ENDOTHELIN-1 INDUCES CD40 BUT NOT IL-6 IN HUMAN MONOCYTES VIA THE PROINFLAMMATORY TRANSCRIPTION FACTOR NF-κB

M. Browatzki, C. A. H. Pfeiffer, J. Schmidt, R. Kranzhöfer

Department of Cardiology, University of Heidelberg, Heidelberg, Germany

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 in-duces 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%(vol/vol) heat-inactivated FCS over night. Approximately 500000 cells were used for specific FACS-analysis of monocyte purity using an anti-human-CD14-PE antibody (Cymbus Biotechnology LTD by Dianova, Hamburg, Germany) on a FACSscan flow cytometer (Becton Dickinson, CA, USA). The remainder of the cells were resuspended in RPMI-1640 with 0.1% human serum 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-κB binding was labeled with γ-^{32}P-ATP (NEN, Boston, MA, USA) using T-4 polynucleotide kinase (Promega). Cell protein (10 μg) and labeled oligonucleotide (50000-70000 cpm) were incubated for binding of active NF-κB 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-κB/DNA binding, antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against the p65 or p50 subunit of NF-κB 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-κB-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 Hybondnitrocellulose membrane (Amersham). Incubation with a polyclonal rabbit antibody for CD40 (Santa Cruz, dilution 1:500) 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-κB (Fig. 1). The specifity of the NF-κB binding to the consensus oligonucleotide was ascertained in two ways: a) an excess of unlabeled 'cold' competitor oligonucleotide re-

duced the signal intensity of the band associated with active NF-κB and b) addition of antibodies against the p65 or the p50 subunit of NF-κB resulted in a further retardation of the mobility of the NF-κB/ oligonucleotide complex ('supershift'). The latter finding demonstrates that NF-κB 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-κB in

Fig.1. Endothelin-1 increases NF-κB binding activity in human monocytes. Human monocytes were stimulated with ET-1 (10 nmol/L) or TNFα (20 ng/mL) as positive control for 1 h. Activation of NF-κB was assessed by electrophoretic mobility shift assay. Specificity of NF-κB binding was ascertained by addition to the reaction mixture of excess unlabelled oligonucleotides or antibodies against p65 or p50 subunits, respectively. The autoradiograph shown is representative of three independent experiments with cells from different donors.
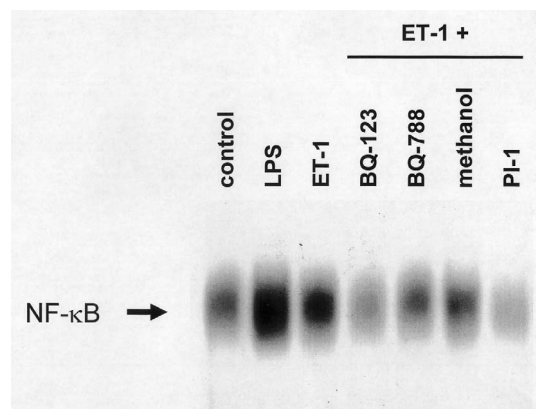

Fig. 2. Endothelin-1 induces NF-κB in human monocytes via the ET-A-receptor. LPS (1 μg/mL) and ET-1 (10 nmol/L) activated NF-κB in human monocytes after 1 hour stimulation. BQ-123 (10 μmol/L), a highly selective ET-A-receptor antagonist, but not BQ-788 (10 μmol/L), which is selective for ET-B receptors, inhibited ET-1-induced NF-κB activation. The solvent methanol (0.25%) had no inhibitory effect. PI-1 (50 μmol/L), an inhibitor of the IκB-α-degrading proteasome complex, also prevented ET-1-induced NF-κB activation. Three experiments from cells of different donors showed comparable results.
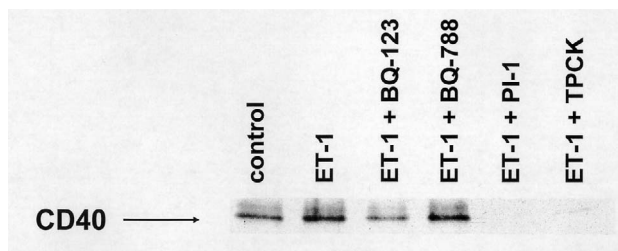
Fig. 3. Endothelin-1 induces CD40 in human monocytes via the ET-A-receptor. Human monocytes were stimulated with ET-1 (10 nmol/L) for 24 hours, and CD40 expression was assessed via Western blot of cell lysates. BQ-123 but not BQ-788 inhibited ET-1-induced CD40 expression. The NF-κB inhibitors PI-1 and TPCK (50 µmol/L each) also prevented CD40 induction by ET-1.
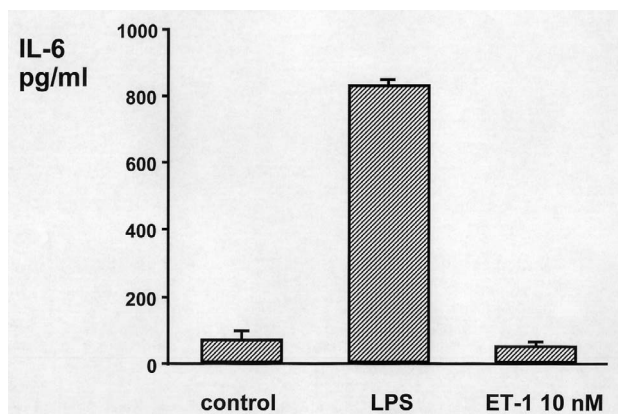

Fig. 4. Endothelin-1 does not stimulate IL-6 release from monocytes. Human monocytes were stimulated with ET-1 (10 nmol/L) or LPS (1 mg/mL) for 24 hours, and IL-6 release was assessed by ELISA of cell culture supernatants.

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 IκB-α-degrading proteasome complex, also prevented NF-κB activation. These findings demonstrate both specifity 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 his 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 also blocked ET-1-induced CD40 expression. This is compatible with a role for NF-κB in regulation of CD40 by ET-1. ET-1 did not increase relase of the cytokine IL-6 as a soluble mediator of inflammation from human monocytes despite the cells were activated by LPS (Fig. 4).

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 (Speciale 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 (Galis 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. 1988, 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 IκB (Henkel et al. 1993). Stimuli that activate NF-κB induce an IκB kinase (IKK) complex leading to phosphorylation of IκB and subsequent to ubiquitination

and degradation of IκB 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 suggest 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.

REFERENCES

Barton M, Haudenschild CC, d'Uscio LV, Shaw S, Munter K, Lüscher TF (1998) Endothelin ETA receptor blockade restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice. Proc Natl Acad Sci U S A 95: 14367-14372

Browatzki M, Schmidt J, Kübler W, Kranzhöfer, R (2000) Endothelin-1 induces interleukin-6 release via activation of the transcription factor NF-κB in human vascular smooth muscle cells. Basic Res Cardiol 95: 98-105

Channick RN, Sitbon O, Barst RJ, Manes A, Rubin LJ (2004) Endothelin receptor antagonists in pulmonary arterial hypertension. J Am Coll Cardiol 43: 62S-67S

Damas JK, Otterdal K, Yndestad A, Aass H, Solum NO, Froland SS, Simonsen S, Aukrust P, Andreassen AK (2004) Soluble CD40 ligand in pulmonary arterial hypertension: possible pathogenic role of the interaction between platelets and endothelial cells. Circulation 110: 999-1005

Ehrenreich H, Anderson RW, Fox CH, Rieckmann P, Hoffman GS, Travis WD, Coligan JE, Kehrl JH, Fauci AS (1990) Endothelins, peptides with potent vasoactive properties, are produced by human macrophages. J Exp Med 172: 1741-1748

Galis ZS, Sukhova GK, Kranzhöfer R, Clark S, Libby P (1995) Macrophage foam cells from experimental atheroma constitutively produce matrix-degrading proteinases. Proc Natl Acad Sci U S A 92: 402-406

Ghosh S, May MJ, Kopp EB (1998) NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol 16: 225-260

Grewal IS, Flavell RA (1998) CD40 and CD154 in cell-mediated immunity. Annu Rev Immunol 16: 111-135

Henkel T, Machleidt T, Alkalay I, Krönke M, Ben-Neriah Y, Baeuerle, PA (1993) Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. Nature 365: 182-185

Hirata Y, Takagi Y, Fukuda Y, Marumo F (1989) Endothelin is a potent mitogen for rat vascular smooth muscle cells. Atherosclerosis 78: 225-228

Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, Christman BW, Weir EK, Eickelberg O, Voelkl NF, Rabinovitch M (2004) Cellular and molecular pathobiology of pulmonary arterial hypertension. J Am Coll Cardiol 43: 13S-24S

Komuro I, Kurihara H, Sugiyama T, Yoshizumi M, Takaku F Yazaki Y (1988) Endothelin stimulates c-fos and c-myc expression and proliferation of vascular smooth muscle cells. FEBS Lett 238: 249-252

Kranzhöfer R, Browatzki M, Schmidt J, Kübler W (1999) Angiotensin II activates the proinflammatory transcription factor nuclear factor-κB in human monocytes. Biochem Biophys Res Commun 257: 826-828

Lerman A, Holmes DR, Bel, MR, Garrat KN, Nishimura RA, Burnett JCjr (1995) Endothelin in coronary endothelial dysfunction and early atherosclerosis in humans. Circulation 92: 2426-2431

Levin ER (1995) Endothelins. N Engl J Med 333: 356-363

Libby P (1995) Molecular bases of the acute coronary syndromes. Circulation 91: 2844-2850

Mackman N (1996) Regulation of tissue factor gene expression in human monocytic and endothelial cells. Haemostasis 26 (Suppl 1): 17-19

Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS (1996) Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. J Clin Invest 98: 2572-2579

Ross R, Masuda J, Raines EW, Gown AM, Katsuda S, Sasahara M, Malden LT, Masuko H, Sato H (1990) Localization of PDGF-B protein in macrophages in all phases of atherogenesis. Science 248: 1009-1012

Ross R (1999) Mechanisms of disease: atherosclerosis-an inflammatory disease. N Engl J Med. 340: 115-126

Schiffrin EL (2001) Role of endothelin-1 in hypertension and vascular disease. Am J Hypertens 14: 83S-89S

Schönbeck U, Libby P (2001) CD40 signaling and plaque instability. Circ Res 89: 1092-1103

Speciale L, Roda K, Saresella M, Taramelli D, Ferrante P (1998) Different endothelins stimulate cytokine production by peritoneal macrophages and microglial cell line. Immunology 93: 109-114

van der Wal AC, Becker AE, van der Loos CM, Das PK (1994) Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation 89: 36-44

Wang Y, Rose PM, Webb ML, Dunn MJ (1994) Endothelins stimulate mitogen-activated protein kinase cascade through either ETA or ETB. Am J Physiol 267: C1130-C1135

Wilson SH, Simari RD, Lerman A (2001) The effect of endothelin-1 on nuclear factor kappa B in macrophages. Biochem Biophys Res Commun 286: 968-972

Zeiher AM, Ihling C, Pistorius K, Schächinger V, Schäfer HE (1994) Increased tissue endothelin immunoreactivity in atherosclerotic lesions associated with acute coronary syndromes. Lancet 344: 1405-1406

Address for correspondence:
Roger Kranzhöfer, M.D.
Department of Cardiology
University of Heidelberg
Im Neuenheimer Feld 410
69120 Heidelberg, FRG
Tel.:   +49-6221-568611
Fax: +49-6221-565515
e- mail: roger_kranzhoefer@med.uni-heidelberg.de