Characterization of heme oxygenase-1 induction in human endothelial cells

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Nicole Bildner

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Erklärung

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Meinen Eltern und meinem Freund Karl

CONTENTS

1 INTRODUCTION

1.1 Background and aim of the work

Endothelial cells play a critical role in the progression, control, and resolution of inflammation. In their normal state, they serve as an antithrombotic and antiinflammatory barrier between blood and tissue, regulating blood flow and permeability of blood vessels to immune cells as well as to micro- and macromolecules. (Hennig et al.1999, Choy et al.2001) Under inflammatory conditions, endothelial cells become activated by exposure to proinflammatory stimuli such as TNF-α, promoting vasoconstriction, vascular permeability, expression of adhesion molecules, coagulation and thrombosis, as well as causing endothelial injury and apoptosis. Injury of the endothelial monolayer by induction of apoptotic cell death, e.g. by TNF-α, plays an important role in the pathophysiology and progression of atherosclerosis (Dimmeler et al. 1998, Rossig et al.2001, Mallat and Tedgui 2001). In order to counteract cell damage caused by the inflammatory response, endothelial cells can upregulate a set of protective proteins, such as the members of the family of heat shock proteins like HSP70 and HSP32 (Heme oxygenase-1, HO-1). Especially HO-1 has come into the focus of interest during the last years due to its prominent cytoprotective properties protecting endothelial cells from apoptosis (Siow et al 1999). In this context, the effects of the atrial natriuretic peptide, ANP, and aspirin on HO-1 in HUVEC were investigated.

ANP is a polypeptide hormone playing an important role in the regulation of blood pressure and cardiovascular homeostasis. The vasoactive peptide is mainly synthesized and secreted by the atrium of the heart in response to atrial stretch and pressure and exerts a variety of potent regulatory effects in the cardiovascular system, such as diuresis, natriuresis and vasorelaxation (Levin et al.1998, Misono 2002). In addition to its well-characterized renal and cardiovascular actions, ANP possesses a much broader biological profile than assumed a couple of years ago. First observations pointing to additional biological functions of ANP, e.g. in the immune system, were the detection of the expression of ANP and its receptors in macrophages (Vollmar and Schulz 1994, Vollmar and Schulz 1995, Kiemer and Vollmar 1997) and in the thymus (Vollmar and Schulz 1990a), indicating that ANP might also influence immunological functions. Recent works confirmed the important role of ANP in the immune system, as it was shown to reduce the expression of important mediators of inflammation in macrophages, such as TNF-α (Kiemer et al.2000a, Tsukagoshi et al.2001), cyclooxygenase-2 (Kiemer et al.2002a) and nitric oxide synthase (Kiemer and Vollmar 1997, Kiemer and Vollmar 1998). In addition to its inhibitory action on inflammatory mediators, induction of protective proteins by ANP was hypothesized to confer protection against TNF-α-induced apoptosis, a central event in inflammatory diseases, such as atherosclerosis, septic shock and rheumatoid arthritis. To date, mechanisms leading to the upregulation of cytoprotective proteins such as heat shock proteins remain unknown and were therefore the subject of the first part of the present work.

Aspirin is a well-known anti-inflammatory drug, introduced for the treatment of inflammatory disorders and joint diseases, such as rheumatoid arthritis, in 1899, based on its analgesic and antiinflammatory effects (Jack 1997). These widely documented properties of aspirin have been ascribed to its ability to inhibit prostaglandin production by inhibition of the enzymatic activity of cyclooxygenases (COX). In recent years, an increasing number of

studies has reported additional protective effects of this non-steroidal antiinflammatory drug against colon cancer (Thun et al.1991) and cardiovascular disease (Mehta 1998, Lauer 2002), as well as its ability to delay the onset of Alzheimer disease (Flynn and Theesen 1999). Inhibition of COX activity can only in part account for these recently observed effects, suggesting that aspirin may exert part of these activities by modulating COX-independent pathways. In this context, aspirin treatment has been demonstrated to result in increased expression of the heat shock protein HSP70 and HSP60 (Jin et al.1999, Amberger et al.1999), inhibition of iNOS expression (Amin et al.1995, Katsuyama et al.1999), inhibition of NF-κB (Kopp et Gosh 1994), suppression of adhesion molecule expression, and down-regulation of MCP-1 (Amberger et al.1999). However, it is widely unknown if aspirin is able to influence the expression of HO-1, especially basal levels of this important heat shock protein. Therefore the aim of the second part of this work was to characterize the effect of aspirin on the heat shock protein HO-1, as a potential new cytoprotective target.

In this context, the following questions were to be answered:

I) Influence of ANP on HUVEC:

1.) Is ANP able to induce the expression of the cytoprotective proteins HO-1 and HSP70 in human umbilical vein endothelial cells, HUVEC?
2.) Which receptor mediates the induction of HO-1 observed after treatment with ANP?
3.) What is the molecular mechanism of HO-1 induction by ANP?
   a) Does ANP influence HO-1 mRNA levels?
   b) Does ANP have an impact on the activity of the transcription factor AP-1?
   c) Does ANP modulate AP-1 activity *via* the phosphorylation status of MAP kinases?
   d) Is there a causal relationship between the effects of ANP on the transcription factor AP-1 and ANP-induced upregulation of HO-1 protein?
4.) Do HUVEC treated with TNF-α under the employed cell culture conditions die by apoptosis or necrosis?
5.) Does the upregulation of HO-1 protein by ANP confer protection against TNF-α-induced cytotoxicity?

II) Influence of aspirin on HUVEC:

1.) Is aspirin able to influence HO-1 expression in HUVEC?
2.) What is the molecular mechanism of the detected upregulation of HO-1 protein levels?
   a) Does aspirin influence HO-1 mRNA levels?
   b) Does aspirin affect AP-1 DNA-binding activity?
   c) Does aspirin affect AP-1 by influencing the activity of MAP kinases?
   d) Is there a causal relationship between the effects of aspirin on the transcription factor AP-1 and aspirin-induced upregulation of HO-1 protein?
3.) Is aspirin able to protect HUVEC against TNF-α-induced apoptosis?

1.2 The heme oxygenase-1

1.2.1 The family of heat shock proteins

Heat shock proteins (HSP), also called stress proteins, play a role in development and normal cell function. Constitutively expressed HSP function as molecular chaperones and participate in protein synthesis, protein folding, oligomeric assembly, transport, and translocalization processes between intracellular compartments, as well as switches between active/inactive protein conformations (Hendrick and Hartl 1993). These processes require the existence of proteins in an unfolded or partially folded conformation, which exposes interactive regions within the proteins that can lead to deleterious protein aggregation. Heat shock proteins bind to these interactive regions, thus preventing aggregation and enabling the folding of polypeptides (Becker and Craig 1994). Besides constitutive expression, stress proteins can also be induced by a variety of stress situations, including hyperthermia, viral infection, exposure to heavy metals, ischemia, anoxia, and oxidative stress (Goldbaum and Richter-Landsberg 2001, Wagner et al. 1999).

Heat shock proteins are classified into subfamilies according to their molecular weight (Goldbaum and Richter-Landsberg 2001). The HSP70 family comprises multiple members. The constitutive cytosolic form HSC70 has a molecular weight of 73kDa and is only moderately inducible. The inducible form HSP70 with a molecular weight of 72 kDa is only weakly constitutively expressed and is strongly induced during oxidative stress conditions (Goldbaum and Richter-Landsberg 2001). Members of the HSP70 family fulfil important chaperone functions. They bind to and stabilize newly synthesized unfolded precursor proteins, thus preventing improper protein interactions, misfolding, and aggregation of these precursors. HSP70 proteins therefore contribute to correct protein synthesis, translocation across biological membranes, and protein folding and assembly into organelles (Becker and Craig 1994). During stress conditions, HSP70 proteins prevent cell damage by binding to damaged or denatured proteins and preventing protein aggregation, thus providing the opportunity for either correct refolding or degradation of these damaged proteins (Becker and Craig 1994). Besides, HSP70 is able to attenuate the inflammatory response by interfering with the activation of the proinflammatory transcription factor NF-κB and consequently with the expression of inflammatory enzymes (Feinstein et al. 1996) and cytokines like TNF-α (Snyder et al. 1992). HSP70 expression is induced in response to various cellular stress stimuli and enables the cell to cope with inflammatory conditions such as ischemia/reperfusion injury (Chen et al. 1997) or sepsis (Hotchkiss et al.1993) and other inflammatory diseases (Kohn et al. 2002, Feinstein et al. 1996), as well as apoptosis (deMeester et al. 1997).

HSP32 is a small stress protein and belongs to the heme oxygenase family of proteins. It is synthesized in response to heat shock, heme, and oxidative stress. Accumulating evidence suggests its role in protecting cells from oxidative stress, as described in detail under 1.2.2.

1.2.2 The heme oxygenase system

1.2.2.1 Physiological function

Heme oxygenase (HO), originally discovered in 1968 by Tenhunen et al., catalyzes the initial and rate limiting step in the oxidative degradation of heme (Tenhunen et al. 1968). Together with the heme synthetic enzyme δ-aminolevulinate synthase it regulates the cellular levels of the prooxidant heme, yielding equimolar amounts of biologically active catabolites. Heme released from oxidized free hemoglobin constitutes a potentially harmful molecule due to its ability to intercalate into cell membranes where it promotes deleterious iron-dependent reactions leading to ROS generation and membrane lipid peroxidation (Ryter and Tyrell 2000). Acting in concert with NADPH-cytochrome P450 reductase as reducing agent, HO catabolizes the breakdown of heme, released mainly from hemoglobin of senescent erythrocytes, from myoglobin or cytochromes (Maines 1997). HO-1 cleaves the α-methene carbon bridge and yields equimolar amounts of biliverdin IXα, carbon monoxide (CO), and iron (Ryter and Tyrell 2000). In mammalian cells, biliverdin is subsequently converted to bilirubin by bilverdin reductase, and released iron is used in intracellular metabolism or sequestered into ferritin **(figure 1)**.

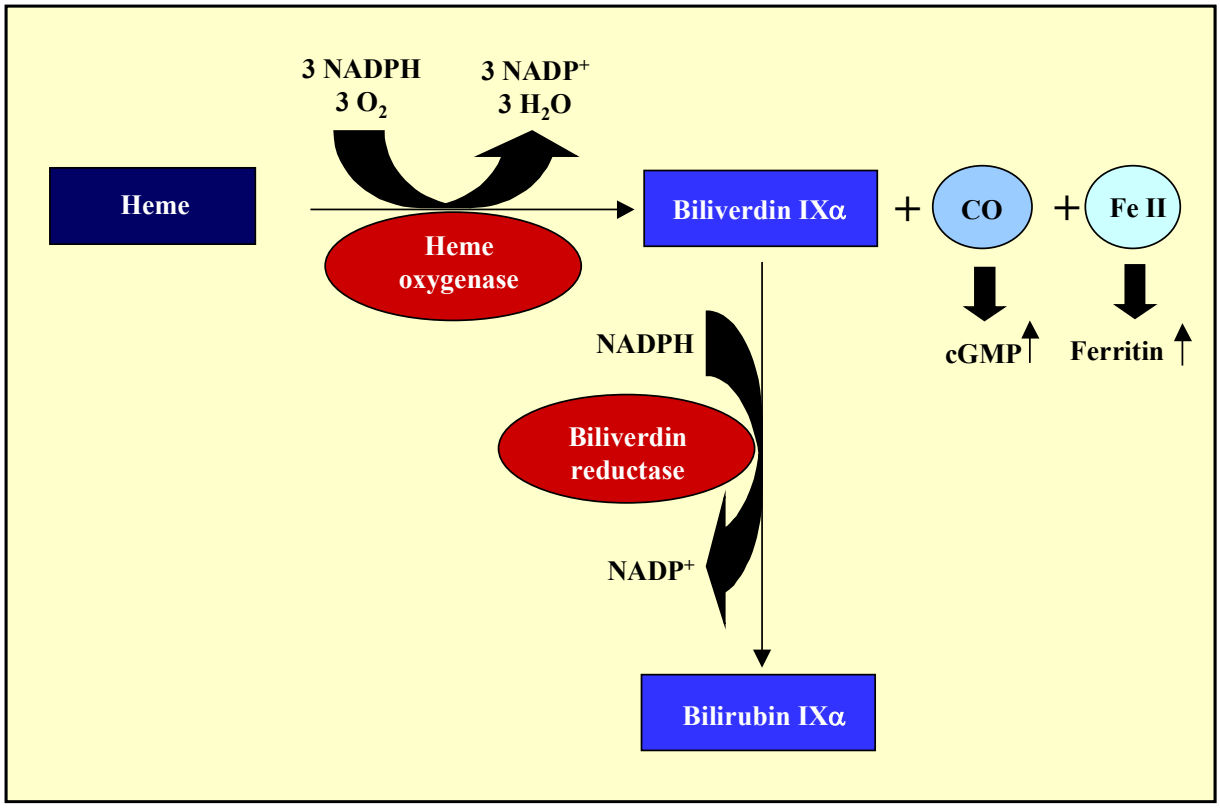


figure 1: heme degradation by heme oxygenase-1

1.2.2.2 The three heme oxygenases

To date, three isoforms of the HO protein have been identified, all of which are encoded by separate genes. HO-1 is the inducible isozyme (Ryter et al. 1999). Under basal conditions, the 32 kDa protein is expressed at low levels in endothelial cells, as well as in kidney, liver, and most abundantly in spleen, where senescent erythrocytes are sequestered and red blood cell hemoglobin is degraded (Maines 1997). Apart from its major substrate heme, HO-1 synthesis is known to be up-regulated by a variety of non-heme inducers, including heavy metals, cytokines, hormones, endotoxins, oxidants, and heat shock (Otterbein and Choi 2000, Choi and Alam 1996). Induction of HO-1 is also highly sensitive to exposure to agents causing oxidative stress, such as UV irradiation, sodium arsenite, hyperoxia and glutathione depletion, indicating that HO-1 induction may be a protective strategy of the cell in the cellular defense mechanism against oxidative and inflammatory damage (Keyse and Tyrrell 1989, Elbirt et al. 1998, Oguro et al. 1998, Otterbein et al. 1999). HO-2 is a constitutively expressed 36 kDa protein and can be detected mainly in the brain, endothelium and testes. The only inducers of HO-2 identified to date are adrenal glucocorticoids (Maines 1997). The more recently discovered third isoform HO-3, 33 kDa in size, is like HO-2 constitutively expressed, but exhibits only low catalytic activity and may function in heme binding and heme sensing (Immenschuh and Ramadori 2000). It has been found in brain, heart, kidney, liver, testes and spleen (Siow et al.1999).

Each isozyme is highly evolutionary conserved in its amino acid and nucleotide sequence. Between HO-1 and HO-2, however, the similarity of amino acid and nucleotide sequence is merely 43% (Maines 1997). HO-3 is closely related to HO-2 and shares 90% amino acid homology. A 24-amino acid segment is completely conserved among all forms, with only exeption of a single residue, and forms a hydrophobic pocket (heme pocket) that binds the heme pyrrol rings 1 and 2 through electrostatic interactions (Maines 1997).

1.2.2.3 Regulation of heme oxygenase-1

Stimulation of HO-1 expression by most stimuli is primarily controlled at the transcriptional level and involves gene activation and *de novo* enzyme protein synthesis. Several response elements located in the 5‘ distal flanking region and the promotor of the HO-1 gene have been identified and characterized, including consensus sequences necessary for the binding of transcription factors such as activator protein 1, NF-κB, and heat-shock factor 1 (Choi and Alam 1996, Maines 1997, Hartsfield et al. 1998, Hartsfield et al. 1999). AP-1 seems to play a crucial role in the control of the heme oxygenase 1 gene as demonstrated by a multitude of reports stressing the importance of this transcription factor in the induction of HO-1 in response to diverse stimuli (e.g. Camhi et al. 1998, Alam et al. 1995, Wiesel et al. 2000).

Upstream signaling pathways implicated in the regulation of HO-1 expression include redox signaling, protein kinase C, protein kinase A, protein kinase G, as well as the mitogen-activated protein kinases, described in detail under 1.7 (Immenschuh and Ramadori 2000).

1.2.2.4 Cytoprotective potential

Accumulating evidence suggests that HO-1 induction in addition to its role in heme degradation might confer cellular protection against oxidant insults and serve a vital function in maintaining cellular homeostasis. HO-1 has a diverse spectrum of cytoprotective effects that, apart from its ability to remove the pro-oxidant heme molecule, are mostly associated with the different end products of heme catabolism.

The HO product biliverdin is rapidly converted into the bile pigment bilirubin which possesses potent free radical scavenging and antioxidant properties (Stocker et al. 1987, Clark et al. 2000). Bilirubin has been demonstrated to provide cellular protection to neuronal cells exposed to oxidative injury by hydrogen peroxide (Dore et al. 1999) as well as in a model of ischemic heart injury (Clark et al. 2000). Iron, released during the enzymatic degradation of heme, is cytotoxic due to its ability to generate ROS by Fenton chemistry, but is promptly sequestered into the iron storage protein ferritin, the synthesis of which is upregulated by iron. Ferritin exerts antioxidative effects that can be ascribed to its capacity to sequester iron, which lowers the prooxidant state of the cell by removing free iron, and to its ferroxidase activity, catalyzing the oxidation of ferrous iron to ferric iron to enable intracellular storage of iron in biological systems (Balla et al. 1992). HO-1 also upregulates an iron ATPase located in the endoplasmatic reticulum resulting in augmented cellular iron efflux (Ferris et al. 1999, Barañano et al. 2000). CO, the third catalytic product of HO-1 activity, can also mediate important cellular functions and has been shown to exhibit potent cytoprotective effects. This gaseous messenger can promote vasodilatation *via* activation of soluble guanylate cyclase and subsequent formation of cGMP. Therefore CO can maintain blood circulation at sites of inflammation, counteracting the deleterious effects of coagulation and thrombosis (Sammut et al. 1998) *Via* the same mechanism, CO is able to suppress platelet activation or aggregation. Most interestingly, CO has also been reported to protect endothelial cells from undergoing apoptosis (Brouard et al. 2000, Brouard et al. 2002) and to provide protection in models of lung injury (Otterbein et al. 2000), effects that are reported to be mediated *via* cGMP-independent pathways, such as activation of p38 MAPK.

HO-1 induction is observed in a number of disease states, such as ischemic stroke or Alzheimer's disease (Panahian et al. 1999, Papolla et al. 1998). Evidence that the induction of HO-1 has therapeutic implications in the treatment of diseases associated with oxidative stress has been provided by various *in vitro* studies demonstrating a cytoprotective potential of this

heat shock protein in models of lung injury (Otterbein et al. 1999), TNF-α-induced apoptosis (Polte et al. 1997, Petrache et al. 2000) or ischemia/reperfusion injury (Katori et al. 2002). Recent studies in HO-deficient mice have further strengthened the important cytoprotective role of this protein. The mice lacked the ability to reuse iron and were characterized by progressive anemia, tissue iron deposition, chronic inflammation, and delayed growth, as well as increased susceptibility to oxidative stress (Poss and Tonegawa 1997). These results in HO-1 knockout mice were additionally confirmed by a first case of human HO-1 deficiency. The boy displayed the same pathological findings, but compared to HO-1-targeted mice, endothelial cells and the reticuloendothelial system were even more affected (Yachie et al. 1999, Kawashima et al. 2002).

In view of this significant antiinflammatory and cytoprotective potential of HO-1 protein, induction of HO-1 gene expression by non-stressful stimuli appears to be a promising target and a novel approach to the therapeutic treatment of diseases associated with oxidative stress, such as atherosclerosis, septic shock, and rheumatoid arthritis. Cytotoxic injury, mostly the induction of apoptosis, of endothelial cells plays a pivotal role in the pathophysiology of inflammatory diseases such as atherosclerosis (Choy et al. 2001, Guevara et al. 2001, Takahashi et al. 2002) and sepsis (Haimovitz-Friedmann et al. 1997, Munshi et al. 2002). Therefore, the next chapter deals with the characteristics and mechanisms of the two forms of cell death that can be distinguished.

1.3 Forms of cell death

When investigating cell death, two basic forms of death associated with characteristic morphological changes can be distinguished: apoptosis and necrosis.

1.3.1 Necrosis

Necrosis, also referred to as accidential cell death, is a passive death process occuring after major pathological cellular injuries, characterized by failure of cellular homeostasis (Raffray and Cohen 1997). Whereas apoptosis can affect single cells, necrotic cell death mainly occurs in cell groups and is often observed when cells are confronted with severe and sudden injury, such as ischemia, sustained hyperthermia, or high doses of toxic agents. Morphological changes characteristic for necrosis are intense eosinophilia, cell and organelle swelling, vacuolization, as well as simultaneous protein denaturation and hydrolysis (Majno and Joris 1995). Necrotic cells display progressive cell and organelle membrane dysfunction, which disturbs ion homeostasis and finally leads to the collapse of internal homeostasis. Nuclear changes include karyolysis, the non-specific DNA breakdown by increased DNase activity.

Finally, membrane rupture of the necrotic cell results in the liberation of cellular contents which activate the host inflammatory response (Raffray and Cohen 1997).

1.3.2 Apoptosis

1.3.2.1 Overview

Apoptosis, also referred to as programmed cell death, is an evolutionary conserved physiological process of cellular autodestruction. This active, energy requiring process plays a central role in development, maintenance of homeostasis and host defense of multicellular organisms. Apoptotic cell death occurs during normal morphogenesis, tissue remodelling as well as in response to pathogenic infections or other irreparable cell damage and enables the elimination of unwanted cells and cells damaged by disease or toxic agents (Chang and Yang 2000). Due to the crucial physiological role of apoptosis, dysregulation of this process is implicated in a variety of diseases associated with a lack of apoptosis, such as cancer, viral infections or autoimmune disorders, as well as with diseases involving pathologically increased apoptosis, such as AIDS and neurodegenerative disorders like Alzheimer's disease or Parkinson's disease (Thompson 1995).

Apoptosis was first described in 1972 by Kerr et al. and given its name according to the image of leaves dropping off from a tree (*apó*, meaning "from", *ptósis*, meaning "a fall") (Kerr et al. 1972). Cells dying from apoptosis exhibit characteristic morphological changes, including plasma membrane blebbing, followed by cell (body) shrinkage and fragmentation of the cell into membrane-bound apoptotic bodies, which are finally phagocytosed by macrophages or neighbouring healthy cells and eventually degraded in lysosomes. Biochemical changes typical for apoptosis are DNA fragmentation into discrete fragments of multiples of 180 bp, chromatin condensation and exposure of phosphatidylserine residues on the cell surface, which mark the cell for elimination by macrophages. Since plasma membrane integrity is maintained throughout the whole process of apoptosis, intracellular contents are not released into the circulation and potentially harmful inflammatory responses do not occur, in contrast to necrosis, which is accompanied by pronounced inflammation.

1.3.2.2 Mechanisms

Apoptosis is a highly conserved and strictly regulated form of cell death, which is reflected in the stereotypic morphological changes that occur during the execution of the apoptotic programme.

The crucial component of this apoptotic machinery is a family of proteases, called caspases. Caspases are cystein proteases that cleave their target proteins after specific aspartic acid residues (name "caspase" originates from *c*ysteinyl *as*partate-specific protein*ase*) (Green 2000). According to their substrate specificity, the 14 caspases identified to date can be divided into subgroups of which the initiator (caspase-6, -8, -9, -20) and effector caspases (caspase-2, -3, -7) are involved in apoptosis (Chang and Yang 2000). Caspases can be found in all mammalian cells where they are synthesized as inactive proenzymes (zymogenes). Activation occurs by proteolytic processing, mostly by other caspases.

Activation of initiator caspases

Two distinct pathways leading to the activation of initiator caspases can be distinguished. The first is triggered by ligand binding to death receptors, the second involves the mitochondria.

Upon ligation of a death receptor, adaptor proteins, such as FADD, are recruited to the cytoplasmic tail of the receptor *via* their death domain. FADD in turn recruits procaspase-8 and –10 *via* the death effector domain, which brings these zymogenes into proximity with one another and allows the autocatalytic processing of the procaspases to form the mature enzymes (Ashkenazi and Dixit 1998). The activated initiator caspases can then cleave and activate several downstream caspases, the effector caspases. (see 1.4. for more detail). *Via* the cleavage of the proapoptotic bcl-2 family protein Bid, caspase-8 can activate the mitochondrial pathway by inducing cytochrome c release (Green 1998).

The mitochondrial pathway is associated with a drop in the mitochondrial membrane potential, involving the opening of a mitochondrial channel at the inner membrane, called the permeability transition pore (Green 1998). This leads to swelling of the mitochondrial matrix, rupture of the outer membrane and release of cytochrome c into the cytoplasm. Cytochrome c associates with Apaf1, procaspase-9 and dATP to form the apoptosome complex, which results in activation of caspase-9. Cytochrome c release is triggered by various cellular stresses, including cytotoxic drugs, growth factor withdrawal, heat, free radicals, hypoxia, and DNA damage (Chang and Yang 2000, Green 1998).

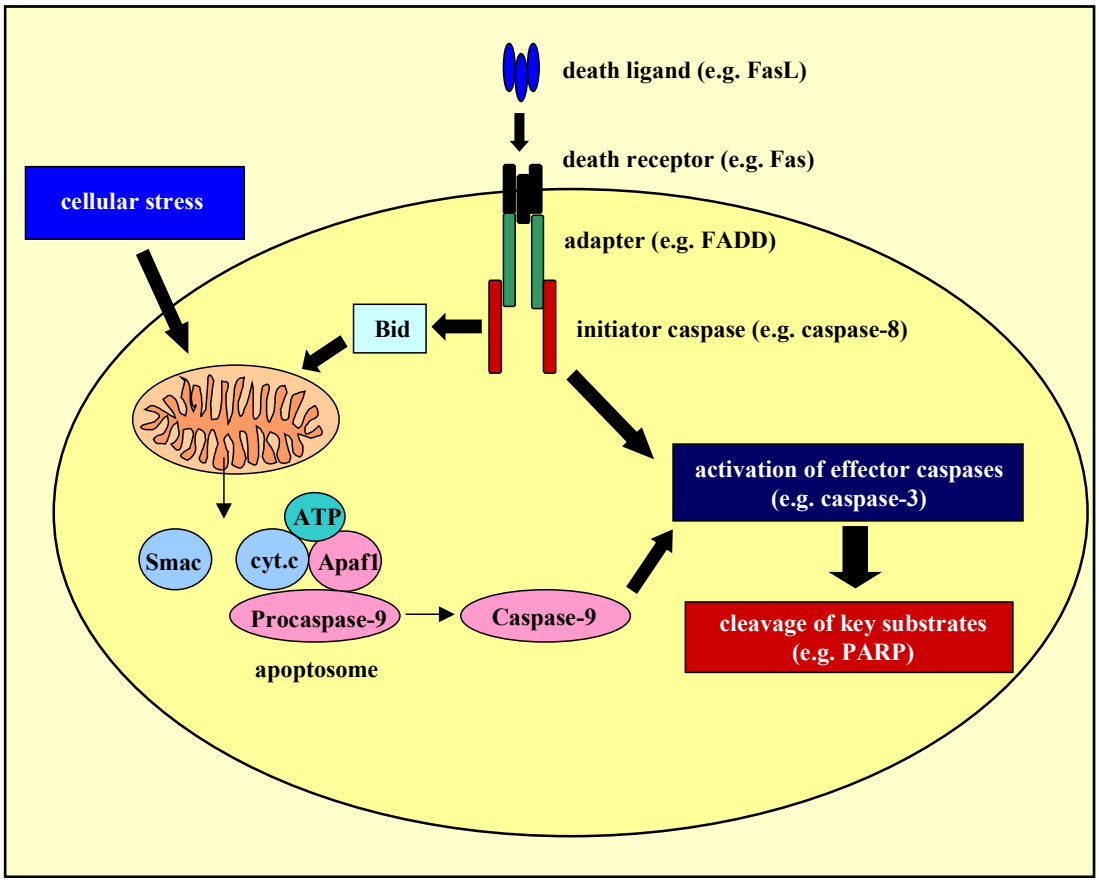


figure 2: pathways of caspase activation and apoptosis

Execution of apoptosis

The initiator caspases activate effector or executioner procaspases, acting in a caspase cascade (Thornberry and Lazebnik 1998, Chang and Yang 2000). Effector caspases cleave a variety of cellular substrates possessing a specific tetrapeptide recognition sequence. The cleavage of these key substrates in the cell finally leads to the execution, packaging and disposal of the cell. Among the targets of effector caspases are several proteins stabilizing the cell structure, such as actin, lamin A, and α-fodrin. Cleavage of these proteins results in destruction of cell structures, such as the cytoskeleton and the nucleus. Moreover, caspases inactivate various regulatory proteins that possess anti-apoptotic properties, including the DNA repair enzyme PARP (poly(ADP-ribose) polymerase) and the antiapoptotic Bcl-2 family proteins Bcl-2 and Bcl-x_L (Chang and Yang 2000, Thornberry and Lazebnik 1998). Taken together, caspases lead to the elimination of the dying cell, by cutting off cell-cell-contacts, reorganization of the cytoskeleton, inhibition of DNA repair and replication, destruction of DNA and nuclear structure, induction of phosphatidylserine exposure which targets the cell for phagocytosis, and disintegration into apoptotic bodies.

1.3.2.3 Apoptosis in vascular disease

Endothelial cells regulate the homeostasis of the vessel wall by influencing vascular tone, adhesion of platelets and monocytes, growth of smooth muscle cells, and production of extracellular matrix (Alvarez et al. 1997, Vane et al. 1990). Injury of the endothelial monolayer by apoptotic cell death is regarded as a critical event in the pathophysiology and progression of inflammatory diseases, such as atherosclerosis, endotoxic shock or ischemia/reperfusion injury (Brouard et al. 2000, Choy et al. 2001). Endothelial apoptosis is an initial event in the development of atherosclerosis, since it leads to increased endothelial cell turnover which critically impairs the function of the endothelium (Hennig et al. 1999). The resulting loss of vascular wall integrity and increase in vascular permeability promotes the migration and deposition of lipids, monocytes and smooth muscle cells into the intima, where they can cause further damage and initiate plaque development (Choy et al. 2001). Infiltrating leucocytes secrete growth factors and cytokines which stimulate smooth muscle cell proliferation. Moreover, endothelial apoptosis, e.g. induced by cytokines like TNF-α, propagates atherogenesis by increased coagulation, since apoptotic endothelial cells become procoagulant by exposure of phosphatidylserine residues and extracellular matrix on the surface (Guevara et al. 2001). Coagulation can further increase proliferation of smooth muscle cells and macrophages.

Taken together, apoptosis of endothelial cells plays a fundamental role in the pathophysiology of inflammatory diseases, as it compromises the physiological functions of the endothelium, important for vascular homeostasis, leading to endothelial dysfunction (Choy et al. 2001). Cytoprotective proteins counteracting proapoptotic events therefore represent a promising target for the therapy of atherosclerosis as they affect atherosclerotic lesion formation at an initial step. The heat shock protein HO-1, discussed in detail under 1.2, possesses potent antiinflammatory (Willis et al. 1996, Poss and Tonegawa 1997) and antiapoptotic properties (Petrache et al. 2000, Brouard et al. 2002) and therefore constitutes such an interesting target. The present work analyzes the mechanisms leading to upregulation of HO-1 in response to ANP and aspirin and investigates whether upregulation of HO-1 can confer protection against apoptosis induced by TNF-α, a cytokine produced during inflammatory conditions. Since TNF-α plays an important role in the propagation of inflammatory diseases the next chapter gives an introduction into effects and signaling mechanisms of this important cytokine.

1.4 Tumor necrosis factor

1.4.1 Overview

Tumor necrosis factor α (TNF-α) is a potent proinflammatory cytokine, produced by many cell types, including macrophages, monocytes, lymphocytes, and fibroblasts, in response to inflammation, infection, injury, and other environmental challenges (Baud and Karin 2001). TNF-α belongs to a growing family of cytokines that, apart from lymphotoxin, LTα, are expressed as membrane-bound surface proteins from which the soluble forms are released by proteolytic cleavage. First reports about the existence of a biological substance, possessing anticancer activity, date back to the 19th century, when Coley observed the shrinkage of tumors in patients undergoing a severe bacterial infection (Idriss and Naismith 2000). However, it was only in 1984 that human TNF was isolated from activated macrophages and T-cells and its cDNA cloned. In the meantime, TNF-α is known to exert a broad spectrum of effects, including lymphocyte and leukocyte adhesion, activation and migration, induction of other cytokines and immunoregulatory molecules, fever, shock, acute phase response, cell proliferation, differentiation, and apoptosis (Baud and Karin 2001, Liu et al. 1996). Therefore, TNF-α plays a key role in the regulation of immune function and the inflammatory response. Sustained elevation of TNF-α has been implicated in a variety of different inflammatory diseases, such as atherosclerosis, sepsis, and rheumatoid arthritis (deMartin et al. 2000).

1.4.2 Signaling by TNF-α

TNF-α exerts its numerous effects by interaction with two distinct cell surface receptors, the TNF-R1 (p55) and the TNF-R2 (p75). These receptors belong to the TNF receptor superfamily, the members of which possess an intracellular tail, a single transmembrane domain and an extracellular ligand-binding domain (Wajant and Scheurich 2001). TNF-α occurs in the circulation as a homotrimer of soluble TNF or as the membrane-bound form. Upon binding to TNF-R1 or TNF-R2 it leads to receptor trimerization, triggering a number of intracellular events that eventually result in such diverse effects as induction of apoptosis, activation of NF-κB or activation of MAP kinases or Akt (Wajant and Scheurich 2001).

The TNF-R1 possesses an intracellular protein-protein interaction motif of about 90 amino acids, called the death domain (DD) (Wallach et al. 1997). After binding of TNF-α to the TNF-R1, several adapter proteins are recruited to the receptor. The first protein recruited to the TNF-R1 *via* its DD is TRADD (TNFR1–associated death domain protein) which serves as a platform for three further signaling proteins, the DD containing RIP (receptor-interacting protein-1) and FADD (Fas-associated death domain protein), and the non-DD containing TRAF-2 (TNF-receptor-associated factor-2) (Chen and Goeddel 2002). These proteins recruit key enzymes to the receptor that mediate the downstream signaling events (figure 3).

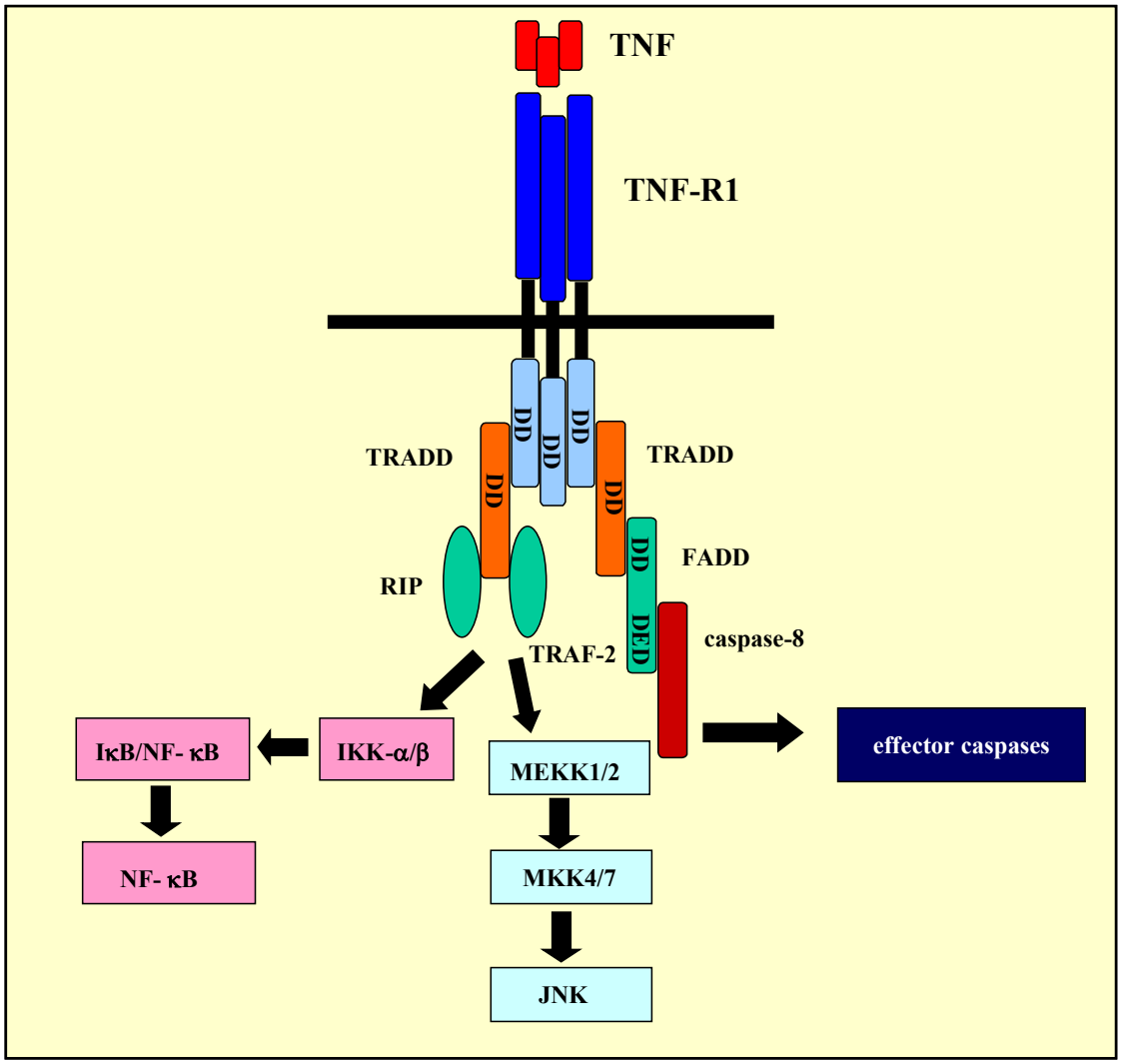


figure 3: signaling by TNF-R1

TRAF-2 and RIP mediate the activation of NF-κB by recruiting the IκB kinase multiprotein complex to the receptor where the catalytic IKKα and IKKβ subunits become activated by phosphorylation, a step that has been associated with different kinases, such as NIK (NF-κB-inducing kinase) and MEKK-1 (Baud and Karin 2001, Wajant et al. 2001). The activated IKK complex in turn phosphorylates IκB, resulting in its ubiquitination, degradation and translocation of the released NF-κB to the nucleus where it initiates the transcription of different genes, frequently involved in acute and chronic inflammatory states, such as adhesion molecules and other cytokines (Baud and Karin 2001). On the other hand, NF-κB activation mediates the expression of a variety of early responsive anti-apoptotic genes after binding of TNF-α to the TNFR-1, such as the bcl-2 member A1 (Ackermann et al. 1999) and members of the inhibitor of apoptosis family, cIAP1 and 2 (Devereaux et al. 1999).

TRAF-2 has also been shown to mediate the activation of the three MAP kinase pathways leading to activation of JNK, p38 MAPK and ERK (Wajant and Scheurich 2001). As outlined in chapter 1.7, all three MAP kinases have been implicated in the regulation of DNA binding activity of the transcription factor AP-1, providing another opportunity for TNF-α to influence the activity of important target genes.

Apart from regulating gene transcription by activation of important transcription factors, another important effect elicited by TNF-α *via* the TNF-R1 is activation of the caspase cascade resulting in apoptosis. TNF-α initiates apoptosis by recruiting FADD to the TNF-R1-TRADD complex. FADD serves as an adapter protein for caspase-8 with which it can interact *via* its death effector domain (Ashkenazi and Dixit 1998, Chen and Goeddel 2002). Recruitment to the receptor leads to autocatalytic activation of caspase-8, initiating the activation of the caspase cascade including effector caspases such as caspase-3 (see chapter 1.3.2.2).

Binding of TNF-α to the TNF-R2 which does not possess a death domain leads to the direct recruitment of TRAF-2 to the receptor. TRAF-2 in turn mediates the activation of the MAP kinases and NF-κB as well as the antiapoptotic components cIAP1 and 2. Since this receptor lacks a death domain, it is not involved in the induction of apoptosis by TNF-α (Wajant and Scheurich 2001).

1.4.3 TNF-α and the endothelium

The endothelium constitutes a major target for proinflammatory cytokines such as TNF-α, which has been reported to play a central role in the pathophysiology and progression of inflammatory diseases, such as atherosclerosis. TNF-α-induced endothelial injury by apoptosis of endothelial cells is regarded as a critical event that can initiate the development of atherosclerosis (Choy et al. 2001) (for the role of apoptosis in vascular disease see 1.3.2.3). Moreover, TNF-α is also able to induce reorganization of the cytoskeleton resulting in formation of intercellular gaps, increase the expression of adhesion molecules, and induce the release of vasoactive substances (Wójciak-Stothard et al. 1998, Madge and Pober 2001). Exposure of endothelial cells to TNF-α, produced during inflammatory processes, thus critically compromises the physiological functions of the endothelium and can therefore serve as the initial trigger for the development of atherosclerosis. Consequently, it is of great interest to elucidate signaling mechanisms leading to the expression of cytoprotective proteins that are able to block TNF-α-induced apoptosis as the initial event in atherosclerotic lesion formation. In this context, ANP and aspirin were tested for their ability to upregulate heat shock proteins and to interfere with TNF-α-induced apoptotic cell death.

1.5 The natriuretic peptides

1.5.1 History

In 1981 de Bold et al. made the interesting observation that i.v. application of atrial muscle extracts into rats resulted in a massive natriuresis and diuresis (de Bold et al. 1981). It was assumed that the atrial extract contains a potent natriuretic factor and soon afterwards the first member of the family of natriuretic peptides, the "atrial natriuretic peptide", ANP, was isolated and cloned (Kangawa and Matsuo 1984). A few years later, a structurally homologous peptide with similar pharmacological spectrum to ANP was isolated from acidic extracts of porcine brain and therefore designated "brain natriuretic peptide", BNP, but was soon discovered to be predominantly expressed in the ventricle (Sudoh et al. 1988). In addition to BNP, another natriuretic peptide was isolated from porcine brain and named "C-type natriuretic peptide", CNP, according to ANP and BNP (Sudoh et al. 1990). In the kidney, the atrial natriuretic peptide gene as well undergoes alternative processing of the precursor, yielding a modified peptide, called urodilatin which was isolated from human urine (Schulz-Knappe et al. 1988, Forssmann et al. 2001). The latest member of the natriuretic peptide family was isolated from the venom of the snake *Dendroospis angusticeps* by Lisy et al. and named DNP, possessing potent natriuretic activity in humans (Lisy et al. 1999).

1.5.2 Structure

The common structural feature of the natriuretic peptides is a 17-amino acid peptide ring, formed by a disulfide-bond between two cystein residues (at positions 105 and 121). The amino acids in the ring structure are highly conserved and this structure appears to be essential for receptor recognition and biological function (Inagami et al. 1987). Due to the strong homology within the ring structure, 10 of these amino acids are identical in all natriuretic peptides, whereas the amino acids either amino-terminal or carboxy-terminal to the ring structure are quite divergent (figure 1).

ANP: ANP is a 28 amino acid peptide. Its amino acid sequence is almost identical throughout mammalian species with the only exeption at position 110, which is methionine in humans, dogs, and cows, compared to isoleucine in rodents, such as mouse, rat, and rabbit.

BNP: BNP is composed of 32 amino acids and its ring structure differs from that of ANP in only 5 amino acids. Its amino acid sequence exhibits only 59% sequence homology between different species and is therefore much less conserved compared to ANP (Rosenzweig and Seidmann 1991).

CNP: CNP contains only 22 amino acids and possesses the primary structure with the greatest difference compared to the other natriuretic peptides, since it completely lacks the C-terminal

tail and ends at the C-terminus after the last cystein participating in the formation on the 17-residue ring (Rosenzweig and Seidmann 1991).

Urodilatin: Urodilatin is the product of the atrial natriuretic peptide gene expressed in the kidney and is generated by alternative biochemical processing of the ANP prohormone. It only differs from ANP in 4 additional amino acids added to the N-terminus (Schulz-Knappe et al. 1988).

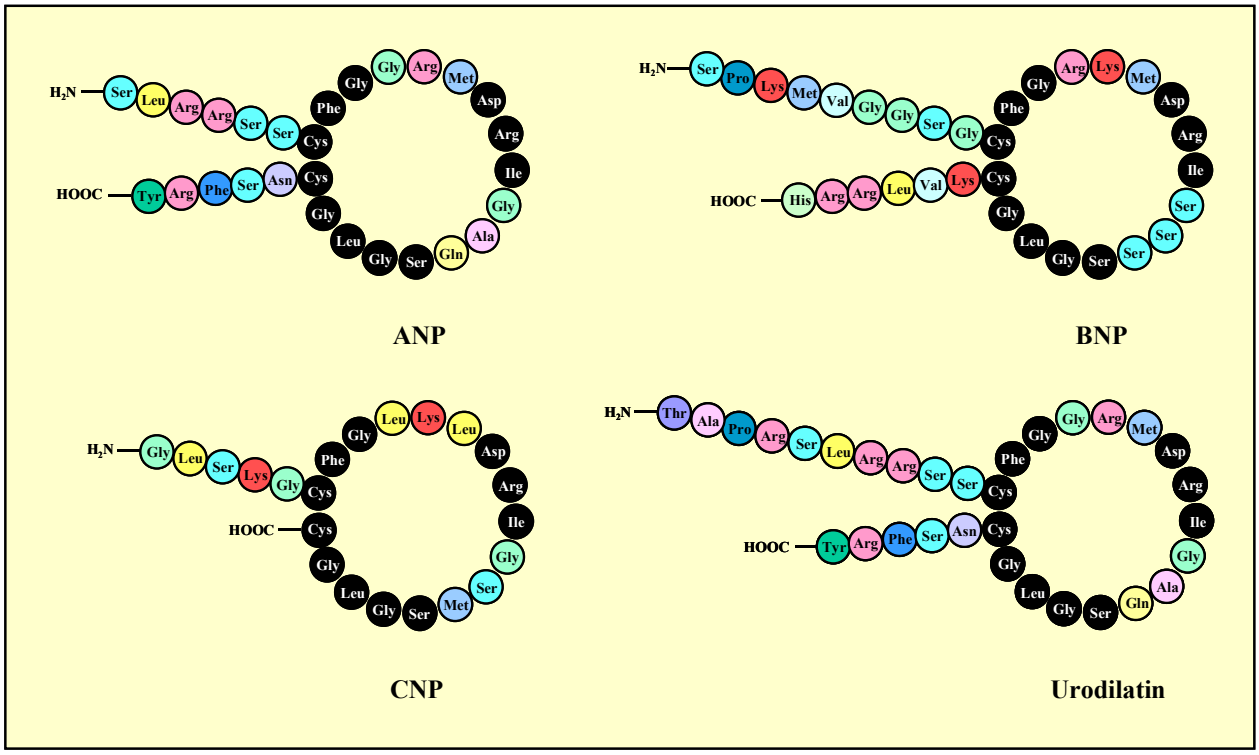


figure 4: structure and amino acid sequence of the human natriuretic peptides (ANP, BNP, CNP and urodilatin). The homologous amino acids are shaded black.

1.5.3 Biosynthesis

Each natriuretic peptide is encoded by a separate gene. As characteristic for peptide hormones, the natriuretic peptides are synthesized as pre-pro-hormones. ANP is synthesized primarily in the cardiac atria as a precursor of 152 amino acids, called prepro-ANP. Cleavage of a signal peptide at the N-terminus yields a prohormone of 126 amino acids that is stored in atrial granules (Rosenzweig and Seidmann 1991). In response to certain stimuli, such as increases in atrial strech or pressure, pro-ANP is cleaved by serine proteases into equimolar amounts of a 98 amino acid amino-terminal fragment (ANP 1-98) and the biologically active 28 amino acid carboxy-terminal fragment, the active ANP (ANP 99-126) (Rosenzweig and Seidmann 1991). The amino-terminal fragment is further cleaved into smaller fragments (ANP 1-30, ANP 31-67, ANP 79-98) which also possess biological activity, but information

regarding their biological significance is limited. The mature ANP is released from atrial myocytes into the circulation by exocytosis (Dagnino et al. 1991). The synthesis of the other natriuretic peptides is analogous to ANP, the synthesis of a precursor protein, followed by cleavage into biologically active fragments.

1.5.4 NP Receptors and Signaling

1.5.4.1 History

The discovery of the natriuretic peptides and their potent natriuretic and diuretic properties raised the question how these effects are mediated. The first step towards an understanding of the signaling mechanisms of ANP and its relatives was the observation that injection of ANP results in formation of cyclic guanosine-3‘, 5‘-monophosphate (cGMP) (Cantin et al. 1984). Further studies were able to demonstrate that cGMP is the second messenger mediating most of the effects of ANP (Leitmann and Murad 1986). Investigation of the binding sites for ANP demonstrated the presence of two binding proteins of different size. Only interaction with one of these proteins leads to the formation of cGMP (Leitmann et al. 1986). The other non-guanylate cyclase coupled receptor was characterized by Maack et al. and termed „clearance receptor“, since it was found to be responsible for the clearance of natriuretic peptides from the circulation (Maack et al. 1987). In 1989 Schulz et al. identified the cDNA sequence of two guanylate cyclase coupled receptors, which were designated NPR-A and NPR-B.

1.5.4.2 Structure and ligand selectivity

Figure 5 shows the structure of the NP receptors which are single transmembrane domain receptors. The two guanylate cyclase-coupled receptors, 120-140 kD in size, consist of an extracellular ligand binding domain, a single transmembrane sequence and an intracellular domain containing a juxtamembrane domain, kinase homology domain, dimerization domain, and the guanylate cyclase catalytic domain (figure 2) (Sharma 2002, Misono 2002). Thus, in these receptors, receptor and effector systems reside in one molecule. The NPR-C, 60 kD in size, is the most abundant receptor of the NPR and possesses a similar extracellular domain compared to the other NPR, but only a truncated intracellular tail of 37 amino acids (Levin 1993, Tremblay et al. 2002).

Due to the fact that the amino acid sequence of the extracellular domain is only 30-40% homologous (Cohen et al. 1996), the three receptors exhibit different ligand selectivity. The NPR-A binds NP in the order ANP>BNP>>CNP, whereby BNP is 10-fold less potent in stimulating this receptor (Misono 2002, Lucas et al. 2000). CNP is the specific ligand for the NPR-B with the highest affinity for this receptor (Suga et al. 1992). The NPR-C exhibits much lower ligand selectivity and binds all three ligands with the following degree of affinity: ANP>CNP>BNP (Maack et al. 1996, Silberbach and Roberts 2001).

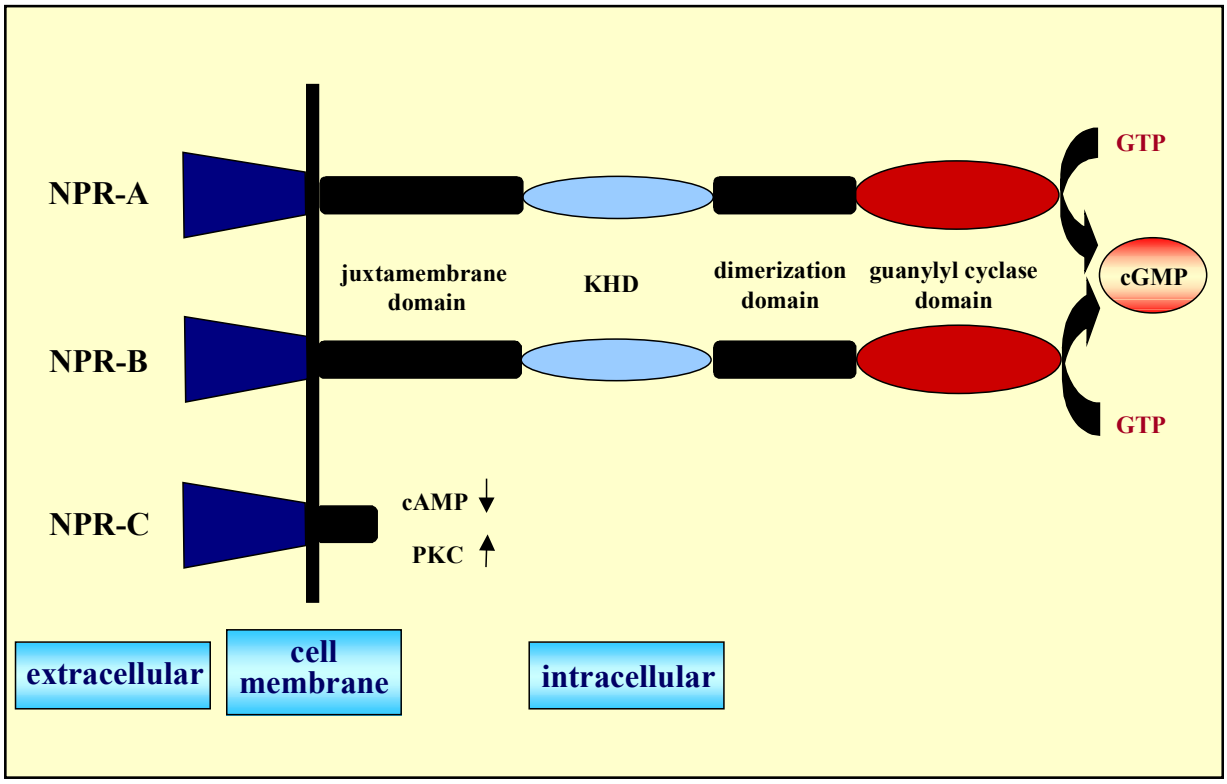


figure 5: model of the structure of the three natriuretic peptide receptors (NPR-A, NPR-B, NPR-C)

1.5.4.3 Signal transduction

The intracellular domain of the two guanylate cyclase-coupled receptors (NPR-A and NPR-B) contains a guanylate cyclase (GC) catalytic domain as well as a kinase-like domain. At the basal state the kinase homology domain (KHD) interacts with the GC-like domain suppressing its activity (Chinkers and Garbers 1989, Lucas et al. 2000, Sharma 2002). In the absence of ligand, the receptors exist in a homo-oligomerized state, each monomer being phosphorylated on key serine and threonine residues within the KHD (Chinkers and Wilson 1992, Potter and Hunter 1999). Oligomerization and phosphorylation are required for high affinity ligand binding. Binding of ligand induces a conformational change of the KHD leading to the binding of ATP to a glycine rich region in the KHD. ATP binding causes an allosteric change in the GC-like domain resulting in the derepression of guanylate cyclase

activity and subsequent conversion of guanosine triphosphate into cGMP (Kurose et al. 1987, Chang et al. 1990, Chinkers et al. 1991, Potter and Hunter 1998). Besides, association of ATP with the KHD results in a reduction in ligand affinity of the extracellular domain (Larose et al. 1991) as well as in dephosphorylation of the KHD (Potter and Garbers 1992, Potter and Hunter 1999, Potter and Hunter 2001), which desensitisizes the receptors to ATP and ligand.

The primary function of the NPR-C is to act as a clearance receptor. After binding of ligand, the receptor complex is internalized, followed by lysosomal hydrolysis of the ligand and subsequent recycling of the receptor to the cell surface (Cohen et al. 1996). However, several studies provide evidence that this receptor has additional biological functions, such as the inhibition of adenylate cyclase activity (Savoie et al. 1995, Anand-Srivastava 1997). Moreover, ANP and NPR-C-selective analogues have been demonstrated to be able to activate phospholipase C. Both effects seem to be mediated *via* a signaling mechanism involving the participation of G-regulatory proteins (Levin 1993, Anand-Srivastava 1996, Murthy et al. 2000). The interaction of the NPR-C with these two intracellular effector systems, adenylate cyclase and protein kinase C, might explain some of the observed NPR-C-mediated effects, such as inhibition of endothelin production (Hu et al. 1992), proliferation and invasion of matrix by endothelial cells (Pedram et al. 1997), activation of endothelial NO synthase in gastric smooth muscle cells (Murthy et al. 1998), inhibition of COX-2 (Kiemer et al. 2002a), or inhibition of MAPK in astrocytes (Prins et al. 1996).

1.5.5 Distribution and known effects of the natriuretic peptides

ANP and BNP are both predominantly found in the heart as cardiac hormones. In adults the highest concentration of ANP is found in the left atrium, followed by the right atrium, the right ventricle and finally the left ventricle (Venugopal 2001). 1% of the total atrial mRNA codes for ANP (Venogupal 2001). Apart from the heart, ANP is also expressed in lower concentrations in the tissues of the lung, brain, kidney, adrenals, gastrointestinal tract, thymus and the eye (Vollmar and Schulz 1990b, Sahai and Ganguly 1992, Salzmann et al. 1998). The main pharmacological actions of ANP include the inhibition of the renin-angiotensin-aldosterone system and enhanced glomerular filtration rate, resulting in diuresis and natriuresis, increased vascular permeability, relaxation of the vascular smooth muscle, resulting in a reduction of blood pressure, as well as antimitogenic effects on endothelial, smooth muscle, and myocardial cells (Levin et al. 1998, Venugopal 2001, Suzuki et al. 2001). The central effects include inhibition of salt appetite and water drinking as well as inhibition of vasopressin secretion (Levin et al. 1998).

BNP is primarily expressed in the ventricles and atria of the heart followed by the brain and the amnion (Venugopal 2001). Like ANP, BNP is secreted to the circulation and exerts

similar biological effects. However, circulating levels of BNP are much lower than ANP levels.

CNP is predominantly synthesized in the nervous system and the endothelium of the blood vessels and exerts primarily autocrine and paracrine actions (Stingo et al. 1992, Venugopal 2001, Silberbach et al. 2001). CNP lacks the renal effects of ANP and BNP and therefore has no natriuretic or diuretic properties. It decreases arterial pressure (Stingo et al. 1992) and has also been implicated in cell growth control (Suga et al. 1993).

1.5.6 Cytoprotective effects of ANP

In the first years after the discovery of the natriuretic peptides, they were mainly associated with the regulation of blood pressure and cardiovascular homeostasis (Levin et al. 1998). Intensive research in the following years demonstrated the expression of the natriuretic peptides and their receptors in a variety of tissues outside the cardiovascular and renal system, including thymus (Vollmar and Schulz 1990a) as well as macrophages (Vollmar and Schulz 1994). The role of ANP in the immune system was further confirmed by the observation that ANP inhibits thymocyte proliferation and thymopoesis (Vollmar et al. 1996, Vollmar 1997). The antiinflammatory potential of ANP became obvious with the finding that ANP reduces the NO production in LPS-stimulated macrophages by inhibiting the expression of the inducible NO synthase at the transcriptional and posttranscriptional level (Kiemer and Vollmar 1997, Kiemer and Vollmar 1998). Other inflammatory systems attenuated in their expression by ANP are cyclooxygenase-2 (Kiemer et al. 2002a) and TNF-α (Kiemer et al. 2000a, Tsukagoshi et al. 2001). Due to these interesting effects, ANP might interfere with diverse pathological conditions associated with inflammatory responses. For instance, ANP has been demonstrated to confer protecion against ischemia/reperfusion injury (Gerbes et al. 1998, Bilzer et al. 1994), the induction of heat shock proteins being a possible mechanism, since ANP has been shown to evoke a heat shock response in rat livers undergoing ischemia and reperfusion (Kiemer et al. 2002b). Protective effects of ANP have also been reported for drug-induced nephrotoxicity (Polte et al. 2002, Capasso et al. 2000, Murakami et al. 1999) and for ventricular hypertrophy (Silberbach et al. 1999, Kishimoto et al. 2001). However, mechanisms mediating the expression of cytoprotective proteins in endothelial cells have as yet not been elucidated.

Taken together, the results of the past years indicate that ANP has a much broader pharmacological profile apart from its cardiovascular functions, suggesting that it plays a role in the immune system and exhibits immunomodulatory, antiinflammatory, and cytoprotective effects. To further elucidate the cytoprotective potential of ANP it is necessary to identify the intracellular targets as well as the signal transduction mechanisms mediating these protective effects. Heat shock proteins, especially HO-1, constitute a possible target for ANP in

endothelial cells and might contribute to its antiinflammatory, antiatherogenic, and cytoprotective potential. However, information on the influence of ANP on HO-1 in endothelial cells is rare and completely lacking concerning signal transduction mechanisms. The first part of the present work therefore investigates the mechanisms leading to upregulation of HO-1 after ANP treatment and the cytoprotective potential of this effect.

1.6 Aspirin

Aspirin and its active metabolite salicylate are the oldest known members of the family of nonsteroidal antiinflammatory drugs (NSAIDs), aspirin being the most widely used NSAID. The world production of aspirin has been estimated to amount to many thousand tons a year, with an average consumption of about 80-100 tablets per person per year in industrialized nations (Vane et al. 1990, Vane and Botting 1998).

1.6.1 History

The history of aspirin dates back until about 3,500 years ago, when in ancient Egypt, the Ebers papyrus already recommended the use of salicylate containing plants for the therapy of rheumatic pains. 1,000 years later, Hippocrates described the use of willow bark, containing salicylic acid, to reduce the pain of childbirth and to reduce fever (Vane et al. 1990). In the 18^{th} and 19^{th} century the first clinical trials were performed with willow bark, but it took until 1859 that the active compound, salicylic acid, was synthesized chemically by Herrmann Kolbe, resulting in the broad application of the drug as an external antiseptic, antipyretic, and antirheumatic drug (Vane and Botting 1998). In 1897, Felix Hoffmann, working as a chemist at Bayer, synthesized acetylsalicylic acid, by acetylating the hydroxyl group on the benzene ring of salicylic acid in order to eliminate the unpleasant taste of salicylic acid. This new drug was introduced in 1899 under the name aspirin, the “a” coming from *acetyl* and the “spir” coming from *Spirea ulmania*, the plant from which salicylic acid was originally isolated (Jack 1997). By the early 1900s, the therapeutic spectrum of aspirin was known to include antipyretic, antiinflammatory, and analgesic effects. Later, several other drugs were discovered that shared some or all of these effects, such as phenacetin, acetaminophen (paracetamol) and phenylbutazone. Due to the similarities in their therapeutic actions, these drugs were classified into one group, the aspirin-like drugs, also called nonsteroidal antiinflammatory drugs, to clearly distinguish them from the also antiinflammatory glucocorticoids (Vane et al. 1990, Jack 1997).

1.6.2 Known effects

Despite their diverse chemical structure NSAIDs possess the same therapeutic properties. They relieve the swelling, redness, and pain of inflammation, reduce general fever, and cure headache. Moreover, they also share the same side effects, causing gastric upset, delay the birth process, and in high doses damage the kidney. The antithrombotic effect is now recognized as a therapeutic action and used for the therapy of cardiovascular disease (Vane et al. 1990, Vane et al. 1998). The fact that this chemically diverse group of drugs all exert the same therapeutic actions and side effects strongly suggested that these drugs possess a common mode of action.

It was not until 1971 that Vane et al. found the inhibition of prostaglandin synthesis by inactivation of the enzyme cyclooxygenase to be responsible for the therapeutic actions of aspirin (Vane 1971). The enzymatically active cyclooxygenase, also called prostaglandin endoperoxide synthase (PGHS) was isolated in 1976 (Hemler and Lands 1976) and cloned in 1988 (Merlie et al. 1988) and catalyzes the first step in the biosynthesis of prostaglandins. It is a membrane-bound heme- and glycoprotein with a molecular weight of 71 kDa and two catalytic sites. The site exhibiting cyclooxygenase activity cyclases arachidonic acid and adds the 15-hydroperoxy group to yield prostaglandin G_2 (PGG_2). At the peroxidase site, PGG_2 is converted to prostaglandin H_2 (PGH_2), which is subsequently converted to the prostaglandins D_2, E_2, $F_{2\alpha}$, I_2 (prostacyclin) and TXA_2 by specific synthases. Both cyclooxygenase and peroxidase activity are combined in the same bifunctional protein molecule (Amann and Peskar 2002).

Two COX isoforms encoded by different genes are known to exist. The first isoform, COX-1, is constitutively expressed in the endoplasmatic reticulum of most cells and possesses physiological functions, playing an important role in tissue homeostasis. The inducible isoform, COX-2, is expressed in very low levels in the cells and can be upregulated by proinflammatory stimuli, such as LPS, and by cytokines, such as TNF-α, as well as growth factors (Xie et al. 1992). COX-2 produces prostaglandins that are involved in the inflammatory and mitogenic response. Aspirin inhibits both isoforms of COX by irreversible acetylation of a specific serine residue (serine 530 of COX-1, serine 516 of COX-2), abolishing the binding of arachidonic acid at the cyclooxygenase active site (Vane et al. 1998, Awtry and Loscalzo 2000). Since COX-2 is considered as a crucial mediator of inflammation, it has been suggested that the antiinflammatory actions of NSAIDs are due to the inhibition of COX-2, whereas the adverse effects, irritation of the stomach, renal toxicity, can be attributed to inhibition of the constitutive COX-1.

1.6.3 New therapeutic aspects and limits of the prostaglandin hypothesis

In recent years, the theory that inhibition of prostaglandin synthesis solely accounts for the therapeutic effects of aspirin has been questioned due to the observation that doses of aspirin that are effective in the treatment of inflammatory diseases are much higher than those required for inhibition of COX. Moreover, salicylic acid, lacking an acetyl group, was shown to be a weak inhibitor of COX, but is equally effective against arthritis compared to aspirin (Abramson and Weissmann 1989, Weissmann 1991). Finally, in recent years an increasing number of studies has reported additional protective effects of this non-steroidal anti-inflammatory drug against colon cancer (Thun et al. 1991, Sandler et al. 1998, Gupta et DuBois 1998), its ability to delay the onset of Alzheimer disease (Flynn and Theesen 1999, Breitner 1996), as well as protective effects in cardiovascular disease (Mehta 1998, Diener 1999). Inhibition of COX activity can only in part account for these recently observed effects, suggesting that aspirin may exert part of its antiinflammatory and antitumor activity by modulating cyclooxygenase-independent pathways.

Especially the beneficial therapeutic effect of aspirin in vascular disease is increasingly recognized (Numano et al. 1995, Lauer et al. 2002). The protection of the vasculature conferred by aspirin is primarily ascribed to the prevention of thrombus formation, but also to the aspirin-mediated protection of endothelial cells against the cytotoxic effects of activated platelets (Numano et al. 1995). In addition to its inhibitory action on platelet activation, representing a central event in vascular damage, aspirin has also been demonstrated to exert direct cytoprotective effects against oxidant-induced endothelial damage (Podhaisky et al. 1997). To further understand these cytoprotective actions of aspirin, it is necessary to elucidate the intracellular targets and the signal transduction mechanisms mediating these effects. In this context, new targets for the pharmacologic actions of aspirin have been described. Aspirin has been reported to interfere with enzyme systems, such as the mitogen-activated protein kinases (Tegeder et al. 2001) as well as with the activation of transcription factors, such as NF-κB (Kopp and Gosh 1994), which frequently initiates the transcription of different genes, such as adhesion molecules and cytokines, implicated in acute and chronic inflammatory states. Moreover, aspirin treatment has been demonstrated to result in inhibition of iNOS expression (Amin et al. 1995, Katsuyama et al. 1999), suppression of adhesion molecule expression and down-regulation of MCP-1 (Amberger et al. 1999), altogether important mediators of the inflammatory response. In several cell models, including endothelial cells, aspirin has been shown to increase the expression of the heat shock proteins HSP70 and HSP60 (Jin et al. 1999, Amberger et al. 1999). However, apart from one report describing an inhibitory action of aspirin on LPS/IFN γ-induced HO-1 expression (Chen et al. 2002), information on the influence of aspirin on this important heat shock protein, especially on basal HO-1 levels, is completely missing. Therefore, the second part of the present work

investigated the influence of aspirin on HO-1 expression, the signal transduction mechanisms involved and its potential cytoprotective action.

1.7 The MAP kinase family

Chapter 1.2 deals with heme oxygenase as a potential target, mediating cytoprotection against apoptosis of endothelial cells. This heat shock protein is regulated at the transcriptional level, mainly *via* the transcription factor AP-1. The members of the MAP kinase family are critically involved in the regulation of this transcription factor. The following chapter therefore gives an introduction in the complex network of this enzyme family.

The mitogen-activated protein kinases represent an evolutionary conserved signal transduction system activated in response to a great variety of extracellular stimuli, such as cytokines, growth factors, neurotransmitters, hormones, cellular stress, and cell adherence (Widmann et al. 1999). By regulating intracellular processes such as gene expression, cell proliferation, differentiation, survival or death, this family of serine/threonine protein kinases enables the cell to respond to such external stimuli (Barr and Bogoyevitch 2001). Mitogen-activated protein kinases are expressed in all eukaryotic cells. To date, more than twelve MAP kinases have been identified in mammalian cells, that form a complex network of signaling and belong to three different subfamilies: the extracellular-signal-regulated kinases (ERK), the c-jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK), and the p38 MAPK (Widmann et al. 1999) **(figure 6)**.

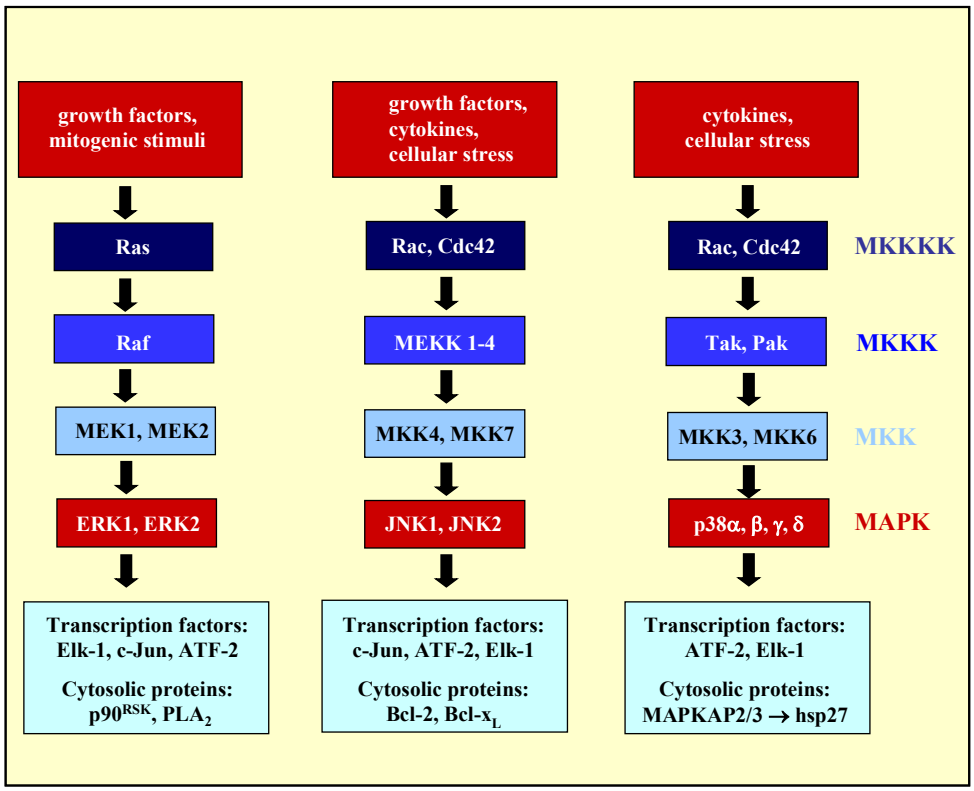


figure 6: mitogen-activated protein kinase (MAPK) signal transduction pathways

All MAP kinases characteristically phosphorylate substrates on serine or threonine residues adjacent to proline residues and are therefore termed proline-directed serine/threonine kinases. Another characteristic feature of the MAP kinases is their activation by a phosphorylation cascade that consists of at least three kinases (Widmann et al. 1999). The first component of this pathway is a MAPK kinase kinase (MKKK). MKKK are serine/threonine kinases that are activated either by phosphorylation through a MAPK kinase kinase kinase (MKKKK) or by interaction with small GTP-binding proteins of the Ras or Rho family. Upon activation, MKKK phosphorylate and activate the next downstream MAPK kinase (MKK) (Widmann et al. 1999). MKK are dual-specificity enzymes that recognize and phosphorylate a threonine-X-tyrosine motif (X can be different amino acids depending on the respective MAP kinase) in an amino acid sequence called the activation loop of their MAP kinase substrates. This phosphorylation induces a conformational change of the activation loop, aligning the catalytic amino acid residues and enabling substrate binding and catalytic activity. Phosphorylation on both threonine and tyrosine is required for activation of MAP kinases, which then in turn phosphorylate and activate substrates on serine and threonine residues. Substrates for MAP kinases include transcription factors, other protein kinases, phospholipases, and cytoskeleton-associated proteins (Widmann et al. 1999).

In addition to the regulation by phosphorylation of a characteristic motif in the activation loop by the upstream kinases, MAP kinase activity is controlled by a family of dual-specificity MAPK phosphatases (MKP), which remove the phosphates of the critical tyrosine and threonine residues. The MKP can be devided into two groups. Either, they are encoded by growth factor or stress-inducible genes and are predominantly expressed in the nucleus, or they are not transcriptionally regulated and located in the cytosol. Different MKP specifically dephosphorylate and inactivate the members of the different MAPK subfamilies (Pearson et al. 2001).

1.7.1 Extracellular signal-regulated kinase (ERK)

This subfamily includes the kinases ERK-1 and ERK-2, which are ubiquitously expressed 44- and 42-kDa isoforms, activated by extracellular stimuli, such as growth factors (EGF, PDGF) or mitogenic stimuli. Many different receptor types are capable of activating the ERK pathway, such as tyrosine kinase receptors, G-protein coupled receptors, and cytokine receptors. Ligand binding of these receptors mostly leads to the recruitment of Ras to the receptor *via* different adapter proteins, promoting formation of Ras:GTP (Downward 1996) at the plasma membrane, which recruits the MKKK Raf1 to the membrane, where it is activated by phosphorylation at tyrosine, serine and threonine residues (Marais and Marshall 1996). This in turn leads to the activation of MEK-1/2, the MKK in the phosphorylation cascade of the MAPK. MEK-1 and MEK-2 act as dual threonine/tyrosine kinases, phosphorylating the Thr-Glu-Tyr sequence in the activation loop of the catalytic domain of ERK-1/2 (Whitmarsh and Davis 1996).

Activated ERK-1/2 phosphorylate substrate proteins at serine or threonine residues located within a proline-directed motif. Cytosolic proteins reported to be phosphorylated by ERK are the p90 S6 kinase RSK, MAP kinase-interacting kinase (Mnk), cytosolic phospholipase A_2 and the juxtamembrane region of the EGF receptor (Lin et al. 1993, Seger and Krebs 1995, Whitmarsh and Davis 1996). Besides, ERK-mediated phosphorylation of the EGF receptor, Raf1 and Mek1 reduces their activity, providing a negative feedback mechanism for the control of the activity of the ERK pathway (Whitmarsh and Davis 1996). Apart from the modulation of cytoplasmatic proteins, activated ERK translocates to the nucleus, where it can phosphorylate a number of different transcription factors, such as Elk1, c-Myc, Ets1 or STAT proteins (Widmann et al. 1999).

ERK activation is associated with enhanced cell proliferation (Seger and Krebs 1995, Pages et al. 1993) and differentiation (Qui and Green 1992) and implicated in cell cycle regulation in some cell types (Tanemoto et al. 1992). Moreover, activation of ERK is reported to confer protection against apoptosis in several cell types (Xia et al. 1995, Gardner and Johnson 1996). This cytoprotective effect is suggested to occur by inhibition of cytosolic caspase activation and phosphorylation of the proapoptotic Bcl2-family protein Bad (Cross et al. 2000).

1.7.2 The c-jun N-terminal kinase (JNK)

This subfamily of the MAP kinases was identified in 1991 and comprises isoforms that are the products of 3 different genes. Differential splicing and selection of alternative exons results in a total of 10 different JNK isoforms (Gupta et al. 1996). JNK1 and JNK2 are expressed ubiquitously, whereas JNK3 is restricted predominantly to the brain and testes (Yang et al. 1997). JNK have been demonstrated to be activated in response to various extracellular stimuli, such as growth factors, cytokines, and cellular stress, like heat shock, hyperosmolarity, UV-radiation, DNA damage, and ischemia/reperfusion (Barr and Bogoyevitch 2001). JNK can be activated through different receptor types, including the TNF receptor family, G-protein coupled receptors, tyrosine kinase receptors, and cytokine receptors. Activation of the JNKs occurs by phosphorylation at threonine and tyrosine in the Thr-Pro-Tyr motif within the activation loop by MKK4 and MKK7 (Widmann et al. 1999). Upstream of MKK-4/7, several MKKKs have been implicated in the activation of the JNK pathway, including MEKK-1-4. These MKKKs phosphorylate MKK4/7 and activate their kinase activity (Barr and Bogoyevitch 2001). Further upstream, the small GTP-binding proteins of the Rho family, especially Rac and Cdc42, have been reported to function as upstream activators of the JNK pathway (Barr and Bogoyevitch 2001, Widmann et al. 1999).

The majority of the JNK substrates identified to date are transcription factors, such as c-jun, ATF-2, Elk-1, p53, DPC4 and NFAT4, resulting in efficient transcription of the target genes, since JNKs have the ability to activate transcription factors as well as to stabilize them (Widmann et al. 1999). In addition to transcription factors and nuclear proteins, further

substrates have been idenfied to be phosphorylated by JNK, such as the antiapoptotic bcl proteins Bcl-2 and Bcl-x_L (Kharbanda et al. 2000, Shia et al. 2001), suggesting a role for JNK in the regulation of apoptosis.

Activation of JNK is generally associated with stress responses, inflammation, and apoptotic cell death, and has been shown to be involved in apoptosis induction in response to growth factor withdrawal, stress, DNA damage, and binding of FasL on the cell surface (Widmann et al. 1999). A role for JNK in apoptosis has been described in several in vitro models (Xia et al. 1995, Hu et al. 1999, Wang et al. 1998). Initiation of c-jun-dependent transcription during apoptosis is suggested to lead to the expression of pro-apoptotic proteins, such as Fas ligand (Kasibhatla et al. 1998). However, there are also investigators reporting that JNKs are not involved in the apoptotic response (Liu et al. 1996) and can even enhance cell survival under certain circumstances (Roulston et al. 1998).

1.7.3 p38 MAPK pathway

The p38 family consists of four homologous isoforms (α, β, γ, δ) that are the products of different genes. HUVEC produce p38 α, β, γ protein, whereas p38 δ is not found in these cells (Herlaar and Brown 1999). Different p38 MAPK are activated by cellular stress, such as UV irradiation, osmotic shock, heat shock, lipopolysaccharide, protein synthesis inhibitors, by certain cytokines, such as TNF-α or IL-1, and by G-protein coupled receptors (Whitmarsh and Davis 1996, Herlaar and Brown 1999). Similar to ERK and JNK, p38 MAPK is activated by phosphorylation on threonine and tyrosine in the Thr-Gly-Tyr activation motif (Raingeaud et al. 1995). MKK3 and MKK6 are the protein kinases responsible for this phosphorylation step, and these MAPKK themselves are in turn targets for the upstream regulators MKKK, such as Tak1 or the p21-activated kinase (Pak), that has been shown to be activated by the small G-proteins of the Rho family, Cdc42 and Rac1 (Bagrodia et al. 1995, Zhang et al. 1995). Other MAPKKK are discussed to play a role in MKK3/6 activation, including Tak1, Ask1, and Sprk (Widmann et al. 1999).

Upon activation, p38 MAPK can phosphorylate and activate a number of different substrates, such as MAPK-activated protein (MAPKAP) kinase 2 and 3 which in turn phosphorylate small heat shock proteins such as the 27-kDa heat shock protein HSP27 (Rouse et al. 1994), an F-actin binding protein involved in cytoskeleton structure. Other substrates targeted by p38 MAPK are transcription factors. p38 MAPK phosphorylates ATF2, Elk1, as well as Chop, a member of the C/EBP family of transcription factors.

Like JNK, p38 MAPK is implicated to play a role in apoptosis (Schwenger et al. 1997, Brenner et al. 1997, Takahashi et al. 2002). Moreover, it is suggested to affect many different biological functions, such as the production of cytokines in hematopoetic cells, cytokine-

stimulated cell proliferation, platelet activation or cardiac hypertrophic growth (Widmann et al. 1999). Therefore, p38 MAPK plays a major role in cytokine signaling and production and is associated with stress responses, inflammation and apoptosis.

All three MAP kinases are involved in the regulation of the transcription factor AP-1, that plays a pivotal role in the transcriptional regulation of HO-1. Therefore, the next chapter outlines the regulation of AP-1 activity by MAP kinases.

1.7.4 Regulation of the transcription factor AP-1 by MAP kinases

The redox-sensitive, inducible transcription factor AP-1 comprises a family of related transcription factors, the majority of which consists of either c-jun/c-fos heterodimers or c-jun/c-jun homodimers. AP-1 has been implicated in the regulation of a wide range of genes involved in a great variety of biological processes, such as cell proliferation, differentiation, inflammatory processes, and apoptosis. AP-1 activity has been reported to be regulated by several members of the MAP kinase family at the level of transcription of jun and fos genes as well as by posttranslational modifications. Due to the described role of AP-1 in HO-1 induction (Camhi et al. 1998, Lavrovsky et al. 1994), the following chapter will give an overview of the regulation of AP-1 activity by MAP kinases.

ERK activation leads to the phosphorylation of the transcription factor Elk1, which stimulates its ability to bind to the serum response elements (SRE) in the promotor of the c-fos gene, forming ternary complexes with serum response factor and DNA and activating transcription. Therefore, activated ERK stimulates AP-1 activity through induction of c-fos expression, since increased amounts of c-fos protein can combine with c-jun after translocation to the nucleus to form AP-1 dimers (Karin 1995, Pearson et al. 2001). Moreover, ERK phosphorylation and activation of the S6 kinase p90rsk results in translocation of this kinase to the nucleus, where it regulates c-fos and c-jun activity (Widmann et al. 1999). c-jun is also a direct target for ERK, but phosphorylation by ERK does not occur at the N-terminal stimulatory sites, but instead at one of the inhibitory sites located next to the C-terminal DNA binding domain, therefore resulting in decreased DNA binding (Karin 1995, Pearson et al. 2001).

p38 MAPK is reported to activate ATF-2 by phosphorylation at Thr-69 and Tyr-71 within its N-terminal activation domain, resulting in increased transcriptional activity and therefore induces c-fos expression (Widmann et al. 1999, Barr and Bogoyevitch 2001). Elk1 is also phosphorylated by p38 MAPK on several residues within the C-terminal transactivating domain (Widmann et al. 1999, Barr and Bogoyevitch 2001, Pearson et al. 2001).

JNK regulates AP-1 activity by phosphorylating c-jun and activating transcription factor-2 (ATF-2), stimulating their ability to heterodimerize and activate transcription via the TPA

(12-O-tetradecanoylphorbol-13-acetate) response element (TRE), therefore inducing increased c-jun expression (Karin 1995). Elk1, involved in the induction of the c-fos gene, is another direct target of JNK (Widmann et al. 1999, Barr and Bogoyevitch 2001). Moreover, JNK-mediated phosphorylation of serine 63 and 73 within the N-terminal activation domain of c-jun leads to a posttranslational upregulation of c-jun activity. This phosphorylation results in increased stability of c-jun and increases its DNA binding affinity as well as transcriptional activity as either homodimer or heterodimer with c-fos (Karin 1995, Pearson et al. 2001, Barr and Bogoyevitch 2001).

Given the pivotal role of AP-1 in the transcriptional control of the heme oxygenase gene, the influence of ANP and aspirin on MAPK and AP-1 activity were investigated in the present work.

2 MATERIALS AND METHODS

2.1 Materials

Rat ANP 99-126 ("ANP") was purchased from Calbiochem (Schwalbach, Germany). 8-Br-cGMP was from Sigma (Taufkirchen, Germany), and cANF from Saxon Biochemicals (Hannover, Germany). Tumor Necrosis Factor-α was obtained from Sigma-Aldrich Chemie GmbH, (Taufkirchen, Germany) and etoposide from Calbiochem (Schwalbach, Germany). Cell culture medium (M 199) and penicillin/streptomycine were from PAN (Aidenbach, Germany). Fetal calf serum (FCS) was from Biochrom (Berlin, Germany) and Endothelial Cell Growth Medium (ECGM®) from Promocell (Heidelberg, Germany). Endothelial Cell Growth Supplement was purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Plastic ware was from Peske, Aindling-Pichl, Germany. The enhanced chemoluminescence protein detection kit was from NEN (Köln, Germany), Complete® from Roche (Mannheim, Germany). Antiserum against the "von Willebrand-factor" was obtained from Serotec LTD (Wiesbaden, Germany). Anti-phospho-JNK and anti-phospho-ERK monoclonal mouse anti-human antibodies and anti-phospho-p38 polyclonal rabbit anti-human antibody were from Cell Signaling (Frankfurt/M., Germany), peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were from Jackson Immunolab (Dianova, Hamburg, Germany) and anti-p38 antibody from Calbiochem (Schwalbach, Germany). Anti-HSP70 antibody was obtained from Santa Cruz (Heidelberg, Germany) and anti-HSP32 (HO-1) monoclonal mouse anti-human antibody from BD Transduction Laboratories (Heidelberg, Germany). The primers for RT-PCR for GAPDH and HO-1 were purchased from MWG-Biotech AG (Ebersberg, Germany). All other materials were obtained from either Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) or Merck-Eurolab (Munich, Germany).

2.2 Cell culture

2.2.1 Solutions

PBS (phosphate buffered saline):

Na_2HPO_4	1.48 g (8.0 mmol/L)
KH_2PO_4	0.43 g (1.5 mmol/L)
NaCl	7.20 g (160 mmol/L)
H_2O	ad 1,000 mL

PBS (phosphate buffered saline)$^+$:

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.15 g
KH_2O_4	0.20 g
$MgCl_2$ x 6 H_2O	0.10 g
$CaCl_2$	0.10 g
H_2O	ad 1,000 mL

Trypsin/EDTA:

Trypsin (diluted 1:250 in PBS)	0.05 g
EDTA (Sigma)	0.20 g
PBS	ad 100 mL

2.2.2 Cell isolation of HUVEC

Human umbilical vein endothelial cells (HUVEC) were isolated by digestion of umbilical veins with 0.1 g/L of collagenase A (Roche, Mannheim, Germany) according to the method described by Jaffe et al. (Jaffe et al. 1973). Cells were either grown in endothelial cell growth medium (ECGM, Promocell, Heidelberg, Germany) or in M199 (PAN, Aidenbach, Germany) supplemented with 20% heat-inactivated fetal calf serum (FCS), 1 x endothelial cell growth supplement (ECGS, Sigma, Taufkirchen, Germany) and penicillin (100 U/mL)/ streptomycine (100 ng/mL). In order to compensate for inter-individual differences cells of at least two umbilical cords were combined for experiments. Cells were grown at 37°C and 5% CO_2 in an Heracell incubator (Heraeus Instruments, Munich, Germany).

2.2.3 Cultivation of HUVEC

2.2.3.1 Passaging

After reaching a confluent state, the cells were subcultered 1:3 in culture flasks or seeded in plates for experiments. For passaging of HUVEC, the medium was removed and the cells were washed three times with PBS. After addition of 1 ml or 2 ml trypsin/EDTA per 25 cm^2 or 75 cm^2 flask, respectively, the cells were incubated for 1-2 min at 37°C. HUVEC were carefully detached by gently tapping and the digestion was stopped by addition of M199 containing 10% FCS. The cells were centrifuged at 218 x *g* at 4°C for 10 min, the supernatant was discarded and the cells were resuspended in M199 supplemented with 20% heat-inactivated FCS, 1x endothelial cell growth supplement and penicillin (100 U/mL)/ streptomycine (100 ng/mL). For experiments, cells of passage three or four were grown until confluence in 6-, 12-, 24- or 96-well plates. Experiments were performed in M199 containing 10% FCS, in the absence of endothelial cell growth supplement, and the cells were allowed to adapt to the medium for 2 h before the start of the respective experiments.

2.2.3.2 Freezing, storage and thawing

For long-time storage, confluent HUVEC were detached with trypsin/EDTA, centrifuged at 218 x *g* at 4°C for 10 min, and the pellet was resuspended in 1.5 ml freezing medium (M199 supplemented with 20% heat-inactivated FCS, 1x endothelial cell growth supplement, penicillin (100 U/mL), streptomycine (100 ng/mL) and 10% [v/v] DMSO) per 75 cm^2 flask. The cell suspension was transferred to cryovials and frozen at –20°C for one day, afterwards at –86°C for one week, and then stored at –196°C in liquid nitrogen. This procedure was done in order to ensure gradual freezing of the cells. For thawing, HUVEC were warmed up and the cell suspension transferred into M199 supplemented with 10% FCS. In order to remove DMSO, cells were centrifuged at 218 x *g* at 4°C for 10 min. The supernatant was discarded and the cells resuspended in complete culture medium and transferred to a 75 cm^2 culture flask.

2.3 Characterization of HUVEC by means of von Willebrand factor

2.3.1 Immunfluorescence

2.3.1.1 Solutions

Methanol/acetone: mixture 1:1

PBS: see under 2.2.1

2.3.1.2 Antibody

Sheep anti-human von Willebrand Factor (Serotec LTD, Wiesbaden, Germany), FITC labeled

2.3.1.3 Experimental procedure

HUVEC were grown in 24-well plates until confluence. Cell Culture medium was removed and the cells were washed twice with PBS. Cells were fixed and permeabilized with ice cold methanol/acetone 1:1 at 4°C for 5 min. After washing with PBS, 200 µl sheep serum (20% [v/v] in PBS) was added in order to saturate unspecific binding sites. The plates were incubated for 30 min at room temperature. After addition of 10 µl von Willebrand antibody, incubation was continued for 30 min on ice in the dark. Cells were again washed with PBS and examined with a Zeiss Axioskop MC 80 DX microscope (Zeiss, Munich, Germany) with a 400- or 1,000-fold magnification (excitation: 450 nm = blue, emission: 520 nm = green).

2.3.2 Flow cytometry

2.3.2.1 Solutions

FACS buffer:

NaCl	8.12 g
KH_2PO_4	0.26 g
Na_2HPO_4	2.35 g

KCl	0.28 g
Na_2EDTA	0.36 g
LiCl	0.43 g
Na-azide	0.20 g
H_2O	ad 1,000 mL, pH 7.37

PBS: see under 2.2.1

Trypsin/EDTA: see under 2.2.1

2.3.2.2 Experimental procedure

HUVEC were grown in 24-well plates until confluence. Cell culture medium was removed and the cells were washed three times with PBS and trypsinized with 200 µl per well for 1-2 min at 37°C. The digestion was stopped by addition of M199 containing 10% FCS, and the cell suspension was transferred into FACS tubes. After centrifugation (218 x *g*, 4°C, 10 min) the cell pellet was washed twice with PBS. Cells were incubated with 0.25% paraformaldehyde for 1 h at 4°C to fix the cells. Subsequently, the cells were centrifuged (218 x *g*, 4°C, 10 min) to remove the paraformaldehyde, and the pellet was resuspended in 1 ml 2% Tween ([v/v] in PBS). After permeabilization for 15 min at 37°C, the cells were again centrifuged and washed twice with PBS. Afterwards, the cells were incubated with 10 µl of 20% sheep serum ([v/v] in PBS) for 30 min at room temperature to saturate unspecific binding sites, followed by incubation with von Willebrand antibody for 30 min on ice in the dark. Afterwards, the cells were washed once more with PBS and resuspended in 300 µl PBS for flow cytometric analysis. For the adjustment of instrument settings, control cells were prepared according to the procedure described above, but without addition of the antibody. For storage, cells were fixed with paraformaldehyde. The purity of the cells was judged by measuring the antibody treated cells indicating the percentage of von Willebrand positive cells. L929 cells treated as decribed above served as negative control.

2.4 Western blot analysis of proteins

2.4.1 Solutions and antibodies

RIPA buffer (lysis buffer) :

NaCl	150 mmol/L	
Tris-HCl	50 mmol/L	
Nonidet P40	1.00% (m/v)	
Deoxycholat	0.25% (m/v)	
SDS	0.10% (m/v)	
+ Na_3VO_4	1 mmol/L	
+ PMSF	1 mmol/L	added
+ Complete®	Protease inhibitor, 25x stock solution	freshly
NaF	50 mmol/L	before
$Na_4P_2O_7$	50 mmol/L	use

SDS sample buffer (5x):

Tris-HCl (pH 6.8) 3.125 mol/L	2 mL
Glycerol	10 mL (50% (v/v))
SDS 20% (m/v)	5 mL
DTT 16% (m/v)	2.5 mL
Pyronin Y 5% (m/v)	0.1 mL
H_2O	ad 20 mL

Resolving gel 10%:

Acrylamide 30%-bisacrylamide 0.8% solution (Roth)	5.0 mL
1.5 mol/L Tris-base pH 8.8 (Roth)	3.75 mL
10% SDS	0.15 mL
H_2O dest.	6.1 mL

The solution was degassed for 10 min, since polymerization requires anaerobic conditions. Afterwards, the polymerization reaction was started by addition of:

15 µl TEMED (cross linker)

75 µl APS 10% (m/v) (radical starter)

Stacking gel:

Acrylamide 30%-Bisacrylamide 0.8% solution (Roth)	1.7 mL
1.25 mol/L Tris-base pH 6.8	1 mL
10% SDS	0.1 mL
H_2O	7 mL

The solution was degassed for 10 min. Afterwards, the polymerization reaction was started by addition of:

20 µl TEMED (cross linker)

100 µl APS 10% (m/v) (radical starter)

Electrophoresis buffer:

Tris base	3 g
Glycin	14.4 g
SDS	1 g
H_2O dest.	ad 1,000 mL

Anode buffer I pH 10.4:

Tris base	30 g
Methanol	200 mL
H_2O dest.	800 mL

Anode buffer II pH 10.4:

Tris base	3 g
Methanol	200 mL
H_2O dest.	800 mL

Cathode buffer pH 7.6:

ε-Amino-n-caproic acid	5.2 g
Methanol	200 mL
H_2O	800 mL

Tris buffered saline solution containing Tween, pH 8.0 (TBS-T):

Tris base	3 g	(0.02 mol/L)
NaCl	11.1 g	(0.2 mol/L)
Tween 20	1 mL	(0.1 %)
H_2O dest.	ad 1,000 mL	

Primary antibodies:

Anti-HSP32 (HO-1) monoclonal mouse anti-human antibody
Anti-HSP70 polyclonal goat anti-human antibody
Anti-phospho-p38 MAPK polyclonal rabbit anti-human antibody
Anti-phospho-ERK monoclonal mouse anti-human antibody
Anti-phospho-JNK/SAPK monoclonal mouse anti-human antibody
Anti-α-tubulin rabbit antibody

Secondary antibodies:

Peroxidase-conjugated goat anti-rabbit IgG
Peroxidase-conjugated goat anti-mouse IgG
Peroxidase-conjugated donkey anti-goat IgG

2.4.2 Sample preparation

For detection of proteins, whole cell lysates of HUVEC were prepared. HUVEC were cultured in 12-well plates until confluence and were either left untreated or stimulated with ANP (10^{-9} – 10^{-6} mol/L), 8-Br-cGMP (10^{-3} mol/L) or cANF (10^{-6} mol/L). After the indicated times, the medium was removed, cells were washed three times with ice cold PBS and lysed by addition of 100 µl RIPA buffer. In some cases, Western blots were performed after pretreatment of HUVEC with U0126 (50 µmol/L), PD 98059 (50 µmol/L), or SP 600125 (10

µmol/L) for 1 h. After addition of RIPA buffer, the cells were scraped off the plates with a cell scraper, the lysate was transferred into reaction tubes (Eppendorf), and sonicated (Sonoplus, Bandelin, Germany) for approx. 5 sec. The homogenized samples were clarified by centrifugation at 21,910 x *g* for 10 min at 4°C and the resulting supernatants were collected and subsequently quantified for protein according to the method of Lowry (Lowry 1951). 5x sample buffer was added to the remaining probes and the samples were heated to 95°C for 5 min to achieve protein denaturation. Afterwards, the samples were either subjected to SDS/PAGE or stored at -20°C until further analysis.

2.4.3 SDS polyacrylamide electrophoresis

The separation of proteins was carried out by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (Laemmli 1970). The separation of proteins solely according to their size is achieved by addition of SDS which complexes proteins in a constant weight ratio and masks their charge by its own strongly negative charge resulting in identical charge densities on the surface. Disulphide bridges are cleaved by reducing agents (DTT). The molecular weight of the separated proteins was determined by comparison with a molecular weight standard (biotinylated protein marker, Cell Signaling, 6.5 kDa – 165 kDa). The electrophoresis was carried out with a BioRad Mini Protean II Cell (BioRad, München, Germany).

2.4.4 Semidry blotting

Using a trans-blot semidry system (BioRad), the separated proteins were electrophoretically transferred to a polyvinylidenfluoride membrane (Immobilon-P, 0.45 µm pore size, Amersham) which was incubated for 5 min in methanol, for 5 min in water, and stored in anode buffer II before usage. Six sheets of blotting paper were soaked with anode buffer I and three sheets with anode buffer II and rolled onto the blotter without bubbles. The membrane and the gels were added and covered with nine sheets of blotting paper moistened with cathode buffer. Blotting was carried out for 1 h at 64 mA. Afterwards, the membrane was dried at 80°C for appox. 30 min. Unspecific binding sites were saturated by shaking the membrane in 5% (m/v) Blotto (BioRad) in TBS-T.

2.4.5 Incubation with antibodies

Antibody solutions were prepared 1:1,000 or 1:10,000 in 1% Blotto in TBS-T. The blots were incubated with the first antibody overnight at 4°C under constant shaking. Following three washes with TBS-T, the second horseraddish peroxidase-conjugated antibody was added and incubated for at least 1 h at room temperature. After another three washes with TBS-T, the protein bands were visualized by a chemoluminescence reaction using an enhanced chemoluminescence protein detection kit (NEN, Cologne, Germany), containing the enzyme substrate luminol and chemical enhancers, and a Kodak Image station (Kodak digital science, Stuttgart, Germany). Luminol is oxidized in the presence of H_2O_2 and peroxidase, releasing light (428 nm) which is aquired by the Kodak Image station.

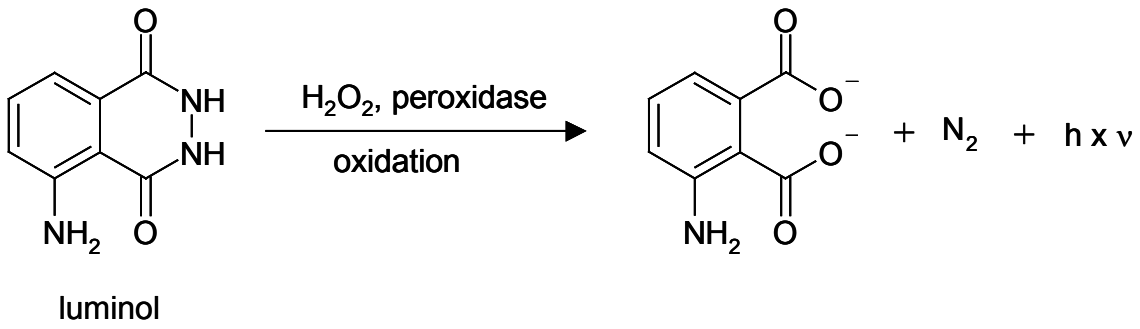


figure 7: principle of the chemoluminescence reaction

2.5 cGMP measurement

2.5.1 Solutions

IBMX (3-Isobutyl-1-methylxanthine):

- inhibitor of phosphodiesterase, preventing rapid degradation of generated cGMP
- stock solution: 0.5 mol/L in DMSO
- final concentration: 0.5 mmol/L

SNP (sodium nitroprusside):

- activator of soluble guanylate cyclase
- stock solution: 1 mg/mL, was prepared freshly before usage
- final concentration: 10 µg/mL

2.5.2 Experimental procedure

For determination of cGMP content, performed with a commercially available kit (Amersham Pharmacia), HUVEC were cultured in 24-well plates until confluence. The cell culture medium was replaced with 270 µl M199 containing 10% FCS and IBMX in a final concentration of 0.5 mmol/L. After preincubation for 10 min at 37°C, cells were either left untreated or treated with ANP (10^{-6} mol/L), aspirin (100 µmol/L) or SNP (10 µg/mL) for up to 4 h. Subsequently, 30 µl lysis buffer A, contained in the kit, were added to each well giving a final volume of 300 µl. The plate was incubated for 10 min on a rocking platform at room temperature and stored at –86°C until further analysis.

The cGMP kit employed is based on a cGMP competitive enzyme immunoassay system. Briefly, the 24-well plate was thawed on ice, cells were scraped with a cell scraper, cleared by centrifugation (21,910 x *g*, 4°C, 10 min) and transferred onto a 96-well plate. By acetylation with a mixture of 1 volume of acetic anhydride and 2 volumes of triethylamine, the sensitivity of the assay was increased from 50 fmol/well to 2 fmol/well. 20 µl of the acetylation reagent were added to each sample and mixed on a microtitre plate shaker for 5 min. In the meantime, 100 µl of a specific cGMP antiserum were pipetted into a pre-coated microtitre plate. Afterwards, 50 µl of the acetylated samples were transferred into the appropriate wells of the immunoassay microtitre plate and incubated at 4°C for 2 h, followed by addition of 100 µl of peroxidase-conjugated cGMP and incubation at 4°C for 1 h. Subsequently, the wells were washed 4 times with wash buffer and 200 µl enzyme substrate were pipetted into the wells. After incubation on a horizontal shaker for 30 min at room temperature the reaction was stopped by addition of 100 µl of 1 M sulphuric acid to the wells and optical density was read at 450 nm with an SLT Spectra ELISA reader (SLT Labinstruments, Crailsheim, Germany).

The assay is based on a competition reaction between unlabeled cGMP produced by the cells and a fixed quantity of peroxidase-labeled cGMP for a limited number of binding sites on a cGMP-specific antibody bound to an immunoassay microtitre plate. Detection is achieved by measuring the OD of the coloured product of the peroxidase reaction. With fixed amounts of antibody and peroxidase-labeled cGMP, the OD at 450 nm is inversely proportional to the concentration of unlabeled cGMP in the samples.

Principle of the EIA:

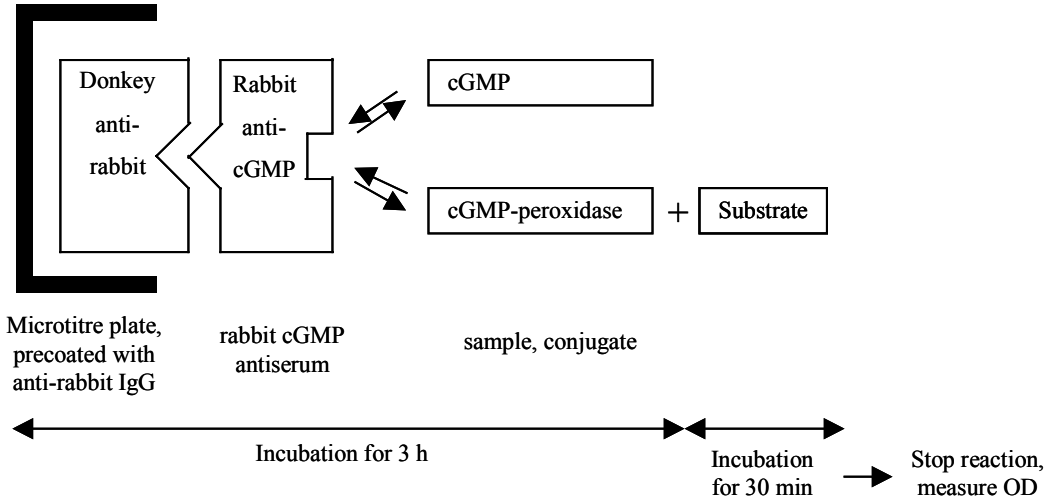


figure 8: principle of the cGMP EIA (according to amersham pharmacia instruction manual)

2.6 Detection of mRNA

2.6.1 Isolation of RNA

For isolation of total RNA (totRNA), HUVEC were grown in 25 cm^2 flasks until confluence and were either left untreated or treated with ANP (10^{-6} mol/L) in the presence or absence of U0126 (50 µmol/L) which was added to the cells 30 min before stimulation with ANP for up to 6 h. totRNA was extracted using a commercially available RNA isolation kit (RNeasy®, Qiagen, Hilden, Germany). Briefly, cells were washed with PBS and lysed with a highly denaturing guanidine isothiocyanate containing buffer which inactivates RNases and ensures isolation of intact RNA. Cells were disrupted by centrifugation through a shredder column (Qiagen). After addition of ethanol 70% (v/v) the homogenized samples were applied to RNeasy® mini spin columns contained in the kit. The addition of ethanol 70% creates suitable conditions for the selective binding of RNA to the silica-gel based membrane of the RNeasy®

column. After several washing steps to wash away contaminants, totRNA was eluted in RNase free water. This isolation procedure yields RNA with a size distribution comparable to that obtained by centrifugation through a CsCl gradient, since RNA molecules longer than 200 nucleotides are isolated. Small RNAs do not bind quantitatively under the conditions of this kit.

All steps were carried out wearing protective gloves with materials that were taken directly from the original packaging or freshly autoclaved to avoid contamination of the samples with RNases. RNases are very stable and active enzymes that require no cofactors for their function and degrade RNA very quickly.

2.6.2 Determination of RNA concentration

Nucleic acids have an UV light absorption maximum at 260 nm due to the aromatic ring systems of the bases contained in the RNA. Therefore, RNA concentrations of the samples were determined by measuring the OD at 260 nm with a Perkin Elmer spectrophotometer. The purity of the RNA was evaluated according to the ratio E_{260}/E_{280} which should be 2.0 +/- 0.3 since pure RNA has a ratio of 2.0. Proteins have an absorption maximum at 280 nm based on the absorption of aromatic amino acid residues. A high absorbance at 280 nm resulting in a low A_{260}/A_{280} ratio therefore indicates the presence of contaminating proteins or phenol.

2.6.3 Agarose gel electrophoresis

Solutions:

Ethidium bromide stock solution: 10 mg/mL

0.01 M NaH_2PO_4

Experimental procedure:

The integrity of RNA was evaluated by agarose gel electrophoresis. 2.5 µg RNA as determined by OD measurement were adjusted to a volume of 20 µl with RNase-free water. 4 µl blue/orange 6x loading dye (Promega, Mannheim, Germany), were added and the samples were run on a 1.2% (m/v) agarose gel in 0.01 M NaH_2PO_4 electrophoresis buffer pH 7.0 at 90 V for approx. 3 hours. Immediately before casting of the gel, 5 µl ethidium bromide stock solution (final concentration: 0.5 µg/mL) were added. This fluorescence dye intercalates

with RNA and therefore allows visualization of the bands in UV light (254 nm). The 28 S and the 18 S band should ideally be visible at a ratio of 2:1. This represents a measure of quality and intactness of the isolated RNA. RNA bands were visualized using a Kodak Image station (Kodak digital science, Stuttgart, Germany).

2.6.4 Reverse transcription

Reverse transcription was performed using a reverse transcription system kit (Promega, Mannheim, Germany) and a GeneAmp PCR System 9700 (PE Applied Biosystems, Weiterstadt, Germany). 2.5 µg RNA were dried in a Speed Vac®Plus SC 110A (Thermo Life Sciences GmbH, Egelsbach, Germany), dissolved in 10 µl RNase-free water and put on ice. After addition of the reaction mixture (see below), the samples were incubated at 42°C for 25 min. The reaction was stopped by heating at 99°C for 5 min, the samples were cooled down on ice and stored at –20°C.

Reaction mixture for one sample:

$MgCl_2$ (25 mmol/L)	4	µl (5mmol/L)
10x Transcription buffer	2	µl
dNTP mix (10 mmol/L)	2	µl (1 mmol/L/dNTP)
RNAsin ribonuclease inhibitor (40 U/mL)	0.5	µl = 20 U (1 U/µl)
Oligo$(dT)_{15}$primer (500 µg/mL)	1	µl = 0.5 µg (0.5 µg/µg RNA)
AMV reverse transcriptase (20 U/µl)	0.75	µl = 15 U (15 U/µg RNA)
	10.25	µl

AMV RT: Avian Myeloblastosis Virus Reverse Transcriptase catalyzes the polymerization of DNA using DNA, RNA or RNA:DNA hybrids as template. It requires a primer as well as Mg^{2+} or Mn^{2+}.

2.6.5 Polymerase Chain Reaction

2.6.5.1 Used primers

	sense primer	antisense primer
GAPDH	ACC-TAA-CTA-CAT-GGT-TTA-CAT-GTT	GGT-CTT-ACT-CCT-TGG-AGG-CCA-TGT-G
HO-1	CAG-GCA-GAG-AAT-GCT-GAG-TTC	GCT-TCA-CAT-AGC-GCT-GCA

table 1: primers used for PCR

DNA sequences are indicated from 5`to 3`terminus according to convention

2.6.5.2 Conditions

	pretreatment	denaturing	annealing	extension	posttreatment	cylcles
GAPDH	93°C; 1.06 min	93°C; 24 sec	55°C; 30 sec	73°C; 1 min	73°C; 10 min	30
HO-1	95°C; 2 min	94°C; 30 sec	58°C; 1 min	72°C; 1 min	72°C; 10 min	30

table 2: conditions for PCR experiments

cDNA was amplified using Taq DNA Polymerase. DNA polymerases are enzymes that polymerize ssDNA to dsDNA, provided that there is a short double-stranded region as initiator available. This is achieved by addition of strand-specific primers, one being complementary to the coding strand, one being complementary to the non-coding strand.

As a first step, the DNA double-strand is separated by heat denaturation allowing the primers to bind to their target sequence (annealing). The resulting double strand serves as starting point for the Taq DNA Polymerase, synthesizing the complementary strand (extension). The repetition of this procedure leads to a doubling of the DNA after each cycle and an exponential duplication of the DNA sequence.

The reaction mixture was prepared on ice and PCR was run with a GeneAmp PCR System 9700 (PE Applied Biosystems, Weiterstadt, Germany) according to the conditions mentioned

in the table above. cDNA was denatured at 93°C or 95°C resp. (pretreatment), followed by 30 cycles of denaturing, annealing (primer hybridization), and extension (synthesis of the DNA strand). During posttreatment all DNA fragments were finished by the DNA polymerase.

Reaction mixture for 1 sample:

BSA (1%)	0.5 µl (0.005%)
$MgCl_2$	6 µl (1.5 mmol/L)
dNTP (dATP, dCTP, dGTP, dTTP)	1 µl (1.0 mmol/L)
10x buffer	10 µl
Oligo sense primer	1 µl
Oligo antisense primer	1 µl
Taq Polymerase	0.5 µl (2.5 U)
H_2O	60 µl

Taq Polymerase: thermostable enzyme from Thermophilus aquaticus, replicates DNA at 74°C, catalyzes the polymerization of nucleotides into dsDNA in the 5'→3' direction in the presence of Mg^{2+}

Amplification products were separated on a 2.5% agarose gel (m/v) in TAE buffer (90 V, approx. 3 h). The size of the PCR products was determined by comparison to DNA fragments of well-defined size (phIX174 DNA/*Hin*f I Markers, 24 to 726 base pairs, Promega, Mannheim, Germany) loaded on the same gel.

TAE buffer 50x:

Tris base	242	g (2 mol/L)
EDTA sodium salt	100	mL 0.5 mol/L EDTA (pH 8.0) (0.05 mol/L)
Glacial acetic acid	57.1	mL
H_2O	ad 1,000	mL

2.7 Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays were kindly performed by Nina Weber and Brigitte Weiss.

2.7.1 Isolation of nuclear and cytosolic protein

Solutions:

Buffer A:		**Buffer B:**	
HEPES pH 7.9	10 mmol/L	HEPES pH 7.9	20 mmol/L
KCl	10 mmol/L	NaCl	0.4 mmol/L
EDTA	0.1 mmol/L	EDTA	1.0 mmol/L
EGTA	0.1 mmol/L	EGTA	1.0 mmol/L
in H_2O		Glycerol (100%) in H_2O	25%
DTT	1.0 mmol/L		
PMSF	0.5 mmol/L		

DTT stock solution: 1 ml of a 1 mol/L solution was aliquoted and stored at –20°C

PMSF stock solution: 1 ml of a 50 nmol/L solution in isopropanol was aliquoted and stored at –20°C.

Experimental procedure:

HUVEC were cultured in 6-well plates until confluence and were either left untreated or stimulated with ANP (10^{-9} – 10^{-6} mol/L) for the indicated times. Nuclear extracts were prepared as described previously (Kiemer et al. 2002). Briefly, cells were washed with PBS and detached with a cell scraper. After resuspension in 400 µl hypotonic buffer A, cells were allowed to swell on ice for 15 min. Nonidet P-40 (10%, 25 µl) was added, followed by 10 sec of vigorous vortexing. Samples were centrifuged at 12,000 x *g* for 30 sec. The supernatants (containing the cytosolic protein) were removed and frozen at –20°C. The nuclear pellet was extracted with 50 µl hypotonic buffer B by shaking at 4°C for 15 min. After centrifugation at 12,000 x *g* the supernatants were frozen at –85°C. Protein concentrations were measured according to the method of Lowry (Lowry 1951).

2.7.2 Binding reaction and electrophoretic separation

EMSA was performed using a 22-mer double-stranded oligonucleotide probe containing a consensus binding-sequence for AP-1 (5`-CGC TTG ATG AGT CAG CCG GAA-3`), 5'end-labeled with [γ ^{32}P]-ATP (10 µCi) using T4 polynucleotide kinase. Specificity of the DNA-protein complex was confirmed by competition with a 100-fold excess of unlabeled AP-1 and AP-2 (5`-GAT CGA ACT GAC CGC CCG CGG CCC GT-3`) binding sequences, respectively.

Equal amounts of nuclear protein (10 – 20 µg) were incubated (20 min, room temperature) in a 15 µl reaction volume containing 10 mmol/L Tris-HCl pH 7.5, 5 x 10^4 cpm radiolabeled oligonucleotide probe, 2 µg poly (dIdC), 4% glycerol, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 50 mmol/L NaCl, and 0.5 mmol/L DTT. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis (4.5% non-denaturing polyacrylamide gel, 100 V) and signals were detected by phosphorimaging.

Determination of cytotoxicity

2.8 Fluorescence microscopy after Hoechst staining

Solution:

Hoechst 33342 stock solution: 1 mg/mL in PBS

Principle of staining with Hoechst 33342:

Hoechst 33342 is a fluorescence dye, which freely passes the plasma membrane of intact cells as well as damaged cells, therefore it enters viable and apoptotic cells without the need for fixation. Hoechst 33342 specifically stains adenine-thymidine-rich regions of double stranded DNA and possesses an excitation maximum at 360 nm and an emission maximum at 450 nm. After staining with Hoechst, apoptotic cells can be identified by the typical hallmarks of apoptosis: chromatin condensation, nuclear fragmentation, cytoplasmatic blebbing and formation of apoptotic bodies. The most noticeable feature is the condensed chromatin which is detected by strongly increased fluorescence due to a higher concentration of dye in these areas. Healthy cells are characterized by a weaker, evenly distributed fluorescence compared to the apoptotic cells, as they possess intact nuclei and cytosol and moreover are able to eliminate the dye from the cytosol.

Experimental procedure:

HUVEC were grown in 24-well plates until confluence and were either left untreated or treated with TNF-α (10 ng/mL) or etoposide (200 µmol/L) in the presence or absence of ANP (10^{-6} mol/L) or aspirin (100 µmol/L) resp. for 16 h. ANP and aspirin were added to the cells 4 h before addition of TNF-α or etoposide. After 16 h, cells were incubated with Hoechst 33342 in a final concentration of 10 µg/mL at 37°C for 5 min. Photographs were taken with a Zeiss Axioskop MC 80 DX microscope (Zeiss, Munich, Germany) with a 200-fold magnification.

2.9 Haemacolor staining

HUVEC (24-well plates) were either left untreated or stimulated with TNF-α (10 ng/mL) or etoposide (200 µmol/L) in the presence or absence of ANP (10^{-6} mol/L) or Aspirin (100 µmol/L) for 16 h. ANP and Aspirin were added to the cells 4 h before addition of TNF-α or Etoposide. After 16 h cells were washed three times with PBS^{+} and stained with Haemacolor® (Merck, Munich, Germany) according to the manufacturer's instructions. Photographs were taken with a Zeiss Axioskop MC 80 DX microscope (Zeiss, Munich, Germany) with a 100- or 400-fold magnification.

2.10 Caspase-3-like activity measurement

2.10.1 Principle of the activity measurement

The activation of the downstream caspase-3, which is considered to be one of the key effector caspases (Hengartner 2000), can be determined with an activity assay using the synthetic peptide substrate DEVD (asp-glu-val-asp). A fluorophor, bound to this substrate, is cleaved after the aspartate residue by activated caspase-3, resulting in an increase in fluorescence (Thornberry 1994). Since other downstream caspases belonging to the same subgroup, such as caspase-7, possess similar substrate specificity, their activity is measured as well. Consequently, this assay does not exclusively acquire caspase-3 activity, and the measured activity is therefore named "caspase-3-like activity" considering caspase-3 to be the main effector caspase. The DEVD substrate is labeled with a fluorescence dye, called 7-amino-4-

trifluoromethyl coumarin (AFC), allowing the detection of the cleavage by caspase-3. The release of this fluorophor results in a shift from blue to green fluorescence, measured at an extinction wavelength of 390 nm and an emission wavelength of 505 nm. AFC is a highly sensitive fluorophor with satisfactory stability to allow convenient handling.

The reaction has a linear progression over at least 2 hours, provided the enzyme is saturated with substrate. Since caspases need a thiol group for their catalytic function, they are susceptible to changes of redox potential, and the thiol group can be oxidized by agents such as air oxygen and traces of metal ions. Therefore, the substrate buffer contained dithiothreitol (DTT) as a reducing agent.

2.10.2 Solutions

Lysis buffer:

$MgCl_2$ x 6 H_2O	5	mmol/L
EGTA	1	mmol/L
Triton X-100	0.1	%
HEPES 50 mM pH 7.5	25	mmol/L

Buffer B:

HEPES	50	mmol/L
Sucrose	1	% (m/v)
CHAPS	0.1	% (m/v)

→ adjusted to pH 7.5 with 4 M NaOH

Substrate buffer:

Buffer B	8 ml
DEVD-AFC (10 mmol/L)	45 µl (50 µmol/L)
DTT (1 mol/L)	100 µl (10 mmol/L)

2.10.3 Experimental procedure

2.10.3.1 Treatment of cells

HUVEC were cultured in 12-well plates until confluence and were either left untreated or treated with TNF (10 ng/mL) or etoposide (200 µmol/L) in the presence or absence of ANP (10^{-6} mol/L) or aspirin (100 µmol/L), respectively. ANP and aspirin were added to the cells simultaneously with the cytotoxic agent or preincubated for 4 hours. After 16 hours, the medium was removed and cells were washed with ice-cold PBS, followed by addition of 70 µl cold lysis buffer. Subsequently, the plates were frozen at –85°C until measurement of caspase activity.

2.10.3.2 Caspase-3-like activity assay

The frozen plates were allowed to thaw on ice, cells were scraped with a cell scraper and collected in Eppendorf tubes, followed by centrifugation at 21,910 x *g* (4°C, 10 min) to clarify lysates. Supernatants were transferred to microtiter plates (Greiner, Frickenhausen, Germany) and caspase-3-like activity was measured according to the method originally described by Nicholson (Nicholson et al. 1995). Wells contained either 10 µl of sample (triplicates) or extraction buffer in case of blank . The Ac-DEVD-AFC cleavage reaction was started by addition of 90 µl of substrate buffer containing the fluorigenic peptide substrate. Plates were incubated at 37°C, and formation of free 7-amino-4-trifluoro-methylcoumarin (AFC) was kinetically acquired by fluorescence measurement (excitation at 385 nm, emission at 505 nm) every 30 minutes with a fluorometer microplate reader (Fluostar, BMG GmbH, Offenburg, Germany). Caspase-3-like activity was calculated on the basis of an external AFC (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) standard curve and expressed in relation to the protein content of the corresponding samples, measured using the Pierce Assay (see below).

2.10.3.3 Protein determination – Pierce Assay (BCA assay)

The underlying reaction of this photometric assay is the Biuret reaction of proteins with copper under alkaline conditions and the interaction of two molecules of bicinchoninic acid (BCA) with one copper (I) ion (Cu^{+}). This reaction yields a water soluble purple complex possessing a strong absorbance at 562 nm (Smith et al. 1985).

Solutions:

BCA Protein Assay Reagent (Pierce, Rockford, USA):

Reagent A: Na_2CO_3, $NaHCO_3$, BCA reagent, and Na-tartrate in 0.2 mol/L NaOH

Reagent B: 4% aqueous $CuSO_4$ solution

$\Rightarrow$ 20 ml reagent A + 400 µl reagent B

BSA standard: 2 mg/ml stock solution

Experimental procedure:

200 µl of the BCA working solution were added to 10 µl of each sample. The plates were incubated at 37°C for 30 minutes, followed by measurement of the absorbance at 562 nm in a Lambda Bio 20 photometer. Protein content was calculated on the basis of a standard curve, obtained by diluting the BSA standard (0-2,000 µg/mL, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) at a ratio of 1:2.

2.11 Propidium iodide staining

2.11.1 Principle

DNA fragmentation is considered to be a characteristic hallmark of apoptosis. Therefore, apoptotic cells can be detected and quantified by propidium iodide staining of their subdiploid DNA content in hypotonic buffer, followed by fluorescence activated cell sorting (FACS). Propidium iodide is a fluorescence dye that intercalates with DNA and can be measured at an excitation wavelength of 535 nm and an emission wavelength of 617 nm. Apoptotic nuclei appear as a broad subdiploid peak which is easily discriminable from the narrow peak of cells with normal diploid DNA content.

2.11.2 Solutions

HFS (Hypotonic fluorochrome solution):

Triton X-100	0.1%
Sodium citrate	0.1%
Propidium iodide stock solution	50 µg/mL
in PBS	

Propidium iodide stock solution: 5 mg/mL

PBS: see under 2.2.1

2.11.3 Experimental procedure

For determination of subdiploid DNA content, HUVEC were grown in 24-well plates until confluence and were treated with TNF-α (10 ng/mL) or etoposide (200 µmol/L) in the presence or absence of ANP (10^{-6} mol/L) or aspirin (100 µmol/L) which were added to the cells either simultaneously with the cytotoxic agent or 4 h before TNF-α or etoposide, respectively. After 16 h, flow cytometric determination of subdiploid DNA content was undertaken by a modified procedure as described by Nicoletti (Nicoletti et al. 1991). Culture medium, containing dead cells that had detached from the wells, was collected in FACS tubes, centrifuged at 600 x *g* for 10 min at 4°C and washed twice with PBS to remove residual medium. The cell pellet was resuspended in 250 µl HFS. In the meantime, wells containing living cells and dying cells that have not yet detached, were washed twice with PBS and 250 µl HFS were added, as well as the corresponding samples containing detached cells. Plates were incubated at 4°C overnight. The following day, residual adherent cells were detached by pipetting up and down, and the samples were analyzed by flow cytometry. Cell death was calculated as % dead cells in relation to TNF-α treated cells, the apoptosis rate of which was set as 100%.

2.12 MTT-Test

In order to determine necrotic cell death in addition to apoptosis, an MTT test was performed assessing also cells dying independently of apoptotic characteristics. This assay determines the activity of mitochondrial enzymes. Only living cells are capable of reducing [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] (MTT) to the blue coloured formazane, a reaction carried out under ATP consumption by NADPH-dependent enzymes. This reaction can not take place in dead cells. After dissolution in DMSO, the absorption of the generated formazane can be measured at 570 nm.

Reaction:

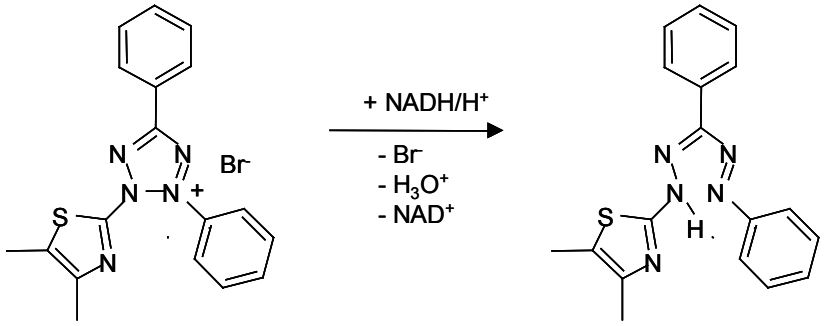


figure 9: conversion of MTT into formazane by NADPH-dependent enzymes

Solutions:

5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] (Sigma, Taufkirchen, Germany) in PBS, sterile.

DMSO

Experimental procedure:

For determination of mitochondrial activity, HUVEC were grown in 96-well plates until confluence and were stimulated with TNF-α (10 ng/mL) or etoposide (200 µmol/L) in the presence or absence of ANP (10^{-6} mol/L), which was added to the cells either simultaneously or 4 h before TNF-α. After 18 h, 5 µl MTT reagent was added followed by incubation at 37°C for 6 h. During this time, HUVEC converted tetrazolium bromide into blue formazane according to their viability. Afterwards, 250 µl DMSO were added to the wells, and generated

formazane crystals were dissolved by rocking on a shaker for at least 2 h in the dark. Absorption was measured with an SLT Spectra ELISA reader (SLT Labinstruments, Crailsheim, Germany) at 570 nm.

3 RESULTS

3.1 ANP

3.1.1 Induction of HO-1 protein expression by ANP

3.1.1.1 Characterization of HUVEC by Flow Cytometry and Immunfluorescence

HUVEC were prepared as described in *Materials and Methods*. Since this cell model constitutes a primary cell culture, the cells had to be characterized. The identity and purity of the cells had to be verified, i.e. the presence of other cell types (e.g. fibroblasts, smooth muscle cells) had to be excluded. A typical hallmark for endothelial cells is the presence of von Willebrand factor, a large glycoprotein (MW ~ 220 kD) of complex multimeric structure, that mediates attachment of platelets to the subendothelium after vascular injury. Von Willebrand protein in HUVEC is present in the endoplasmatic reticulum, stored in Weibel Palade bodies, and also in extracellular matrix filaments. It is not found in smooth muscle cells or fibroblasts.

The identity of HUVEC was demonstrated by staining of fixed and permeabilized cells with a FITC-labeled anti-human von Willebrand Factor-antibody, followed by microscopic investigations as well as flow cytometry *(Math. and Meth.)*. Fluorescence microscopy of the cells revealed the typical staining of the rod-shaped Weibel Palade bodies in a granular pattern **(figure 10)**. The rods were present throughout the cytoplasm, with greater concentrations being located at the periphery of the cells. L929, a mouse fibroblast cell line that served as negative control, treated according to the same protocol showed no staining at all. Flow cytometric analysis of stained cells allowed the quantification of the amount of von Willebrand positive cells. **(figure 11).** >95 % of isolated cells stained positive for von Willebrand factor. The fluorescence intensity of L929 increased as well after treatment with the antibody, but to a much lesser extent compared to HUVEC. This observation can be ascribed to unspecific binding of the antibody to the cell surface and can clearly be distinguished from specific binding to the intracellular localized von Willebrand factor by virtue of the different fluorescence intensities. Unspecific binding of the antibody results in only weak increases in fluorescence intensity, whereas specific binding to von Willebrand factor yielded very high fluorescence values.

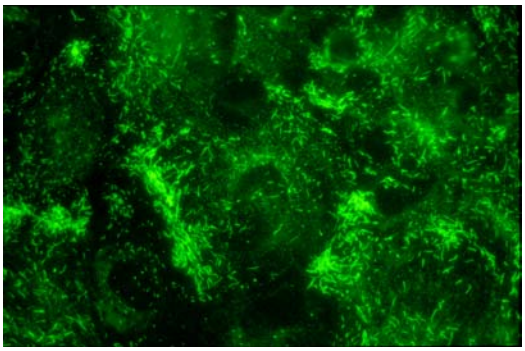


Figure 10: Characterization of HUVEC by means of von Willebrand factor

HUVEC were grown in 24-well-plates on cover slips, fixed and permeabilized with an Acetone/MeOH 1:1 mixture and photographed with a 400-fold magnification after staining with a FITC-conjugated anti-human von Willebrand Factor antibody *(Math. and Meth.)*. The picture presented above shows one out of three independent experiments from different cell preparations.

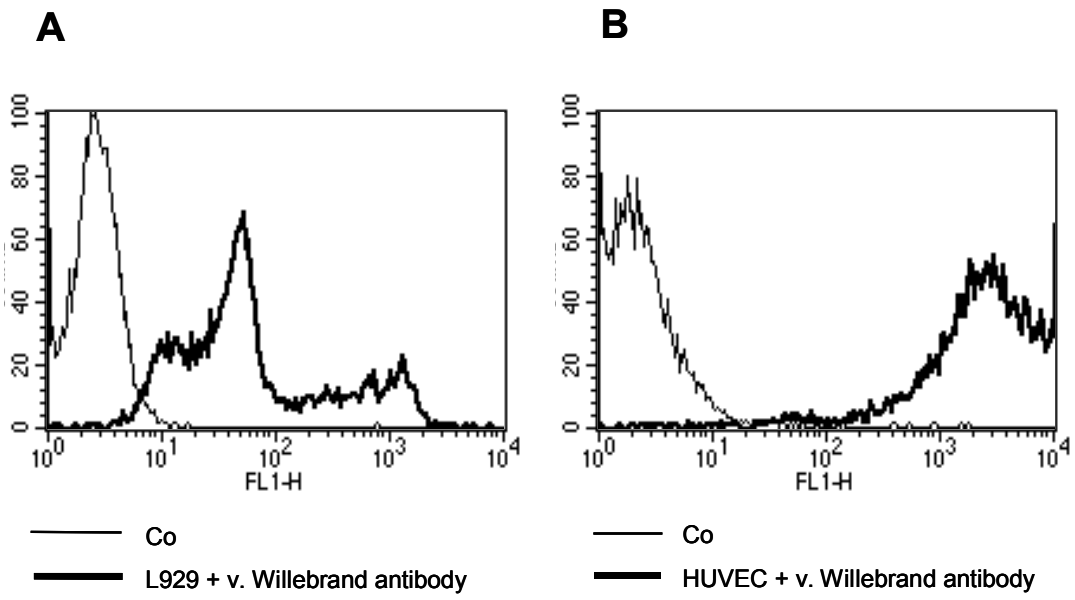


Figure 11: Flow cytometric detection of von Willebrand protein

L929 (panel A) and HUVEC (panel B) were trypsinized, fixed and permeabilized, followed by staining with a FITC-conjugated sheep anti-human von Willebrand antibody and analyzed by flow cytometry *(Math. and Meth., 2.4.2)*. The identity of the cells was verified by measuring the fluorescence of the cells at 530 nm (FL 1). The figure shows one representative experiment out of three different experiments from different cell preparations where the fluorescence intensity of the

cells is displayed versus the cell count. Co: basal fluorescence of L929 or HUVEC, respectively, without staining.

3.1.1.2 Evidence for the existence of functional NP receptors

cGMP levels were determined after 20 min stimulation with ANP (10^{-6} mol/L) by EIA according to *Materials and Methods*. Treatment with ANP lead to a significant increase of total (extracellular and intracellular) cGMP levels compared to untreated control cells **(figure 12).** LY 83583 (10^{-6} mol/L), an inhibitor of the soluble guanylate cyclase, did not reduce this ANP induced rise in cGMP, demonstrating that increased cGMP levels originated from the particular guanylate cyclase. SNP (10 µg/mL), an activator of the soluble guanylate cyclase, served as positive control to test the reliability of the EIA and the inhibitor LY 83583. After 20 min SNP activated the soluble guanylate cyclase, resulting in significantly elevated cGMP levels. This increase was almost completely abrogated by preincubation with LY 83583, demonstrating the specific effect of the inhibitor on the soluble guanylate cyclase.

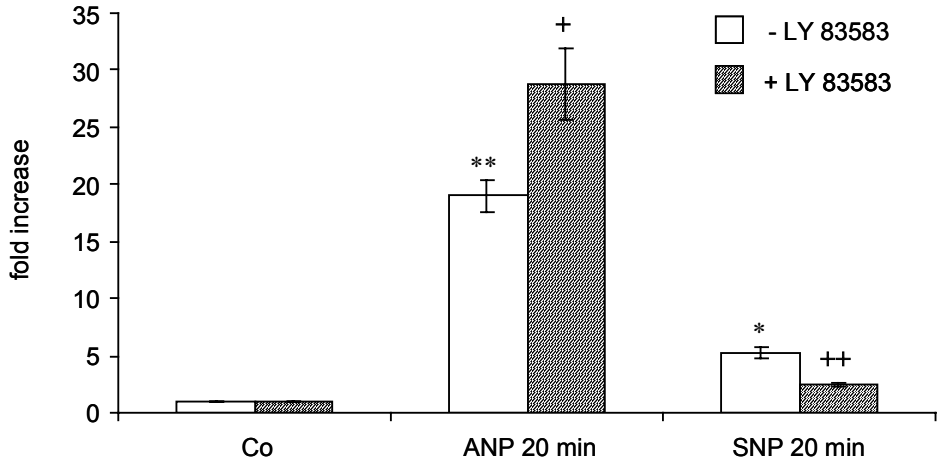


Figure 12: cGMP production in HUVEC after stimulation with ANP

cGMP production was determined in control cells (Co) and cells treated with ANP (10^{-6} mol/L) or with SNP (10 µg/mL) for 20 min with a commercially available kit as described under *Materials and Methods*. LY 83583 (10^{-6} mol/L), was added to the cells 10 min before ANP. Results presented above are expressed as x-fold increase in cGMP levels compared to the cGMP production in untreated control cells and represent mean ± SEM of one representative measurement out of two independent experiments performed in triplicates. ** $p<0.01$ and * $p<0.05$ represent significant differences compared to control, ++ $p<0.01$ and + $p<0.05$ significantly different compared to cells treated without LY 83583.

3.1.1.3 Time- and concentration dependent induction of HO-1 protein by ANP

In order to determine the potency of ANP to induce HO-1 protein in HUVEC, cells were treated with ANP for different periods of time. Time and concentration courses were performed to find the maximum induction of HO-1. HUVEC were treated with different concentrations of ANP (10^{-9} mol/L - 10^{-6} mol/L) for up to 16 h and HO-1 protein was determined by Western blot analysis *(Math. and Meth., 2.5)*. ANP dose- and time-dependently induced the expression of HO-1 protein with a maximum induction occurring at a concentration of 10^{-6} mol/L between 4 and 6 hours **(figure 13 and 14)**. Therefore the following experiments aiming to characterize mechanisms by which ANP leads to HO-1 upregulation were performed with this concentration and a stimulation time of 5 hours.

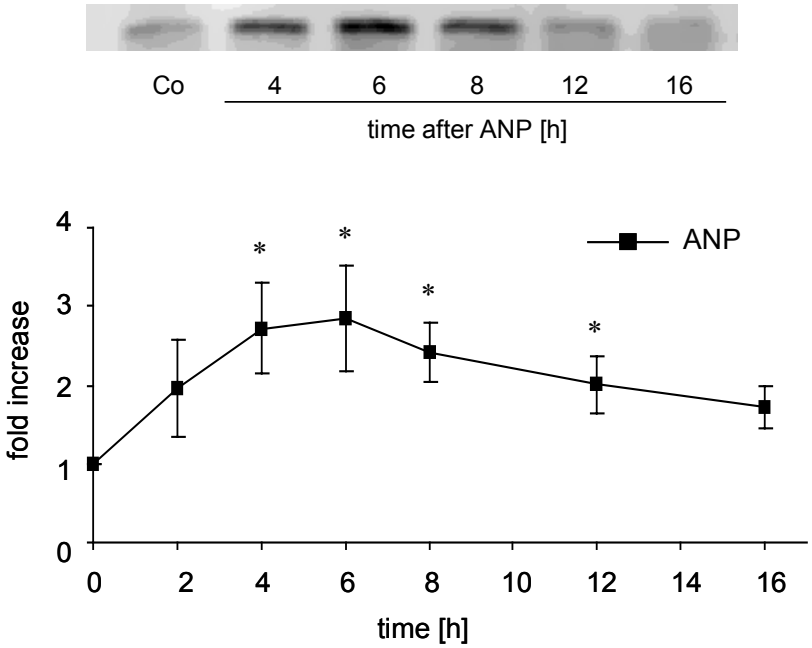


Figure 13: Time-dependent induction of HO-1 protein by ANP

HUVEC were either left untreated or treated with ANP (10^{-6} mol/L) for different periods of time (2 h - 16 h) and expression of HO-1 protein was analyzed by Western blot as described under *Materials and Methods*. Results show one representative blot out of three independent experiments with different cell preparations. The graph presented below shows the densitometric evaluation of Western blots, expressed as x-fold of values obtained for control cells. Data represent means ± SEM out of three independent experiments with different cell preparations with ∗ $p<0.05$ significantly different compared to the values seen in untreated cells (Co).

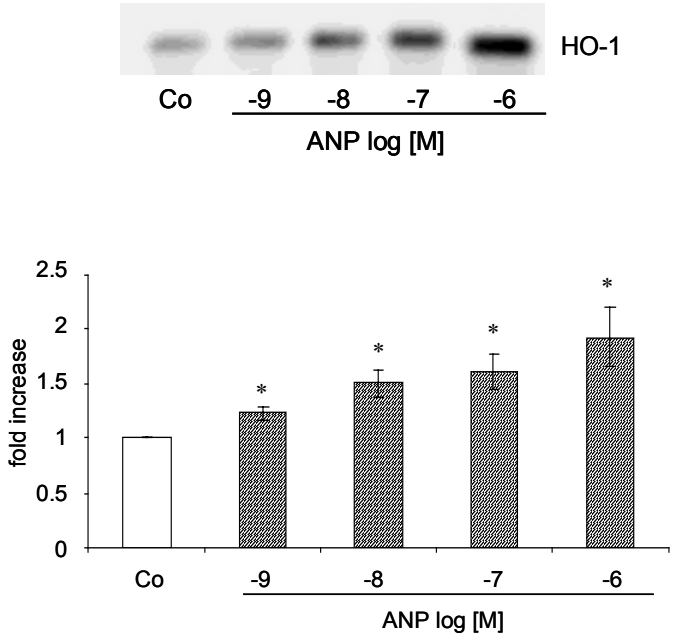


Figure 14: Dose-dependent induction of HO-1 protein by ANP

HUVEC were cultured in medium alone (Co) or were treated with different concentrations of ANP (10^{-6} - 10^{-9} mol/L) for 5 h. Western blots were performed as described under *Materials and Methods*. Data show one representative out of four independent Western blots with different cell preparations. The graph shows the densitometric evaluation of Western blots, expressed as x-fold induction compared to untreated cells. Data represent means ± SEM out of four independent experiments with * $p<0.05$ significantly different compared to control.

3.1.2 No induction of HSP70 after treatment with ANP

Apart from HO-1, another important member of the family of heat shock proteins was investigated, HSP70. In order to investigate the effect of ANP on HSP70 protein expression, cells were treated with ANP (10^{-6} mol/L) for different periods of time and HSP70 protein levels were determined by Western blot. In contrast to HO-1, ANP had no effect on HSP70 expression up to 12 hours **(figure 15)**.

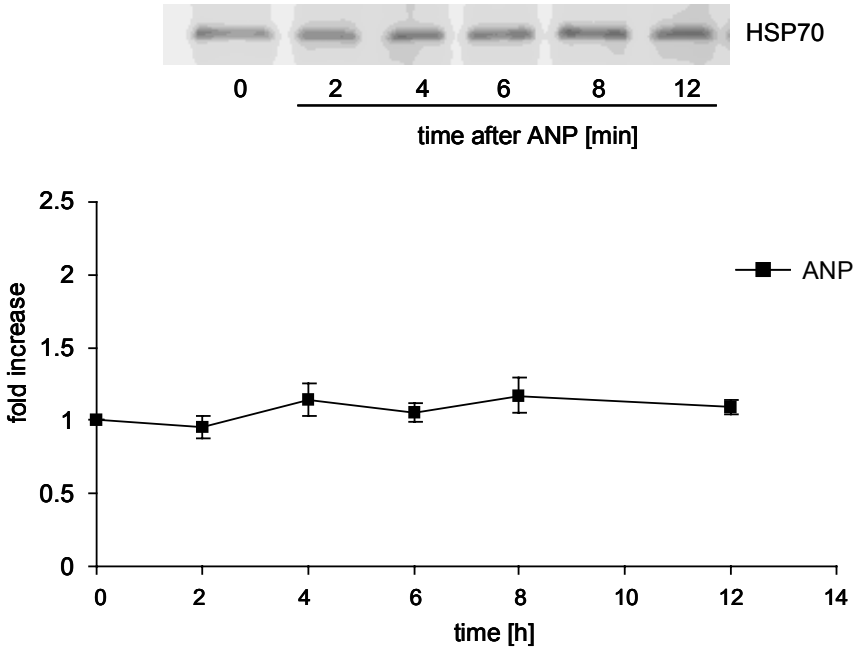


figure 15: No effect of ANP on HSP70 protein expression

HUVEC were either left untreated or treated with ANP (10^{-6} mol/L) for different periods of time (2 h – 12 h) and expression of HSP70 protein was determined by Western blot as described under *Materials and Methods*. Results show one representative blot out of three independent experiments with different cell preparations. The graph below shows the densitometric evaluation of three Western blots, expressed as x-fold of values obtained for untreated control cells. Data represent means ± SEM out of three independent experiments.

3.1.3 Receptor specificity of the ANP effect on HO-1

Since ANP has been reported to exert its effects *via* the guanylate cyclase-coupled A receptor (NPR-A) (Tremblay et al. 2002) or *via* its clearance receptor (NPR-C) (Levin et al. 1991) the receptor specificity of the ANP effect on HO-1 needed to be determined. For this purpose, the influence of the cell-permeable cGMP analogue 8-Br-cGMP as well as of the specific ligand of the NPR-C, cANF, on the induction of HO-1 protein were investigated by performing Western blots after treatment of the cells with these substances.

3.1.3.1 cGMP analogue 8-Br-cGMP

If the effect of ANP on HO-1 protein expression is mediated *via* the guanylate cyclase A-receptor, the cGMP analogue 8-Br-cGMP should be able to mimic this HO-1 upregulation. In fact, 8-Br-cGMP (10^{-3} mol/L) could induce the expression of HO-1 over time with the first significant induction occurring after 4 h **(figure 16)**.

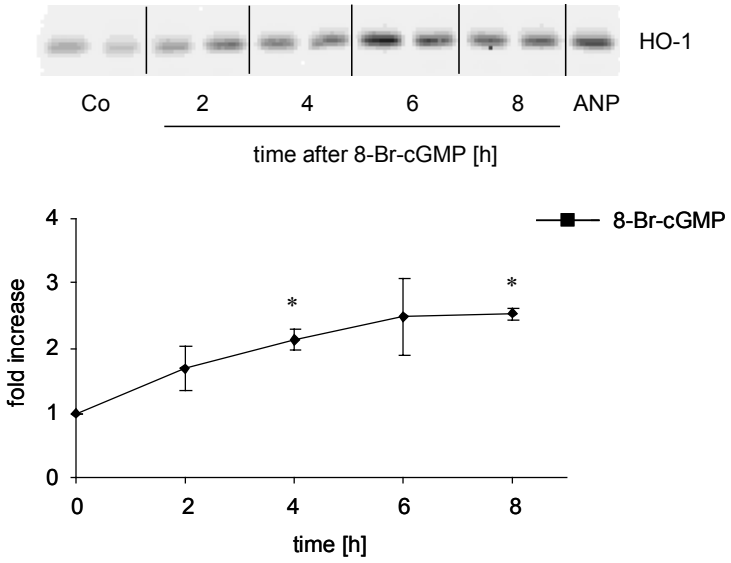


Figure 16: Induction of HO-1 protein by 8-Br-cGMP

HUVEC were treated with 8-Br-cGMP (10^{-3} mol/L) for up to 8 h and HO-1 protein expression was determined by Western blot. Upper panel shows one representative out of three independent experiments with different cell preparations with similar results. The graph shows the densitometric evaluation of three Western blots, data being expressed as x-fold of values obtained for untreated control. Data represent means ± SEM out of three independent experiments with different cell preparations. ∗ $p<0.05$ significantly different compared to the values of control cells (Co).

3.1.3.2 NPR-C ligand cANF

The specific NPR-C ligand cANF had no influence on HO-1 protein expression in HUVEC, as demonstrated in **figure 17**.

These results suggest that the induction of HO-1 protein expression by ANP is mediated *via* the guanylate cyclase coupled NPR-A with cGMP as second messenger, as the cGMP analogue 8-Br-cGMP was able to mimick the effect of ANP on HO-1 expression. On the

contrary, the NPR-C appears not to be involved, since the specific NPR-C ligand cANF did not influence HO-1 protein expression.

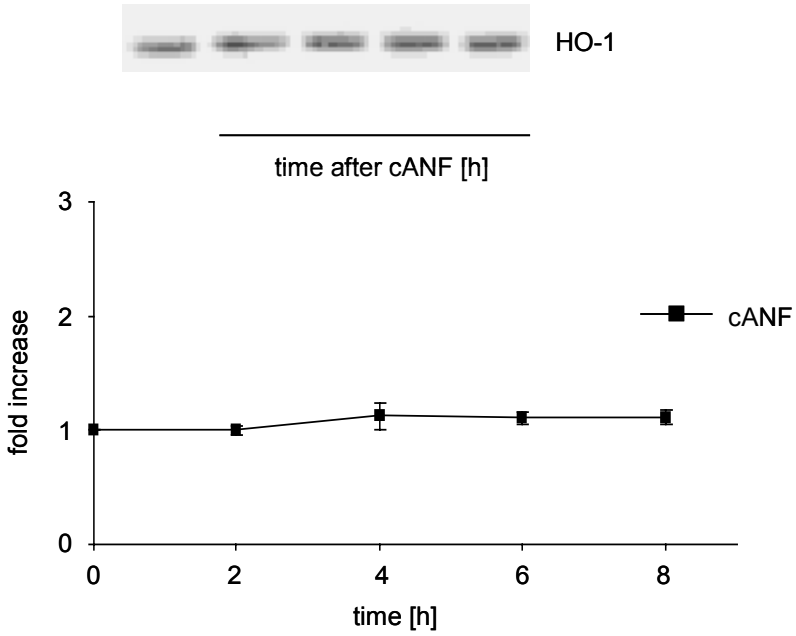


figure 17: Influence of the specific NPR-C ligand cANF on HO-1 expression

HO-1 expression of control cells (Co) and of cells treated with cANF (10^{-6} M) for different periods of time was determined by Western blot as described under *Materials and methods*. Results show one representative out of three independent experiments with different cell preparations. The histogramm shows the densitometric evaluation of signal intensities of three independent Western blots ± SEM, expressed as x-fold increase compared to untreated cells.

3.1.4 Molecular mechanism involved in the HO-1 induction by ANP

The following experiments were performed in order to elucidate the molecular mechanisms involved in the induction of HO-1 protein by ANP. The first objective was to determine whether the regulation of this heat shock protein occurs at the transcriptional level. For this purpose, semi-quantitative RT-PCR experiments to determine HO-1 mRNA were carried out as follows.

3.1.4.1 Influence of ANP on HO-1 mRNA

To verify the intactness of the isolated totRNA, the samples were separated on agarose gels and stained with ethidium bromide before reverse transcription was performed. In case of intact RNA the 28 S and 18 S bands of the ribosomal RNA could be detected at a ratio of 2 : 1

In the following PCR experiments ANP (10^{-6} mol/L) induced the expression of HO-1 mRNA in a time-dependent manner suggesting that ANP regulates HO-1 on a transcriptional level. **(figure 18)**.

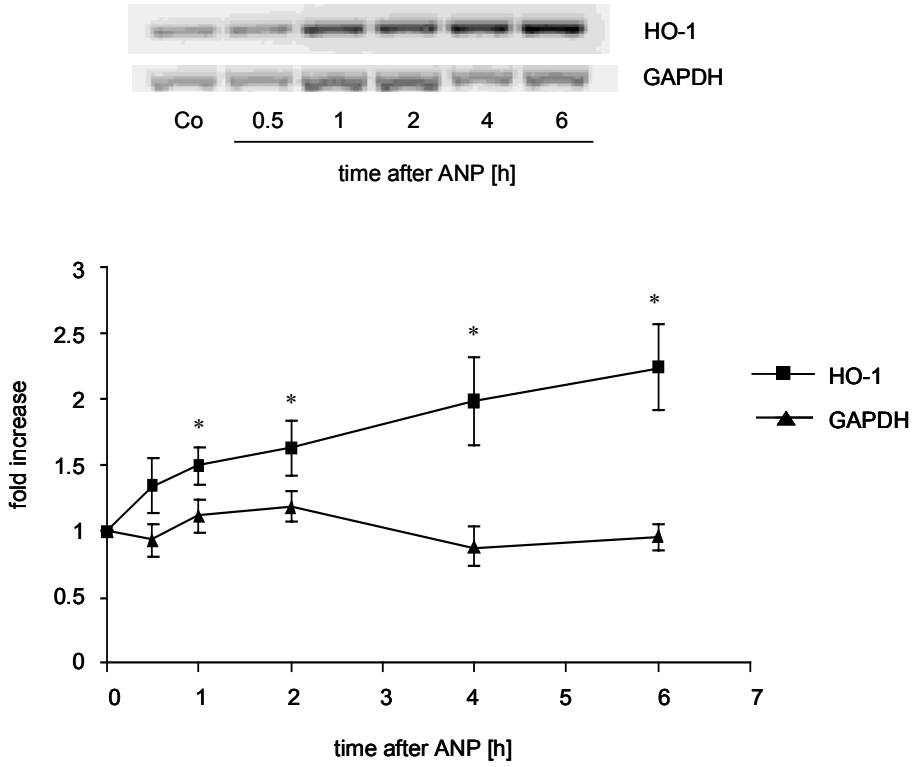


figure 18: Time-dependent induction of HO-1 mRNA expression by ANP

HUVEC were either left untreated (Co) or treated with ANP (10^{-6} mol/L) for up to 6 h. totRNA was isolated and RT-PCR was performed according to *Materials and Methods*. Amounts of cDNA employed in PCR were normalized versus GAPDH. The figure shows one representative agarose gel out of 3 independent experiments, the GAPDH gel is shown below. The graph shows the densitometric evaluation of signal intensities, expressed as x-fold increase of values for untreated control cells. Data show mean ± SEM of four independent experiments with different cell preparations. ∗ $p<0.05$ significantly different compared to control.

3.1.4.2 Influence of ANP on the activator protein-1 (AP-1)

The experiments performed so far suggested a regulation of HO-1 by ANP on the protein as well as the transcriptional level, raising the question which transcription factors are involved in this signal transduction pathway. One transcription factor previously reported to play a pivotal role in the transcriptional induction of HO-1 is the activator protein-1 (AP-1) (Wiesel et al. 2000). Therefore, in order to elucidate if ANP mediates its induction of HO-1 expression *via* AP-1, the activation of this transcription factor, which binds to specific promotor sequences of the HO-1 gene after translocation to the nucleus, was investigated by electrophoretic mobility shift assay (EMSA).

3.1.4.2.1 Activation of AP-1 by ANP

To determine a potential effect of ANP on the DNA-binding activity of AP-1, EMSA with ANP time and concentration courses was performed **(figure 19 and 20)**.

Treatment with ANP in fact resulted in a significant time-dependent activation of this transcription factor, with maximum stimulation occurring after 15 min and a second maximum after 90 min, indicating a biphasic time course for AP-1 activation exerted by ANP. **(figure 19)**

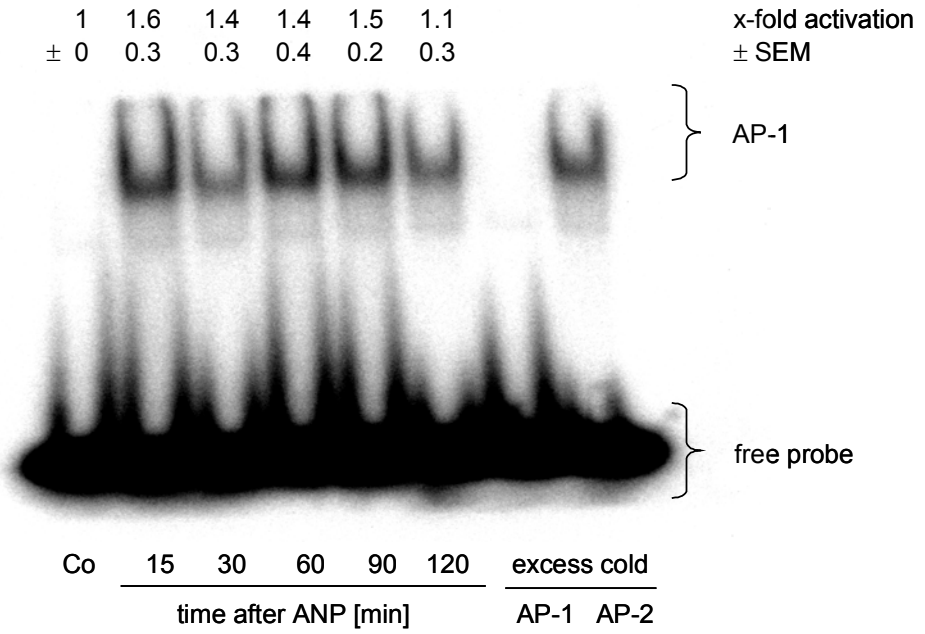


figure 19: time course of AP-1 induction by ANP

HUVEC were either left untreated (Co) or treated with ANP (10^{-6} mol/L) for up to 2 h. Nuclear protein was isolated and binding activity to a radioactively labeled promotor sequence was determined *(Materials and Methods)*. The figure shows one representative EMSA out of three independent

experiments. For each experiment the binding specificity was assessed by addition of a 100-fold excess of unlabeled AP-1 and AP-2 oligonucleotide.

Figure 20 shows the activation of AP-1 in the presence of increasing concentrations of ANP (10^{-8} mol/L – 10^{-6} mol/L) and clearly demonstrates a maximum induction of AP-1 DNA binding activity at a concentration of 10^{-6} mol/L, consistent with the findings at HO-1 protein level, where maximum induction also appeared at an ANP concentration of 10^{-6} mol/L.

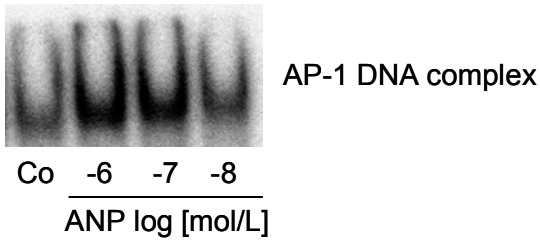


figure 20: Dose-dependent induction of AP-1 DNA binding activity by ANP

HUVEC were treated with different concentrations of ANP (10^{-6} mol/L – 10^{-8} mol/L) for 1 h and activation of AP-1 was determined by EMSA as described under *Materials and Methods*. The figure shows one representative EMSA out of three independent experiments with different cell preparations.

3.1.4.2.2 Inhibition of AP-1 DNA binding acitivity by U0126

The observations described above, that expression of HO-1 is significantly elevated at the mRNA and protein level after treatment with ANP are consistent with an induction of DNA binding activity of the activator protein –1 and point to an involvement of this transcription factor in this signaling pathway. Nevertheless, evidence for a causal link between AP-1 and HO-1 still had to be supplied.

In order to determine a direct causal relationship for AP-1 activation in ANP-induced upregulation of HO-1, we aimed to perform decoy experiments to prevent AP-1 activation. As shown in **figure 21**, addition of scrambled decoy oligonucleotides, performed as control experiment, induced both AP-1 activity as well as HO-1 protein expression, suggesting the activation of these stress-related pathways due to the transfection procedure. Consequently, this approach revealed not to be suitable for proving a causal relationship between AP-1 activation and HO-1 induction.

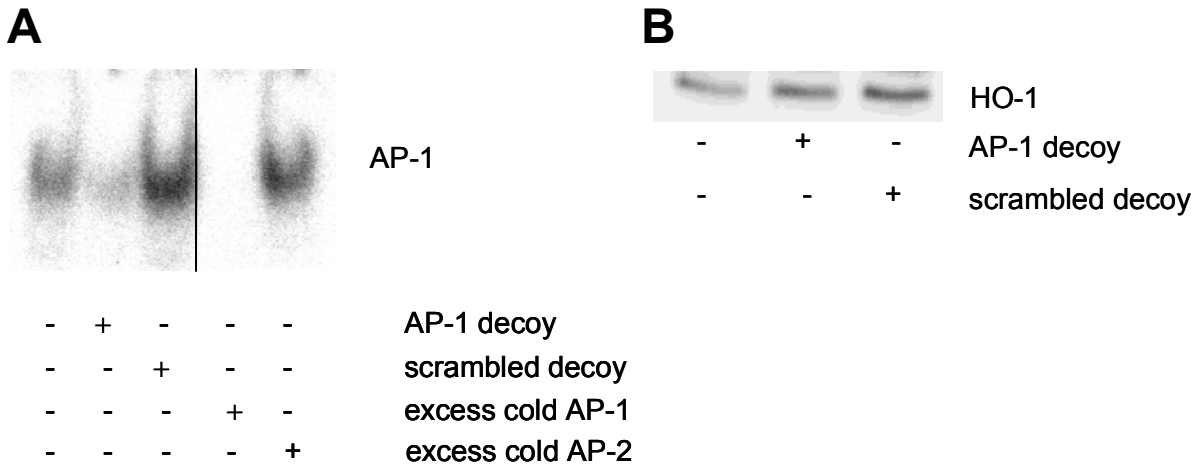


Figure 21: Treatment of HUVEC with AP-1 decoy oligonucleotides

HUVEC were transfected with AP-1 decoy oligonucleotides or scrambled decoy for 4 h or treated with transfection reagent without addition of DNA for the same time (Co). EMSA (left panel, A) and Western blot (right panel, B) was performed as described under *Materials and Methods*. Specificity of the binding reaction was assessed by using an excess of unlabeled AP-1 or AP-2 DNA oligonucleotides to binding reactions of TNF-α-treated cells. Results shown in the left panel represent one representative out of three independent experiments from different cell preparations. The blot illustrated in the right panel shows one representative out of six independent experiments from different cell preparations with similar results.

As an alternative approach to demonstrate a causal link between HO-1 induction and AP-1 activation, experiments were performed with U0126, a specific pharmacologic inhibitor of AP-1 activity.

The ability of U0126 to inhibit AP-1 DNA binding activity was verified by performing EMSA after treatment of the cells with ANP (10^{-6} mol/L) in the presence and absence of U0126 (50 µmol/L). In fact, ANP-induced DNA binding activity of AP-1 could successfully be inhibited by this compound, as shown in the shift in **figure 22**.

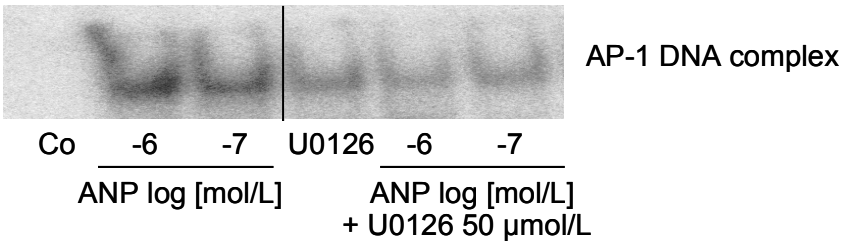


Figure 22: Inhibition of AP-1 DNA binding activity by U0126

HUVEC were either left untreated (Co) or treated with ANP (10^{-7} mol/L – 10^{-6} mol/L) for 1 h in the presence or absence of U0126 (50 µmol/L), which was added to the cells 30 min before ANP. AP-1 DNA binding activity was determined by EMSA as described under *Materials and Methods*. The figure shows one representative shift out of three independent experiments from different cell preparations with similar results.

After the effectivity of U0126 to inhibit AP-1 DNA binding activity had been evidenced, the following experiment was performed to demonstrate the causal relationship between HO-1 induction and ANP induced AP-1 activation.

HUVEC were preincubated with U0126 (50 µmol/L) for 1 h, followed by stimulation with ANP (10^{-6} mol/L) for 5 h, in accordance with the maximum increase in HO-1 protein between 4 and 6 h after ANP addition. Whole cell lysates were prepared and HO-1 protein was detected with a mouse anti-HO-1 antibody.

The following Western blot shows that pretreatment with U0126 in fact completely abrogates ANP-induced HO-1 protein expression **(figure 23)**, demonstrating the involvement of AP-1 in HO-1 upregulation by ANP. U0126 alone did not affect basal HO-1 protein levels.

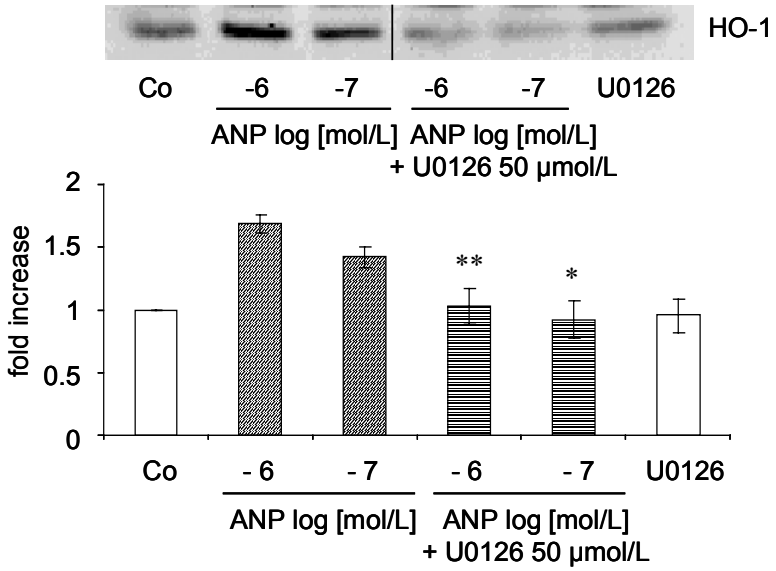


Figure 23: Inhibition of ANP-induced HO-1 protein expression by U0126

Cells were either left untreated (Co) or treated with ANP (10^{-6} mol/L) for 5 h in the presence or absence of U0126 (50 µmol/L), which was added 1 h before ANP. HO-1 protein levels were determined by Western blot *(Materials and Methods)*. The upper panel shows one representative blot out of three independent experiments from different cell preparations. The lower panel illustrates the densitometric evaluation of Western blot whereby data are expressed as x-fold of values of signal intensities obtained for untreated control cells. Data represent means ± SEM of three independent experiments. $**p<0.01$ and $*p<0.05$ represent significant differences compared to the values for ANP-treated cells.

This result was confirmed by RT-PCR experiments performed with U0126 (50 µmol/L). The cells were preincubated with the inhibitor for 1 h, followed by stimulation with ANP (10^{-6} mol/L) for up to 6 h. Consistent with the results at protein level, U0126 completely abrogated the expression of HO-1 mRNA induced by ANP at all time points **(figure 24)**.

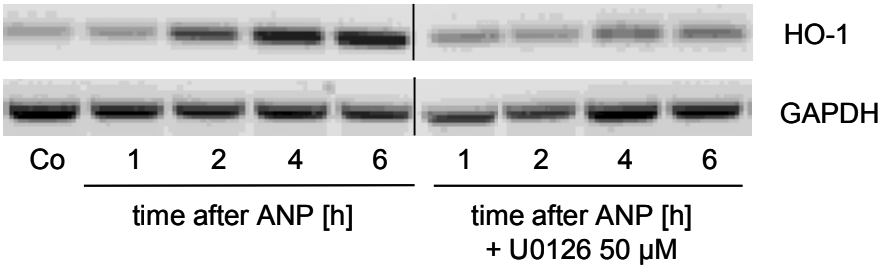


Figure 24: Inhibition of ANP-induced HO-1 mRNA expression by U0126

HUVEC were either left untreated (Co) or treated with ANP (10^{-6} mol/L) for up to 6 h in the presence or absence of U0126 (50 µmol/L), which was added 1 h before ANP. totRNA was isolated and RT-PCR was performed according to *Materials and Methods*. Amounts of cDNA employed in PCR were normalized versus GAPDH. The figure shows one representative agarose gel out of 3 independent experiments, the GAPDH gel is shown below.

3.1.4.3 Involvement of the MAP kinases

The experiments conducted so far revealed an induction of HO-1 mRNA and protein by ANP mediated *via* the transcription factor AP-1. The following experiments aimed to elucidate the mechanisms lying upstream and mediating the activation of AP-1.

Since the family of MAP kinases is reported to mediate the activation of a large number of transcription factors and plays an important role in the regulation of AP-1, the role of this family of kinases in AP-1 activation was investigated. Western blots were performed with whole cell lysates, probed with phospho-specific antibodies, detecting the activated forms of these enzymes.

3.1.4.3.1 Effect of ANP on JNK

Due to the knowledge that AP-1 is predominantly activated *via* c-jun N-terminal kinase (JNK), we investigated JNK for its potential involvement in the activation of AP-1 by ANP. As shown in **figure 25**, ANP significantly elevated JNK activity (p54 and p46) in a time-dependent fashion, with maximal activation occurring after 30 min, suggesting this action to be crucial in the regulation of AP-1 activity.

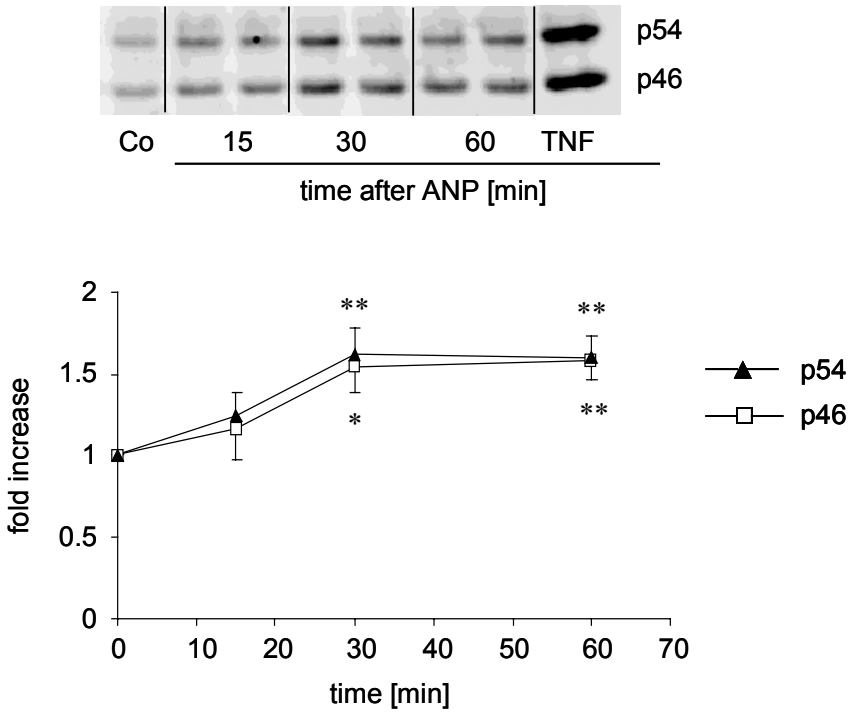


Figure 25: Induction of JNK phosphorylation by ANP

HUVEC were either left untreated (Co) or treated with ANP (10^{-6} mol/L) for 15 min up to 60 min. Phosphorylation of JNK was investigated by performing Western blots with phospho specific antibodies *(Materials and Methods)*. The upper panel shows one representative blot out of six experiments with similar results. The lower panel represents the densitometric quantification of signal intensities of six independent blots. Data show mean ± SEM with ** $p<0.01$ and * $p<0.05$ significantly different from values seen in untreated control cells.

The Western blot results presented above suggest a possible role for JNK in the induction of HO-1 by ANP. To demonstrate a causal relationship between this MAP kinase and HO-1, we investigated the influence of a specific inhibitor of the JNK pathway on HO-1 induction by ANP.

The inhibitor of JNK activity, SP600125 (10 µmol/L) completely abrogated ANP (10^{-6} mol/L)-induced expression of HO-1 protein, when preincubated for 1 h, providing evidence for the crucial role of this MAPK for the upregulation of HO-1 by ANP **(figure 26)**. The inhibitor alone had no effect on HO-1 protein.

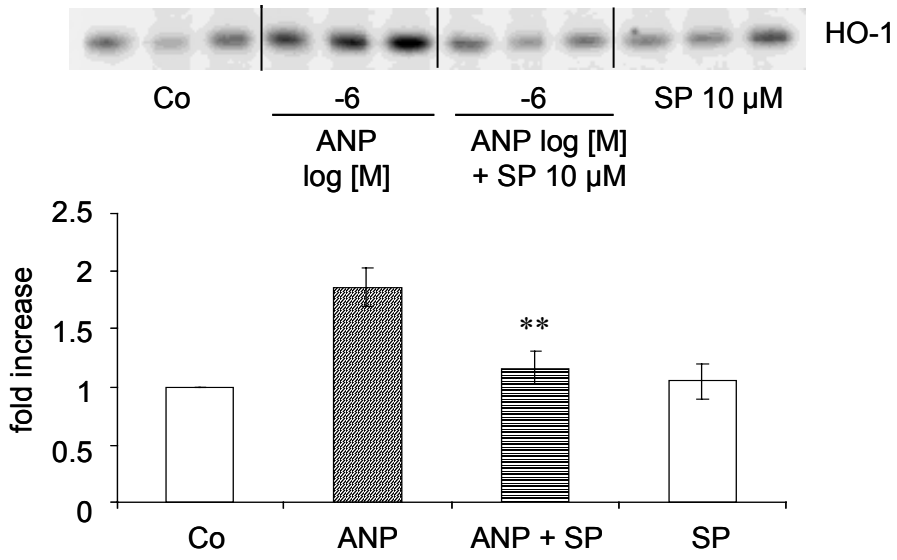


Figure 26: Effect of the specific JNK inhibitor SP600125 on HO-1 induction by ANP

HUVEC were either kept in medium alone (Co), in medium with SP600125 (10 µmol/L), or in medium containing ANP (10^{-6} mol/L) in the presence or absence of SP600125 (10 µmol/L) for 5 h. SP600125 was added to the cells 1 h prior to ANP. Expression of HO-1 protein was analyzed by Western blot as described under *Materials and Methods*. The upper panel shows one representative Western blot out of three independent experiments with different cell preparations. The densitometric quantification of signal intensities is illustrated below. Data are expressed as x-fold increase compared to untreated control and show mean ± SEM of three experiments. ** $p<0.01$ significantly different from values for ANP-treated cells.

3.1.4.3.2 Effect of ANP on ERK

Due to the controversially discussed role of ERK activation in HO-1 induction (Numazawa et al. 1997, Chen et al 2000, Alam et al. 2000), we tested ERK1/2 for its possible participation in ANP-mediated human HO-1 induction. As shown in **figure 27** ANP was also able to significantly induce phosphorylation of this MAPK (p42 and p44) in a time-dependent manner with maximal activation occuring between 15 and 30 min.

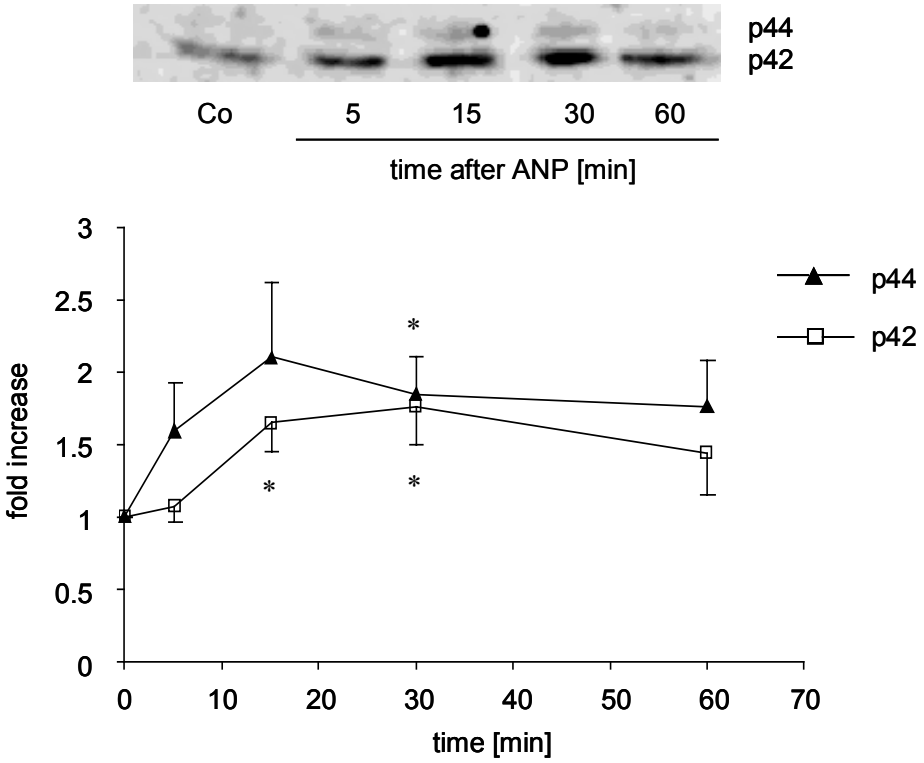


Figure 27: Induction of ERK phosphorylation by ANP

Cells were either left untreated (Co) or treated with ANP (10^{-6} mol/L) for up to 1 h. Whole cell lysates were prepared and ERK phosphorylation was detected by performing Western blots with phospho specific antibodies as described under *Materials and Methods*. The upper panel shows one representative blot out of five independent experiments with similar results. The histogramm shows the densitometric evaluation of Western blots expressed as x-fold of values obtained for untreated cells. Data represent means ± SEM out of five independent experiments with different cell preparations with * $p<0.05$ significantly different compared to untreated cells.

The data presented above suggest a possible participation for ERK1/2 in the induction of HO-1 by ANP in addition to JNK. To demonstrate a causal relationship between ERK1/2 and HO-1, we investigated the influence of a specific inhibitor of the ERK pathway on HO-1 upregulation by ANP.

As shown in **figure 28**, cotreatment of the cells with the MEK1/2 inhibitor PD98059 (50 µmol/L) and ANP (10^{-6} mol/L) resulted in a significantly attenuated HO-1 induction, confirming a participation of ERK1/2 in this signaling pathway. The inhibitor alone did not influence HO-1 protein levels.

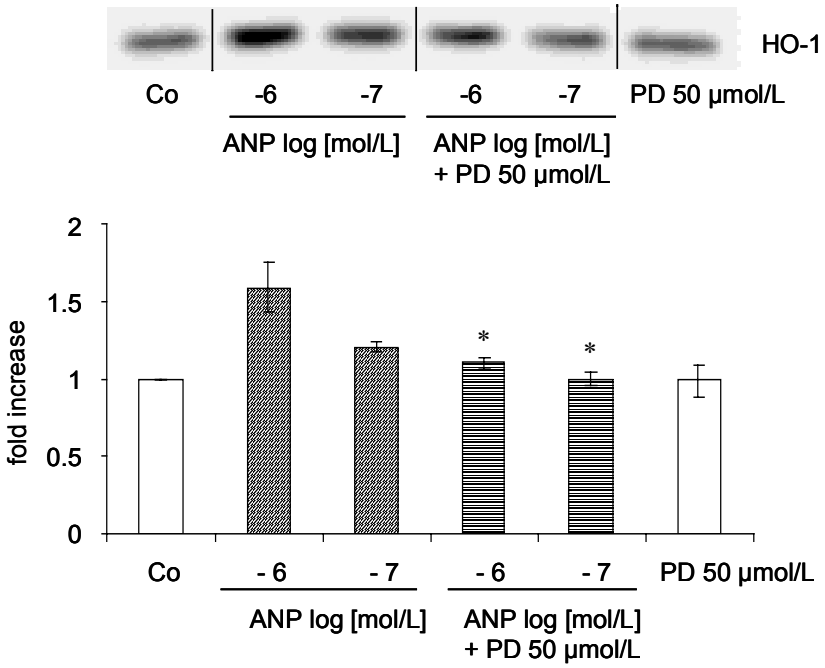


Figure 28: Effect of the specific MEK 1/2 inhibitor PD98059 on HO-1 induction by ANP

HUVEC were either left untreated (Co), or treated with ANP (10^{-6} mol/L) for 5 h in the presence or absence of PD98059 (50 µmol/L), which was added to the cells 1 h prior to ANP. HO-1 protein levels were determined by performing Western blots *(Materials and Methods)*. The upper panel shows one representative blot out of three independent experiments with different cell preparations. In the densitometric quantification illustrated below, values for untreated cells were referred to as 1. Data show mean ± SEM of three experiments with ∗ $p<0.05$ significantly different compared to values for control cells.

3.1.4.3.3 Effect of ANP on p38 MAPK

Another MAP kinase investigated in this context was phospho p38 MAPK.

Western blots were performed according to ERK and JNK/SAPK with whole cell lysates and phospho specific antibodies. In contrast to the other two MAP kinases, ANP did not influence the phosphorylation of p38 MAPK **(figure 29)**.

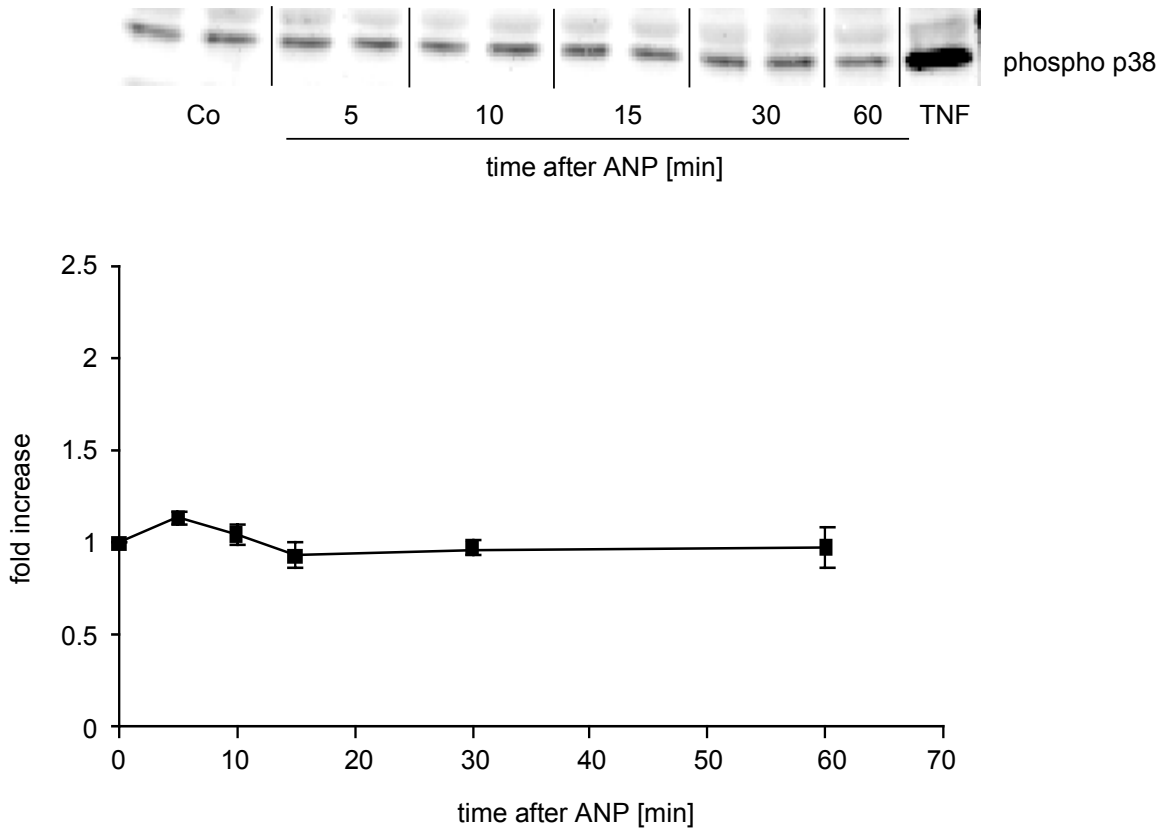


Figure 29: Influence on p38 MAPK phosphorylation by ANP

HUVEC were treated with ANP (10^{-6} mol/L) for 5 min up to 60 min or with TNF (10 ng/mL) for 30 min. Phosphorylation of p38 MAPK was determined by performing Western blots with phospho specific antibodies *(Materials and Methods)*. The upper panel shows one representative blot out of four independent experiments with similar results. The lower panel shows the densitometric evaluation of Western blots expressed as x-fold of values obtained for untreated cells. Data represent means ± SEM out of 4 independent experiments with different cell preparations.

Taken together, these results demonstrate a significant elevation in JNK and ERK 1/2 activity in ANP-treated HUVEC, suggesting the involvement of these two MAP kinases in ANP-mediated human HO-1 induction, whereas no effect could be detected on p38 MAPK.

3.1.5 Potential protective effect of ANP on TNF-α-induced cytotoxicity

HO-1 induction has previously been reported to confer resistance against TNF-α-induced cytotoxicity (Polte et al. 2000, Brouard et al. 2000). After investigation of the signaling pathway leading to increased HO-1 protein expression in ANP-treated cells, the next aim was to clarify whether ANP could confer protection of HUVEC against cytotoxic stimuli *via* induction of this heat shock protein. Since TNF-α can induce both apoptotic and necrotic cell death, the following experiments were performed to investigate typical parameters of apoptosis and necrosis in TNF-α-treated cells and the potential influence of ANP.

3.1.5.1.1 Morphological investigations

The first observation pointing to a possible cytoprotective potential of ANP was that cotreatment of the cells with ANP and TNF abrogated the morphological changes caused by TNF treatment. Untreated HUVEC displayed the typical cobblestone morphology and grew in confluent monolayers. Treatment with TNF-α (10 ng/mL) for 16 h resulted in pronounced changes of cell morphology. The cells became elongated and the monolayer started to form gaps. ANP could almost completely abrogate these TNF-α-induced morphological changes **(figure 30)**.

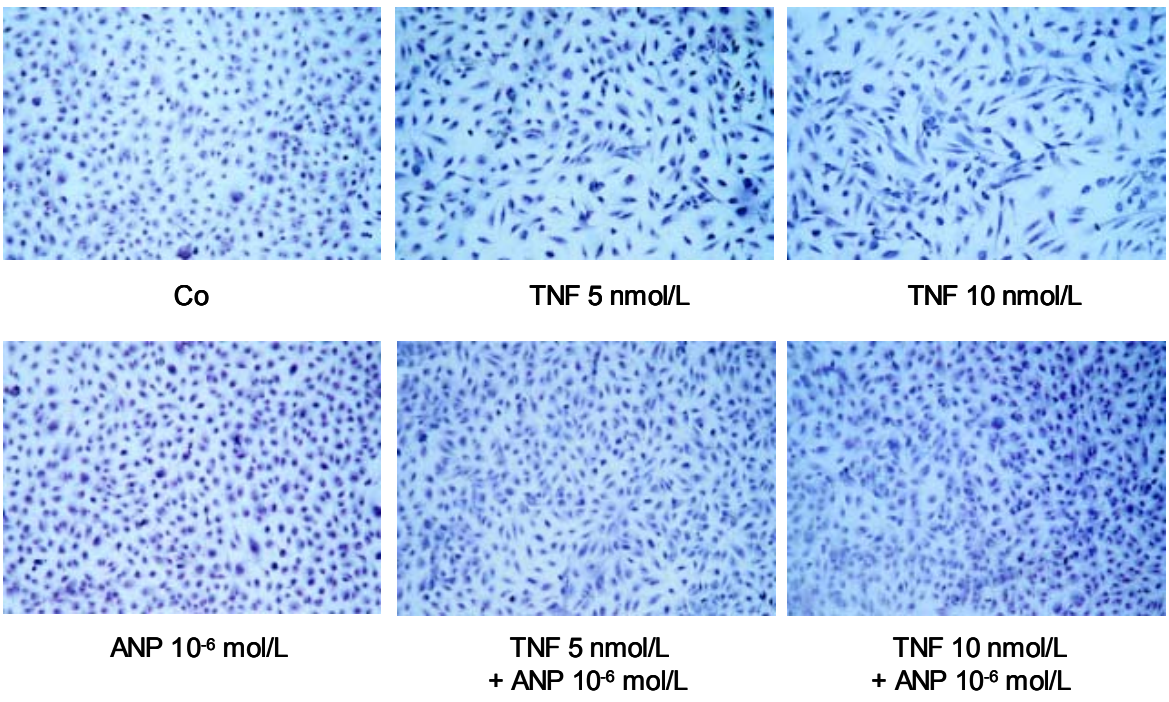


Figure 30: Influence of ANP on TNF-α induced morphological changes

HUVEC were either left untreated (Co) or treated with TNF-α (5 nmol/L, or 10 nmol/L) for 16 h in the absence or presence of ANP (10^{-6} mol/L), which was added to the cells simultaneously. Cells were stained with Haemacolor® according to the manufacturer's instructions. Photographs were taken with a 100-fold magnification and show representative pictures out of three independent experiments.

3.1.5.1.2 Fluorescence microscopy after staining with Hoechst 33342

Besides well-described morphological changes, TNF-α induces cell death which was analysed on the basis of different parameters of cytotoxicity in the following experiments. The first experiment performed in this context aimed to elucidate the mode of cell death induced by TNF-α in HUVEC. In order to differentiate between apoptotic and necrotic cell death, HUVEC were stained with Hoechst 33342 and examined by fluorescence microscopy.

As shown in **figure 31**, HUVEC treated with TNF-α (10 ng/mL) for 16 h exhibited the typical morphological hallmarks of apoptosis: TNF-α lead to pronounced accumulation of cells displaying chromatin condensation, nuclear fragmentation and formation of apoptotic bodies. These characteristic markers for apoptotic cell death could predominantly be observed in cells floating in the culture medium, consistent with the fact that HUVEC become detached when dying. Untreated cells were characterized by a weaker, evenly distributed fluorescence. Cotreatment of the cells with ANP (10^{-6} mol/L) and TNF-α did not seem to reduce the number of cells displaying chromatin condensation. ANP was added to the cells either simultaneously (not shown) or 4 h prior to TNF-α since the maximal induction of the potentially cytoprotective HO-1 protein was observed between 4 and 6 h after treatment with ANP. No difference in the rate of cytotoxicity could be detected between these two treatments.

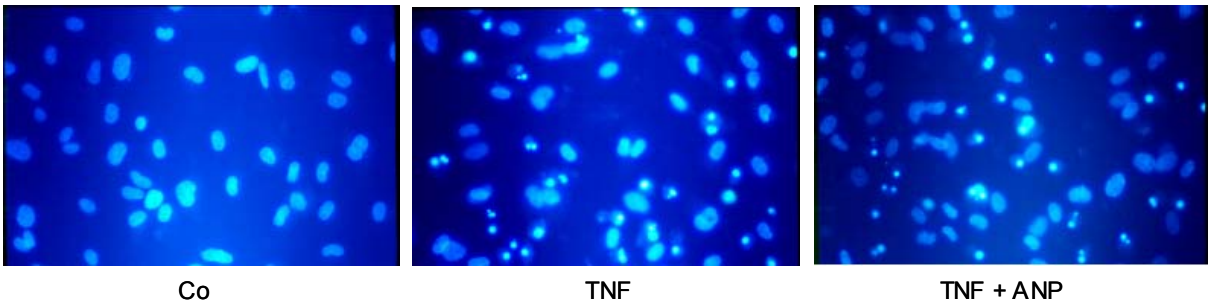


Figure 31: Influence of ANP on TNF-α -induced chromatin condensation

HUVEC were either cultured in medium alone (Co) or in medium containing TNF-α (10 ng/mL) in the presence or absence of ANP (10^{-6} mol/L) for 16 h, whereby ANP was preincubated for 4 h. Cells were stained with Hoechst 33342 as described in *Materials and Methods* and examined using a Zeiss fluorescence microscope. Pictures presented above were taken with a 200-fold magnification and show representative photographs out of three independent experiments.

3.1.5.2 Caspase activity measurement

For the analysis of apoptosis it is required to determine several parameters characteristic for apoptotic cell death. In order to investigate the influence of ANP on TNF-α-induced apoptosis, caspase-3 like activity measurements were performed, as a specific marker for apoptotic cell death. First, a time course for TNF-α-induced caspase activation was carried out to find the suitable time point for measuring enzyme activity.

The time course shown in **figure 32** demonstrated that a significant activation of caspase-3-like activity occurred after 16 h and 20 h, beginning to decline afterwards.

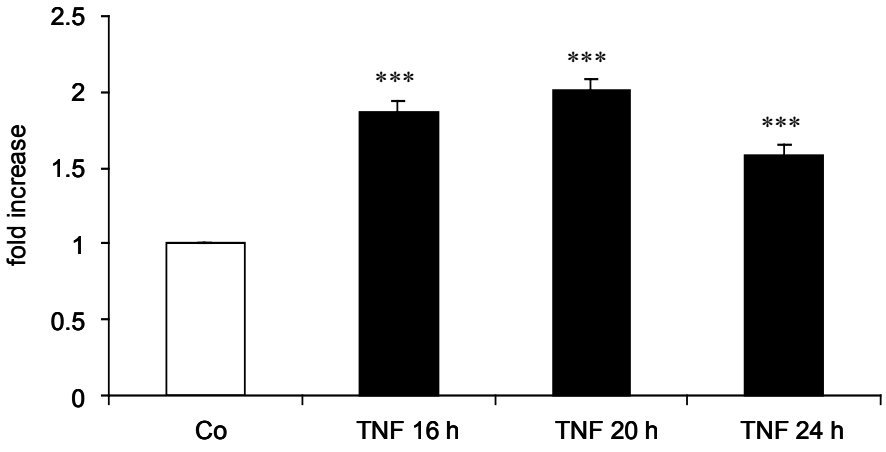


Figure 32: Time course of caspase activation by TNF-α

HUVEC were either left untreated (Co) or treated with TNF-α (10 ng/mL) for up to 24 h. Caspase-3-like activity was determined as described under *Materials and Methods*. Results are expressed as x-fold induction of caspase-3-like activity per µg protein compared to untreated control cells. Data show means ± SEM of two independent experiments performed in sixlets.*** $p<0.001$ significantly different from untreated control cells (Co),

Since an incubation period with TNF-α for 16 h represented a suitable experimental set-up, further measurements were performed at this time point. To test the influence of ANP on TNF-α-induced caspase activation, HUVEC were treated with TNF-α for 16 h in the presence or absence of ANP, which was preincubated for 4 h or added to the cells simultaneously, and enzyme activity measurements were performed.

As shown in **figure 33**, treatment of the cells with TNF-α (10 ng/mL) resulted in marked increases in caspase-3-like activity. Neither simultaneous addition of ANP (10^{-6} mol/L) nor pretreatment for 4 h significantly attenuated caspase-3 activity levels.

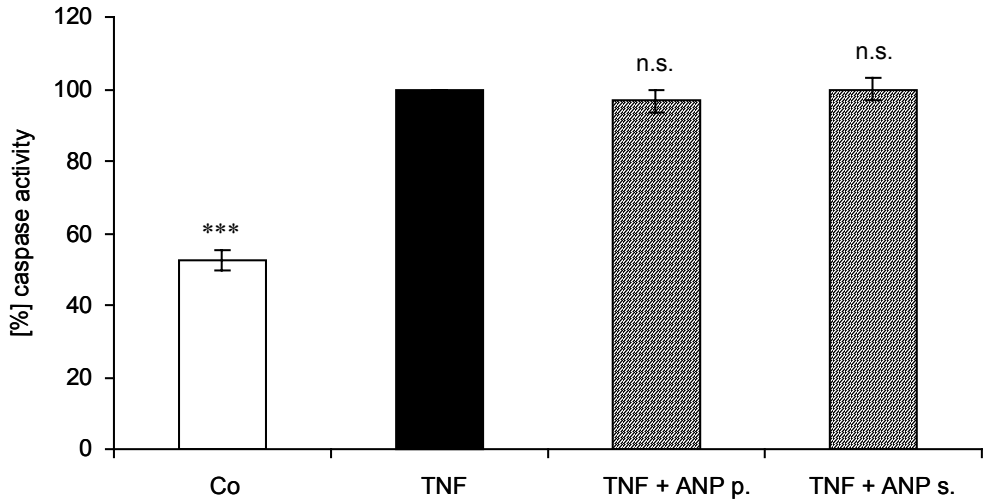


Figure 33: Effect of ANP on TNF- α induced caspase-3 activation

Caspase-3 like activity was determined in untreated cells (Co) and in cells treated with TNF-α (10 ng/mL) for 16 h in the presence or absence of ANP (10^{-6} mol/L), added to the cells 4 h prior to TNF-α (p.) or simultaneously (s.), as described under *Materials and Methods*. Caspase activity in TNF-α-treated cells was referred to as 100%. Data show mean ± SEM of four independent experiments with different cell preparations performed in triplicates with *** $p<0.001$ significantly different compared to the values obtained in TNF-α-activated cells.

3.1.5.3 Propidium iodide staining – Flow Cytometry

Another marker for apoptotic cell death is the fragmentation of the chromatin in multiples of 180 bp occurring downstream of caspase-3 activation. Determination of cells with subdiploid DNA content was performed by flow cytometry after permeabilization and staining with propidium iodide.

These experiments confirmed the results obtained by caspase activity measurement, as described under 3.1.4.2. Treatment of HUVEC with TNF-α (10 ng/mL) for 16 h resulted in a marked induction of apoptotic cell death, as assessed by the percentage of cells with sub-diploid DNA content. ANP pretreatment (10^{-6} mol/L) for 4 h did not significantly reduce this TNF-α-induced apotosis **(figure 34)**.

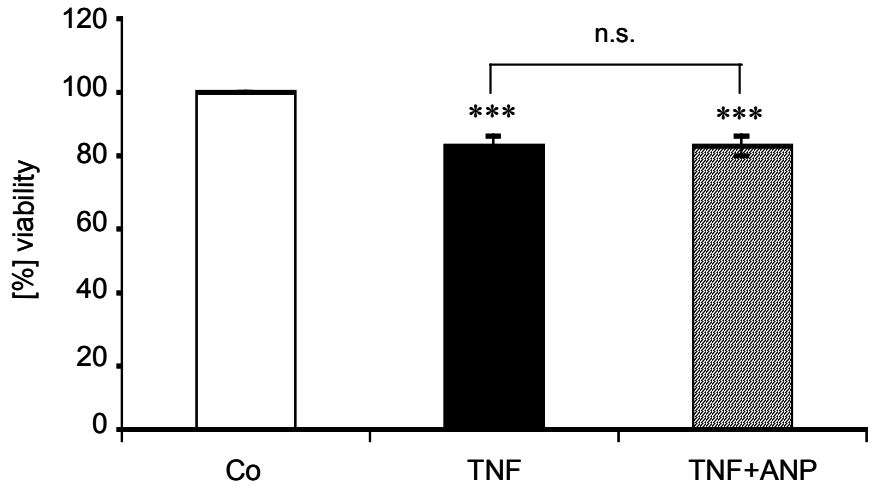


Figure 34: Influence of ANP on TNF-α-induced DNA fragmentation/subdiploid DNA content

HUVEC were either cultured in medium alone (Co) or in medium containing TNF-α (10 ng/mL, 16 h) with or without pretreatment with ANP (10^{-6} mol/L) for 4 h. The percentage of cells with subdiploid DNA content was determined by flow cytometry after staining with propidium iodide as described under *Materials and Methods*. The results illustrated in the graph above represent the fraction of viable cells with regular DNA content, whereby the viability of untreated control cells was referred to as 100%. Data show means ± SEM of five independent experiments with different cell preparations performed in triplicates with *** $p<0.001$ significantly different from the values obtained for control cells, n.s. not significantly different from TNF-α-treated cells.

3.1.5.4 MTT Test

After analysis of typical apoptotic characteristics induced by TNF, the following experiment was performed in order to acquire not only apoptotic but also necrotic cell death. Mitochondrial respiratory activity was assessed by MTT test, recording also cells dying independently from apoptotic characteristics.

Treatment of HUVEC with TNF-α (10 ng/mL) significantly decreased mitochondrial activity. Consistent with the results of the previous experiments, pretreatment with ANP (10^{-6} mol/L) did again not affect this cell death parameter **(figure 35)**.

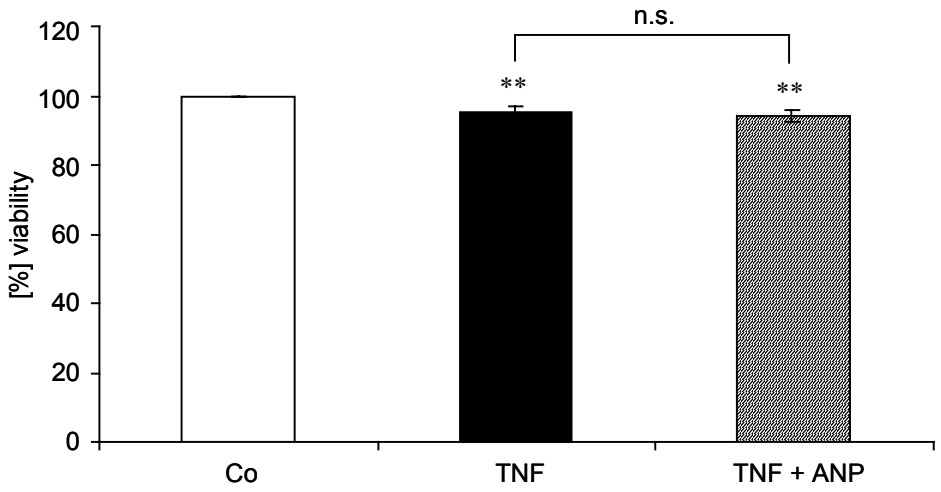


Figure 35: Effect of ANP on TNF-α-induced decrease in mitochondrial activity

Cell viability of untreated cells (Co) and of cells treated with TNF-α (10 ng/mL) for 16 h in the presence and absence of ANP (10^{-6} mol/L, preincubated for 4 h) was assessed by MTT test. Viability of cells is expressed as [%] viability of untreated cells, referred to as 100%. Results illustrated in the graph above represent means ± SEM of four independent experiments from different cell preparations performed in triplicates. ** $p<0.01$ represents significant difference compared to the values seen in untreated cells, n.s. not significantly different from TNF-α-treated cells.

Taken together, the results of the experiments conducted to determine apoptotic and necrotic cell death revealed that ANP-induced HO-1 protein expression does not confer protection against TNF-α-induced cytotoxicity.

3.1.6 Potential protective effect of ANP on etoposide-induced cytotoxicity

As demonstrated in the experiments described above, no protective effect of ANP could be detected in TNF-α-treated cells. This is likely to be due to the inhibition of TNF-α-induced activation of the potentially cytoprotective NF-κB by ANP. Brouard et al. recently reported a crucial role for NF-κB for HO-1 mediated cytoprotection (Brouard et al. 2002). ANP has been identified as an inhibitor of TNF-α-induced NF-κB activation in endothelial cells (Kiemer et al. 2002e). This inhibitory action might be responsible for the lack of protection conferred by ANP-induced HO-1. Therefore the following experiments were performed to investigate the

influence of ANP on the apoptotic effects of etoposide on HUVEC, a compound that does not induce NF-κB, as demonstrated by etoposide time course experiments followed by electrophoretic mobility shift assay (data not shown). Apoptosis was investigated on the basis of Hoechst staining, caspase activity measurements, and propidium iodide staining.

3.1.6.1 Fluorescence microscopy after staining with Hoechst 33342

Figure 36 shows the results of Hoechst staining after treatment of HUVEC with etoposide in the presence and absence of ANP.

Untreated cells were homogeneously stained with the dye and exhibited a weaker, evenly distributed fluorescence. Etoposide (200 µmol/L, 16 h) induced distinct apoptotic cell death in HUVEC as assessed by detection of the characteristic morphological hallmarks of apoptosis (chromatin condensation, nuclear fragmentation, formation of apoptotic bodies). Cotreatment of the cells with ANP (10^{-6} mol/L) did not reduce the number of cells featuring chromatin condensation. Again, no difference could be observed between simultaneous addition of ANP and etoposide and preincubation with ANP for 4 h before addition of etoposide

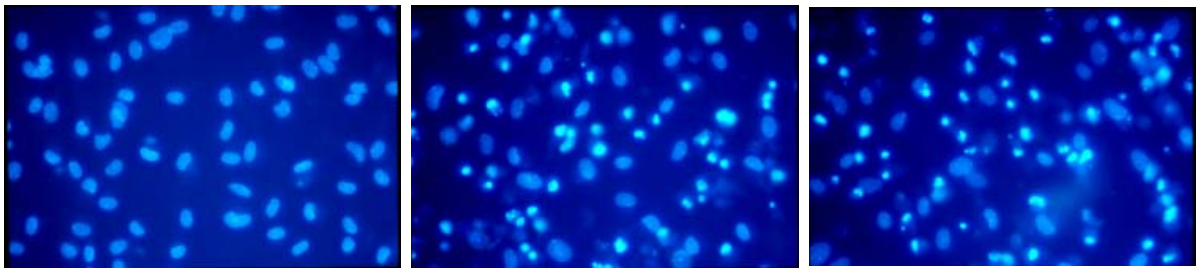


Figure 36: Influence of ANP on etoposide-induced chromatin condensation

HUVEC were either cultured in medium alone (Co) or in medium containing etoposide (200 µmol/L) in the presence or absence of ANP (10^{-6} mol/L) for 16 h, whereby ANP was preincubated for 4 h. Cells were stained with Hoechst 33342 as described in *Materials and Methods* and examined using a Zeiss fluorescence microscope. Pictures presented above were taken with a 400-fold magnification and show representative photographs out of three independent experiments.

3.1.6.2 Caspase activity measurement

The results of caspase activity measurements in etoposide-treated HUVEC are presented in **figure 37**. Etoposide (200 µmol/L) led to a considerable increase in caspase-3-like activity after 16 h. Neither simultaneous addition of ANP (10^{-6} mol/L) nor pretreatment for 4 h significantly inhibited etoposide-induced increases in caspase-3-like activity.

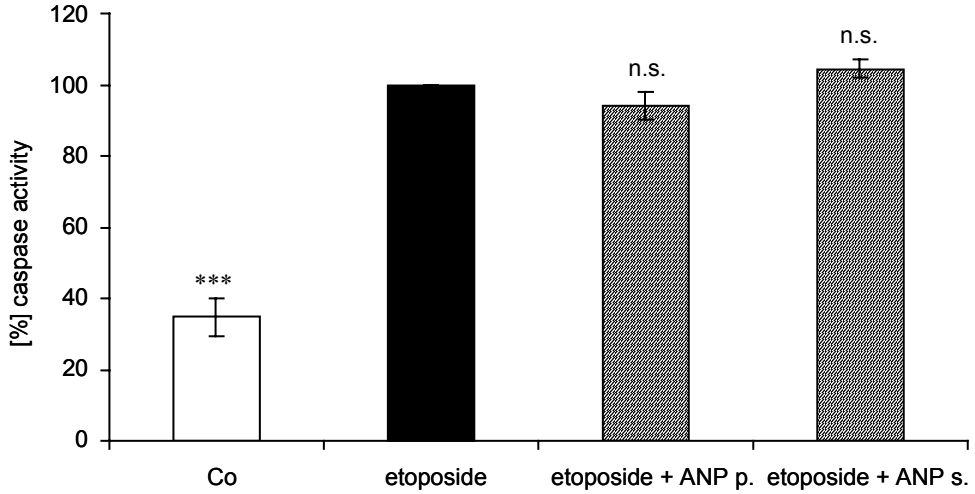


Figure 37: Effect of ANP on etoposide-induced caspase-3 activation

HUVEC were either left untreated (Co) or treated with etoposide (200 µmol/L) in the presence or absence of ANP (10^{-6} mol/L), which was added to the cells 4 h prior to etoposide (p.) or simultaneously (s.). Caspase-3 like activity measurements were performed as described under *Materials and Methods*. Caspase activity in etoposide-treated cells was set as 100%. Results show mean ± SEM of four independent experiments with different cell preparations performed in triplicates. *** $p<0.001$ represents significant difference compared to the values obtained for etoposide-treated cells.

3.1.6.3 Propidium iodide staining – Flow Cytometry

Cells were permeabilized, stained with propidium iodide and the DNA content was determined by flow cytometry. Treatment of HUVEC with etoposide (200 µmol/L) for 16 h lead to a marked increase in the number of cells possessing subdiploid DNA content. ANP (10^{-6} mol/L), added to the cells 4 h prior to etoposide did not significantly attenuate etoposide-induced apoptotic cell death consistent with the results presented above **(figure 38)**.

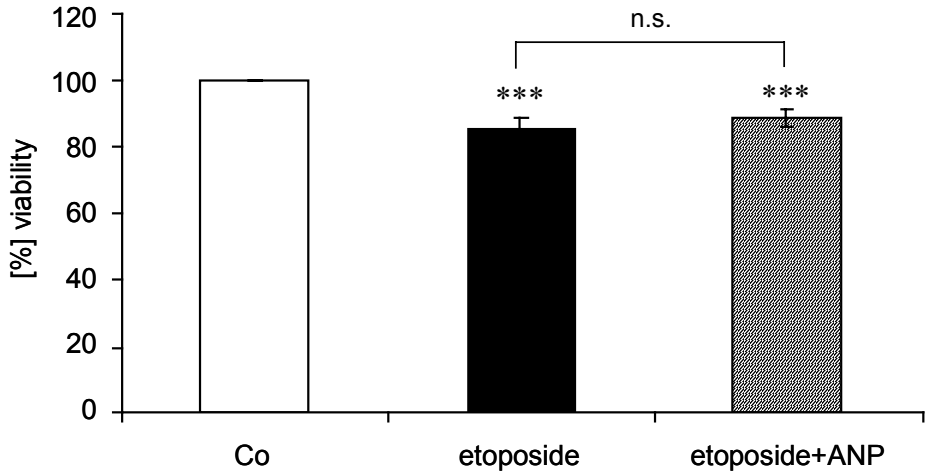


Figure 38: Influence of ANP on etoposide-induced DNA fragmentation/sub-diploid DNA content

Cells were either untreated (Co) or treated with etoposide (200 µmol/L, 16 h). ANP (10^{-6} mol/L) was given to the cells 4 h before etoposide. Sub-diploid DNA content was measured by flow cytometry as described under *Materials and Methods*. Results are expressed as percentage of viable cells, possessing regular DNA-content, with 100% representing values for untreated cells. Data show means ± SEM of five independent experiments from different cell preparations performed in triplicates. *** $p<0.001$ significantly different compared to the values obtained for untreated cells, n.s. not significantly different from etoposide-treated cells.

Taken together, these results demonstrate that upregulation of HO-1 protein expression by ANP does not confer resistance to etoposide-induced apoptosis.

3.2 Aspirin

3.2.1 Induction of HO-1 protein expression by aspirin

In addition to the well-known effects of aspirin in the therapy of pain and inflammation, its beneficial therapeutic effect in vascular desease has been increasingly recognized (Numano et al. 1995, Lauer et al. 2002). Due to the central role of endothelial cell apoptosis in the pathophysiology of atherosclerosis (Choy et al. 2001, Guevara et al. 2001) and due to the cytoprotective virtues of HO-1 protecting endothelial cells from apoptosis (Siow et al. 1999), the following experiments aimed to elucidate whether aspirin influences the expression of HO-1 in HUVEC and to determine its potency to confer protection against endothelial apoptosis.

In order to investigate the effect of aspirin on HO-1, time and concentration courses were performed and protein levels were determined by Western blot.

As shown in **figure 39**, treatment with aspirin (100 µmol/L) resulted in a marked increase in HO-1 protein expression in a time-dependent manner with maximal induction occurring between 4 h and 8 h after treatment.

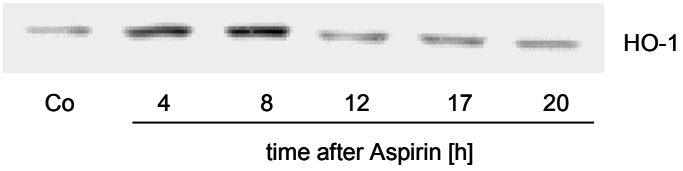


Figure 39: Time-dependent induction of HO-1 protein expression by aspirin

HUVEC were either left untreated (Co) or treated with aspirin (100 µmol/L) for the indicated times. HO-1 protein levels were determined by Western blot. The figure shows one representative blot out of three independent experiments from different cell preparations.

The induction of HO-1 protein by aspirin is also dose-dependent with maximum protein levels being detectable at a concentration of 100 µmol/L **(figure 40)**.

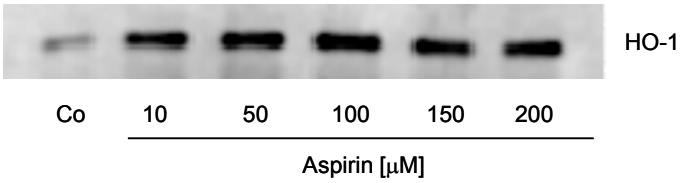


Figure 40: Dose-dependent induction of HO-1 protein expression by aspirin

Cells were treated with different concentrations of aspirin. Western blot was performed as described under *Materials and Methods*. One representative blot out of three independent experiments from different cell preparations is illustrated above.

3.2.2 Molecular mechanism involved in the HO-1 induction by aspirin

In the following experiments, the molecular mechanisms mediating the upregulation of HO-1 protein expression by aspirin were investigated.

3.2.2.1 Influence of aspirin on HO-1 mRNA

The objective of the following experiment was to determine whether the regulation of HO-1 protein expression occurs at the transcriptional level. In this regard, cells were treated with aspirin for different periods of time and totRNA was isolated. The effect of aspirin on HO-1 mRNA was determined by performing semi-quantitative RT-PCR experiments with specific primers for human HO-1. **Figure 41** shows a marked increase in HO-1 mRNA expression by aspirin (100 μmol/L) treatment, suggesting a transcriptional regulation of HO-1.

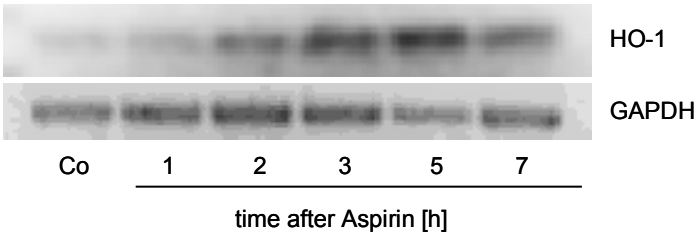


Figure 41: Time-dependent effect of aspirin on HO-1 mRNA expression

totRNA was isolated from untreated HUVEC (Co) or after treatment of cells with aspirin (100 µmol/L) for the indicated times. RT-PCR was performed with specific primers for human HO-1 as described under *Materials and Methods*. Amounts of cDNA employed in PCR experiments were compared to GAPDH. The figure shows one representative agarose gel out of three independent experiments from different cell preparations.

3.2.2.2 Influence of aspirin on activator protein-1

After investigating the regulation of HO-1 by aspirin in HUVEC at the mRNA and protein level, the following experiments aimed to clarify the signaling cascade mediating these effects. Activation of AP-1 transcription factor has previously been suggested to be important in the transcriptional induction of HO-1 (Wiesel et al. 2000). Therefore, we investigated a potential effect of aspirin on the activation of the DNA-binding activity of this transcription factor. For this purpose, nuclear protein was isolated from aspirin treated cells and binding to a radioactively labeled promotor sequence was determined *(Materials and Methods)*.

3.2.2.2.1 Activation of AP-1 by aspirin

To investigate the influence of aspirin on the DNA-binding activity of AP-1, cells were treated with aspirin for different periods of time and EMSA was performed **(figure 42)**.

Treatment of HUVEC with aspirin (100 µmol/L) in fact resulted in a pronounced time-dependent activation of AP-1 DNA-binding activity as assessed by EMSA **(figure 42)**. This effect of aspirin on AP-1 was maximal after 60 min.

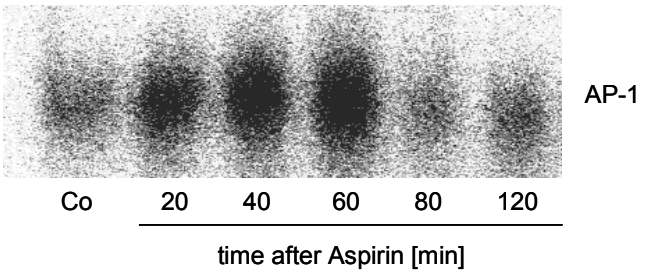


Figure 42: Time-dependent activation of AP-1 DNA-binding activity by aspirin

HUVEC were either left untreated (Co) or treated with aspirin (100 µmol/L) for the indicated times. AP-1 EMSA was performed as described under *Materials and Methods*. Results show one representative EMSA out of three independent experiments from different cell preparations.

The results presented in **figure 43** show that this activation of AP-1 DNA-binding activity by aspirin was dose-dependent, with maximal activation occurring at a concentration of 100 µmol/L.

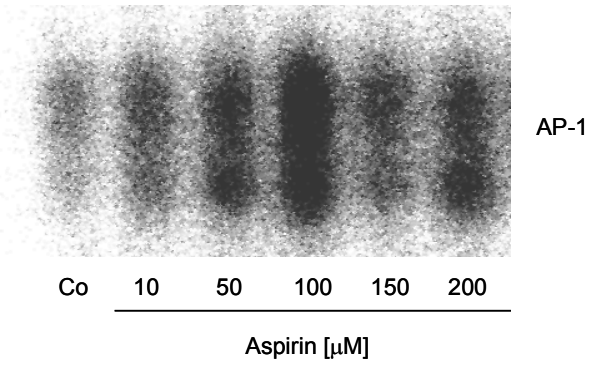


Figure 43: Dose-dependent activation of AP-1 DNA-binding activity by aspirin

Cells were treated with aspirin in different concentrations (10-200 µmol/L) for 1 h. AP-1 DNA-binding activity was assessed by EMSA *(Materials and Methods)*. Results show one representative EMSA out of three independent experiments performed with different cell preparations.

3.2.2.2.2 Inhibition of AP-1 DNA-binding activity by U0126

The results presented above suggest a participation of the transcription factor AP-1 in the signal transduction of aspirin leading to increased expression of HO-1 protein. The direct causal relationship for AP-1 activation in aspirin-induced HO-1 upregulation was demonstrated by the use of U0126, a pharmacological inhibitor of AP-1 activity. HUVEC were treated with aspirin in the presence and absence of U0126 and the effect of this inhibitor on HO-1 protein expression was determined by Western blot.

As shown in **figure 44**, pretreatment of HUVEC with U0126 (50 µmol/L) for 1 h abrogated aspirin-induced (100 µmol/L) HO-1 expression. U0126 alone did not affect basal HO-1 protein levels.

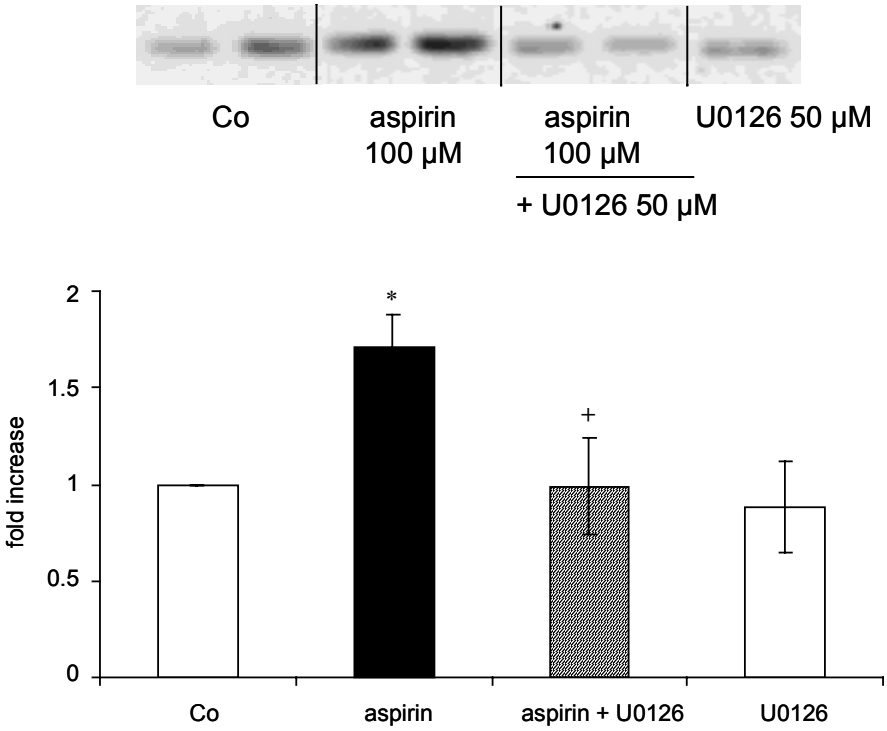


Figure 44: Effect of the AP-1 inhibitor U0126 on aspirin-induced HO-1 expression

HUVEC were either left untreated (Co) or treated with aspirin (100 µmol/L) for 5 h in the presence or absence of U0126 (50 µmol/L), which was added to the cells 1 h prior to aspirin. HO-1 protein levels were determined by performing Western blots as described under *Materials and Methods*. The blot presented in the upper panel shows one representative out of three independent experiments from different cell preparations. The graph in the lower panel illustrates the densitometric evaluation of signal intensities of three Western blots, expressed as x-fold induction compared to untreated control cells (Co). $*p<0.05$ represents significant difference compared to control, $+p<0.05$ significantly different from aspirin-treated cells.

3.2.2.3 Involvement of the MAP kinases

The investigations carried out so far demonstrated that aspirin upregulates HO-1 at the mRNA and protein level, mediated *via* the transcription factor AP-1. The mechanisms upstream of AP-1, involved in the mediation of these effects were studied in the following experiments. In order to investigate a potential participation of MAP kinases in this signaling pathway Western blots were performed with specific antibodies against the activated form of these enzymes.

3.2.2.3.1 Effect of aspirin on JNK/SAPK

Due to the knowledge that AP-1 is activated *via* c-jun N-terminal kinase (JNK), we assessed JNK activation by the use of phospho-specific antibodies. Treatment of HUVEC with aspirin (100 µmol/L) in fact significantly elevated JNK activity in a time-dependent manner as shown in **figure 45**. Maximum activation occurred at approx. 30 min after addition of aspirin.

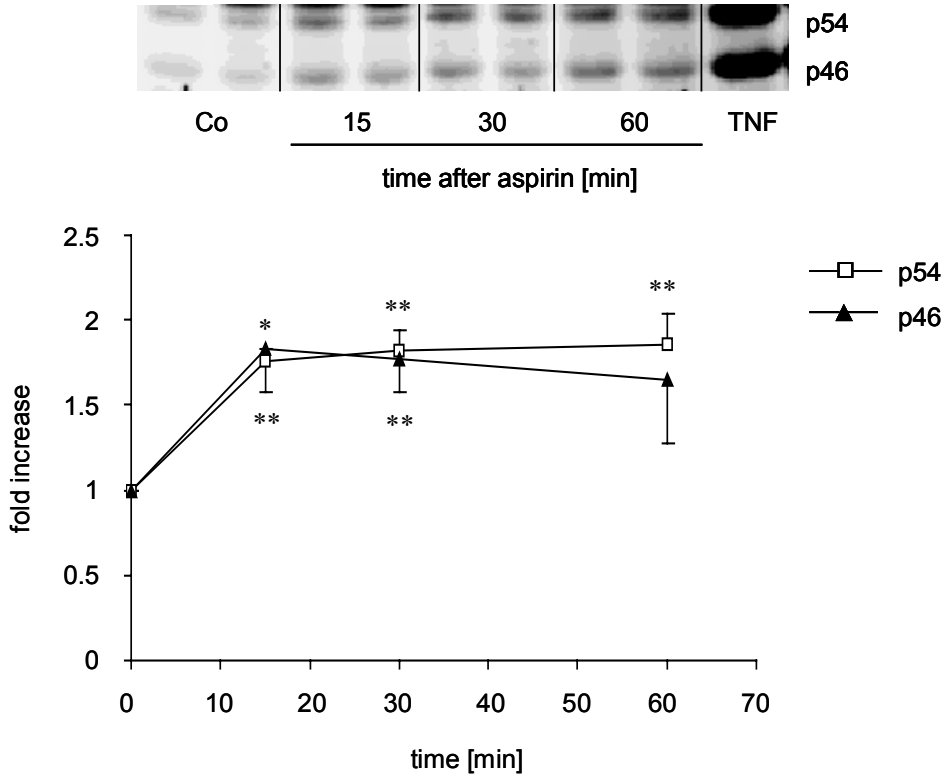


Figure 45: Time-dependent activation of JNK/SAPK by aspirin

HUVEC were treated with aspirin (100 µmol/L) for 15-60 min or with TNF-α (10 ng/mL) for 30 min. Activated JNK (p46 and p54 isoforms) was determined by Western blot as described under *Materials and Methods* using phospho-specific antibodies. Results presented in the upper panel show one representative blot out of three independent experiments from different cell preparations. The lower panel illustrates the densitometric evaluation of three experiments. Data are expressed as x-fold of signal intensities of control cells and show means ± SEM with $*p<0.05$ and $**p<0.01$ representing significant differences compared to the values seen in untreated cells.

The results of the Western blot experiments presented above point to a possible role for the JNK pathway in the induction of HO-1 by aspirin. The participation of JNK in aspirin signaling was confirmed by employing the specific JNK inhibtor SP600125.

Pretreatment of HUVEC with SP600125 (10 µmol/L) for 1 h completely abolished HO-1 expression induced by aspirin (100 µmol/L) treatment as shown in **figure 46**.

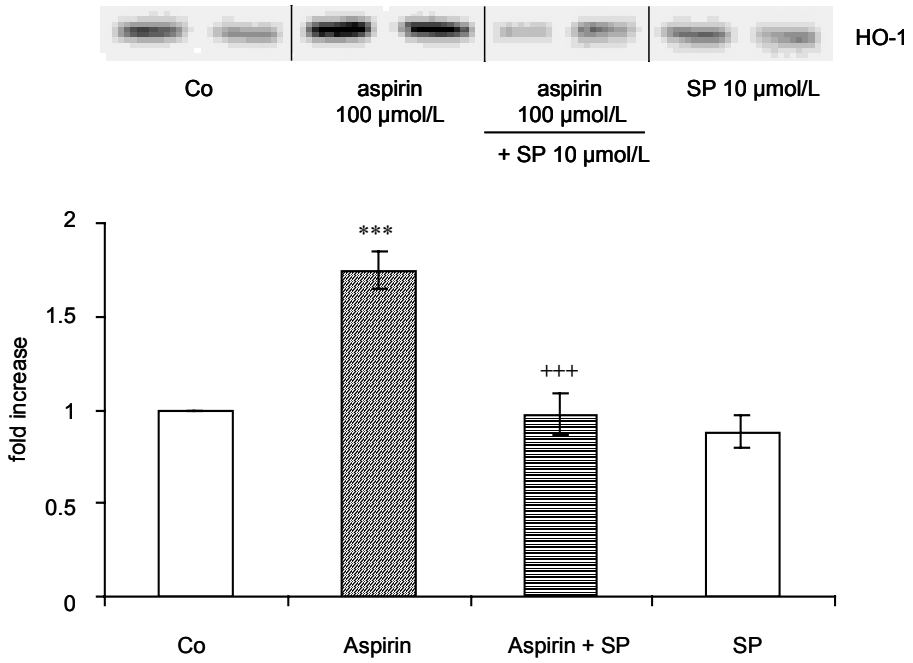


Figure 46: Effect of the specific JNK inhibitor SP600125 on HO-1 induction by aspirin

HUVEC were either left untreated (Co), treated with SP600125 (10 µmol/L, SP) or with aspirin (100 µmol/L, 5 h) in the presence and absence of SP600125 (10 µmol/L, SP), which was added to the cells 1 h prior to addition of aspirin. HO-1 protein levels were determined by Western blot *(Materials and Methods)*. The upper panel shows one representative blot out of three independent experiments from different cell preparations. The lower panel illustrates the densitometric evaluation of three experiments. Data are expressed as x-fold increase in signal intensities compared to the values obtained for untreated control cells and show means ± SEM. ***$p<0.001$ represents significant difference compared to the values seen in untreated cells, +++$p<0.001$ significantly different from cells treated with aspirin alone.

3.2.2.3.2 Effect of Aspirin on ERK

In order to determine whether ERK activation plays a role in aspirin-mediated human HO-1 upregulation ERK1/2 protein levels were analysed by performing Western blot analysis of whole cell lysates using a phospho-specific antibody against the p42 and p44 isoform of ERK. These experiments revealed that aspirin (100 µmol/L) did not affect ERK1/2 activity **(figure 47)** whereas TNF-α, known to induce ERK activity, lead to a significant phosphorylation of both ERK isoforms.

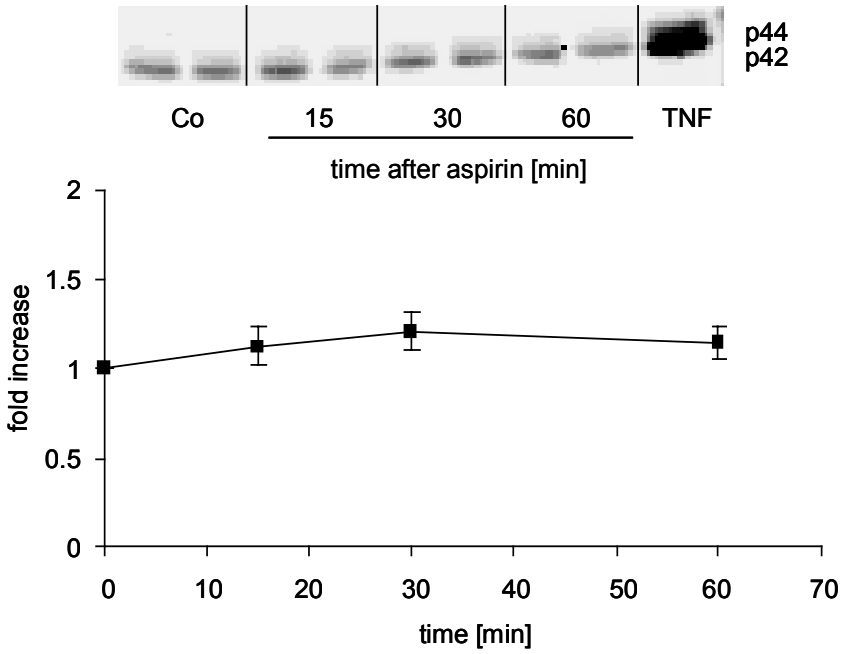


Figure 47: No effect of aspirin on ERK activation

Cells were treated with aspirin (100 μmol/L) for 15-60 min or with TNF-α (10 ng/mL) for 30 min. Activated ERK (p42 and p44 isoforms) was assessed by performing Western blot as described under *Materials and Methods* using phospho-specific antibodies. The upper panel shows one representative blot out of three independent experiments from different cell preparations. The lower panel illustrates the densitometric evaluation of three experiments. Data are expressed as x-fold increase in signal intensities compared to the values obtained for untreated control cells and show means ± SEM.

3.2.2.3.3 Effect of aspirin on p38 MAPK

Another member of the MAPK family investigated in this context was phospho p38 MAPK. Western blots were performed according to ERK and JNK with whole cell lysates and phospho-specific antibodies.

As shown in **figure 48** no effect of aspirin (100 μmol/L) on the activation of this MAPK could be detected.

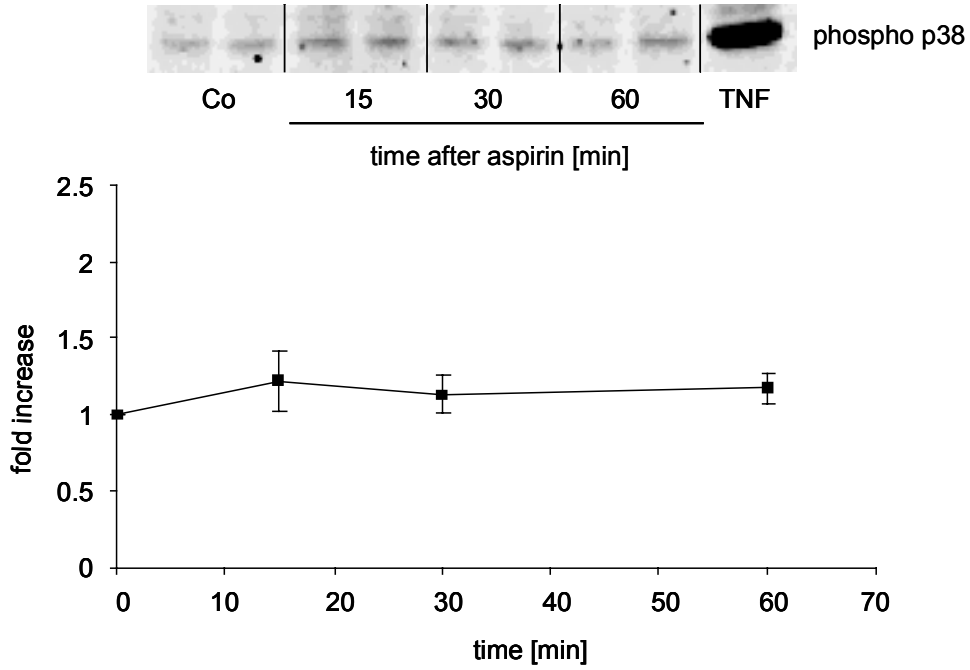


Figure 48: No impact of aspirin on p38 MAPK activation

HUVEC were either left untreated (Co) or treated with aspirin (100 μmol/L) for the indicated times (15-60 min) or with TNF-α (10 ng/mL) for 30 min. Activation of p38 MAPK was assessed by performing Western blots as described under *Materials and Methods* using phospho-specific antibodies. Results presented in the upper panel show one representative blot out of three independent experiments from different cell preparations. The lowel panel shows the densitometric evaluation of three experiments. Data are expressed as x-fold increase in signal intensities compared to the values obtained for untreated control cells and show means ± SEM.

Western blot analysis of the MAPK pathways demonstrated an involvement of JNK in aspirin-induced HO-1 upregulation.

Since aspirin did neither exert an activation of ERK nor of p38 MAPK, a role for these two MAP kinases in HO-1 induction by aspirin could be ruled out.

3.2.2.4 cGMP production after stimulation with aspirin

Due to reports about increases in intracellular cGMP after treatment with aspirin (De la Cruz et al. 2000, Sánchez de Miguel et al. 1998, Schachter et al. 1997) the next investigations aimed to determine whether accumulation of intracellular cGMP regulates aspirin-induced expression of HO-1 protein.

To differentiate between cGMP produced by the soluble and the particulate guanylate cyclase, an inhibitor of sGC, LY83583, was tested for its influence on cGMP production.

As shown in **figure 49**, treatment of HUVEC with aspirin did not affect total (intracellular plus extracellular) cGMP levels, suggesting that this second messenger is not involved in the mediation of aspirin-induced HO-1 upregulation. ANP (10^{-6} mol/L, 20 min) and SNP (10 µg/mL), activators of the particulate and the soluble guanylate cyclase, respectively, served as positive control for the reliability of the EIA and the specificity of the inhibitor LY 83583 (see 3.1.1.2).

Treatment of HUVEC with ANP for 20 min lead to significantly elevated cGMP levels. This increase was not abolished by preincubation with LY 83583, consistent with the activation of the particulate guanylate cyclase by ANP. Exposure of the cells to SNP for 20 min significantly activated the soluble guanylate cyclase, implicating a marked rise in the amount of cGMP, which was almost completely abrogated by LY 83583.

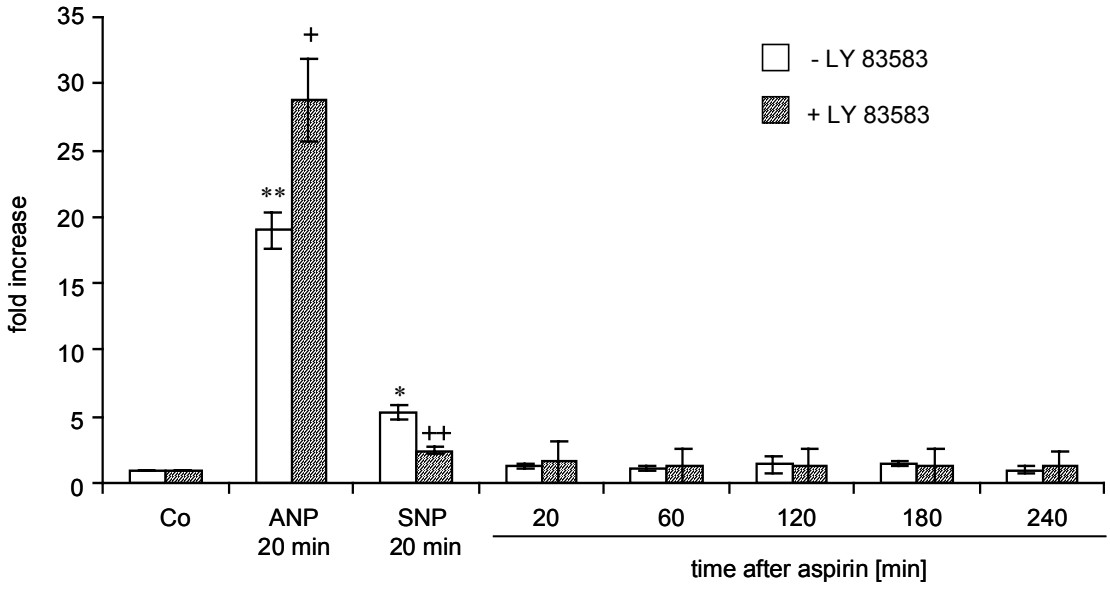


Figure 49: Influence of aspirin on cGMP levels

cGMP levels were determined in untreated cells (Co) and in cells treated with ANP (10^{-6} mol/L, 20 min), SNP (10 µg/mL) and aspirin (100 µmol/L) for the indicated times as described under *Materials and Methods*. IBMX (0.5 mmol/L) was given to the culture medium 10 min before the addition of the respective stimulus to inhibit phosphodiesterase activity. Results illustrated in the upper panel are expressed as x-fold increase compared to the cGMP levels found in untreated control cells and represent one representative out of two independent experiments from different cell preparations performed in triplicates. Data show mean ± SEM of one experiment with ** $p<0.01$ and * $p<0.05$ representing significant differences compared to untreated control cells, ++ $p<0.01$ and + $p<0.05$ significantly different compared to cells treated without LY 83583.

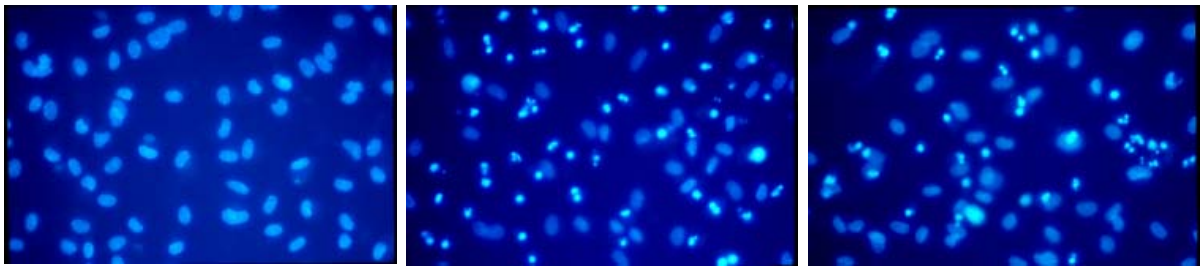
3.2.3 Potential protective effect of aspirin on TNF-α-/etoposide-induced cytotoxicity

The next investigations performed in this context aimed to determine whether upregulation of the potentially protective heat shock protein HO-1 by aspirin treatment was associated with protection against cytotoxic stimuli.

3.2.3.1 Microscopic investigation

In a first experiment it was investigated whether aspirin could affect TNF-α- or etoposide-induced chromatin condensation and nuclear fragmentation representing specific markers for apoptotic cell death. HUVEC were stained with Hoechst 33342 and examined by fluorescence microscopy.

The photographs presented in **figure 50** demonstrate that treatment with TNF-α (10 ng/mL) as well as with etoposide (200 µmol/L) for 16 h lead to a marked induction of apoptosis in HUVEC, associated with distinct condensation of the chromatin, fragmentation of the DNA and occurrence of apoptotic bodies. Untreated cells displayed a weaker, evenly spread fluorescence. Pretreatment of the cells with aspirin (100 µmol/L) for 4 h did not influence the proportion of cells exhibiting the typical hallmarks of apoptosis.



Co TNF TNF + aspirin

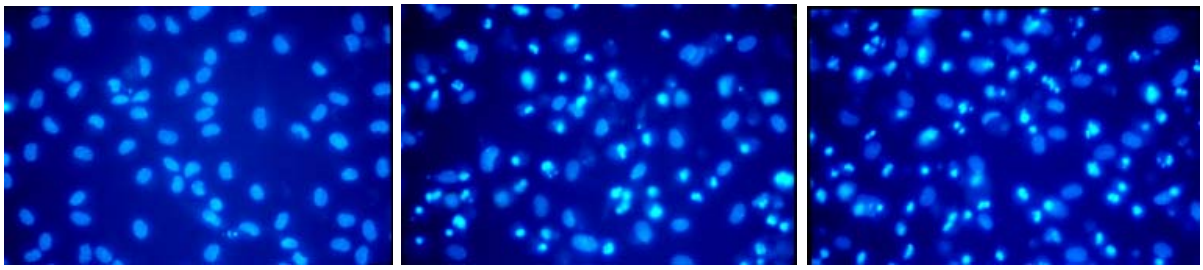
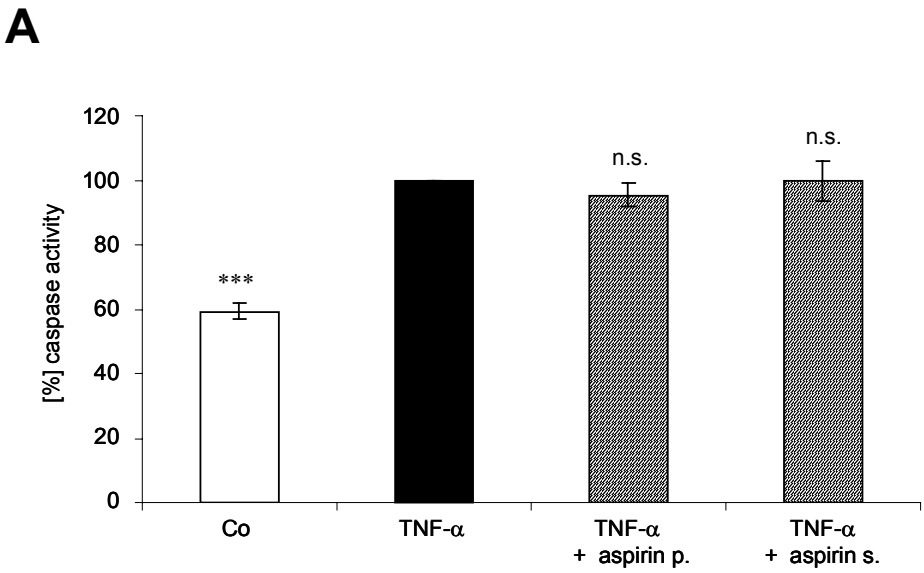


Figure 50: Influence of aspirin on TNF-α- or etoposide-induced chromatin condensation

HUVEC were either cultured in medium alone (Co) or in medium containing TNF-α (10 ng/mL) or etoposide, respectively, in the presence or absence of aspirin (100 µmol/L) for 16 h. Aspirin was preincubated for 4 h. Cells were stained with Hoechst 33342 as described in *Materials and Methods* and examined using a Zeiss fluorescence microscope. Pictures presented above were taken with a 200-fold magnification and show representative photographs out of three independent experiments.

3.2.3.2 Caspase activity measurement

The following measurements of caspase-3-like activity aimed to quantify the influence of aspirin on TNF-α- or etoposide-induced apoptosis. HUVEC were preincubated with aspirin (100 µmol/L) for 4 h and afterwards treated with TNF-α (10 ng/mL) or etoposide (200 µmol/L) for 16 h. Treatment with TNF-α and etoposide lead to a marked increase in caspase-3-like activity. Pretreatment with or simultaneous addition of ANP (10^{-6} mol/L) did not significantly attenuate elevated enzyme activity after TNF-α- **(figure 51, panel A)** or etoposide-treatment **(figure 51, panel B)**, respectively.



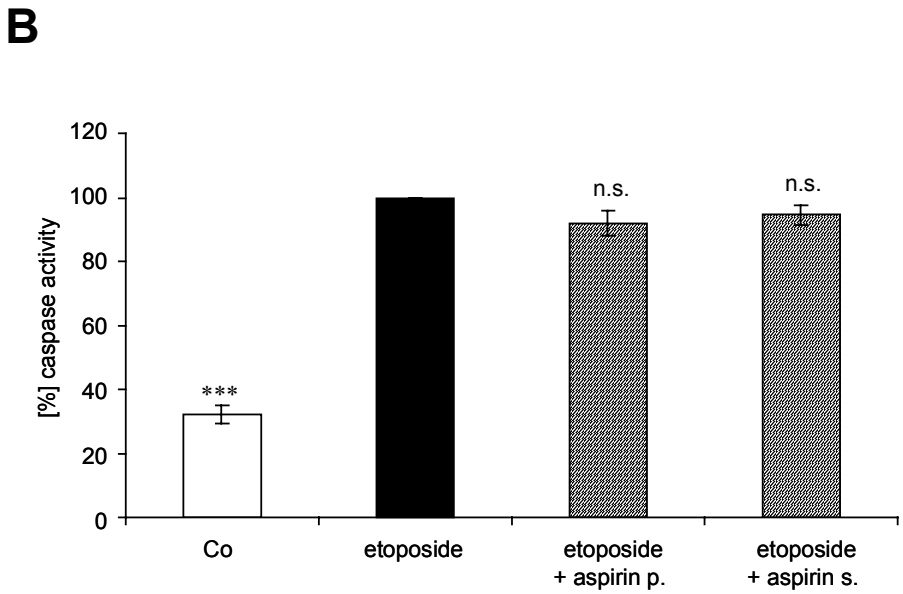


Figure 51: Effect of aspirin on TNF- α-/etoposide-induced caspase-3 activation

Caspase-3-like activity was determined in untreated cells (Co) and in cells treated with TNF-α (10 ng/mL) or etoposide (200 μmol/L), respectively, for 16 h in the presence or absence of aspirin (100 μmol/L) as described under *Materials and Methods*. Aspirin was preincubated for 4 h (p.) or added simultaneously (s.) with apoptosis inducers. Caspase activity in TNF-α- or etoposide-treated cells was referred to as 100%. Data show mean ± SEM of three independent experiments with different cell preparations performed in triplicates with *** $p<0.001$ significantly different compared to the values obtained in TNF-α- or etoposide-activated cells, respectively, n.s. no significant difference from cells exposed to TNF-α or etoposide.

3.2.4 Characterization of NF-κB activation by TNF-α

Brouard et al. recently described that NF-κB activation is crucial for HO-1 to exert protective effects on TNF-α-induced endothelial apoptosis (Brouard et al. 2002). Taking into account that aspirin has been demonstrated to be an inhibitor of NF-κB activation (Weber et al. 1995, Tegeder et al. 2001, Joussen et al. 2002), the lack of protection from TNF-α-induced cytotoxicity conferred by aspirin-induced HO-1 could be ascribed to attenuated NF-κB activation in aspirin-treated cells. The following experiments therefore aimed to elucidate the effect of aspirin on TNF-α-induced NF-κB activation in our cell model.

HUVEC were treated with TNF-α for 1 h in the presence or absence of aspirin (100 μmol/L, preincubated for 30 min) and NF-κB DNA-binding activity was assessed by EMSA.
Figure 52 shows that TNF-α lead to a pronounced activation of NF-κB. Pretreatment with aspirin significantly attenuated the activation of this transcription factor, suggesting this

inhibitory effect to be a possible cause for the lack of protection exerted by aspirin-induced HO-1.

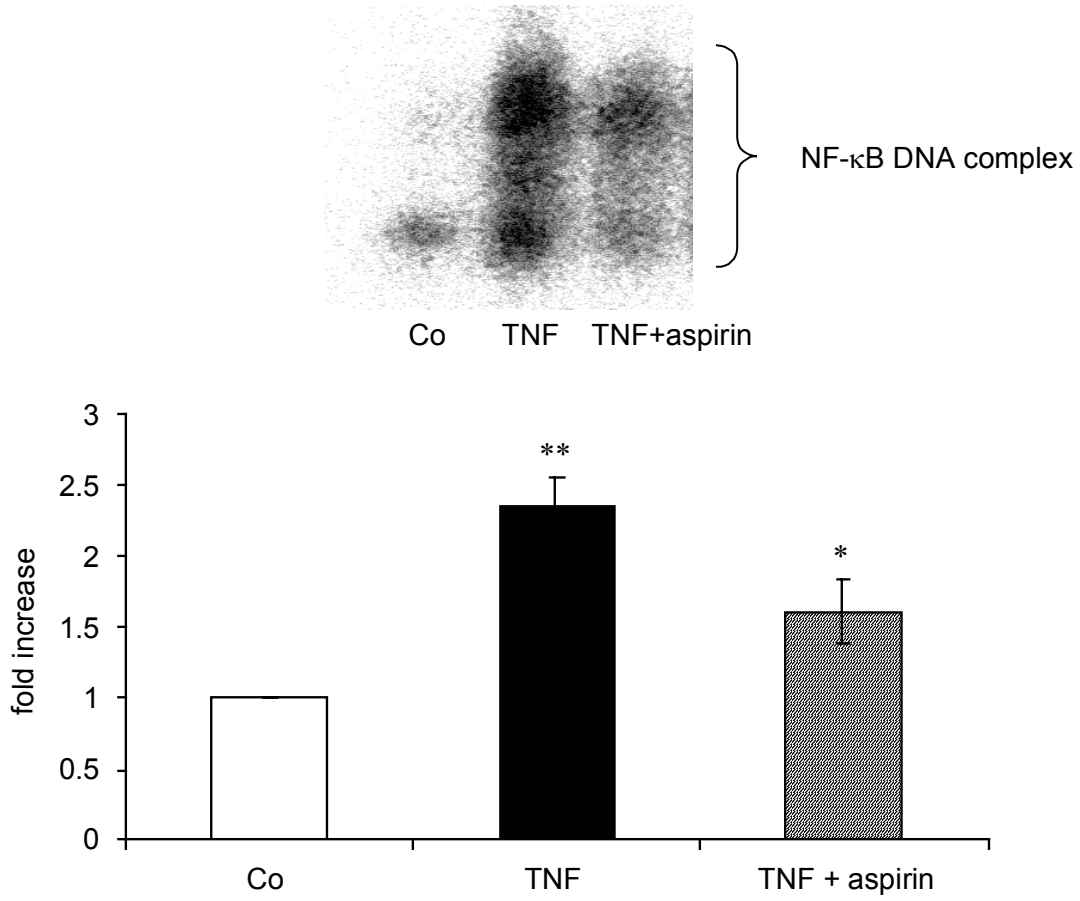


Figure 52: Effect of aspirin on TNF-α-induced activation of NF-κB

EMSA *(Materials and Methods)* was performed with untreated cells (Co) and with cells treated with TNF-α (10 ng/mL) in the presence or absence of aspirin (100 μmol/L, preincubated for 30 min). Results in the upper panel show one representative shift out of five independent experiments from different cell preparations. The graph in the lower panel illustrates the quantitative evaluation of five independent experiments, performed by phosphorimaging. Data are expressed as x-fold increase in signal intensities compared to untreated control cells which were referred to as 1.**$p<0.01$, *$p<0.05$ represent significant differences compared to the values obtained for untreated control cells.

4 DISCUSSION

Due to their exposed localization in the vasculature, endothelial cells play a critical role in the development of pathological conditions, such as inflammation and atherosclerosis. Under inflammatory conditions, endothelial cells become activated by exposure to proinflammatory stimuli, promoting vasoconstriction, leukocyte adhesion and activation, as well as coagulation and thrombosis. The cytokine TNF-α plays a key role in inflammatory disorders by upregulating the expression of adhesion molecules, promoting vascular permeability, inducing the release of vasoactive substances, and causing endothelial injury and apoptosis. Endothelial apoptosis is a critical event in the pathogenesis of atherosclerosis and contributes to the progression of inflammatory diseases by sustaining inflammation and promoting vascular thrombosis. In order to counteract cell damage caused by the inflammatory response, endothelial cells can upregulate a set of protective proteins, such as the members of the family of heat shock proteins. In this context, the effects of ANP and aspirin on the expression of HSP70 and HSP32 in HUVEC were investigated.

4.1 ANP

4.1.1 ANP induces HO-1 expression *via* activation of ERK and JNK MAP kinases and subsequent induction of AP-1 DNA-binding activity

The aim of the first section of this work was to investigate whether ANP could influence the expression of protective genes in endothelial cells and confer protection against TNF-α-induced apoptosis.

4.1.1.1 ANP induces HO-1 expression at the protein and mRNA level, but has no effect on HSP70

The results of the present work demonstrated that ANP is able to upregulate the expression of HO-1 at the mRNA and protein level. The induction of HO-1 has been reported to be a stress inducible cytoprotective response counteracting a variety of pathophysiological states, such as oxidative stress (Poss and Tonegawa 1997a), ischemia-reperfusion injury (Clark et al. 2000), inflammation (Willis et al. 1996), and apoptosis (Foresti et al. 1999). Besides the classical stress-related inducers of HO-1 expression, involving proinflammatory cytokines and heavy metal ions, few other endogenous inducers of HO-1 have been described. Examples are low density lipoproteins (Siow et al. 1999), platelet-derived growth factor (Durante et al. 1999), and dopamine (Berger et al. 2000). However, molecular mechanisms mediating HO-1

induction by these endogenous regulators have not been elucidated yet. Our data therefore present for the first time a systematical characterization of the signaling events responsible for the induction of this cytoprotective protein by a cardiovascular hormone in a human cell system.

Apart from HO-1, another important member of the family of heat shock proteins, HSP70, was investigated. Similar to HO-1, HSP70 has been shown to exert cytoprotective effects in a number of pathophysiological conditions, such as heat stress (Cumming et al. 1996), ischemia/reperfusion injury (Chen et al. 1997), sepsis (Hotchkiss et al. 1993) and other inflammatory diseases (Kohn et al. 2002, Feinstein et al. 1996), as well as apoptosis (deMeester et al. 1997). However, in contrast to its effect on HO-1, ANP was not able to influence the expression of HSP70 protein, as demonstrated by the results of the present work. Apart from heat shock, HSP70 expression is mainly upregulated by stimuli leading to different forms of cellular stress, such as oxidized LDL (Zhu et al. 1994), LPS (Flohé et al. 1999), heavy metal ions (Liu et al. 2001, Wagner et al. 1999) and hydrogen peroxide (Madamanchi et al. 2001). Its increased detection in atherosclerotic plaques suggests that HSP70 might play a critical role in atherosclerosis (Johnson et al. 1995). Exept from one report by Kiemer et al., who demonstrated that ANP prevents the decrease of HSP70 protein during reperfusion of rat livers (Kiemer et al. 2002b), no information exists on the influence of non-stressful stimuli like ANP on this heat shock protein. Therefore, the present work investigated for the first time the effect of ANP on HSP70 expression in a human cell system.

4.1.1.2 cGMP mediates the induction of HO-1

ANP is known to exert its effects by interaction with the natriuretic peptide A receptor coupled to particulate guanylate cyclase and generation of the second messenger cGMP (Tremblay et al. 2002). However, ANP also mediates several effects *via* the clearance receptor NPR-C lacking guanylate cyclase activity (Levin 1993). Therefore, it was of interest to investigate the receptor specificity of the effects observed after treatment of HUVEC with ANP. The cell permeable cGMP analogue, 8-Bromo-cGMP, could mimick the effect of ANP on HO-1 expression indicating that the upregulation of HO-1 by ANP is mediated *via* cGMP. Moreover, the specific NPR-C agonist cANF did not influence HO-1 levels, ruling out a participation of this natriuretic peptide receptor in mediating the ANP effect. Therefore, the data presented in this work demonstrate that ANP induces HO-1 *via* cGMP. Several reports showing an upregulation of HO-1 by cGMP ascribe this observation to an activator of the soluble guanylate cyclase, nitric oxide (Polte et al. 2000, Immenschuh et al. 1999).

Interestingly, however, reports about the dependency of HO-1 induction by NO on cGMP are contradictory. Liang et al. found the HO-1 induction in renal tubular epithelial cells by NO donors to be independent of cGMP (Liang et al. 2000), an observation confirmed by experiments performed in vascular smooth muscle cells (Durante et al. 1997, Hartsfield et al. 1997). Alternative cGMP-independent pathways suggested to mediate HO-1 induction by NO include the involvement of free radicals, S-nitrosothiols or NO-induced glutathione depletion and heme loss (Hartsfield et al. 1997). This discrepancy might reflect cell-type, species- and stimulus-dependent differences in HO-1 induction pathways.

Despite the fact that several groups demonstrated HO-1 induction by cGMP, only the report by Immenschuh et al. (Immenschuh et al. 1998) investigated the signal transduction pathway involved. This respective work, however, was performed in primary rat hepatocytes and reported cGMP to be unable to induce HO-1 in liver endothelial cells. To our knowledge, to date no information exists concerning the molecular mechanisms participating in HO-1 upregulation *via* cGMP-dependent pathways in a human cell system.

4.1.1.3 ANP activates JNK and AP-1 DNA-binding

The activity of the transcription factor AP-1, which has been implicated in the regulation of a wide range of genes participating in the regulation of biological processes, such as cell proliferation, differentiation, inflammatory processes and apoptosis, has been reported to be regulated by several members of the MAPK family at the level of transcription of jun and fos genes as well as by posttranslational modifications. Due to the fact that the HO-1 gene contains AP-1 binding sites (Lavrovsky et al. 1994) suggesting a possible involvement of this transcription factor in HO-1 upregulation by ANP, the effects of ANP on the JNK/AP-1 pathway were investigated. The results of the present work demonstrate an increase in DNA-binding activity of AP-1 in endothelial cells treated with ANP. Only few investigations exist concerning the influence of ANP or its second messenger cGMP on AP-1 activation and data are controversial. cGMP analogues have been shown to activate transcription from AP-1 responsive promoters in thyroid follicular cells as well as in fibroblasts (Pilz et al. 1995). Interestingly however, in other systems ANP has been found to have no impact on basal AP-1 activities, but to exert an inhibitory effect on activated AP-1. This observation was made for LPS–induced AP-1 activation in murine macrophages (Kiemer et al. 2000a) as well as for ischemia-reperfusion-induced AP-1 activity in rat liver (Kiemer et al. 2000b). Moreover, Isono and coworkers were able to demonstrate that ANP abrogated AP-1 DNA-binding activity in endothelin-1-stimulated glomerular mesangial cells (Isono et al. 1998). This respective work additionally reported an inhibitory action of ANP on endothelin-1-induced activation of JNK in the same cell model. The inhibition of JNK activity by ANP has also

been shown for VEGF-stimulated bovine aortic endothelial cells (Pedram et al. 2001). Our work, investigating the effects of ANP on JNK in resident cells, clearly demonstrates an increase in JNK activity of both isoforms by ANP.

These differential roles of ANP, either as an inducer or inhibitor of JNK/AP-1 suggest a highly species- and cell type-specific regulation of this signal transduction pathway and an important difference between resting and activated cells. These controversial data might also be ascribed to the complex regulation of AP-1 activity by the different members of the MAPK family at the level of transcription of jun and fos genes as well as by posttranslational modifications. The fact that different AP-1 complexes are reported to exhibit different transcriptional activities might provide further explanation for the controversial data found concerning regulation and activity of this transcription factor.

4.1.1.4 ANP induces the phosphorylation of ERK, but has no effect on p38 MAPK

Due to the regulatory effects of ERK and p38 on several AP-1 subunits, the influence of ANP on these two MAP kinases was investigated as well. The data presented here demonstrate that ANP induces activation of ERK in a time-dependent manner, whereas no influence on p38 MAPK activity could be detected. Our data for the first time provide evidence that ANP induces ERK activation in human endothelial cells. Previous works reported before that ANP is able to induce activation of ERK in neonatal rat ventricular myocytes (Silberbach et al. 1999). On the other hand, ANP was previously shown to inhibit endothelin-1-induced activation of ERK in rat mesangial cells (Isono et al. 1998), as well as VEGF-induced ERK activity in bovine aortic endothelial cells (Pedram et al. 2001). These results suggest that ANP differentially influences ERK in resident or activated cells.

Reports concerning ANP and p38 MAPK are rare and mostly describe an inhibitory effect of this natriuretic peptide on p38 in activated cells. This observation was reported by Tsukagoshi and coworkers for IFN-γ-treated RAW macrophages (Tsukagoshi et al. 2001), as well as for VEGF-activated bovine aortic endothelial cells (Pedram et al. 2001) and HUVEC exposed to TNF-α (Kiemer et al. 2002c). The only study documenting an activation of basal p38 MAPK after treatment with ANP comes from our laboratory and was performed in a model of ischemia reperfusion injury in the rat liver, preconditioned with ANP, therefore representing a completely different cell model (Kiemer et al. 2002d). To our knowledge, no data exist, reporting an activation of p38 MAPK in human endothelial cells.

4.1.1.5 Role of AP-1, JNK and ERK in the induction of HO-1

After investigation of the effects of ANP on the MAP kinases ERK and JNK, as well as on the transcription factor AP-1, we aimed to causally link these effects to the described ANP-mediated upregulation of HO-1. In order to demonstrate the causal involvement of the JNK/AP-1 and ERK/AP-1 signaling pathway, several inhibitors were tested for their impact on HO-1 induction by ANP. U0126, a pharmacologic inhibitor of AP-1 activity, PD 98059, an inhibitor of the ERK pathway, as well as SP 600125, a JNK inhibitor, were able to abrogate ANP-induced HO-1 upregulation. These results clearly pointed to an involvement of the described pathways mediating the ANP effects and causally connect ANP-induced increases in HO-1 mRNA and protein levels with the JNK/AP-1 and ERK/AP-1 signaling cascade. AP-1 has been demonstrated to play an important role in HO-1 regulation, as shown for thioredoxin-mediated HO-1 induction in rodent macrophages (Wiesel et al. 2000) as well as for HO-1 gene induction in murine macrophages after hyperoxic stress (Lee et al. 2000, Lee et al. 1996). The significance of AP-1 for HO-1 induction mediated by cGMP has been reported in another rodent cell system, i.e. in rat hepatocytes (Immenschuh et al. 1998). On the other hand, Oguro et al. suggested that AP-1 (Oguro et al. 1996) and JNK play a major role in phorone-induced HO-1 expression in rats (Oguro et al. 1998). A role for the ERK/AP-1 pathway in signaling leading to HO-1 upregulation has been described for sodium arsenite-mediated induction of HO-1 in a chicken hepatoma cell line (Elbirt et al. 1998). Interestingly, this work also suggested p38 MAPK to be involved in the signal transduction events leading to increased HO-1 gene expression. HO-1 induction has also been reported to occur independent of AP-1 in curcumin-treated bovine aortic endothelial cells (Motterlini et al. 2000).

Corresponding studies on human HO-1, however, are rare. Only one report by Numazawa et al. suggested a role for both AP-1 as well as ERK in HO-1 induction in human fibroblasts (Numazawa et al. 1997). Besides, Chen and Maines were able to demonstrate a role for ERK in the transcriptional upregulation of human HO-1 by NO (Chen and Maines 2000), but ruled out a participation of JNK as well as of AP-1 in the signal transduction pathway. Another controversial observation was reported by Alam et al. who described HO-1 induction to occur independent of ERK and JNK and to be mediated by p38 MAPK in cadmium-treated MCF-7 mammary epithelial cells (Alam et al. 2000). Taken together, these data reflect the complex regulation of AP-1 and HO-1, being highly dependent on cell-type, species and stimulus.

4.1.2 ANP does not confer protection of HUVEC against TNF-α- and etoposide-induced cytotoxicity

The observation that ANP upregulates the expression of HO-1, reported to play a protective role in inflammation and oxidative stress, led us to focus on potential protective effects of this cardiovascular hormone on cytotoxicity induced by TNF-α, one of the key mediators in inflammatory disease. The results of the present work demonstrate that ANP does not significantly attenuate TNF-α-mediated apoptosis in HUVEC.

HO-1 induction has previously been reported to confer protection of endothelial cells against TNF-α-induced apoptosis (Brouard et al. 2000). However, most of these investigations were performed by expressing large amounts of HO-1 in the respective cells, induced by heavy metals, porphyrins or by viral transfer of the HO-1 gene. By means of such approaches it was indeed possible to demonstrate a cytoprotective effect against oxidant-induced injury (Yang et al. 1999) as well as against oxyhemoglobin-induced endothelial dysfunction (Eguchi et al. 2001). This observation is supported by studies performed in various other cell models. For instance, HO-1 overexpression by gene transfer has been reported to protect against TNF-α-induced apoptosis in murine L929 fibroblasts (Petrache et al. 2000) and to confer protection against heme/hemoglobin-induced toxicity in rabbit coronary microvessel endothelial cells (Abraham et al. 1995). Moreover, Motterlini et al. achieved protection against oxidative stress by curcumin-mediated HO-1 induction in bovine aortic endothelial cells (Motterlini et al. 2000).

On the other hand, there are groups that report that HO-1 expression does not necessarily have cytoprotective effects. This observation was recently described by Redaelli et al. who reported no protection from ischemia/reperfusion induced apoptosis despite the expression of high levels of HO-1 in rat liver grafts (Redaelli et al. 2002). Adenovirus-mediated HO-1 gene expression has even been described to stimulate apoptosis in rat vascular smooth muscle cells (Liu et al. 2002).

These contradictory data, reporting either protection by HO-1 or non-protective effects, might be ascribed to the interplay of other pathways in the signaling events determining cellular survival. In this regard, two signaling pathways were shown to play a crucial role in HO-1-mediated protection against TNF-α-induced cytotoxicity: activation of p38 MAPK (Brouard et al. 2000, Brouard et al. 2002) and activation of NF-κB (Brouard et al. 2002). Brouard et al. found the gaseous molecule CO to mediate the antiapoptotic effect of HO-1 and to act *via* the activation of a transduction pathway involving the activation of p38 MAPK (Brouard et al. 2000). NF-κB mediates the expression of a variety of early responsive anti-apoptotic genes after binding of TNF-α to the TNFR-1. Inhibition of NF-κB therefore promotes apoptosis induced by TNF-α, whereas NF-κB activation protects cells from cell death (Beg and Baltimore 1996, van Antwerp et al. 1996, Wang et al. 1996). Brouard et al. demonstrated that

HO-1/CO cooperates with NF-κB-dependent anti-apoptotic genes to protect endothelial cells from TNF-α-mediated apoptosis and that the ability of HO-1/CO to activate the p38 MAPK pathway was necessary for this effect (Brouard et al. 2002). This observation is further supported by the findings of Madrid et al. who reported that p38 MAPK together with IKK participates in the stimulation of the transactivation potential of the p65 subunit of NF-κB by the serine/threonine kinase Akt (Madrid et al. 2001).

However, both the p38 MAPK (Tsukagoshi et al. 2001, Kiemer et al. 2002) as well as the NF-κB (Kiemer and Vollmar 1998, Kiemer et al. 2000b, Kiemer et al. 2002e) pathway have previously been shown to be inhibited by ANP. Due to the important role in cell survival attributed to these pathways, the HO-1 protein upregulated by ANP might not be sufficient to confer protection against TNF-α.

On this basis, controversial reports in the literature of either cytoprotective or cell damaging effects of ANP might also be explained. Observations exist about ANP to be either an inducer of apoptosis or to protect from cytotoxic cell damage. For instance, ANP was found to protect endothelial cells from lysophosphatidylcholine-induced cytotoxicity (Murohara et al. 1999), to attenuate kidney damage induced by different stimuli (Polte et al. 2002, Murakami et al. 1999), to reduce hepatic ischemia reperfusion injury (Gerbes et al. 1998), to prevent Kupffer cell-induced oxidant injury in the rat liver (Bilzer et al. 1999), and to reduce apoptosis in serum-deprived PC12 cells (Fiscus et al. 2001). On the other hand, there are reports that describe the induction of apoptosis in rat aortic endothelial cells (Suenobu et al. 1999) and in cardiac myocytes (Wu et al. 1997). These contradictory observations might be ascribed to the interplay of protective as well as deleterious signaling pathways modulated by ANP.

Since TNF-α alone already induces NF-κB activity and therefore the expression of antiapoptotic proteins, we tested another well-known apoptosis-inducing drug, etoposide, in the same experimental setting for its influence on NF-κB and cell viability. In contrast to TNF-α, etoposide does not activate NF-κB in HUVEC, as shown by our experiments. Besides, to our knowledge, no reports exists reporting the activation of NF-κB after etoposide in endothelial cells. Therefore, the NF-κB-regulated antiapoptotic mechanisms, described in response to TNF-α, do not influence apoptosis elicited by etoposide. Nevertheless, the results of the present work indicate that ANP does not confer significant protection against etoposide-mediated apoptosis in HUVEC, either.

Although ANP was not able to protect HUVEC against TNF-α- and etoposide-induced apoptosis, it had a strong effect on morphological changes occuring after treatment with TNF-α. The pronounced changes of cell morphology, including elongation of the cells and formation of gaps in the endothelial monolayer, were almost completely abrogated by ANP, indicating that ANP has a regulatory effect on TNF-α-exposed HUVEC. The mechanisms

underlying this effect were investigated in another project by Nina Weber and published by Kiemer et al. (Kiemer et al. 2002c).

4.2 Aspirin

4.2.1 Aspirin induces HO-1 expression *via* activation of JNK/SAPK and subsequent induction of AP-1 DNA binding activity

In recent years, an increasing number of studies have reported protective effects of aspirin against colon cancer and cardiovascular disease, as well as its ability to delay the onset of Alzheimer disease. Inhibition of COX activity can only in part account for these recently observed effects, suggesting that aspirin may exert part of its anti-inflammatory and antitumor activity by modulating cyclooxygenase-independent pathways. Therefore, the aim of the second part of the present work was to investigate the signaling pathway leading to aspirin-induced upregulation of HO-1, a potential new target through which aspirin might exert protective effects on endothelial cells. Aspirin has previously been shown to confer protection of the vascular endothelium against oxidative stress (Podhaisky et al. 1997). This effect, in addition to platelet inhibition, might represent an effective approach to influence the triggering events of atherosclerosis and to reduce the incidence of occlusive cardio- and cerebrovascular diseases. However, data on the mechanisms of this protective effect are rather sparse.

4.2.1.1 Aspirin induces HO-1 expression at the mRNA and protein level

In the present study, we characterized the effects of aspirin on the heat shock protein HO-1 and provided data indicating that aspirin can induce the expression of heme oxygenase mRNA and protein. These findings provide an interesting new target through which aspirin might be able to interfere with pathological conditions, since HO-1 has been reported to confer protection in a variety of different experimental settings. This might indeed be of relevance to the therapeutic actions of aspirin *in vivo*, since we were able to demonstrate a marked induction of HO-1 with doses as low as 10 μM aspirin, which is in a dose range achieved upon systemic administration during anti-inflammatory therapy (1-2 mM) (Abramson and Weissmann 1989, Furst et al. 1987, Cianferoni et al. 2001, Kopp and Gosh 1994). In addition to aspirin, other pharmacological inducers of HO-1 have been described.

The highly cytotoxic drug doxorubicin, for instance, has been documented to exert hepatoprotective effects by inducing HO-1 (Ito et al. 2000). Moreover, polyphenolic compounds, such as curcumin, have been identified as inducers of HO-1 (Scapagnini et al. 2002). Our findings that aspirin upregulates HO-1 might be connected to findings demonstrating an aspirin-induced increase in ferritin synthesis in bovine aortic endothelial cells (Oberle et al. 1998). Since HO-1 is known to liberate iron by heme catabolism (Maines 1997, Immenschuh and Ramadori 2000, Suematsu and Ishimura 2000) and free iron in turn induces ferritin synthesis (Eisenstein and Munro 1990), we suggest that upregulation of HO-1 might be responsible for aspirin-induced ferritin production. Besides its effect on HO-1, there exists also evidence indicating an influence of aspirin on other members of the family of heat shock proteins. For instance, aspirin has been described previously to induce HSP72 *in vivo* in rat gastric mucosa (Jin et al. 1999). Data on the influence of aspirin on heat shock proteins, however, are rare and contradictory, as shown by another report demonstrating an inhibition of heat-induced HSP72 expression in chicken testis (Mezquita et al. 2001) whereby mM concentrations of aspirin were employed. Moreover, Chen et al. recently reported an inhibitory effect of aspirin on LPS/IFN-gamma-induced HO-1 protein expression in RAW macrophages (Chen et al. 2002).

4.2.1.2 Aspirin activates AP-1 and JNK

Due to the reported role of the transcription factor AP-1 in HO-1 induction (Numazawa et al. 1997) and our own results described under 4.1., our findings that aspirin induces HO-1 protein as well as mRNA levels raised the question whether AP-1 might as well be involved in signaling by aspirin. The results presented in this work demonstrate that aspirin induces AP-1 DNA-binding as well as JNK activity in HUVEC. These observations differ from the data published on this issue by other investigators, the majority of which investigated the effects of aspirin on cells activated by diverse stimuli. To date, there is only one further group reporting a stimulatory effect on the transcription factor AP-1 by aspirin-like drugs in human T lymphocytes (Flescher et al. 1995). On the other hand, numerous studies have documented an inhibitory action of aspirin on AP-1 DNA-binding activity in different *in vitro* and *in vivo* systems. For instance, aspirin was shown to inhibit AP-1 activity elicited by UV-B radiation in transgenic mice (Huang et al. 1997) as well as angiotensin II-induced AP-1 activity in a transgenic rat model (Muller et al. 2001). Similar findings have been reported in another rodent cell system, i.e. in a mouse epidermal cell line by Chen et al. who demonstrated aspirin to interfere with arsenite-induced AP-1 activation (Chen et al. 2001). However, all these studies have demonstrated inhibitory effects of aspirin in cell systems activated by diverse stimuli and reports on basal effects of aspirin are very limited.

Respective studies in human cell systems are rather rare and there are no data on the effects of aspirin on AP-1 in human endothelial cells. A recent study by Abiru and coworkers provides evidence indicating that aspirin inhibits hepatocyte growth factor-induced AP-1 activity in human hepatoma cells (Abiru et al. 2002). Murono et al. reported aspirin to suppress AP-1 DNA-binding activity enhanced after treatment of human cervical cancer cells with EBV latent membrane protein 1 (Murono et al. 2000).

Data on the influence of aspirin on JNK activity are extremely contradictory and most works on the influence on JNK investigated the aspirin metabolite sodium salicylate. Sodium salicylate was demonstrated to activate JNK in human peripheral blood eosinophils (Wong et al. 2000) as well as in COS-1 and HT-29 human colon adenocarcinoma cells (Schwenger et al. 1999). However, the latter group could detect no activation in normal human diploid FS4 fibroblasts (Schwenger et al. 1999), an observation consistent with the findings of Chen et al. in mouse epidermal JB6 cells after treatment with aspirin (Chen et al. 2001). On the other hand, other investigators reported an inhibitory effect of aspirin on JNK activity in a variety of cell systems, such as UV B radiation-activated transgenic mice (Huang et al. 1997). Sodium salicylate was shown to suppress JNK activation by TNF-α in normal human diploid FS4 fibroblasts (Schwenger et al. 1997) as well as in LPS-stimulated macrophages (Vittimberga et al. 1999).

However, all of these studies were performed with high concentrations of aspirin and sodium salicylate, mostly ranging from 0.5 up to 5 mM or 20 mM, respectively, whereas our experiments were carried out with an aspirin concentration of 100 μM, raising the possibility that the effect of aspirin on AP-1 might be dependent on the concentration used. This idea is supported by the findings of Muller et al. who documented a strictly dose-dependent effect of aspirin on the transcription factors AP-1 and NF-κB (Muller et al. 2001). Another possible explanation for these contradictory results may lie in the fact that actions of aspirin occur to be cell type-specific. Consistent with this idea, examples of cell type-specific actions of aspirin and salicylates have been presented by Schwenger et al. who reported a coordinate activation of p38 MAPK and JNK in COS-1 and HT-29 cells, but merely an effect on p38 MAPK and no activation of JNK in FS 4 fibroblasts (Schwenger et al. 1999). Moreover, Weyand et al. failed to demonstrate an inhibitory effect of aspirin on NF-κB in a recent study in an inflammatory mouse model although this effect has been widely documented in a variety of different cell systems (Weyand et al. 2002). Most importantly, it always has to be distinguished between basal effects and effects on activated cells. Studies reporting an inhibitory effect of aspirin on JNK activity were performed in cells treated with different stimuli whereas our experiments investigated aspirin effects on basal JNK activity levels.

4.2.1.3 Aspirin has no effect on the phosphorylation of ERK and p38 MAPK

Since ERK and p38 participate in the regulation of several AP-1 subunits, we also tested the possibility that aspirin might exert part of its effect on AP-1 activity by influencing these two MAP kinases. The results of the present work clearly indicate that aspirin does neither interfere with the phosphorylation status of ERK nor with p38 MAPK activation in HUVEC.

The observations concerning ERK activity are in agreement with the findings of other investigators. For instance, Dong et al. investigated the influence of aspirin on ERK activity in TPA-activated mouse epidermal JB6 cells and found the phosphorylation of this MAP kinase not to be influenced by aspirin (Wong et al. 2000). The same observation has been described in transgenic mice exposed to asbestos (Ding et al. 1999). In other cell systems, aspirin has been reported to exert an inhibitory effect on ERK phosphorylation, such as in hepatocyte growth factor-activated human hepatoma cells (Abiru et al. 2002), in *formyl*methionyl-leucyl-phenylalanine-stimulated neutrophils (Pillinger et al. 1998) and in transgenic mice exposed to UV B radiation (Huang et al. 1997). However, it should be noted that the majority of the studies focused on the effects of aspirin on cells activated with the respective stimuli, whereas only few works assessed the influence of aspirin on basal levels of phosphorylated ERK, as it was the case in our experiments.

Data concerning the effects of aspirin on p38 MAPK are rather controverse. Aspirin has been reported to induce p38 MAPK activity in normal human diploid FS4 fibroblasts (Schwenger et al. 1999), an effect that has been observed in the same cell system after treatment with sodium salicylate (Schwenger et al. 1997). Moreover, sodium salicylate has been shown to activate p38 MAPK in human peripheral blood eosinophils (Wong et al. 2000). On the other hand, Wang et Brecher presented results indicating that salicylate has no effect on p38 MAPK activity (Wang and Brecher 1999) consistent with the observations of Chen et al. in arsenite-activated mouse epidermal JB6 cells treated with aspirin (Chen et al. 2001). An inhibitory effect of aspirin on p38 MAPK phosphorylation has been documented in different stimulated cell models (Huang et al. 1997, Paccani et al. 2002). Taken together, these diverging observations again support the idea that effects of aspirin might be extremely dependent on the concentration used as well as on the cell system in which the respective investigation is performed.

4.2.1.4 Role of JNK/AP-1 in the induction of HO-1 by aspirin

In view of the results presented so far, we aimed to establish a causal relationship between upregulation of HO-1 mRNA and protein by aspirin and the activation of the JNK/AP-1 pathway. In order to examine the role of JNK/AP-1 in the signaling leading to HO-1

upregulation, we tested U0126, a pharmacologic inhibitor of AP-1 activity, as well as SP 600125, a JNK inhibitor, for their ability to attenuate HO-1 induction by aspirin. Both inhibitors were able to abrogate aspirin-induced HO-1 upregulation, suggesting the JNK/AP-1 pathway to play a major role in the signaling events responsible for increased HO-1 protein and mRNA levels after treatment with aspirin.

As discussed under 4.1.1.5, AP-1 has previously been documented to play a central role in the regulation of HO-1 expression, as evidenced for thioredoxin-mediated HO-1 induction in rodent macrophages (Wiesel et al. 2000), as well as for cGMP-mediated HO-1 induction in rat hepatocytes (Immenschuh et al. 1998) and HO-1 induction in human fibroblasts (Numazawa et al. 1997). Interestingly, Hill-Kapturczak et al. reported the polyphenolic compound curcumin, a well-known inhibitor of AP-1, to induce HO-1 in human renal proximal tubule cells (Hill-Kapturczak et al. 2001).

4.2.2 Aspirin is not able to protect HUVEC against TNF-α- or etoposide-induced cytotoxicity

Due to the fact that aspirin was able to induce the expression of HO-1, we considered the possibility that aspirin could confer protection against TNF-α-induced cytoxicity *via* this heat shock protein. The results of the present work, however, provide evidence that aspirin does not significantly interfere with apoptosis induced by TNF-α, a central signaling molecule in inflammatory states, in HUVEC.

As described under 4.1.2, cytoprotective effects have been attributed to HO-1 expression in a variety of cell systems exposed to different deleterious stimuli, such as endothelial cells treated with TNF-α (Brouard et al. 2000).

On the other hand, there is evidence indicating that HO-1 expression does not necessarily elicit cytoprotective effects, such as a recent study by Redaelli et al. who detected no protection from ischemia/reperfusion-induced apoptosis despite the expression of high levels of HO-1 in rat liver grafts (Redaelli et al. 2002) and a work by Liu et al showing the induction of apoptosis in rat vascular smooth muscle cells by adenovirus-mediated HO-1 gene expression (Liu et al. 2002). As mentioned under 4.1.2, these controversial data, reporting either protection by HO-1 or non-protective effects, might be accounted for by the interplay of other pathways. The two signaling components demonstrated to play a pivotal role in HO-1-mediated protection against TNF-α-induced cytotoxicity are activated p38 MAPK (Brouard et al.2000, Brouard et al. 2002) and activated NF-κB (Brouard et al. 2002).

Aspirin has been demonstated to interfere with both p38 MAPK and the NF-κB activity. While reports concerning the influence of aspirin on p38 MAPK are quite contradictory, as mentioned under 4.1.3.3, its inhibitory effect on NF-κB has been widely documented in a great variety of cell systems, such as in TNF-α-stimulated HT-29 human colon adenocarcinoma cells and COS-1 African green monkey kidney cells (Schwenger et al. 1997, Alpert et al. 1999), in LPS- or TNF-α-treated Jurkat cells (Kopp and Gosh 1994, Yin et al. 1998) and in LPS-stimulated macrophages (Vittimberga et al. 1999). We also observed the inhibition of NF-κB DNA-binding activity in TNF-α-treated cells in our cell model.

On account of the pivotal role of these pathways and the evidence for their inhibition by aspirin, the HO-1 protein upregulated by aspirin might not be sufficient to confer protection against TNF-α. Besides, the influence of aspirin on other pathways might also play an important role in the regulation of cell viability. One example are the MAPK. ERKs are characteristically associated with cell proliferation and have previously been demonstrated to protect cells from apoptosis in a number of cell systems (Xia et al. 1995), whereas JNK (Tournier et al. 2000) and p38 MAPK (Schwenger et al. 1997) are able to promote apoptosis in several experimental settings. In view of these findings, the lack of effect of aspirin on ERK together with the activation of JNK observed in the present study, might also contribute to the inability of aspirin to protect HUVEC against apoptosis elicited by TNF-α.

These observations might as well provide a possible explanation for the controversial reports in the literature describing aspirin as an either cytoprotective or apoptosis-inducing drug. Aspirin has previously been shown to protect endothelial cells from H_2O_2-induced cytotoxicity (Podhaisky et al. 1997). The induction of ferritin was suggested to contribute to this cytoprotective action (Oberle et al. 1998). Further evidence in favour of a cytoprotective action of aspirin was provided by a study on arsenite-induced apoptosis in mouse epidermal JB6 cells (Chen et al. 2001) as well as by Grilli et al. who demonstrated neuroprotective properties of aspirin against toxicity elicited by glutamate in rat primary neuronal cultures (Grilli et al. 1996). In an *in vivo* rat model, aspirin was found to protect from angiotensin II-induced organ damage (Muller et al. 2001). On the other hand, aspirin has been reported to induce apoptosis in gastric (Tomisato et al. 2001) and oral (Slomiany and Slomiany 2001) mucosal cells, as well as in HeLa cells (Callejas et al. 2002). Consistent with these findings, Schwenger et al. documented that sodium salicylate induces apoptosis in normal human diploid FS4 fibroblasts (Schwenger et al. 1997), an observation also described in human peripheral blood eosinophils (Wong et al. 2000).

In another set of experiments, we examined the influence of aspirin on etoposide-induced apoptosis in HUVEC. As mentioned under 4.1.2, compared to TNF-α, etoposide has the advantage that this chemotherapeutic drug is not an activator of NF-κB in our cell model, as confirmed by the literature and our own experiments. The data presented in this work demonstrate that aspirin can not confer protection to HUVEC exposed to etoposide. In the

literature, one report exists documenting protection of colon carcinoma cells from etoposide-induced apoptosis by aspirin (Ricchi et al. 2002). This might reflect cell type-specific differences in the regulation of apoptosis by aspirin.

4.3 Outlook

The results on the influence of ANP and aspirin on TNF-α- and etoposide-treated HUVEC suggest that HO-1 upregulation alone might not be sufficient to confer significant protection to HUVEC treated with different apoptosis-inducing agents. Protection seems to require the induction of additional protective pathways, such as NF-κB and p38, shown to be inhibited by ANP and aspirin. This observation raises the question what physiological function the induction of HO-1 by ANP and aspirin might serve. Although HO-1 has mainly been described to play an important role in cytoprotection, other regulatory functions of this inducible protein have been reported. In this context, HO-1 has been demonstrated to reduce vasoconstriction and inhibit cell proliferation during vascular injury (Duckers et al. 2001) as well as to attenuate the expression of COX-2, a well-known mediator of inflammatory responses (Haider et al. 2002). Since the antiproliferative potential of ANP is well-known (Appel 1992) and ANP has been shown to reduce COX-2 induction (Kiemer et al. 2002a), a causal relationship between these regulatory actions of ANP and HO-1 induction might be suggested. Antiproliferative effects of aspirin and salicylates have been reported as well (Marra and Liao 2001, Marra et al. 2000). Moreover, HO-1 has been suggested to modulate NO production by NO synthase (Maines 1997). An inhibitory effect on the expression of the inducible NO synthase has also been demonstrated for ANP (Kiemer and Vollmar 1998, Kiemer and Vollmar 2001a, Kiemer and Vollmar 2001b), an effect that might be mediated by HO-1 induction by ANP. Aspirin has been shown to inhibit iNOS expression in a variety of cell models (Amin et al. 1995, Katsuyama et al. 1999).

Taken together, these data demonstrate that there are various potential targets apart from cytoprotection, through which ANP- and aspirin-mediated HO-1 induction might confer regulatory effects to endothelial cells.

5 SUMMARY

5.1 ANP

In the present work, we could demonstrate a significant time- and dose-dependent induction of HO-1 protein and mRNA levels by ANP which was mediated *via* binding to its particulate guanylate-cyclase-coupled receptor. Concerning the molecular mechanism leading to the upregulation of HO-1, we were able to show that ANP increased the DNA-binding activity of the transcription factor AP-1. In Western blot experiments performed with phospho-specific antibodies for the MAP kinases JNK, ERK and p38, we could further demonstrate an activation of JNK as well as ERK after treatment with ANP, whereas it had no effect on p38 MAPK. The causal relationship between the observed ANP-induced increases in AP-1, JNK and ERK activity and the induction of HO-1 was proven by the use of specific pharmacological inhibitors. Taken together, we demonstrated that ANP induces HO-1 mRNA and protein expression *via* binding to its particulate guanylate-cyclase-coupled receptor, activation of the MAP kinases JNK and ERK, and subsequent activation of the transcription factor AP-1.
The cytokine TNF-α as well as the topoisomerase inhibitor etoposide induce apoptosis in human endothelial cells. Despite the induction of HO-1, neither simultaneous addition nor preincubation with ANP was able to confer significant protection of HUVEC against TNF-α- or etoposide-induced apoptotic cell death.

5.2 Aspirin

In the present work, we could report for the first time that aspirin induces the expression of HO-1 at protein and mRNA level in a time- and dose-dependent manner. The aspirin concentrations effective in upregulating HO-1 were within the dose range of plasma levels achieved during antiinflammatory therapy. Moreover, we were able to elucidate the molecular mechanism leading to the observed HO-1 induction. Aspirin was shown to increase the DNA-binding activity of AP-1 as well as JNK activation, whereas it did not affect the MAP kinases ERK and p38. The causal involvement of the effects of aspirin on AP-1 and JNK in the induction of HO-1 was demonstrated by employing specific pharmacological inhibitors.

Moreover, we report here that aspirin was not able to protect endothelial cells against TNF-α- and etoposide-induced apoptosis.

Taken together, the data presented in the present work provide for the first time a characterization of the molecular mechanisms leading to the induction of HO-1 in human endothelial cells after treatment with ANP, as well as aspirin for which the influence on this heat shock protein was shown for the first time. Heat shock proteins are of central importance for the protein homeostasis of the cell due to their function as molecular chaperones under physiologic as well as stress conditions. Therefore, it is of vital importance to elucidate the mechanisms controlling the expression of heat shock proteins and the present work represents an important contribution to the understanding of the regulation of the heat shock protein HO-1. Although the upregulation of HO-1 by ANP and aspirin was not sufficient to protect endothelial cells against apoptosis induced by the cytokine TNF-α, this heat shock proteins might unfold its cytoprotective potential by acting on other attractive targets, such as COX-2 or NO synthase, important mediators of the inflammatory response.

6 APPENDIX

6.1 Abbreviations

AIDS	Aquired immuno deficiency syndrome
ANP	Atrial natriuretic peptide
AP-1	Activator protein 1
APS	Ammonium persulphate
ATP	Adenosine-5'-triphosphate
BNP	Brain natriuretic peptide
bp	Basepair
BSA	Bovine serum albumine
cAMP	cyclic Adenosine-5'-monophosphate
cDNA	complementary DNA
cGMP	cyclic Guanosine-5'-monophosphate
CNP	C-type natriuretic peptide
Co	Control
COX	Cyclooxygenase
Da	Dalton
dATP	2'-Desoxyadenosine-5'-triphosphate
dCTP	2'-Desoxycytosine-5'-triphosphate
dGTP	2'-Desoxyguanosine-5'-triphosphate
DMSO	Dimethylsulphoxide
DNA	Desoxyribonucleic acid
dNTP	Desoxynucleosidtriphosphate
dsDNA	double strand DNA
DTT	Dithiothreitol
ECL	Enhanced Chemoluminescence reagent
EDTA	Ethylene diamine-N,N,N',N'-tetra acid
EGF	Endothelial growth factor
EGTA	
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular related kinase
EtBr	Ethidium bromide
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
FITC	Fluoresceinisothiocyanate
FCS	Fetal calf serum
GAPDH	Glyceraldehyde phosphate dehydrogenase
GTP	Guanosine-5'-triphosphate
h	Hour

HFS	hypotonic fluorochrome solution
HO-1	Heme oxygenase-1
HSP	Heat shock protein
HUVEC	Human Umbilical Vein Endothelial Cells
IBMX	Isobutylmethylxanthine
IFN γ	Interferon gamma
IκB (α/β)	Inhibitory protein kappa (α/β)
iNOS	inducible NO synthase
JNK	c-jun N-terminal kinase
kDa	kilo Dalton
L	Liter
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
m	milli (10^{-3})
M	Molar
MAPK	mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MeOH	Methanol
MW	molecular weight
μ	micro (10^{-6})
min	Minute
mRNA	messenger Ribonucleic acid
% (m/v)	mass per volume per cent
n	nano (10^{-9})
NF-κB	Nuclear Factor κB
NP	Natriuretic peptides
NPR	Natriuretic peptide receptor
NSAID	non steroidal antiinflammatory drug
NTP	ATP, CTP, GTP, or TTP
OD	Optical density
PAA	Polyacrylamide
PAGE	Polyacrylamide-gel-electrophoresis
PBS	Phosphate buffered saline solution
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonylfluoride
RNA	Ribonucleic acid
Rnase	Ribonuclease
rpm	Rotations per minute
RT	Room temperature
SAPK	stress-activated protein kinase

sec	Second
SEM	Standard error of the mean value
SDS	Sodium dodecyl sulfate
SMC	Smooth muscle cells
SNP	Sodium nitroprusside
ssDNA	single strand DNA
TAE	Tris, acetate, EDTA buffer
TBS-T	Phosphate buffered saline solution with Tween
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylendiamine
TNF-α	Tumor necrosis factor alpha
totRNA	total RNA
TRIS	Tris-hydroxymethyl-aminomethan
TXA_2	Thromboxane A_2
UV	ultra violet
V	Volt
VEGF	Vascular endothelial growth factor
% (v/v)	volume per volume per cent
W	Watt

6.2 Alphabetical order of companies

Alexis Biochemicals	(Grünberg, Germany)
Amersham	(Braunschweig, Germany)
Beckmann Instruments	(Munich, Germany)
Becton Dickinson	(San Jose, CY, USA/Heidelberg, Germany)
Biochrom	(Berlin, Germany)
Biometra	(Göttingen, Germany)
BioRad Laboratories	(Munich, Germany)
Calbiochem	(Schwalbach, Germany)
Carl Roth	(Karlsruhe, Germany)
Cell Signaling	(Frankfurt/M, Germany)
Eppendorf	(Maintal, Germany)
Fluostar, BMG GmbH	(Offenburg, Germany)
Gibco/BRL	(Eggenstein,Germany)
Greiner	(Nürtingen, Germany)
Heraeus Instruments	(Munich, Germany)
Jackson Immunolab	(Hamburg, Germany)
Kodak digital science	(Stuttgart, Germany)

Merck-Eurolab	(Munich, Germany)
Millipore	(Eschborn, Germany)
MWG-biotech	(Ebersberg, Germany)
NEN	(Cologne, Germany)
NUNC	(Wiesbaden, Germany)
PAN	(Aidenbach, Germany)
PE Applied Biosystems	(Weiterstadt, Germany)
Peske	(Aindling-Pichl, Germany)
Promega	(Mannheim, Germany)
Promocell	(Heidelberg, Germany)
Qiagen	(Hilden, Germany)
Roche	(Mannheim, Germany)
Roth	(Nürnberg,Germany)
Santa Cruz	(Heidelberg, Germany)
Saxon Biochemicals	(Hannover, Germany)
Schleicher & Schüll	(Dassel, Germany)
Serotec LTD	(Wiesbaden, Germany)
Sigma	(Taufkirchen, Germany)
SLT Labinstruments	(Crailsheim, Germany)
Thermo Life Sciences	(Egelsbach, Germany)
Zeiss	(Munich, Germany)

6.3 Publications

Parts of this work are published or are in preparation for publication:

Poster:

Nina C. Weber, Nicole Bildner, Angelika M. Vollmar, Alexandra K. Kiemer, 2002, ANP inhibits TNF-α-induced expression of adhesion molecules in endothelial cells *via* induction of IκB. Naunyn-Schmiedeberg's Archives of Pharmacology, Suppl.1 to Vol.365, March 2002.

Alexandra K. Kiemer, Nina C. Weber, Nicole Bildner, Angelika M. Vollmar, 1999, ANP verhindert TNF-α-induzierte Veränderungen von Morphologie und Zytoskelett in Endothelzellen. Proceedings 7. Tagung für Zell- und Gewebeschädigung in Weinheim.

Alexandra K. Kiemer, Nicole Bildner, Nina C. Weber, Angelika M. Vollmar, HO-1 induction by ANP is mediated via MAPK activation and does not confer protection against apoptosis. VIII. NO Forum der deutscHSPrachigen Länder, Frankfurt 2002.

Original publications:

Alexandra K. Kiemer, Nina C. Weber, Robert Fürst, Nicole Bildner, Stefanie Kulhanek-Heinze, Angelika M. Vollmar, 2002. Inhibition of p38 MAPK activation via induction of MKP-1: Atrial Natriuretic Peptide reduces TNF-α-induced actin polymerization and endothelial permeability. *Circulation research, May 2002*.

Alexandra K. Kiemer, Nicole Bildner, Nina C. Weber, Angelika M. Vollmar, 2002. Characterization of Heme Oxygenase 1 (HSP 32) induction by Atrial Natriuretic Peptide in human endothelial cells. *Endocrinology, submitted for publication*.

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Curriculum vitae

Personal data

Name:	Nicole Jutta Erika Bildner, Mohaupt
Date and place of birth:	07.12.1972, Berlin
Married since:	25.05.1998
Citizenship:	German

Education

1979 – 1983:	Elementary school, Kirchheim
1983 – 1992:	Grammar school Kirchheim
06/1992:	Abitur

Studies:

WS 1992 – SS 1997:	Study of pharmacy at the LMU Munich
08/1994:	First state examination pharmacy
10/1997	Second state examination pharmacy
11/1997 – 04/1998:	Internship at the Rumford-Apotheke, Munich
05/1998 – 10/1998:	Internship at the Klinge Pharma Inc., Munich
11/1998 – 02/1999:	Work in the same department at Klinge Pharma Inc.
03/1999:	Third state examination pharmacy and certificate as a pharmacist
03/1999 – 11/2002:	Ph.D. dissertation on "Characterization of Heme oxygenase-1 induction in human endothelial cells" in the research group of Prof. Dr. A. M. Vollmar, Department of pharmacy and drug research, Ludwig-Maximilians-Universität