole blood. Blood (70-100 ml) was collected by venipuncture, anticoagulated with NH4-heparine and adjusted with phosphate buffered saline (PBS 1x) to a total volume of 100 ml. Blood was loaded on Biocoll 400 (Biochrom-Seromed, Berlin, Germany) and centrifuged for 20 min at 650g at room temperature. The upper cell band containing the PBMC was aspirated, transferred to a 50 ml tube and the cells were washed twice in PBS (pH 7.4). Separation of monocytes and lymphocytes was achieved by adherence of monocytes to uncoated plastic culture flasks for two hours. Afterwards cells were incubated in fresh RPMI-1640 medium (Life Technologies, Karlsruhe, Germany) containing 10% albumin, L-glutamine and antibi-
otics. For experiments the monocytes were stimulated with the respective stimuli, and for inhibition experiments cells were preincubated with the inhibitors for 1 hour.

**ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)**

Protein extracts from human monocytes were prepared and EMSA performed as previously described (Kranzhöfer et al. 1999). Briefly, for electrophoretic mobility shift assays, a double-stranded oligonucleotide (Promega, Madison, WI, USA) representing the consensus sequence for NF-kB binding was labeled with γ-32P-ATP (NEN, Boston, MA, USA) using T-4 polynucleotide kinase (Promega). Cell protein (10 µg) and labeled oligonucleotide (50,000-70,000 cpm) were incubated for binding of active NF-kB for 20 minutes at room temperature. Immediately after binding, the protein/DNA complexes were separated from unbound oligonucleotide by electrophoresis on a native 5% polyacrylamide gel in TBE. Autoradiography was performed with the dried gels using Hyperfilm (Amersham, Buckinghamshire, UK). For testing of specificity of NF-kB/DNA binding, antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against the p65 or p50 subunit of NF-kB were added to the proteins, resulting in further retardation of electrophoretic mobility ('super-shift'), or a 160 fold molar excess of unlabeled 'cold' competitor oligonucleotide was added to the binding reaction, leading to a decrease in NF-kB-bound radioactivity.

**DETERMINATION OF CD40 BY WESTERN BLOT**

Cell lysates (10 µg protein per lane) were separated on SDS-PAGE minigels and electrophoretically transferred to Hybond nitrocellulose membrane (Amersham). Incubation with a polyclonal rabbit antibody for CD40 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was followed by incubation with a secondary antibody (donkey anti Ig, Amersham, dilution 1:5000) coupled to horseradish peroxidase. Antibody binding was visualized using the ECL chemiluminescence system (Amersham).

**DETERMINATION OF IL-6 RELEASE**

Monocytes were plated on 96-well tissue culture dishes (250,000 cells per well) and stimulated for 24 h. The cell culture supernatant was assayed for IL-6 concentration 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.

**RESULTS**

Peripheral blood mononuclear cells contained between 19 and 25% monocytes. Isolation of monocytes by adherence to uncoated plastic culture flasks resulted in a monocyte population of ≥ 80% purity (data not shown).

ET-1 caused rapid activation of NF-kB (Fig. 1). The specificity of the NF-kB binding to the consensus oligonucleotide was ascertained in two ways: a) an excess of unlabeled 'cold' competitor oligonucleotide reduced the signal intensity of the band associated with active NF-kB and b) addition of antibodies against the p65 or the p50 subunit of NF-kB resulted in a further retardation of the mobility of the NF-kB/oligonucleotide complex ("supershift"). The latter finding demonstrates that NF-kB in monocytes exists predominantly as a p65/p50 heterodimer. TNFα (20 ng/mL) or LPS (1 µg/mL) used as positive control stimuli also induced a strong activation of NF-kB in
monocytes. The specific ET-A-receptor antagonist BQ-123 blocked ET-1-induced NF-κB binding activity whereas the highly selective ET-B-receptor antagonist BQ-788 (10 µmol/L each) did not (Fig. 2). Methanol (0.25%) as solvent for BQ-788 did not show any intrinsic inhibitory effect. Preincubation with PI-1 (50 µmol/L), a specific inhibitor of the IkB-α-degrading proteasome complex, also prevented NF-κB activation. These findings demonstrate both specificity of NF-κB activation by ET-1 and involvement of the ET-A-receptor.

Since NF-κB governs the regulation of a plethora of molecules involved in inflammation, we investigated the effect of ET-1 on expression of CD40 and IL-6 in monocytes. CD40, which mediates together with its ligand CD154 inflammatory processes through direct cell-cell contact, was upregulated by ET-1 (Fig. 3). This effect was blocked by BQ-123, but not by BQ-788, suggesting involvement of ET-A receptors. The NF-κB inhibitors PI-1 and TPCK (50 µmol/L each) also prevented CD40 induction by ET-1.

Endothelin-1 does not stimulate IL-6 release from monocytes. Human monocytes were stimulated with ET-1 (10 nmol/L) or LPS (1 mg/mL) for 24 hours, and IL-6 release was assessed by ELISA of cell culture supernatants. BQ-123 blocked ET-1-induced NF-κB expression from human monocytes. The specific ET-A-receptor antagonist BQ-123, but not BQ-788, inhibited ET-1-induced CD40 expression. The NF-κB inhibitors PI-1 and TPCK (50 µmol/L each) also prevented CD40 induction by ET-1.

DISCUSSION

Monocytes play a key role in the inflammatory response and function as effector cells that mediate tissue damage through the release of proinflammatory cytokines (Special et al. 1998), growth factors (Ross et al. 1990), oxygen radicals, and matrix metalloproteinases (Rajagopalan et al. 1996). Particularly acute coronary syndromes result from thrombosis triggered by disruption of an atherosclerotic plaque with high inflammatory activity (van der Wal et al. 1994). Rupture prone regions show a high number of activated macrophages. These cells express proteinases capable of digesting collagen and elastin which stabilize the fibrous cap (Galili et al. 1995). Moreover, monocytes secrete active tissue factor, a potent procoagulant that initiates the coagulation cascade (Mackman 1996).

Synthesis of most of these factors depends on the proinflammatory transcription factor NF-κB. Our data demonstrate activation of NF-κB by ET-1 in human monocytes. Increased ET-1 levels were found in the coronary and systemic circulation of humans with coronary dysfunction, suggesting involvement of this peptide in early atherosclerosis (Lerman et al. 1995) as well as in advanced 'unstable' atherosclerotic plaques leading to acute coronary syndromes (Zeiher et al. 1995). The main cellular sources of ET-1-secretion are endothelial cells and macrophages themselves (Levin 1995, Ehrenreich et al. 1990). The concept of ET-1, a peptide commonly involved in regulation of vascular tone, as an important mediator in atherogenesis is further supported by animal studies showing reduced atherosclerosis in mice treated with an endothelin receptor antagonist (Barton et al. 1998). At the cellular level ET-1 acts as a strong mitogen promoting smooth muscle cell proliferation via induction of the mitogen activated protein kinase cascade (Hirata et al. 1989, Komuro et al. 1998, Wang et al. 1994). These effects of ET are mediated by two types of G-protein-coupled receptors, ET-A and ET-B. Only few data exist concerning the expression of ET-A- or ET-B-receptors on the surface of mononuclear cells. Wilson et al. described an ET-B-receptor-dependent activation pathway in the human monocytic tumor cell line THP-1 (Wilson et al. 2001). However, this cell line was established from a 1-year-old boy with acute monocytic leukemia and may express a receptor pattern different from that of normal monocytes. The stimulatory effect of ET-1 on NF-κB activation in human monocytes demonstrated here was specific and mediated by the ET-A-receptor, since BQ-123, a highly selective ET-A-receptor antagonist completely inhibited this effect. This is in contrast to the observations made in THP-1 cells, and delineates that it is difficult to extrapolate data derived from neoplastic cells to normal cells. The signaling pathway by which ET-1 leads to NF-κB activation in human monocytes needs to be further investigated. NF-κB is a redox-sensitive proinflammatory transcription factor involved in multiple immune and inflammatory responses. It is activated primarily through dissociation of an inhibitor protein IkB (Henkel et al. 1993). Stimuli that activate NF-κB induce an IkB kinase (IKK) complex leading to phosphorylation of IkB and subsequent ubiquitination...
and degradation of IkB by a multicatalytic 20S proteasome complex. The proteasome inhibitor PI-1 prevented ET-1-induced NF-κB activation in this study.

NF-κB is a key transcription factor that regulates a whole plethora of inflammatory pathways (Ghosh et al. 1998), especially the induction of proinflammatory cytokines such as IL-6. Surprisingly, ET-1 did not induce IL-6 in human monocytes despite strong NF-κB activation. In contrast, ET-1 stimulated expression of the cell surface molecule CD40 on monocytes. At present, it remains unclear why ET-1 differentially induces CD40 but not IL-6. CD40 is part of a receptor-ligand system and can act together with its counterpart CD154 (formerly CD40 Ligand) as a powerful inducer of cell-cell contact-mediated inflammatory responses (Grewal and Flavell 1998). Growing evidence suggests an important role in the pathogenesis of atherosclerosis and its complications (Schönbeck and Libby 2001). ET-1 could promote atherogenesis through stimulation of monocytes to express CD40.

The involvement of the CD40/CD154 system in the pathogenesis of pulmonary arterial hypertension is less clear. A recent report found increased plasma levels of soluble CD154 in patients with pulmonary arterial hypertension (Damas et al. 2004). To date, however, pulmonary arterial hypertension is the only disease for which a beneficial effect of ET receptor antagonism with the non-selective receptor blocker bosentan has proven benefit for the patients (Channick et al. 2004). Part of the effects of bosentan could be the result of an anti-inflammatory action on monocytes and smooth muscle cells.

This study demonstrates the capability of ET-1 to induce an inflammatory response in human monocytes which can be reversed by selective ET-receptor antagonism. Inhibition of ET-induced inflammatory activation may be a therapeutic principle for the prevention of atherosclerosis and the treatment of pulmonary arterial hypertension.

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