Dissertation

Cyanate is a novel inducer of endothelial dysfunction: A potential link between inflammation, smoking and uremia

submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Graz, ---------- ----------

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TABLE OF CONTENTS

ABBREVIATIONS

ACh	acetylcholine chloride
ADMA	asymmetric dimethylarginine
ADP	adenosine diphosphate
ATP	adenosine-5'-triphosphate
BAY	BAY 11-7082
BH_4	tetrahydrobiopterin
BSA	bovine serum albumin
BW	bodyweight
C5a	complement component 5a
Ca^{2+}	calcium
CaM	calmodulin
CD	cluster of differentiation
cGMP	cyclic guanosine monophosphate
CKD	chronic kidney disease
CML	carboxymethyl-lysine
CRP	C-reactive protein
CVD	cardiovascular disease
DAN	2, 3-diaminonaphthalene
DTPA	diethylene triamine pentaacetic acid
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
ERK1/2	extracellular signal-regulated kinase 1/2
ESRD	end-stage renal disease
FAD	flavin adenine dinucleotide
FAK	focal adhesion kinase
FBS	fetal bovine serum
Fe	iron (heme)
FITC	fluorescein isothiocyanate
FMN	flavin mononucleotide
GLT	gliotoxin
H_2O_2	hydrogen peroxide
HBr	hydrobromic acid
HCAEC	human coronary artery endothelial cells
HCit	homocitrulline, carbamyllysine
HCl	hydrochloric acid
HDL	high-density lipoprotein

HNCO	isocyanic acid
HOCl	hypochlorous acid
HOSCN	hypothiocyanic acid
HPLC	high performance liquid chromatography
hsp90	heat shock protein 90
ICAM-1	intercellular adhesion molecule-1
IL-1	interleukin-1
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-8	interleukin-8
IP-10	interferon gamma-induced protein 10
IκB	inhibitory κB
JAMs	junctional adhesion molecules
JNK	c-jun N-terminal kinase
KBr	potassium bromide
kDa	kilo Dalton
LC-MS/MS	liquid chromatography – tandem mass spectrometry
LDL	low-density lipoprotein
LDL-c	LDL-cholesterol
LFA-1	lymphocyte function-associated antigen 1
L-NNA	N5-[imino(nitroamino)methyl]-L-ornithine
Lys	lysine
Mac-1	macrophage-1 antigen
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MIG	monokine induced by gamma interferon
MIP-1α	macrophage inflammatory protein-1alpha
MLCK	myosin light chain kinase
MPO	myeloperoxidase
mRNA	messenger RNA
MTT	3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NAT	2, 3-naphthotriazole
NE	norepinephrine
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NH_2	amino group
NO	nitric oxide
NOS	nitric oxide synthase

O_2	oxygen
O_2^-	superoxide anion
OCN^-	cyanate
$ONOO^-$	peroxynitrite anion
PBS	phosphate buffered saline
PDVF	polyvinylidene difluoride
PE	phosphatidylethanolamine
PECAM-1	platelet endothelial cellular adhesion molecule-1
PGI_2	prostaglandin I_2 (prostacyclin)
PMNL	polymorphonuclear leukocytes
ppbv	part per billion by volume
ppmv	part per million by volume
pptv	part per trillion by volume
PS	phosphatidylserine
RANTES	regulated upon activation, normal T-cell expressed, and secreted
ROS	reactive oxygen species
S1177	serine-1177
SCN^-	thiocyanate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sICAM-1	soluble intercellular cell adhesion molecule-1
SMC	smooth muscle cells
SNP	sodium nitroprusside
SOD	superoxide dismutase
TNF-α	tumor necrosis factor-alpha
VCAM-1	vascular cell adhesion molecule-1
VE-cadherin	vascular endothelial- cadherin
VLA-4	very late antigen-4

SUMMARY

Many studies have evaluated the impact of protein carbamylation on structure and/or function of proteins, enzymes and hormones. The irreversible carbamylation of proteins by the reactive electrophile "cyanate" has been demonstrated to predict cardiovascular risk and is thought to promote vascular dysfunction; however, the underlying mechanisms remain unclear.

Endogenous sources of cyanate include the slow breakdown of urea *in vivo* and myeloperoxidase (MPO)-catalyzed oxidation of thiocyanate, which has recently been introduced as the preferred substrate of MPO. Exogenous sources of cyanate also exist as it was shown that humans are significantly exposed to cyanate, a major constituent of smoke, generated when coal, fuel, biomass, or tobacco is burned.

Although humans are exposed to considerable amounts of cyanate from various sources, very little is known about the impact of cyanate on human health. In particular, the effect of cyanate on the endothelium, regulator of vascular homeostasis, has not been investigated yet.

In the first part of this PhD thesis, we show that cyanate induces the expression of inflammatory mediators in human coronary artery endothelial cells (HCAEC). Cyanate induced a time- and concentration-dependent increase in intercellular cell adhesion molecule-1 (ICAM-1) expression depending on activation of the mitogen activated protein kinase p38 (p38 MAPK) and nuclear factor kappa B (NF-κB) signaling pathways. Additionally, cyanate could promote the release of inflammatory chemokines, IL-8 and MCP-1 in a concentration-dependent manner when compared to untreated cells. Cyanate-activated endothelial cells displayed enhanced neutrophil adhesion which was reversed upon NF-κB inhibition.

Mice receiving cyanate in drinking water exhibited marked endothelial ICAM-1 expression in the aorta as evident by immunohistochemical staining. In addition, elevated plasma levels of carbamyllysine (a marker for cyanate exposure which reflects the extent of plasma protein carbamylation), was observed. Interestingly, in patients with end-stage renal disease, a significant correlation between plasma carbamyllysine (HCit) levels and soluble ICAM-1 levels was observed.

In the second part of this work, prompted by the recent finding that smoke serves as an efficient and direct route for isocyanic acid/cyanate delivery, we focused on exploring the effect(s) resulting from chronic inhalation of cyanate which remain yet unknown. Smokers are particularly exposed to significant levels of isocyanic acid/cyanate resulting from not only pyrolysis of tobacco itself but also urea a major tobacco additive. Additionally, thiocyanate, a detoxification product of hydrocyanic acid present in tobacco smoke, is oxidized by the leukocyte enzyme MPO, augmenting the cyanate pool *in vivo*.

In mice, chronic inhalation of cyanate induced significant plasma protein carbamylation (as revealed by mass spectrometry), corresponding to levels previously observed in humans with cardiovascular disease.

Notably, cyanate treatment markedly attenuated arterial vasorelaxation of aortic rings in response to acetylcholine, without affecting sodium nitroprusside-induced relaxation. Moreover, total endothelial nitric oxide synthase (eNOS), phospho-eNOS (S1177) and levels of the active dimeric form of eNOS were significantly reduced in aortic tissue of cyanate-treated mice, which was accompanied by a marked decrease in production of nitric oxide.

Resembling our findings in mice, cyanate treatment of HCAEC decreased eNOS protein expression in a concentration- and time-dependent manner, whereas eNOS protein expression was not altered when low molecular weight substances (i.e. cyanate) were removed from cell culture medium, ruling out a role of carbamylated (lipo)proteins in modulating eNOS.

We show for the first time that inhalation of cyanate disrupts endothelial function by altering eNOS activity. This data suggest that cyanate, a potentially harmful constituent in tobacco smoke, induces endothelial dysfunction *in vivo* and *in vitro*, and might hence contribute towards increased cardiovascular risk of smokers.

The findings of this current work denote cyanate as a potential inducer of endothelial dysfunction, thereby linking inflammation, smoking, and uremia.

ZUSAMMENFASSUNG

Die irreversible Carbamylierung von Plasmaproteinen durch reaktives Zyanat korreliert stark mit dem kardiovaskularen Risiko, die tatsächlichen Mechanismen sind aber nicht klar. Endogen entsteht Cyanat durch den langsamen Zerfall von Harnstoff oder durch enzymatische Oxidation von Thiocyanat. Cyanat wird aber auch über die Atemluft aufgenommen, so entsteht Cyanat z.B. beim Verbrennen von Biomasse und Tabak. Obwohl seit längerem bekannt ist, dass wir beträchtlichen Mengen von Cyanat ausgesetzt sind, ist die direkte Wirkung von Cyanat auf die Funktionalität von Endothelzellen bislang nicht erforscht.

In dieser Doktorarbeit sollte nun untersucht werden, ob physiologisch relevante Konzentrationen von Cyanat Endothelzellen aktivieren können, ein entscheidender Schritt bei der Entstehung von Atherosklerose.

Im ersten Teil der Arbeit zeigen wir, daß Cyanat selektiv die Expression von interzellulären Adhäsionsmolekülen (ICAM-1) in humanen arteriellen Endothelzellen induziert, was zu einer deutlich erhöhten leukozytären Adhesion führt. Die induzierte Expression von ICAM-1 wird mittels MAP-Kinasen und NF-κB induziert. Die Zugabe von Cyanat zum Trinkwasser von Mäusen induziert eine signifikante Carbamylierung von Plasmaproteinen, die vergleichbar ist mit dem Carbamylierungsgrad von Plasmaproteinen in Dialysepatienten. In den mit Cyanat behandelten Mäusen konnte eine signifikant erhöhte ICAM-1 Expression in der Gefäßwand von Aorten festgestellt werden. Interessanterweise zeigte sich, dass lösliche ICAM-1 Proteine (sICAM-1) signifikant mit dem Carbamylierungsgrad von Plasmaproteinen in Dialysepatienten korrelieren, was die physiologische Relevanz unserer Arbeit unterstreicht.

Nachdem vor allem Raucher erhöhten Cyanat Konzentrationen ausgesetzt sind, untersuchten wir im zweiten Teil der Doktorarbeit, ob chronisch inhaliertes Cyanat die Gefäßreaktivität beeinträchtigen kann. Die Inhalation von Zyanat führte zu einer signifikanten Reduktion der vaskulären Reaktivität, die mit einer deutlich verringerten Expression und Aktivität der endothelialen NO-Synthase einherging. Diese *in vivo* Resultate konnten wir *in vitro* bestätigen, wo eine Kultivierung humaner arterieller Endothelzellen in Gegenwart von Cyanat signifikant die eNOS Protein Expression reduzierte.

Die Ergebnisse dieser Arbeit zeigen ganz klar, daß Cyanat eine endotheliale Dysfunktion induzieren kann und somit möglicherweise das kardiovaskulare Risiko bei Rauchern und Patienten mit Nierenerkrankungen erhöht.

I. INTRODUCTION

Endothelium

A monolayer of endothelial cells forming the inner lining of all blood vessels within the vascular tree, covering glycocalyx (glycosaminoglycans/proteoglycans) and underlying basement membrane constitute the endothelium (Granger, Senchenkova 2010a). The human body contains approximately ten trillion endothelial cells covering a surface area of up to 7000 m^2; hence the endothelium is the largest organ in the body with a strategic position between blood vessel walls and blood stream. Therefore, endothelial cell structure and functional integrity are fundamental in maintenance of vessel wall and circulatory functions (Jaffe 1987, Galley, Webster 2004).

Endothelial cells actively and reactively participate in haemostasis and immune and inflammatory reactions. Being the chief regulator of vascular homeostasis, the endothelium maintains the balance between vasodilation and vasoconstriction, inhibition and stimulation of smooth muscle cell (SMC) proliferation and migration, as well as thrombogenesis and fibrinolysis through the secretion of a large variety of mediators as outlined in ***Figure I-1*** (Luscher, Barton 1997).

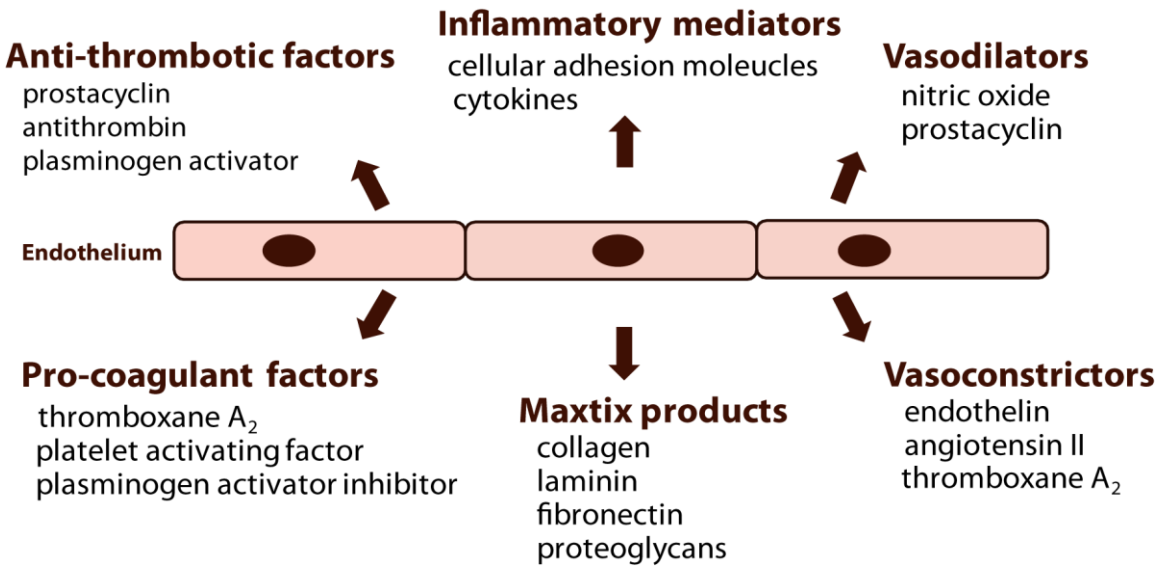

Figure I-1. Array of endothelial functions. *Through the secretion of a large variety of mediators endothelial cells influence various functions throughout the body including control over coagulation, and fibrinolysis to maintain a non-thrombogenic blood-tissue interface, arterial vessel tone as well as leukocyte infiltration into underlying tissue through a highly regulated process involving adhesion molecules and cytokines.*

Endothelial Activation and Dysfunction

Endothelial dysfunction was initially identified as impaired vasodilation to specific stimuli as acetylcholine or bradykinin. Thereafter, a broader understanding of endothelial dysfunction developed to include an overall pro-inflammatory and pro-thrombotic state in which activated endothelial cells undergo a variety of changes leading to characteristic alterations in microvascular function. These endothelial alterations include impaired vasomotor function, thrombus formation, leukocyte adhesion and emigration, increased vascular permeability and angiogenesis (Malyszko 2010, Endemann, Schiffrin 2004). Furthermore, detachment and apoptosis of endothelial cells (anoikis) are associated phenomena as activated endothelial cells exhibit reduced glycocalyx (endothelial surface layer) thickness, and increased rates of apoptosis and detachment from the basement membrane (Granger, Senchenkova 2010a).

The pathophysiology of endothelial dysfunction is complex and comprises multiple manifestations; however, some of them seem to be common in most disease conditions. Among the features of endothelial dysfunction, a few will be discussed below such as vasomotor dysfunction manifested as reduced vasodilator response, up-regulation of pro-inflammatory proteins (i.e. adhesion molecules and chemoattractants) and enhanced vascular permeability.

Vasomotor Dysfunction

In response to humoral and mechanical stimuli, endothelial cells regulate vascular tone through the synthesis and release of powerful vasoactive substances such as nitric oxide (NO), prostacyclin (PGI_2) and endothelin (Galley, Webster 2004). Impaired vasomotor responses are generally characterized by a reduced responsiveness to vasodilators and an enhanced sensitivity to vasoconstrictors (Granger, Senchenkova 2010b).

NO, a potent vasodilator, is considered to be the ‘‘protector of the vascular wall’’. Homeostatic mechanisms of NO include vasodilation, inhibition of platelet adhesion and aggregation, reduction of endothelial adhesion molecule expression and hence leukocyte adherence, and inhibition of SMC proliferation (Cooke, Tsao 1994). Endothelium-derived NO also prevents the uptake of low-density lipoprotein (LDL) (Ross 1999), limits the formation of oxidized LDL and inhibits the release and action of the vasoconstrictor endothelin (Vanhoutte 2009). When the protective role of NO is hampered, endothelial

dysfunction ensues. Decreased production or activity of NO, manifested as impaired endothelium-dependent vasodilation, is one of the earliest signs of atherosclerosis (Ludmer et al. 1986, Davignon, Ganz 2004).

NO is synthesized by a family of enzymes termed nitric oxide synthase (NOS), catalyzing the reaction of L-arginine, NADPH and oxygen to the free radical NO, citrulline and NADP in the presence of tightly-bound cofactors: tetrahydrobiopterin (BH_4), FAD, FMN and iron protoporphyrin IX (heme). Proper NOS enzymatic activity requires binding to various cofactors and formation of NOS dimers, wherein NOS first binds to the cofactors FAD and FMN, and the addition of L-arginine, BH_4 and heme promotes NOS dimerization (Alderton, Cooper & Knowles 2001).

In vascular endothelial cells, a calcium-dependent, constitutive isoform of NOS known as endothelial NOS (eNOS) is responsible for NO production (Moncada 1997, Moncada 1999). NO, having a half-life of a few seconds, once formed, rapidly diffuses to the underlying SMC to activate guanylate cyclase (Villanueva, Giulivi 2010), thereby generating cGMP which in turn reduces intracellular calcium leading to muscle relaxation and vessel dilation (Galley, Webster 2004).

For full enzymatic activity eNOS requires proper dimerization and intracellular localization to caveolae, mediated in part by protein-protein interactions with caveolin and heat shock protein 90 (hsp90). Notably, eNOS activity is regulated by changes in cytosolic calcium concentrations; wherein calmodulin binds calcium, and the calcium-calmodulin complex interacts with eNOS dimers resulting in increased enzyme activity (Govers, Rabelink 2001, Huang 2009). eNOS is also regulated at the post-translational level. This includes phosphorylation by the serine/threonine kinase Akt at serine-1177 (S1177) which is a key regulator of eNOS activity and hence a promising therapeutic target to treat endothelial dysfunction (Kolluru, Siamwala & Chatterjee 2010).

Several potential mechanisms account for reduced NO bioavailbilty. This can be broadly separated into three categories: reduced eNOS expression levels, reduced eNOS enzymatic activity, and rapid removal/inactivation of NO (e.g. during inflammation, cytokines as TNF-α can activate endothelial NADPH oxidase to produce superoxide anion (O_2^-) which in turn inactivates NO) (Granger, Senchenkova 2010b) ***(Figure I-2)***.

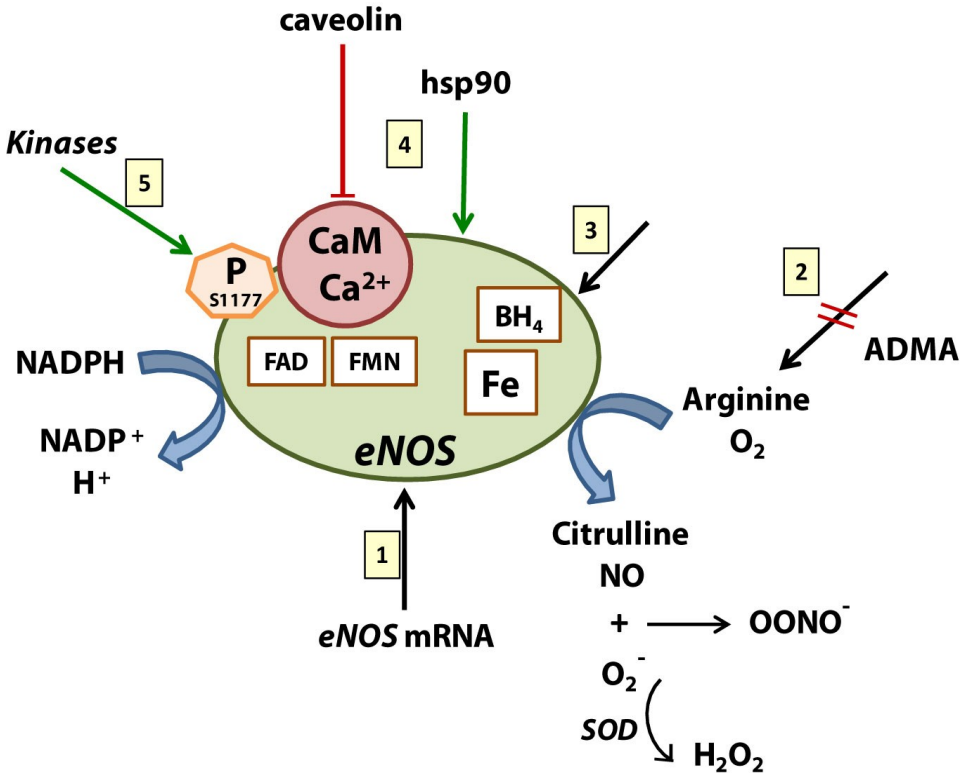

Figure I-2. Regulation of endothelial nitric oxide synthase (eNOS) activity and mechanisms for endothelial dysfunction. *Vascular supply of nitric oxide (NO) might be affected at various levels such as:(1) eNOS mRNA or protein expression; (2) availability of its substrate arginine, which may be competed by asymmetric dimethylarginine (ADMA) (3) availability of its cofactors such as* tetrahydrobiopterin *(BH_4) (4) protein-protein interaction essential for intracellular localization to caveolae, for instance with caveolin "inhibitory" or heat shock protein 90 (hsp90) "stimulatory" (5) phosphorylation at* serine-1177 (*S1177) by various kinases (stimulatory) and 6) reaction of NO with superoxide anion (O_2^-) to form peroxynitrite anion ($ONOO^-$), which is catalyzed by superoxide dismutase (SOD). Ca^{2+}; calcium, CaM; calmodulin, Fe; heme, mRNA; messenger RNA, O_2; oxygen.*

Up-regulation of Pro-inflammatory Molecules

Stimulated endothelial cells begin to express *adhesion molecules* and secrete various *chemoattractants* necessary for leukocyte recruitment and subsequent infiltration into underlying tissue in a multistep cascade (Walzog, Gaehtgens 2000) as illustrated in ***Figure I-3***.

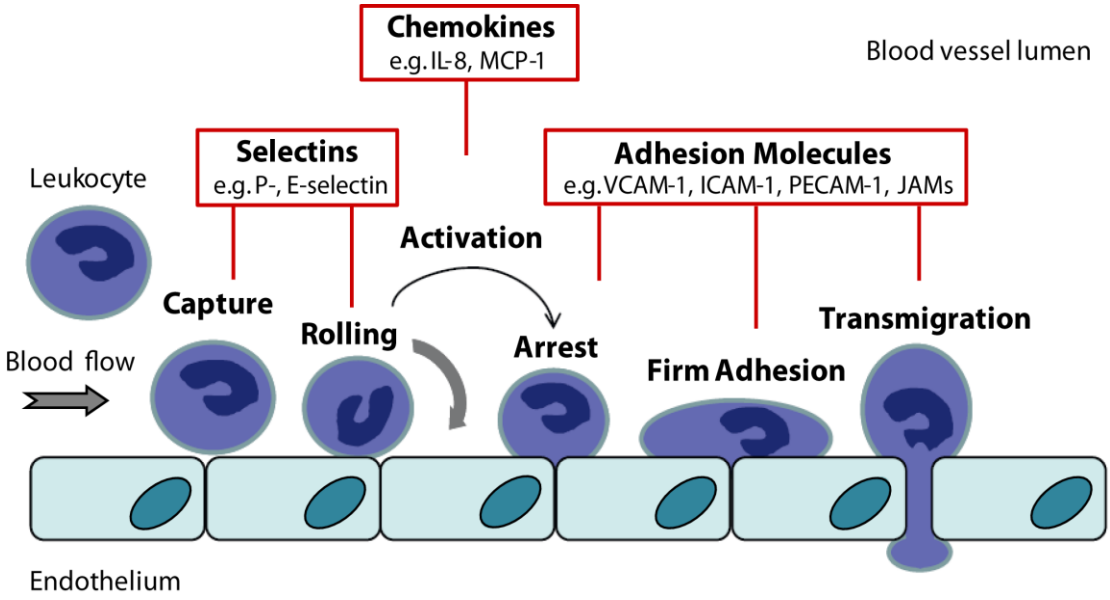

Figure I-3. Multistep cascade of leukocyte adhesion and transmigration. *Pro-inflammatory signaling leads to increased adhesion molecule expression on endothelial cells. Selectins facilitate initial "capture" and "rolling" of leukocytes via loose bonds; whilst vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) mediate "firm adhesion". Chemokines secreted by endothelial cells activate leukocyte integrins, thus securing the firm "arrest" of leukocytes on the endothelium via integrin-adhesion molecule interactions. Subsequently "transmigration" of adherent leukocytes across the endothelium into the underlying tissue is implemented by platelet endothelial cellular adhesion molecule-1 (PECAM-1) and junctional adhesion molecules (JAMs) (Langer, Chavakis 2009).*

a- adhesion molecules

Leukocyte rolling, a prerequisite for firm adhesion, is orchestrated by a distinct family of adhesion molecules residing on both endothelial cells and leukocytes, known as the selectin family of adhesion molecules (P-, E-, and L-selectin). E- and P-selectin are expressed on stimulated endothelial cells, with the latter also being found on activated platelets (Bevilacqua et al. 1987, McEver et al. 1989). In contrast, L-selectin is constitutively expressed on leukocytes only, and is shed upon activation (Kishimoto et al. 1989).

Several counter ligands for E- and P-selectin have been identified on leukocytes, such as sialyl Lewis^x, P-selectin glycoprotein ligand-1, E-selectin ligand-1 and CD44 (Beauharnois et al. 2005, Sperandio, Gleissner & Ley 2009). After cell activation, selectin molecules are rapidly removed from the surface, by internalization and lysosomal targeting (P- and E-

selectin) or by shedding/proteolytic cleavage (L-, E-selectin) resulting in soluble selectin isoforms detectable in circulation (Blankenberg, Barbaux & Tiret 2003).

Firm adhesion between leukocytes and endothelial cells is mediated by the immunoglobulin superfamily of adhesion molecules located on the surface of endothelial cells, and their corresponding ligands (integrins) located on most leukocytes. This family includes intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) which mediate firm adhesion of leukocytes to the endothelium, whereas platelet endothelial cellular adhesion molecule-1 (PECAM-1) and junctional adhesion molecules (JAMs), are concentrated at endothelial cell-cell junctions and have been implicated in leukocyte infiltration into underlying tissues (Carlos, Harlan 1994, Nourshargh, Krombach & Dejana 2006, Ebnet et al. 2004).

ICAM-1 is widely expressed at a basal level and is up-regulated by pro-inflammatory cytokines in leukocytes and endothelial cells. Ligands for ICAM-1 (CD54) include leukocyte specific β_2 integrins; $\alpha_L\beta_2$ integrin (CD11a/CD18, LFA-1), $\alpha_M\beta_2$ integrin (CD11b/CD18, Mac-1) and $\alpha_X\beta_2$ integrin (CD11c/CD18) (Blankenberg, Barbaux & Tiret 2003). VCAM-1 (CD106) is transcriptionally induced on endothelial cells and is also expressed by other cell types (e.g. macrophages and dendritic cells). It interacts with $\alpha_4\beta_1$ integrin also known as CD49d/CD29; VLA-4. VCAM-1 engagement induces signals in endothelial cells triggering shape change, thereby allowing leukocyte emigration (Matheny, Deem & Cook-Mills 2000). Soluble circulating forms of VCAM-1 and ICAM-1 have been shown to increase in various inflammatory disorders (Gearing, Newman 1993) as a result of increased proteolytic cleavage of endothelium-bound forms secondary to endothelial cell activation or injury (Ley 1992).

Transendothelial migration, the final step of leukocyte recruitment and infiltration into underlying tissue, is governed by PECAM-1 (CD31) and JAMs, which are expressed by endothelial cells as well as a variety of circulating leukocytes. Through dynamic interactions with their leukocyte counter-receptors (PECAM-1 with $\alpha_v\beta_3$ integrin, JAM-A with LFA-1, JAM-B with VLA-4, and JAM-C with Mac-1), they secure the transendothelial migration of leukocytes (Walzog, Gaehtgens 2000). Endothelial ICAM-1 and VCAM-1 have also been implicated in leukocyte transmigration. ICAM-1 can colocalize with ring-like LFA-1 clusters on neutrophils during transmigration (Shaw et al. 2004). Additionally, a “cup-like” transmigratory structure containing ICAM-1- and

VCAM-1-enriched vertical microvilli-like projections was shown to surround transmigrating neutrophils during diapedesis (Carman, Springer 2004). Transenothelial migration is either "paracellular" which requires transient junctional disruption as leukocytes migrate between adjacent cells or "transcellular" wherein a leukocyte moves through the body of an endothelial cell (e.g. via the transient formation of a pore/cup). Factors affecting the route preference are still largely unknown (Aghajanian et al. 2008).

b- chemoattractants

Throughout this multistep adhesion paradigm, chemoattractants direct the movement of circulating leukocytes to sites of inflammation/injury. Two groups of chemoattractants have been identified, namely classical chemoattractants and chemokines.

Classical chemoattractants include bacterial-derived N-formyl peptides (Schiffmann, Corcoran & Wahl 1975), complement factors (e.g. C5a) (Gerard, Gerard 1994), small anti-microbial peptides such as defensins (Hoover, Chertov & Lubkowski 2001, Pinheiro da Silva, Machado 2012), and various lipid molecules including leukotriene B4, (Goldman, Goetzl 1982), prostaglandins (Till et al. 1979, Monneret et al. 2001, Politis et al. 1991) and lysophospholipids (Gräler, Goetzl 2002, Lin et al. 2006).

Chemokines (chemoattractant cytokines) are distinguished according to the arrangement of the first two of four conserved cysteines, which are either separated by one amino acid (CXC chemokines) or adjacent (CC chemokines). CXC chemokines mainly attract polymorphonuclear leukocytes to sites of acute inflammation, while CC chemokines attract mononuclear cells to sites of chronic inflammation (Baggiolini, Dewald & Moser 1994, Charo, Taubman 2004).

Not only do chemoattractants play a role in firm adhesion by rapidly activating integrins on leukocytes (Detmers et al. 1990), but they also guide directional leukocyte migration (i.e. chemotaxis) along a chemokine gradient that is soluble or immobilized to the extracellular matrix (Middleton et al. 2002). Chemokines are immobilized on endothelial cells through interaction with cell surface glycosaminoglycans/proteoglycans (e.g. heparan sulfate) which sequester/bind chemokines, thereby increasing their local concentrations (Middleton et al. 2002, Tanaka, Adams & Shaw 1993, Hoogewerf et al. 1997). Additionally, this interaction promotes presentation of chemokines to their G protein-coupled receptors on target cells, thereby enhancing various biological functions including integrin activation,

shape change, and leukocyte migration (Kuschert et al. 1999, Murdoch, Finn 2000). Binding sites for various chemokines including IL-8, RANTES, MIP-1α and MCP-1, have been identified on endothelial cell surface (Hoogewerf et al. 1997, Rot et al. 1996, Hub, Rot 1998).

IL-8 (CXCL8), the prototype of CXC chemokines, was initially characterized as a neutrophil chemotactic and activating factor (Baggiolini, Moser & Clark-Lewis 1994). In response to inflammatory stimuli, IL-8 is secreted by multiple cell types including endothelial cells which can store IL-8 in special vesicles (Weibel-Palade bodies) thus allowing its immediate appearance at the tissue-blood interface (Hoogewerf et al. 1997, Rot et al. 1996, Hub, Rot 1998). Moreover, IL-8 can resist proteolytic degradation and bind to matrix glycosaminoglycans, thereby prolonging its biological activity at sites of inflammation (Baggiolini, Moser & Clark-Lewis 1994). IL-8 was shown to up-regulate neutrophil CD11a/CD18 complex and establish a chemotactic gradient, leading to the recruitment and activation of neutrophils (Strieter et al. 1993), a key event in acute inflammation (Walz et al. 1991). IL-8 was also reported to be a potent chemoattractant for T-cells (Larsen et al. 1989, Taub et al. 1996), and was recently shown to activate monocytes, and may direct their recruitment to vascular lesions (Huo et al. 2001).

MCP-1 (CCL2), the most thoroughly characterized CC chemokine, is a potent agonist for monocytes, dendritic cells, memory T cells, and basophils (Charo, Taubman 2004). MCP-1 is implicated as a key player in the recruitment of monocytes (foam cell precursors) into early atherosclerotic lesions, wherein MCP-1 was shown to up-regulate the expression and activation of β_2 integrins on monocytes, hence promoting monocyte adhesion to extracellular matrix proteins (Melgarejo et al. 2009).

In endothelial cells, the expression of pro-inflammatory genes necessary for recruitment of inflammatory cells to the vessel wall (e.g. selectins, ICAM-1, VCAM-1 and various inflammatory cytokines) is typically mediated by nuclear factor-kappa B (NF-κB) signaling ***(Figure I-4)*** (Kuldo et al. 2005).

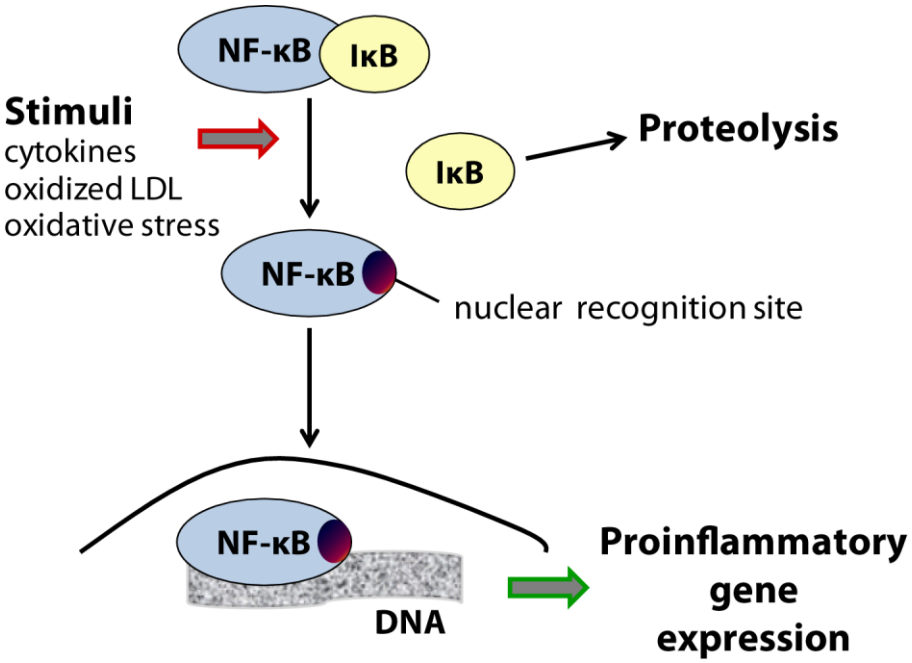

Figure I-4. Nuclear factor-kappa B (NF-κB) pathway. *In the absence of a stimulus, NF-κB is bound to an inhibitory subunit, IκB (inhibitory κB) which masks its nuclear recognition site sequestering it in the cytoplasm in a non-activated state. In response to various stimuli, IκB is released mostly by activation of an IκB kinase which phosphorylates IκB leading to its ubiquitination and subsequent proteolytic degradation, thereby revealing the nuclear recognition site. This prompts NF-κB to translocate to the nucleus where it binds to target DNA resulting in expression of various pro-inflammatory genes such as cell adhesion molecules, cytokines and chemokines (Vermeulen et al. 2002, Warboys et al. 2011).*

NF-κB is a redox-sensitive transcription factor closely linked to cardiovascular health and disease due to its control on multiple immune and inflammatory processes (Sun, Oberley 1996). Nevertheless, upstream to NF-κB lays the mitogen-activated protein kinase (MAPK) pathway, a key regulator in many cellular processes including apoptosis, proliferation, and inflammation. Three well-characterized MAPK subfamilies have been identified, namely: the c-Jun N-terminal kinase (JNK), the p38, and the extracellular signal-regulated kinase (ERK) family. Both JNK and p38 are preferentially activated by inflammatory cytokines and stress, whereas the ERK pathway is activated by growth factors (Herlaar, Brown 1999, Johnson, Lapadat 2002). Several pro-inflammatory genes implicated in atherosclerosis (e.g. genes encoding VCAM-1, E-selectin and IL-8) require the activation of both pathways for transcription to occur indicating the contribution of both pathways in inflammation and lesion development at susceptible sites (Warboys et al. 2011).

Increased Vascular Permeability

Under resting conditions, the endothelium is fairly permeable to small-molecule solutes while restricting the movement of larger molecules, thereby serving as a semipermeable barrier that actively participates in blood-tissue exchange of plasma fluid, proteins and cells. When this barrier function is altered, plasma fluid and protein leak into the interstitium resulting in interstitial edema (Komarova, Malik 2010).

Key players in maintaining endothelial barrier integrity are illustrated in ***Figure I-5***. During inflammatory injury, accumulated inflammatory mediators and immune cells activate endothelial cell signaling pathways, which target structural elements (i.e. actin/myosin) that regulate vascular permeability, thereby diminishing barrier function (Kvietys, Granger 2012).

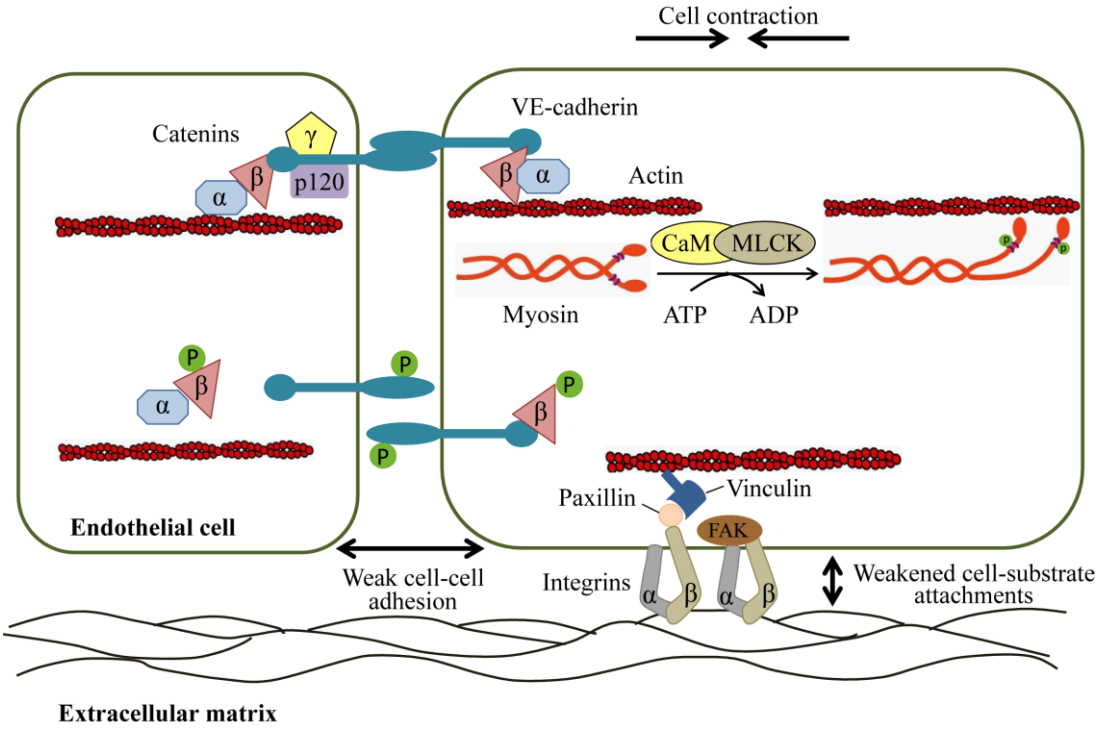

Figure I-5. Schematic diagram illustrating endothelial barrier structure. *Endothelial cells are connected together by the junctional adhesive molecule vascular endothelial (VE)-cadherin, which binds to another VE-cadherin molecule from a neighboring cell and connects to the actin cytoskeleton via a family of catenins (α, β, γ and p120). This endothelial lining is tethered to the extracellular matrix through focal adhesions mediated by transmembrane integrins composed of α and β subunits, focal adhesion kinase (FAK), and cytoskeleton-linking proteins (paxillin and vinculin). Endothelial barrier integrity is maintained by VE-cadherin-mediated cell-cell adhesions and focal-adhesion-supported cell-matrix attachment. Dynamic interactions among these*

structural elements control the opening and closing of the paracellular pathways for fluid, proteins and cells. In particular, Ca^{2+}/calmodulin (CaM)-dependent myosin light chain kinase (MLCK) catalyses phosphorylation of myosin light chains, triggering binding of the myosin heavy chain motor domains to actin and their cross-bridge movement. This reaction promotes cytoskeleton contraction and cell retraction. In parallel, phosphorylation of VE-cadherin and/or catenins dissociates the junction complex from its cytoskeletal anchor, leading to diminished cell-cell adhesion. Both cytoskeletal and junctional responses act together causing endothelial hyperpermeability (Kumar et al. 2009).

Adhesion and transendothelial migration of leukocytes in inflamed vessels has been linked to endothelial barrier dysfunction in acute inflammation. Activated neutrophils are considered important inducers of endothelial hyperpermeability. Neutrophil-derived proteases (elastase or cathepsin G) were shown to disrupt interendothelial junction complexes and induce endothelial cell retraction, thereby increasing vascular permeability (Hermant et al. 2003). Alternatively, neutrophil engagement to endothelial adhesion molecules (i.e. LAF-1/ICAM-1 interaction) can elicit the development of actin-myosin based endothelial cell tension as well as disrupt endothelial cell junctions (Shen et al. 2010).

Endothelial Dysfunction and Cardiovascular Disease

Endothelial dysfunction has been implicated in the pathophysiology of different forms of cardiovascular disease (CVD) including hypertension, atherosclerosis, coronary and peripheral artery diseases, heart failure, and chronic renal failure (Vanhoutte et al. 2009). Hence, the starting point of CVD lies in endothelial damage/injury.

Atherosclerosis

Atherosclerosis, the most common cause of CVD, is complex in origin. Variables involved in its pathogenesis include hemodynamic, thrombotic, and metabolic factors, along with intrinsic characteristics of the vessel wall and environmental/behavioral factors such as Western diet, sedentary lifestyle and smoking (Ross 1993).

Progression of atherosclerosis is closely related to the presence and extent of cardiovascular risk factors as hypertension, smoking, obesity, diabetes, increased platelet

reactivity, LDL-cholesterol (LDL-c), triglycerides, homocysteine, C-reactive protein (CRP), and fibrinogen (Berenson et al. 1998, Kullo, Gau & Tajik 2000). In the presence of risk factors, endothelial dysfunction was found before development of structural coronary atherosclerosis (Luscher, Barton 1997, Reddy et al. 1994), and was considered an early marker preceding angiographic or ultrasonic evidence of plaques (Luscher, Barton 1997, Reddy et al. 1994). Such a dysfunction favors vasospasm, thrombosis, leukocyte penetration, cellular growth, and a cascade of inflammatory events promoting the development of macrophage-enriched atherosclerotic lesions (Ross 1999, Stocker, Keaney 2004) as illustrated in ***Figure I-6***. Hence, endothelial dysfunction has become not only a hallmark, but indeed a predictor of CVD (Vanhoutte et al. 2009).

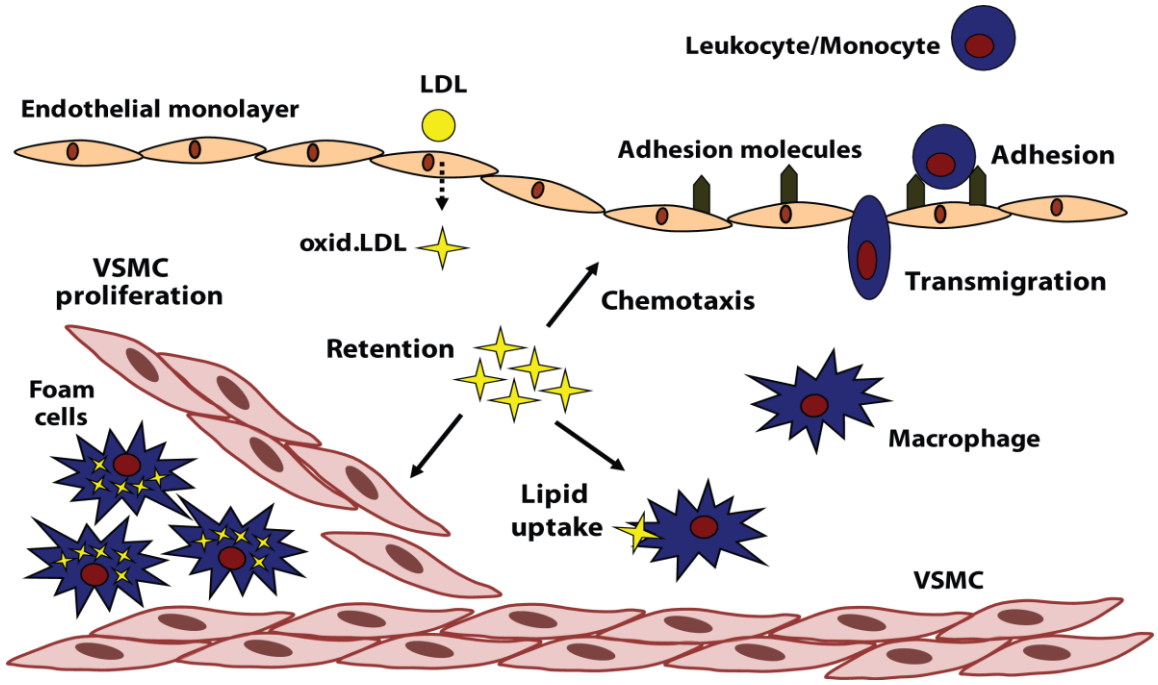

Figure I-6. Development of atherosclerosis. *Atherosclerosis begins with endothelial injury or dysfunction characterized by enhanced endothelial permeability. Low-density-lipoprotein (LDL) becomes entrapped in the subendothelial space, where it is subject to modification by resident vascular cells. LDL becomes oxidized (oxid. LDL), resulting in endothelial cell activation and stimulation of leukocyte chemotaxis. In turn, activated endothelial cells express adhesion molecules that capture leukocytes (monocytes). Recruited monocytes differentiate into macrophages within the vessel wall. These macrophages then take up modified LDL to yield mature lipid-laden macrophages (foam cells), which subsequently produce growth factors and cytokines leading to the proliferation of vascular smooth muscle cells (VSMC) and ultimately the development of atherosclerotic plaques (Proctor, Vine & Mamo 2002).*

Renal Disease

In renal failure, endothelial dysfunction, atherosclerosis and cardiovascular complications are almost universal (Malyszko 2010). Most recently, endothelial vasomotor dysfunction was shown to independently associate with the progression of renal dysfunction in patients with coronary artery disease (Nakamura et al. 2011).

In patients suffering from renal disease, endothelial dysfunction may have a dual role. From one side, it is a crucial step underlying the development of CVD and on the other side, activation, dysfunction, and disintegration of endothelial cells in renal glomerular capillaries pave the way for progression of chronic kidney disease (CKD) (Fliser et al. 2011). However, individuals with CKD are more likely to die of CVD (premature death) than develop kidney failure (Shulman et al. 1989, Tonelli et al. July 2006).

Risk of cardiovascular mortality in dialysis patients was found to be 30-fold higher compared to age-matched controls (Tonelli et al. July 2006, PARFREY, FOLEY 1999), and cardiac mortality of dialysis patients aged 45 years or younger is more than 100-fold increased in comparison to the general population (Cheung et al. 2000). However, the increased progression and development of CVD in patients with kidney disease cannot be explained solely by the traditional risk factors. Therefore, non-traditional risk factors have recently emerged such as oxidative stress and endothelial dysfunction, in a milieu of constant low-grade inflammation (Yao et al. 2004).

Numerous signs of endothelial dysfunction have been reported in dialysis patients such as impaired endothelium-dependent vasodilation and reduced NO bioavailability (Passauer et al. 2005, Ueda et al. 2007). Nevertheless, increased soluble adhesion molecules and various pro-inflammatory cytokines such as IL-6 and TNF-α (Suliman et al. 2006, Jacobson et al. 2002, Al-Koussi et al. 1994, Bolton et al. 2001) indicate endothelial activation and contribute to the chronic inflammatory state in end-stage renal disease (ESRD) patients (Stenvinkel, Alvestrand 2002).

Several factors present in CKD have been linked to endothelial cell injury and oxidative stress including lipoprotein modification(s) (Holzer et al. 2011b, Holzer et al. 2011a, Apostolov et al. 2010, Apostolov et al. 2012) and accumulation of uremic toxins such as asymmetric dimethylarginine (ADMA), homocysteine, indoxyl sulfate, creatinine, and urea (Herget-Rosenthal et al. 2009, Dou et al. 2007).

Of particular interest, urea is a major source for the formation of cyanate, as 0.8% of urea is transformed to cyanate *in vivo* (Dirnhuber, Schutz 1948). Furthermore, cyanate spontaneously formed from urea was shown to increase as renal function declines; thus proposing cyanate itself as a potential toxin (Kraus, Kraus 1998, Kraus, Kraus 2001), based primarily upon its ability to carbamylate amino acids and proteins.

Cyanate and Protein Carbamylation

Protein carbamylation, an irreversible post-translational protein modification, is mediated by cyanate; a highly reactive electrophile which reacts with nucleophilic amino groups (NH_2) of proteins, in particularly ε-NH_2 groups of lysine residues to produce ε-carbamyllysine, also known as homocitrulline (HCit) ***(Figure I-7)***. Notably, lysine side-chains are the main target for carbamylation on proteins due to the high abundance and relatively lower pK_a value of ε-amino lysine moieties when compared with alternative nucleophilic targets (Stark, Stein & Moore 1960, Stark 1965).

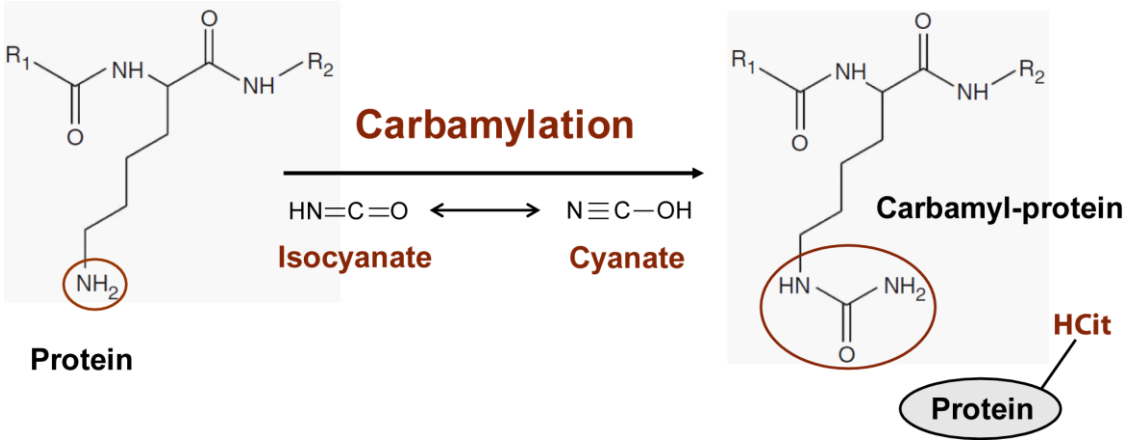

Figure 1-7. Cyanate induces protein carbamylation. *The irreversible reaction of isocyanate (the active form of cyanate) with nucleophilic ε-lysine residue on target proteins results in the formation of carbamylated homocitrulline (HCit)-bound proteins with altered function(s).*

The irreversible reaction of isocyanate, the active form of cyanate, with free NH_2 groups on polypeptides induces protein conformational changes by altering the overall charge distribution, resulting in a consequent loss of function (Kraus, Kraus 1998, Kraus, Kraus 2001). In addition, cyanate reacts with α-amino groups of free amino acids, hence interferes with protein synthesis, as carbamylated amino acids can no longer form peptide

bonds. This may contribute to a decrease in the free amino acid pool resulting in protein malnutrition, inhibition of protein synthesis, weight loss, and altered enzyme activities common among patients with ESRD (Kraus, Jones & Kraus 1998).

Several lines of evidence support the detrimental consequence of protein carbamylation on structure and/or function of various target proteins - enzymes, and hormones - such as insulin (Oimomi et al. 1987), 6-phospho-d-glucuronate dehydrogenase (Ganea, Harding 1996), matrix metalloproteinase-2 (Kraus et al. 2001) and erythropoietin (Mun, Golper 2000). Importantly, carbamylated albumin was shown to interfere with oxidative functions of polymorphonuclear neutrophils (respiratory burst), hence partly explaining the increased occurrence of inflammatory and infectious complications in patients with CKD (Jaisson et al. 2007).

An emerging and significant body of research has recently focused on carbamylation of lipoprotein(s) owing to its relevance to atherogenesis. *In vitro* carbamylated LDL and uremic LDL were reported to induce multiple pro-atherosclerotic effects including monocyte adhesion, adhesion molecules over-expression, vascular smooth muscle cell proliferation and endothelial cell apoptosis (Apostolov et al. 2007, Ok et al. 2005, Asci et al. 2008). Likewise, cardioprotective properties of high-density lipoprotein (HDL) were severely altered upon carbamylation. For instance, carbamylated HDL was shown to display (i) loss of anti-inflammatory and anti-oxidative properties (Holzer et al. 2012), (ii) increased binding affinity to the HDL receptor scavenger receptor B-I, hence inducing intracellular cholesterol accumulation and lipid-droplet formation in macrophages (Holzer et al. 2012), and (iii) enhanced aortic endothelial cell apoptosis, a critical hallmark of atherosclerotic lesions (Wang et al. 2007). In addition, impaired cholesterol efflux ability was exhibited by uremic HDL isolated from patients on maintenance hemodialysis (Holzer et al. 2011a).

Until recently, the potential role for protein carbamylation in human health and disease has mainly been investigated in the context of uremia; however, a novel concept of inflammation-derived carbamylation has recently emerged, in which the phagocytic enzyme myeloperoxidase (MPO) plays a novel role in protein carbamylation (Wang et al. 2007); thus proposing a uremia-independent alternative and dominant mechanism for cyanate formation and hence protein carbamylation, at sites of inflammation.

MPO is a heme protein highly expressed by neutrophils, monocytes as well as lipid rich-macrophages in atheromatous lesions (Sugiyama et al. 2001, Daugherty et al. 1997, Daugherty et al. 1994). In the presence of hydrogen peroxide (H_2O_2), MPO catalyzes the oxidation of halides/pseudohalides as chloride and thiocyanate into hypochlorous acid (HOCl) and hypothiocyanous acid (HOSCN), respectively, wherein thiocyanate was indicated as the preferred substrate for MPO (van Dalen et al. 1997, van der Veen, de Winther & Heeringa 2009).

Of great importance, the MPO-catalyzed oxidation of thiocyanate was finally introduced as a catalytic source for cyanate formation *in vivo* at sites of inflammation and atherosclerotic plaques (Wang et al. 2007). Moreover, our group recently showed that the MPO-product HOCl itself rapidly decomposes thiocyanate and urea, thereby contributing to considerable amounts of cyanate *in vivo* (Holzer et al. 2012). This is of particular importance in smokers due to their:

(i) higher endogenous levels of thiocyanate (Husgafvel-Pursiainen et al. 1987, Morgan et al. 2011)

(ii) increased blood leukocyte count (Nieto et al. 1992, Schwartz, Weiss 1991) in particular granulocytes (Smith et al. 2003)

(iii) enhanced MPO expression (van Eeden, Hogg 2000)

In addition to the endogenous sources of cyanate mentioned above, humans are exposed to significant amounts of isocyanic acid (HNCO)/cyanate from exogenous sources, namely smoke (Roberts et al. 2011). HNCO/cyanate was reported to be a major constituent of smoke resulting from combustion/pyrolysis of biomaterial including:

a- coal, a common fuel used in open indoor fires for cooking and heating especially in rural areas (Wang et al. 2010), in which HNCO levels ≥10 ppbv in such homes could be expected (Roberts et al. 2011)

b- tobacco ingredients and urea (a major tobacco additive) incorporated to enhance flavour in some cigarettes (Hansson et al. 2004). Although exact levels of HNCO in tobacco smoke have not yet been reported, it was estimated that a smoker is exposed to 1.9 mg of HNCO per cigarette (140 ppmv) based solely on the amount

of urea added (4 mg⁄g tobacco), 93% of which is pyrolyzed to HNCO (Baker, Bishop 2004)

c- diesel urea-selective catalytic reduction exhaust systems which incorporate urea as a catalyst in a 1-3% by volume urea/fuel ratio may produce up to 50 ppmv of HNCO (Kröcher, Elsener & Koebel 2005)

d- wildfires which are the largest global source of HNCO to date e.g. HNCO levels reached 200 pptv in Boulder, Colorado during the recent 2010 Fourmile Canyon fire (Roberts et al. 2011)

Intriguingly, HNCO has an atmospheric lifetime of several decades due to its relative stability against reaction with hydroxyl radicals (Tsang 1992), a major process for removal of trace organic species from the atmosphere (Finlayson-Pitts, Pitts 2002).

Notably, measurements of aqueous solubility demonstrate that HNCO is highly soluble as it dissociates at physiological pH; hence smoke provides a rapid route for the uptake and absorption of cyanate directly into the blood stream (Roberts et al. 2011). HNCO exposure levels within the 1 ppbv range in inhaled breath produces an equilibrium aqueous concentration of 100 μM, a concentration sufficient to drive protein carbamylation (Wang et al. 2007). This clearly suggests that smoke-related HNCO/cyanate exposure significantly contributes to major negative health problems.

Aim of the Thesis

Although humans are exposed to considerable amounts of cyanate from various sources, very little is known about the impact of cyanate on human health. In particular, the effect of cyanate on the endothelium, regulator of vascular haemostasis, has not been investigated yet.

The present doctoral thesis aimed to elucidate the possible harmful effects of cyanate exposure *in vitro* and *in vivo* with a particular focus on endothelial function. Hence, the question at stake is "Does cyanate trigger endothelial dysfunction? "

For that purpose we set out to evaluate different aspects of endothelial dysfunction including:

- Expression of endothelial activation markers (cell adhesion molecules)
- Release of inflammatory mediators (i.e chemokines)
- Endothelial-leukocyte interaction (leukocyte adhesion)
- Expression of endothelial nitric oxide synthase (eNOS)
- Vascular reactivity (endothelium-dependent vasodilation response)

In **Part I** we investigated the effect(s) of cyanate on human coronary artery endothelial cells with a particular focus on renal disease patients in an attempt to highlight the clinical relevance of cyanate exposure in human disease. Furthermore, animal studies were performed to assess the expression of endothelial adhesion molecule in the aorta.

In **Part II**, we focused on exploring the effect(s) resulting from chronic inhalation of cyanate. Mice were allowed to inhale cyanate for 3 weeks, after which vessel reactivity and NO release, as well as eNOS protein expression were investigated.

II. MATERIAL AND METHODS

Material

Reagents	
Name	**Company**
$^{13}C_6$-homocitrulline	Ascent Scientific
$^{13}C_6$-lysine	Euroiso-Top
2′,7′-dichlorofluorescein diacetate (2′,7′-DCF-DA)	Sigma
Acetylcholine chloride (ACh)	Sigma
Ammonium formiate (for HPLC)	Sigma
Annexin V Binding Buffer	BD Bioscience
Antibody diluent, background reducing solution	Dako
BAY 11-7082 (NF-κB inhibitor)	Merck
Bromophenol Blue	Sigma
BSA	PAA
Butylated hydroxyl toluene (BHT)	Sigma
$CaCl_2$	Merck
Calcein-AM	Molecular Probes
Carboxymethyl-lysine	PolyPeptide Labs
CellFix	BD Bioscience
Cellfix solution	BD Bioscience
cOmplete Mini protease inhibitor cocktail tablets	Roche Diagnostics
D-glucose	Sigma
Diethylenetriaminepentaacetic acid (DTPA)	Sigma
Dimethyl sulfoxide (DMSO)	Merck
Ethylenediaminetetraacetic acid (EDTA)	Roth
FACS Lysing Solution	BD Bioscience
FACS-Flow	BD Bioscience
FITC Annexin V	BD Bioscience
Formamide	Merck
Gliotoxin (NF-κB inhibitor)	Merck
Glycerol	Sigma
HEPES (1M)	PAA
Hexane	Roth
Homocitrulline	Bachem
Hydrobromic acid	Sigma

Hydrogen peroxide (H_2O_2)	Sigma
Isoflurane	Baxter
Isopropanol	Sigma
KCl	Merck
Ketamine HCl/Xylazine HCl solution	Sigma
KH_2PO_4	Sigma
Lithium-Citrat buffer	Biochrom
L-lysine	Sigma
Methanol (for HPLC)	Sigma
$MgCl_2$	Sigma
N-(4-Amino-5-cyano-6-ethoxypyridin-2-yl)-2-(2,5-dimethoxyphenyl)acetamide (JNK Inhibitor VIII)	Merck
N5-[imino(nitroamino)methyl]-L-ornithine (L-NNA)	Sigma
Na_3PO_4	Sigma
NaCl	Roth
$NaHCO_3$	Merck
NaN_3	Roth
NaOH	Roth
Non-fat dried milk	Maresi GmbH
Paraformaldehyde	Sigma
PD98059 (ERK1/2 inhibitor)	Merck
Phenol	Sigma
Phenyl acetate	Sigma
Phosphate buffered saline (PBS) with or without Ca^{2+} and Mg^{2+}	PAA
PhosSTOP phosphatase inhibitor cocktail tablets	Roche Diagnostics
Potassium bromide	Sigma
Potassium cyanate	Sigma
Propidium Iodide (PI) Staining Solution	BD Bioscience
RIPA buffer	Sigma
SB202190 (p38 MAPK inhibitor)	Merck
SB203580 (p38 MAPK inhibitor)	Merck
SDS	Sigma
Sodium cyanate	Sigma
Sodium nitrite	Roth
Sodium nitroprusside (SNP)	Sigma

SP600125 (JNK inhibitor II)	Merck
Thiazolyl Blue Tetrazolium Bromide	Sigma
TRIS	Roth
Triton X-100	Invitrogen
Tween 20	Merck
U0126 (ERK1/2 inhibitor)	Merck
Water (for HPLC)	Sigma
Antibodies	
Anti-mouse CD54	Abbiotec
FITC anti-human CD106	BD Bioscience
FITC mouse IgG1 isotype control	BD Bioscience
HRP-conjugated anti-mouse IgG	Thermo Scientific
HRP-conjugated anti-rabbit IgG	Sigma
Monoclonal anti-β-actin	Sigma
Mouse anti-eNOS (pS1177)	BD Bioscience
Non-immune rabbit IgG	Thermo Scientific
PE anti-human CD54	BD Bioscience
PE anti-human CD62E	BD Bioscience
PE mouse IgG1 isotype control	BD Bioscience
Rabbit anti-eNOS	BD Bioscience

Fixative solution was prepared by mixing distilled water (9 mL), FACS-Flow (30 mL) and CellFix (1 mL). Inhibitors were dissolved in DMSO and further diluted to produce a final concentration of the solvent of less than 0.1%.

Sodium cyanate was tested for endotoxin using the Limulus Amoebocyte Lysate assay (Marsche et al. 2007). Endotoxin levels of sodium cyanate preparations used (1 mg/mL) were below 0.03 EU/mL.

Methods

Culture of endothelial cells

Human coronary artery endothelial cells (HCAEC) were purchased from Lonza (Verviers, Belgium) as cryopreserved tertiary cultures. Endothelial cells were cultured in EGM-2 MV Bullet medium (Lonza) containing 5% FBS in humidified 5% CO_2. The medium was changed every second day and cells were passaged when they reached 80 – 90% confluence; the cultures were used within 4 passages for experiments. All experiments were performed without serum starvation.

Cell viability assay

To assess the effects of cyanate on cell viability, a MTT (3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) reduction assay was performed. MTT is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable cells.

After cyanate treatment, HCAEC were incubated with fresh medium containing MTT (0.5 mg/mL) for 4 hours at 37°C. To dissolve the formazan crystals, medium was removed and cells were lysed using acid-isopropanol (0.04 N HCI in isopropanol) on a shaker (1400 rpm) for 10 min. Absorbance was evaluated at 560 nm using a microplate spectrophotometer (xMark™, BioRAD, USA).

Flow-cytometric detection of surface inflammatory markers

The surface expression of ICAM-1, VCAM-1 and E-selectin on HCAEC was assessed by means of flow cytometry as described (Hadad et al. 2011).

After differential treatments, HCAEC were harvested using a detachment buffer (25 mM HEPES, 10 mM EDTA in PBS). The cells were then stained with directly labelled antibodies against ICAM-1 (PE anti-CD54; 1:40), VCAM-1 (FITC anti-CD106; 1:40) or E-selectin (PE anti-CD62E, 1:40) for 30 min at 4°C in dark. The negative isotype-matched

control was FITC mouse IgG1 or PE mouse IgG1. After immunofluorescence staining, cells were rinsed, fixed and analyzed using a FACSCalibur flow cytometer (Becton-Dickinson, Mountain View, CA, USA).

Detection of apoptosis (Annexin V/PI staining)

Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for phosphatidylserine (PS), thereby useful in identifying apoptotic cells with exposed PS, whereas propidium iodide (PI) is a standard flow cytometric viability probe used to distinguish viable from nonviable cells.

After cyanate treatment, HCAEC were gently trypsinized and washed. The resulting cell pellet was resuspended in 50 µL binding buffer containing 5 µL FITC Annexin V and 2 µL PI. After 15 min incubation in the dark at room temperature, the stained cell suspension was diluted with 100 µL binding buffer and immediately analyzed by flow cytometry (FACSCalibur). Annexin V-positive/ PI-negative cells were counted as early apoptotic cells while the Annexin V-positive/PI-positive cells were regarded as late apoptotic/dead cells.

Measurement of intracellular reactive oxygen species

The level of intracellular reactive oxygen species (ROS) was examined using the cell permeable non-fluorescent dye 2′,7′-DCF-DA which in the presence of ROS is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (2′,7′-DCF).

HCAEC seeded in 96-well plates were treated with cyanate (1 mM) for up to 24 hours. Cells were then loaded with 5 µM 2′,7′-DCF-DA for 30 min at 37°C. ROS production was determined by measuring the fluorescence of 2′,7′-DCF, using excitation and emission filters of 485 and 535 nm, respectively (FlexStationII, Molecular Devices, Sunnyvale, CA).

Preparation of human polymorphonuclear leukocytes

Blood was sampled from healthy volunteers after an informed consent, according to a protocol approved by the Institutional Review Board of the Medical University of Graz.

Polymorphonuclear leukocytes (PMNL, containing eosinophils and neutrophils) were prepared by means of dextran sedimentation of erythrocytes followed by centrifugation on Histopaque gradients as described previously (Schratl et al. 2007, Schratl et al. 2006). All separation steps were performed at room temperature. The resulting purity and viability of neutrophil preparations was typically greater than 95%. Isolated PMNL were immediately used for adhesion assays.

Leukocyte adhesion assay

HCAEC monolayers grown on 96-well plates were treated for 48 hours with cyanate (1 mM) in the presence or absence of a NF-κB inhibitor BAY-11 7082. Freshly isolated neutrophils were stained using the fluorescent dye calcein-AM (1 mM). Fluorochrome-labeled neutrophils (10^5/well) were then added onto the confluent monolayers and left to adhere for a period of 30 min at 37°C. After incubation, total flouresence (FL*t*) was measured on FlexStationII using excitation and emission filters of 485 and 530 nm, respectively. Non-adherent leukocytes were removed by washing twice with PBS, and the flourescence of the remaining adherent cells (FL*r*) was measured. The fluorescence of unlabelled cells was used as blank (FL*b*) (De Clerck et al. 1994).

Adhesion was calculated as the percentage of adherent cells on the basis of the total cells added.

$$\% \text{ adhesion} = \frac{FLr - FLb}{FLt - FLb} \times 100$$

Chemokine assessment in cell culture supernatants

Chemokines (IL-8, RANTES, MIG, MCP-1, IP-10) were estimated in the supernatants of cyanate-treated HCAEC by flow cytometry using cytometric bead array human chemokine kit (BD Bioscience; Vienna, Austria) according to the manufacturer's protocol. HCAEC were treated with cyanate (1 or 2 mM), and supernatants were collected 12 and 24 hours later and immediately stored at -70°C until analysis.

Isolation and carbamylation of LDL

LDL was isolated by a rapid one-step density gradient ultracentrifugation method, with modifications (Sattler, Mohr & Stocker 1994). Plasma was isolated by centrifugation at 400 g for 15 min at 8°C. Plasma density was adjusted to 1.24 g/L with potassium bromide (KBr). In order to improve separation capacity, longer centrifuge tubes (16 x 76 mm, Beckman) were used. A two-layer density gradient was produced by layering the density-adjusted plasma (1.24 g/mL) underneath a KBr-density solution (1.063 g/mL). Centrifugation tubes were sealed and centrifuged at 90,000 g for 4 hours at 8°C. After centrifugation, the LDL band was clearly separated and isolated by puncturing the tube with a syringe. LDL was desalted by gel filtration on Sephadex PD-10 columns (Amersham Biosciences; Uppsala, Sweden) and later used for cell culture experiments.

To induce carbamylation, LDL (1 mg protein/mL) was incubated with potassium cyanate (1, 2 or 10 mM) in PBS (pH 7.4) containing 100 μmol/L DTPA for 4 hours at 37°C. Control LDL was incubated under same conditions in the absence of cyanate. Modified LDL preparations were passed over Sephadex PD-10 columns to remove residual un-reacted cyanate. Under these conditions, from ~0.5 to 50% of apolipoprotein lysine residues were carbamylated as estimated by mass spectrometry analysis.

Removal of cyanate from preconditioned cell culture medium

Serum-containing cell culture medium was incubated for 48 hours with 1 mM sodium cyanate (or vehicle) to induce protein carbamylation (preconditioned medium). Subsequently, low molecular weight substances (i.e. cyanate) were removed either by dialysis (molecular-weight cut-off of 3 kDa) or gel filtration on Sephadex PD-10 columns.

Preperation of HCAEC lysates for Western blot

After treatment, cells were washed with ice-cold PBS, snap-frozen in liquid nitrogen and lysed in IPB buffer (0.3% Triton X-100, 150 mM NaCl, 25 mM KCl, 1 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4, freshly supplemented with protease and phosphatase inhibitors). The protein content of the samples was determined using BCA protein assay (Novagen, Darmstadt, Germany) according to the manufacturer's instructions.

Blood collection from renal patients and control subjects

Blood was taken from 23 end-stage renal disease (ESRD) patients on hemodialysis (HD) prior to dialysis session and from 19 age matched control subjects at the time of routine laboratory investigations in agreement with the Ethics Committee of the Medical University of Graz. Blood (5 mL) was collected in standard sterile polystyrene vacuum tubes containing 5 mM EDTA.

Plasma analysis of cholesterol, triglycerides, urea, C-reactive protein, fibrinogen, creatinine and uric acid was performed by the Clinical Institute of Medical and Chemical Laboratory Diagnostics at the Medical University of Graz using commercially available kits (Diasys, Holzheim, Germany). For the determination of sICAM-1 plasma concentrations, a platinum ELISA kit for human sICAM-1 was used (eBioscience; Vienna, Austria). Plasma levels of homocitrulline (HCit) and carboxymethyl-lysine (CML) were quantified by LC-MS/MS analysis (performed by Michael Holzer, Ph.D. from the Institute of Experimental and Clinical Pharmacology, Medical University of Graz).

Quantification of homocitrulline and carboxymethyl-lysine

Protein samples were hydrolyzed with a fast, low-volume hydrolysis method as described (Damm et al. 2010). Briefly, protein samples (3 – 20 µg) were placed into Qsert vials (Waters; Vienna, Austria) and 10 µL of internal standard was added (containing 10 ng $^{13}C_6$-HCit and 1 µg $^{13}C_6$-lysine). Hydrobromic acid with 0.25% phenol was added to a final concentration of 6 N, vials were flushed with argon, sealed and hydrolyzed at 160°C for 5 min. Afterwards, hydrobromic acid was evaporated in a Speedvac. Protein hydrolysates were resuspendend in 100 µL 0.2 mol/L Li-Citrat buffer (pH 2.2) and derivatized with the EZ:faast Kit (Phenomenex; Aschaffenburg, Germany) according to the manufacturer's instructions.

Electrospray ionization liquid chromatography tandem mass spectrometry (LC-MS/MS) with online HPLC was used for HCit and CML quantification as described (Holzer et al. 2011b). In brief, LC-MS/MS calibration curves were prepared by varying lysine, CML and HCit levels with fixed amounts of internal standards. The calibration curves had a linearity

range from 50 pg - 100 ng for HCit and CML (R^2: 0.998 and R^2: 0.989) and from 100 ng - 3 µg for lysine (R^2: 0.997). The HPLC column (250x4 mm, AAA-MS HPLC column, Phenomenex, Aschaffenburg, Germany) was equilibrated for 15 min with 100% solvent A (10 mmol/L ammonium formiate in water) at 35°C. After equilibration, the sample (10 µL) was injected onto the HPLC column at a flow rate of 0.25 mL/min. Compounds were eluted with a discontinuous gradient starting with 83% solvent B (10 mmol/L ammounium formiate in methanol) for 13 min followed by 68% of solvent B for 4 min. The HPLC column effluent was introduced into an API 200 triple quadrupole mass spectrometer. Ions were generated by electrospray ionization in the positive-ion mode with multiple reactions monitoring of parent and characteristic daughter ions. Following transitions were monitored indicated by their mass-to-charge ratio (m/z): m/z 318→127 for HCit; m/z 324→132 for $^{13}C_6$-HCit; m/z 461→170 for CML; m/z 361→170 for lysine; m/z 367→175 for $^{13}C_6$-lysine. Following mass spectrometry analysis, the generated calibration curves were used to quantify HCit, CML and lysine.

***In vivo* animal studies**

C57BL/6 mice (males, 20 – 22 g, 5 weeks old) were purchased from Charles River (Sulzfeld, Germany). Mice were housed in sawdust-floor plastic cages under controlled temperature (22°C), humidity (40%) and a 12:12-hour light-dark cycle with *ad libitum* access to food and water in a pathogen free animal facility. Experimental protocols were approved by the Animal Care Committee of the Austrian State Department of Science and Research.

Mice were exposed to cyanate either by peroral administration (peroral model) or inhalation (inhalation model):

a- Peroral model

Mice were equally assigned to three groups which received normal drinking water (control group), drinking water containing 0.2 mg/mL sodium cyanate (low-cyanate group) or drinking water containing 1 mg/mL sodium cyanate (high-cyanate group). Treatment continued for a period of 9 weeks after which mice were deeply anesthetized with

isoflurane and 0.5 mL blood was collected by cardiac puncture (using 3.8% citrate as an anticoagulant). Plasma was stored at -70°C for further analysis. Following blood collection, mice were killed by cervical dislocation. The ascending part of the aortic arch, closer to the lesser curvature, was removed and cleaned of adipose and connective tissue under a dissection microscope. Tissue samples were immediately fixed in 4% paraformaldehyde and later embedded in paraffin for immunohistochemical staining.

Analysis of plasma parameters

Malondialdehyde, total cholesterol and urea concentrations were measured by assay kits obtained from Cayman (Ann Arbor, MI, USA) and BioVision (Mountain View, CA, USA), respectively. Carbamyllysine (HCit) quantification was performed by LC-MS/MS as described above.

Immunohistochemical staining of endothelial ICAM-1

Serial cross sections (5 μm) of the aortic arch, proximal to the origin of innominate artery, were generated on a microtome and processed by standard technique for all mice. In brief, endogenous peroxidase was blocked with 3% H_2O_2 and immunolocalization at the inner curvature of the cross sections was visualized using the Ultravision-labeled polymer-horseradish peroxidase detection system specific for rabbit antibodies from Thermo Scientific (Fremont, CA, USA) according to the manufacturer's protocol. Rabbit anti-CD54 to detect ICAM-1 (1:100) and non-immune rabbit IgG (isotype control; 1:100) were diluted in an antibody diluent (Dako, CA, USA). Between incubation steps, slides were washed in Tris-buffered saline containing 0.05% (vol/vol) Tween 20. Slides were counterstained with Mayer's hemalaun from Merck (Darmstadt, Germany). Cross sections were studied to quantify endothelial ICAM-1 immunostaining at the luminal surface. Percentage coverage with ICAM-1 was measured using Xcellence imaging software Version 1.1 from Olympus soft imaging solutions (Munich, Germany).

b- Inhalation model

Mice were assigned equally to two groups and were exposed to either water (bi-distilled sterile water; control group), or cyanate aqueous solution (5 mg/mL sodium cyanate;

cyanate-treated group). Treatment was delivered using a compressor-assisted nebulizer (PARI JuniorBOY® S, PARI GmbH, Starnberg, Germany) to generate fine aerosol particles with a mass median diameter of 2.9 µm at the air flow rate of 12 L/min according to the manufacturer. Aerosol particles of such a fine size deposit primarily in the lung periphery (Koshkina et al. 2001), hence presenting a suitable means for cyanate inhalation. Nebulizations were delivered daily in the morning over 2.5 hours as five consecutive 30-min aerosols (with short pauses in between). During exposure mice were housed in sealed chambers under laminar flow conditions and treatment continued for a period of 3 weeks Thereafter, mice were deeply anesthetized with an intraperitoneal injection of ketamine (80 mg/kg BW) and xylazine (12 mg/kg BW), and the chest and peritoneal cavity were opened. Blood was collected by cardiac puncture and plasma was isolated and stored at -70°C for further analysis (HCit quantification as described above). Aortas were removed, cleaned of surrounding tissue under a dissection microscope and processed for subsequent assays. The descending thoracic aorta was either immediately used for vascular function studies or snap-frozen in liquid nitrogen and stored at -70°C for Western blot analysis. For immunohistochemistry, the ascending aorta was isolated and fixed immediately in 4% paraformaldehyde.

Vascular function studies: Assessment of endothelium-dependent relaxation

Aortic rings approximately 2 mm in length were cut from descending thoracic aorta and the arterial rings were positioned in small wire myograph chambers (Danish MyoTechnology, Aarhus, Denmark), which contained Krebs buffered solution (KBS) (114 mM NaCl, 4.7 mM KCl, 0.8 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 11 mM D-glucose pH 7.4) aerated with 5% CO_2/95% O_2 at 37°C. The myograph chambers were connected to force transducers for isometric tension recording (PowerLab, ADInstruments). An initial preload of 10 mN was applied, and the rings were allowed to stabilize for 30 min. KBS containing 60 mM KCl was used to determine maximum contractility of the tissue. When the developed tension attained its peak value, the rings were relaxed by rinsing with the buffer. Next the rings were pre-contracted with increasing concentrations of norepinephrine (NE) (1 nM – 1 µM) to produce 80% of the maximum contraction achieved by 60 mM KCl, followed by a cumulative addition of

acetylcholine chloride (ACh) (1 nM – 1 µM). Endothelium-independent relaxation was examined by exposure of rings to increasing concentrations of sodium nitroprusside (SNP), a NO-donor. The relaxation values were expressed as a percentage of the NE-induced contraction. To check for NO production, samples were taken from the bath solution surrounding the aortic rings only after the concentration-response curve to ACh.

Vascular function studies were carried out in collaboration with Shailaja Prabhakar Rao, M.Sc. at the Institute of Molecular Biology and Biochemistry, Medical University of Graz.

Nitrite determination

As an indicator for NO production, nitrite was determined according to a previously described fluorometric HPLC method (Li, Meininger & Wu 2000) utilizing the reaction of nitrite with 2,3-diaminonaphthalene (DAN).

After the ACh-induced aortic ring relaxation, 100 µL of the bath solution was incubated at 24°C with 10 µL of 316 µmol/L DAN (in 0.62 mol/L HCl) for 10 min, followed by addition of 10 µL of 2.8 mol/L NaOH. This reaction mixture was directly used for chromatographic separation (injection volume: 20 µL) of the formed 2, 3-naphthotriazole (NAT). Nitrite standards (range: 0 – 2 µmol/L) were derivatized accordingly. NAT was isocratically separated on a 5-µm ODS hypersil column (150 x 4.6 mm) guarded by a 5-µm ODS hypersil column (10 x 4.6 mm; Uniguard holder) with a 30 mmol/L sodium phosphate buffer (pH 7.5) containing 50% methanol (flow rate: 0.8 mL/min). Fluorescence was monitored at an excitation wavelength of 375 nm and an emission wavelength of 415 nm. The HPLC apparatus consisted of an L-2200 autosampler, L-2130 HTA pump and L-2480 fluorescence detector (VWR Hitachi, Tokyo, Japan). Detector signals were recorded with a personal computer. The program EZchrom Elite (Scientific Software Inc., San Ramon, CA USA) was used for data acquisition and analysis. The detection limit for nitrite was 10 pmol/mL.

Determination of NO was performed by Seth Hallström, Ph.D. at the Institute of Physiological Chemistry, Medical University of Graz.

Immunohistochemical staining of carbamylated epitopes

To detect carbamylation locally in aortic tissue, a previously characterized antibody specific against carbamyllysine (rabbit anti-HCit) was used (Holzer et al. 2012). The ascending aortas fixed in 4% paraformaldehyde were later embedded in paraffin and serial sections (5 µm thickness) were processed by heat antigen retrieval in epitope retrieval solution pH 9 (Eubio, Vienna, Austria). Standanrd staining technique was preformed as described above using rabbit anti-HCit (1:100) and the negative control rabbit IgG (1:100; Thermo Scientific).

Western blot analysis

Protein expression of eNOS and phospho-eNOS (S1177) in mouse aorta samples and HCAEC lysates was estimated via Western blot.

The thoracic aortas were homogenized in ice-cold RIPA buffer supplemented with a mixture of protease and phosphatase inhibitors and mouse aortic protein was extracted as described previously (Cacicedo et al. 2011). The protein content of the aortic samples was determined by BCA protein assay and equalized by protein content for loading.

Denatured proteins were resolved on 4% – 20% SDS-PAGE reducing gels (Invitrogen, CA, USA), transferred to Immun-Blot™ PVDF membranes (0.2µm) from Bio-Rad Lab. (CA, USA) and later probed with rabbit anti-eNOS (1:1000) in 5% fat-free milk or mouse anti-eNOS (pS1177) (1:1000) in 5% BSA overnight at 4°C. Membranes were further incubated with HRP-conjugated anti-rabbit (1:10,000) or anti-mouse IgG (1:1000) for 2 hours at room temperature and protein bands were visualized with Immobilon™ Western chemiluminescence HRP substrate (Millipore Corp., MA, USA) according to the manufacturer's instructions. In case of re-blotting, immunoblots were stripped by incubation for 30 min in stripping buffer (Tris-HCl 62.5 mmol/L, pH 6.9, 2% SDS, 2-mercapatoethanol 100 mmol/L) at 50ºC.

To determine eNOS dimer protein expression, samples were not denatured by heat and low-temperature SDS-PAGE at 4°C (cold electrophoresis) under reducing conditions was performed as previously described (Hemmens et al. 2000, Klatt et al. 1995).

Immunoblotting for eNOS with detection and visualization was continued as mentioned above. Quantification of bands was performed using ImageJ software (NIH) for all blots.

Statistical analyses

Data are shown as mean ± SEM for n observations. Comparison of multiple groups was performed using One-Way ANOVA with Tukey's Multiple Comparison post-hoc test.

Data from hemodialysis and control subjects are shown as median with the interquartile range and Mann Whitney test was used to test for differences. Correlations were determined using Spearman rank correlation.

Student t test was used to test for differences between control and cyanate-treated mice (inhalation model) while for vascular function studies Two-Way ANOVA followed by a Bonferroni´s post-hoc test was used.

Significance was accepted at $P < 0.05$. Statistical analyses were performed with Prism Version 4.03 (GraphPad Software, USA).

III. RESULTS

PART I: Cyanate induces endothelial inflammation

In **Part I**, I investigate the effect of cyanate on human endothelial cells with a particular focus on renal disease patients in an attempt to highlight the clinical relevance of cyanate exposure in human disease.

Cyanate treatment does not affect endothelial cell viability

Cyanate treatment (1 and 2 mM) for 48 hours had no effect on viability of HCAEC ***(Figure 1A)*** and did not induce apoptosis ***(Figure 1B)***.

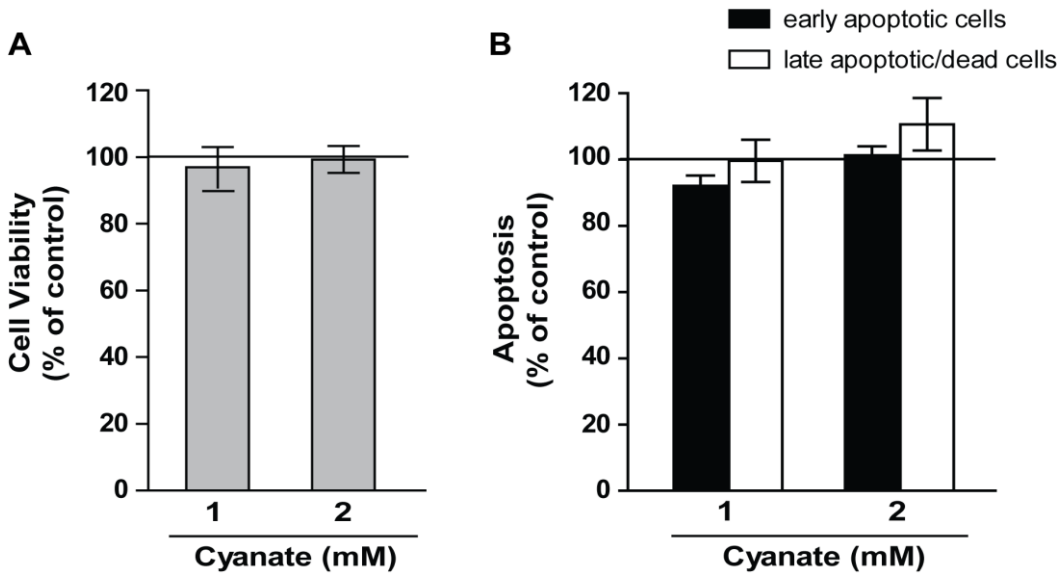

Figure 1. Cyanate treatment has no effect on viability of endothelial cells. *HCAEC were treated with increasing concentrations of sodium cyanate for 48 h.* ***(A)*** *Cell viability was assessed performing a MTT (3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) reduction assay.* ***(B)*** *Apoptosis was measured by flow cytometry after Annexin V/propidium iodide staining. Apoptotic cells are displayed as the percentage of total cells acquired after cyanate treatment. Annexin V-positive/ PI-negative cells were counted as early apoptotic cells while the Annexin V-positive/PI-positive cells were regarded as late apoptotic/dead cells. Control was set at 100% and values are expressed as % of control. Results are shown as mean ± SEM (n = 3-5). * $p<0.05$ versus control.*

Cyanate induces ICAM-1 expression in primary endothelial cells

As vascular endothelial cells might be exposed locally to high cyanate concentrations, I treated HCAEC for 48 hours with increasing concentrations of cyanate. A concentration-

dependent increase in ICAM-1 expression was observed, whereas expression of VCAM-1 ***(Figure 2A)*** and E-selectin ***(Figure 2C)*** were unaltered.

Cyanate-induced ICAM-1 expression was time-dependent and increased substantially from 24 to 48 hours ***(Figure 2B)***.

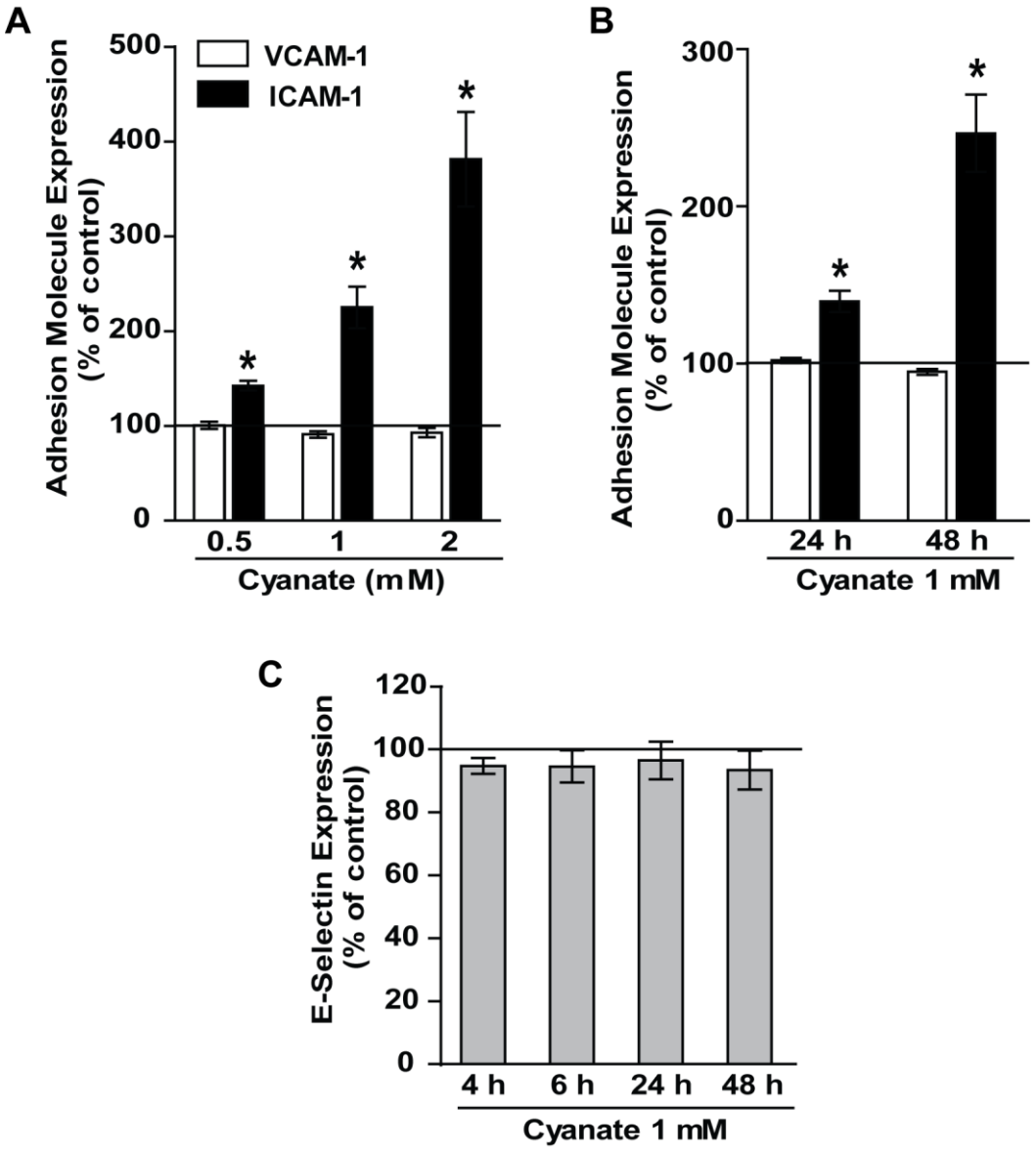

Figure 2. Flow-cytometric quantification of adhesion molecule expression in endothelial cells. ***(A)*** *HCAEC were treated for 48 h with increasing concentrations of sodium cyanate (0.5 up to 2 mM) added to cell culture medium. Subsequently, expression of cell adhesion molecules was assessed by flow cytometry.* ***(B)*** *ICAM-1 and VCAM-1 expression in HCAEC treated with 1 mM sodium cyanate for 24 h or 48 h.* ***(C)*** *E-selectin expression in HCAEC treated with 1 mM sodium cyanate for the indicated time points. Control was set at 100% and values are expressed as % of control. Results are shown as mean ± SEM (n = 3-5). * $p<0.05$ versus control.*

Cyanate-induced (lipo)protein carbamylation does not mediate endothelial ICAM-1 expression

Since all experiments on HCAEC were performed in complete medium containing serum, cyanate-driven carbamylation of (lipo)proteins may have contributed to ICAM-1 expression. To test whether carbamylated (lipo)proteins are involved in ICAM-1 expression, serum-containing cell culture medium was incubated with 1 mM cyanate for 48 hours in the absence of cells to induce protein carbamylation (preconditioned medium). Afterwards, to remove low molecular weight substances (i.e. cyanate); an aliquot of the preconditioned medium was gel-filtered on PD-10 columns. Cells were then treated with preconditioned medium or filtered preconditioned medium for 48 hours. In contrast to the cyanate-containing preconditioned medium, the filtered medium failed to induce any ICAM-1 expression ***(Figure 3)***.

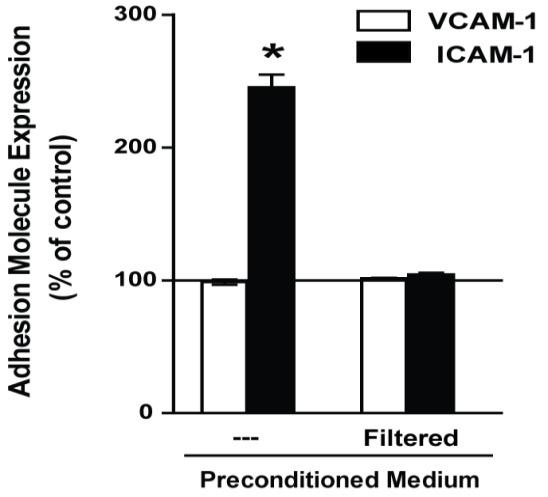

Figure 3. Carbamylated (lipo)proteins do not induce endothelial ICAM-1 expression. *Growth medium was incubated with 1 mM sodium cyanate for 48 h (preconditioned medium), and low molecular weight substances were subsequently removed by gel filtration (PD-10 columns). HCAEC were then treated for 48 h with preconditioned medium or filtered preconditioned medium. Adhesion molecule expression was determined by flow cytometry. Control was set at 100% and values are expressed as % of control. Results are shown as mean ± SEM (n = 3). * $p<0.05$ versus control.*

It is important to refer to previous studies in which carbamylated LDL was shown to induce adhesion molecule expression in endothelial cells (Apostolov et al. 2007). So in a further set of experiments, LDL was incubated with cyanate (1, 2 or 10 mM) for 48 hours to induce carbamylation. Subsequently, upon treating HCAEC with carbamylated LDL, no

change in adhesion molecule expression ***(Figure 4A)*** was observed, clearly indicating that carbamylated LDL did not contribute to ICAM-1 expression under these experimental conditions. However, one cannot rule out that more extensively carbamylated LDL may trigger adhesion molecule expression (Apostolov et al. 2007). Notably, the extent of carbamylation of the LDL preparations was confirmed by mass spectrometry analysis for the carbamyllysine content ***(Figure 4B)***.

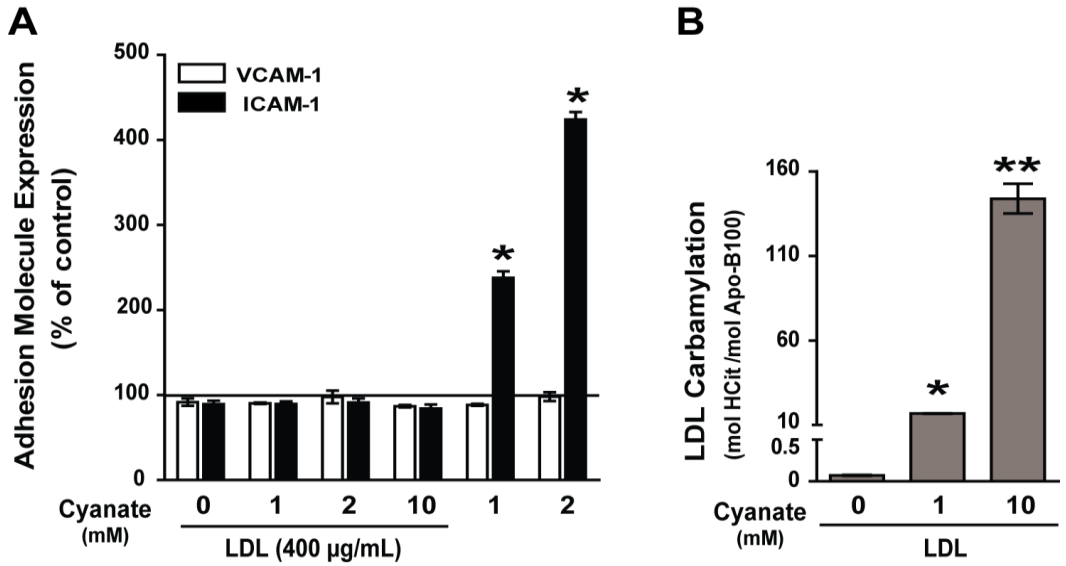

Figure 4. Effect of carbamylated LDL on ICAM-1 expression in HCAEC. (A) *LDL was exposed to cyanate (1, 2 or 10 mM) for 48 h to induce protein carbamylation followed by gel filtration to remove residual cyanate. HCAEC were then treated with control LDL, carbamylated LDL (400 µg/mL) or cyanate (1 - 2 mM) for 48 h. Adhesion molecule expression was determined by flow cytometry. Control was set at 100% and values are expressed as % of control.* ***(B)*** *Quantification of carbamyllysine (HCit) content in carbamylated LDL preparations. The carbamyllysine content in LDL exposed to cyanate for 48 hours was quantified by LC-MS/MS. Results are shown as mean ± SEM (n = 3). * $p<0.05$; ** $p<0.001$ versus control.*

Cyanate-induced ICAM-1 expression is mediated by the p38 MAPK - NF-κB signaling pathways

To elucidate the molecular mechanisms involved in endothelial activation; cyanate-induced ICAM-1 expression in the presence of various pathway inhibitors was examined.

NF-κB is a well-characterized transcription factor crucial in ICAM-1 expression. To examine the contribution of NF-κB, experiments were carried out in the presence of two specific NF-κB inhibitors (BAY 11-7082 and gliotoxin). Upon NF-κB inhibition, cyanate-

induced ICAM-1 expression was completely abolished, indicating a direct involvement of NF-κB in cyanate-induced ICAM-1 expression ***(Figure 5A)***. Further upstream different inhibitors of the MAPK family members; ERK1/2, p38 MAPK and JNK were used. As shown in ***Figure 5B***, the stress-sensitive p38 MAPK inhibitors (SB203580 or SB202190) markedly suppressed cyanate-induced ICAM-1 expression in HCAEC, while inhibitors of the JNK or ERK1/2 signaling pathways showed no effect.

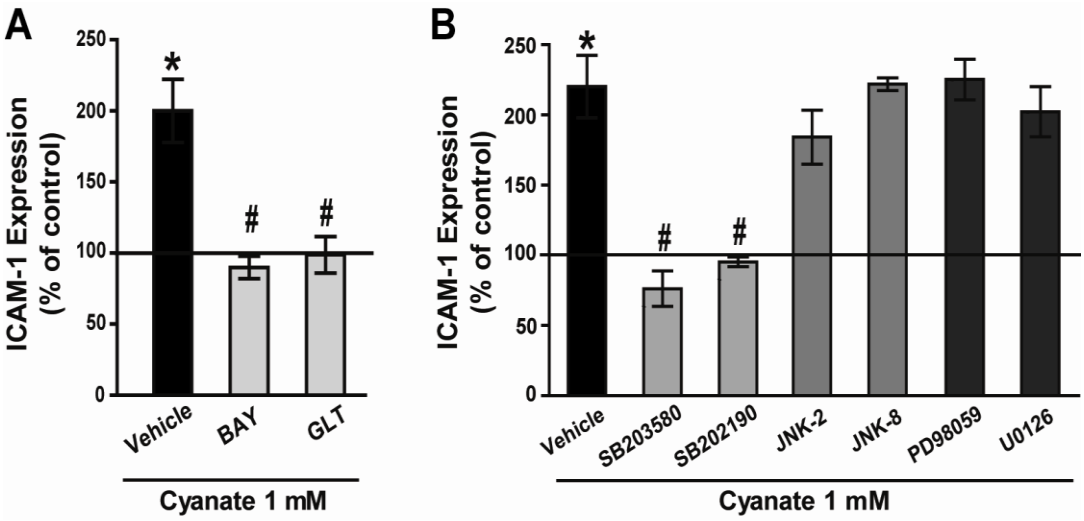

Figure 5. Cyanate-induced ICAM-1 expression is mediated through p38 MAPK and NF-kB signaling. *HCAEC were treated for 48 h with sodium cyanate (1 mM) in the presence of* ***(A)*** *NF-κB inhibitors: BAY 11-7082 (BAY; 5 µM) or gliotoxin (GLT; 1µM).* ***(B)*** *MAPK family member inhibitors: p38 MAPK inhibitors (SB203580; 5 µM or SB202190; 2 µM), JNK inhibitors (JNK-2 inhibitor; 5 µM or JNK-8 inhibitor; 5 µM) or ERK1/2 inhibitors (PD98059; 10 µM or U0126; 5 µM). Subsequently, ICAM-1 expression was determined by flow cytometry. Control was set at 100% and values are expressed as % of control. Results are shown as mean ± SEM (n = 3-5).* $p<0.05$ versus control; # $p<0.05$ versus cyanate-treated cells.*

Cyanate-stimulated endothelial cells produce inflammatory chemokines

Furthermore, I investigated levels of chemokines in the supernatants of cyanate-treated endothelial cells at 12 and 24 hours by flow cytometry using BD™ cytometric bead array human chemokine kit specific for IL-8, RANTES, MIG, MCP-1 and IP-10.

HCAEC treated with cyanate (1 and 2 mM) for up to 24 hours showed a concentration-dependent increase in MCP-1 and IL-8 levels ***(Figure 6)***, whereas levels of RANTES, MIG and IP-10 were below the limit of detection.

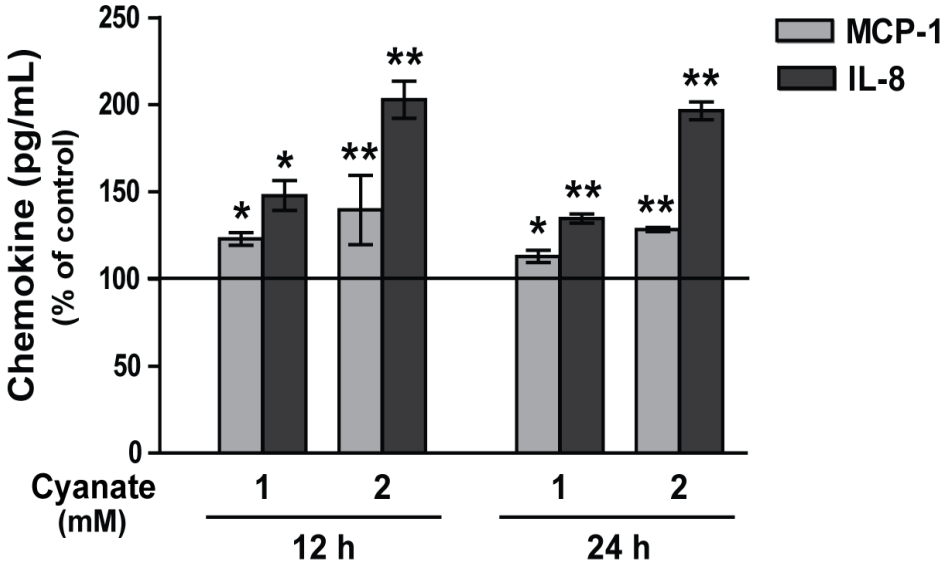

Figure 6. Cyanate stimulates MCP-1 and IL-8 secretion in endothelial cells. *HCAEC were treated with 1 mM sodium cyanate, supernatants were collected 12 and 24 h later for determination of chemokine levels by flow cytometry using a cytometric bead array kit. Control was set at 100% and values are expressed as % of control. Results are shown as mean ± SEM (n = 3-5).* $p<0.05$; ** $p<0.01$ versus control.*

Cyanate promotes leukocyte-endothelial interaction

As endothelial activation and expression of inflammatory mediators is required for leukocyte-endothelial interaction, consequently I was interested to see whether cyanate-stimulated ICAM-1 expression could induce leukocyte adhesion, a key event in the development of atherosclerosis.

For that purpose human polymorphonuclear leukocytes (neutrophils) were freshly isolated, labeled with fluorochrome and allowed to adhere to cyanate-treated endothelial cells (1 mM; 48 hours). As expected a significant increase of neutrophil adhesion to cyanate-stimulated HCAEC was observed in comparison to control un-stimulated cells ***(Figure 7)***. Importantly, this increase in neutrophil adhesion was completely reversed in the presence of the NF-κB inhibitor BAY 11-7082.

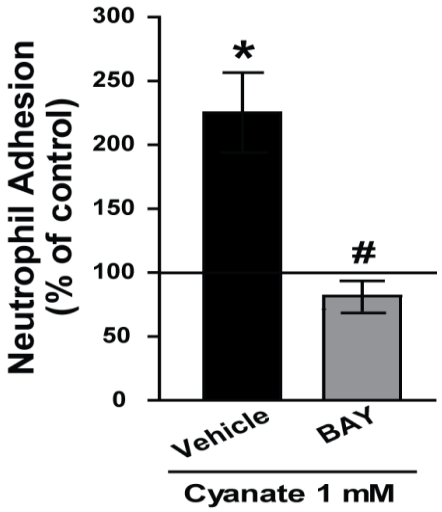

Figure 7. Cyanate enhances leukocyte adhesion to endothelial cells. *HCAEC monolayers were grown on 96-well plates and treated for 48 h with 1 mM sodium cyanate in the presence or absence of a NF-κB inhibitor (BAY 11-7082; 5 μM). Subsequently, fluorochrome-labeled neutrophils were added to the confluent monolayers and percentage of adhesion was determined after a 30 min incubation period at 37°C. Control was set at 100% and values are expressed as % of control. Results are shown as mean ± SEM (n = 3-5).* $p<0.05$ versus control; $^{\#}p<0.05$ versus cyanate-treated cells.*

Cyanate induces endothelial ICAM-1 expression *in vivo*

To verify the physiologic relevance of these *in vitro* observations, I examined whether oral administration of cyanate increases endothelial ICAM-1 expression in mice.

For this purpose, male C57BL/6 mice were assigned to three groups, which received normal drinking water (control), drinking water containing 0.2 mg/mL sodium cyanate (low-cyanate) and drinking water containing 1 mg/mL sodium cyanate (high-cyanate), respectively, for a period of 9 weeks.

General characteristics of mice are given in ***Table 1***. Plasma levels of total cholesterol and urea were not altered. Mass spectrometry analysis of plasma proteins was utilized to evaluate plasma protein carbamylation as a marker for cyanate exposure, and revealed elevated carbamyllysine levels in cyanate-treated mice compared to controls. To investigate the possible involvement of lipid peroxidation, plasma levels of malondialdehyde were measured, but no significant difference was detected between treatment groups ***(Table 1)***.

Table 1. Biochemical characteristics of mice receiving cyanate in drinking water for 9 weeks

Characteristic	Control	Low-cyanate	High-cyanate
Total Cholesterol (mg/dL)	108 ± 4	123 ± 10	118 ± 14
Urea (mg/dL)	74 ± 5	78 ± 4	88 ± 7
Malondialdehyde (nmol/mL)	28.5 ± 5.5	31 ± 3.7	32.5 ± 4.8
Carbamyllysine (µmol HCit /mol Lys)	2.9 ± 0.8	276.8 ± 30.8 *	1311 ± 128.2 *

*Plasma levels of total cholesterol, urea and malondialdehyde were measured using commercial kits; carbamyllysine quantification was by LC-MS/MS and expressed as homocitrulline (HCit) µmol/mol Lysine (Lys). Results are shown as mean ± SEM; (n=5 mice per group). * p< 0.05 versus control.*

Consistent with the previous *in vitro* findings in cyanate-treated HCAEC, a significant increase in the expression of ICAM-1 in vascular endothelial cells of the aortic arch was observed in mice upon oral administration of cyanate ***(Figure 8A and 8B)***.

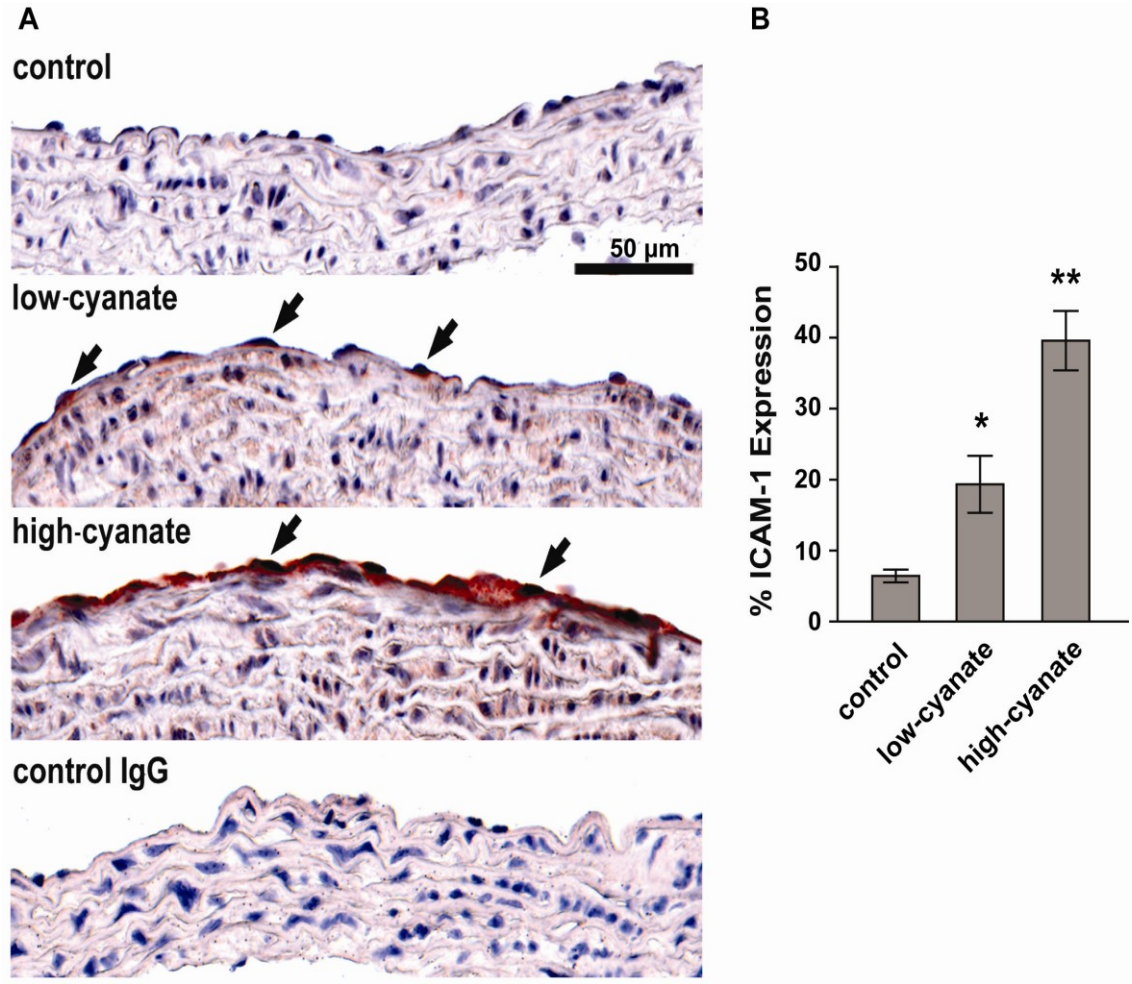

Figure 8. Oral administration of cyanate induces ICAM-1 expression in mice aorta. (A) *Sections of paraffin-embedded aortic arches were stained with polyclonal anti-CD54 or control IgG where positive immunohistochemical staining is indicated by a red immunoreaction product. In contrast to control mice, mice receiving low cyanate (0.2 mg/mL) and mice receiving high cyanate (1 mg/mL) in drinking water, expressed visible levels of ICAM-1 in vascular endothelial cells (black arrows). Control IgG showed no staining. Images are representatives of the treatment groups (n=5 mice/group). Scale bar indicates 50 µm.* ***(B)*** *Quantification of endothelial ICAM-1 immunostaining in each group expressed as mean ± SD. * $p<0.05$, ** $p<0.001$ versus control.*

In an effort to highlight the clinical relevance of the previous findings, experiments were conducted on plasma from chronic renal disease patients, as elevated MPO activity and high urea concentrations are common features among this population, thereby contributing to increased cyanate formation in such patients. Therefore, I next assessed whether plasma carbamyllysine levels in patients with end-stage renal disease (ESRD) correlate with plasma sICAM-1 concentrations, a proteolytic cleavage product of vascular ICAM-1

(Leeuwenberg et al. 1992, Witkowska 2005). The general characteristics of both groups are shown in **Table 2**.

Table 2. Clinical chemistry of control subjects and hemodialysis patients

	control	**hemodialysis**
n	19	23
age (yr)	53 (45 - 68)	68 (48 - 74)
male/female	9/10	13/10
plasma parameter		
Cholesterol (mg/dL)	188 (176 - 195)	161 (124 - 196) *
Triglycerides (mg/dL)	122 (83 - 169)	147 (90 - 200)
Urea (mg/dL)	28 (25 - 31)	118 (98 - 143) †
C-reactive protein (mg/L)	1 (0 - 3)	9 (3 - 17) †
Fibrinogen (mg/dL)	272 (211 - 403)	490 (411 - 619) †
Creatinine (mg/dL)	0.92 (0.84 - 1.14)	6.91 (6.28 - 9.81) †
Uric acid (mg/dL)	4.8 (4.4 - 5.4)	5.8 (5.1 - 7.1) †

*Plasma parameters were determined using commercially available kits. Results are given as median with the interquartile range. Significance was accepted at † $p<0.01$; * $p<0.05$ (Mann Whitney test).*

Increased sICAM-1, HCit and carboxymethyl-lysine (CML) in renal patients

Plasma sICAM-1 concentrations from ESRD patients on maintenance hemodialysis and age-matched healthy control subjects were estimated by ELISA. Significantly higher sICAM-1 concentrations were observed in hemodialysis patients in comparison to control subjects ***(Figure 9A)***. Patients on hemodialysis showed elevated plasma levels of both, HCit and CML ***(Figure 9B)***.

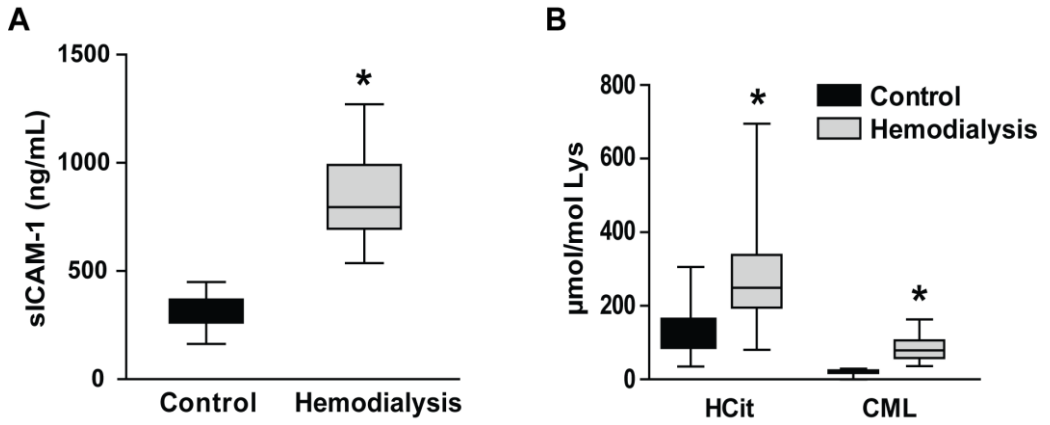

Figure 9. Increased plasma levels of sICAM-1, carbamyllysine and carboxymethyl-lysine in end-stage renal disease patients on hemodialysis. (A) *Plasma levels of sICAM-1 were quantified by ELISA whereas* ***(B)*** *plasma levels of carbamyllysine (HCit) and carboxymethyl-lysine (CML) were quantified by LC-MS/MS from a total of 23 hemodialysis patients and 19 control subjects. * p<0.001.*

Notably, a significant correlation between plasma levels of sICAM-1 and plasma protein carbamylation (HCit content) was observed, whereas no correlation was found with plasma levels of the major advanced glycation end product CML, the formation of which is closely linked to increased local oxidative stress (Suzuki et al. 1999). In addition, sICAM-1 correlated with C-reactive protein (CRP) in hemodialysis patients, but not with creatinine or uric acid ***(Table 3)***.

Table 3. Correlation matrix of sICAM-1, HCit, CML, CRP, creatinine and uric acid in hemodialysis patients

	sICAM-1	HCit	CML	CRP	Creatinine
sICAM-1	-	-	-	-	-
HCit	0.588†	-	-	-	-
CML	-0.026	-0.143	-	-	-
CRP	0.609†	0.360	-0.296	-	-
Creatinine	-0.304	-0.090	0.421*	-0.531†	-
Uric acid	-0.163	0.004	0.084	-0.045	0.400*

*sICAM-1, soluble ICAM-1; HCit, carbamyllysine; CML, carboxymethyl-lysine and CRP, C-reactive protein. Spearman rank correlation coefficients are noted at * P<0.05; † P<0.01.*

PART II: Cyanate impairs vascular reactivity

To gain further insight on the effect(s) of cyanate on endothelial function, an inhalation model for cyanate exposure in mice was used. As it was recently established that smoke contains sufficient amounts of isocyanic acid (HNCO)/cyanate, wherein measurements of aqueous solubility demonstrate that HNCO is highly soluble (dissociates at physiological pH); hence smoke provides a route for the uptake and absorption of cyanate directly into the blood stream (Roberts et al. 2011).

Inhalation of cyanate induces protein carbamylation

Five-week old male C57BL/6 mice were exposed to cyanate for a period of 3 weeks (2.5 hours/day). The cyanate solution was delivered by a nebulizer forming a fine aerosol suitable for inhalation. To investigate the extent of plasma protein carbamylation as a marker for cyanate exposure, carbamyllysine (HCit) levels were measured by mass spectrometric analysis of plasma proteins which revealed a 5-fold increase in HCit in cyanate-treated mice ***(Figure 10A)*** reaching levels previously observed in humans with CVD (Wang et al. 2007, El-Gamal et al. 2012). Significant cyanate-induced protein carbamylation was observed in aortic tissue of cyanate treated mice ***(Figure 10B)***.

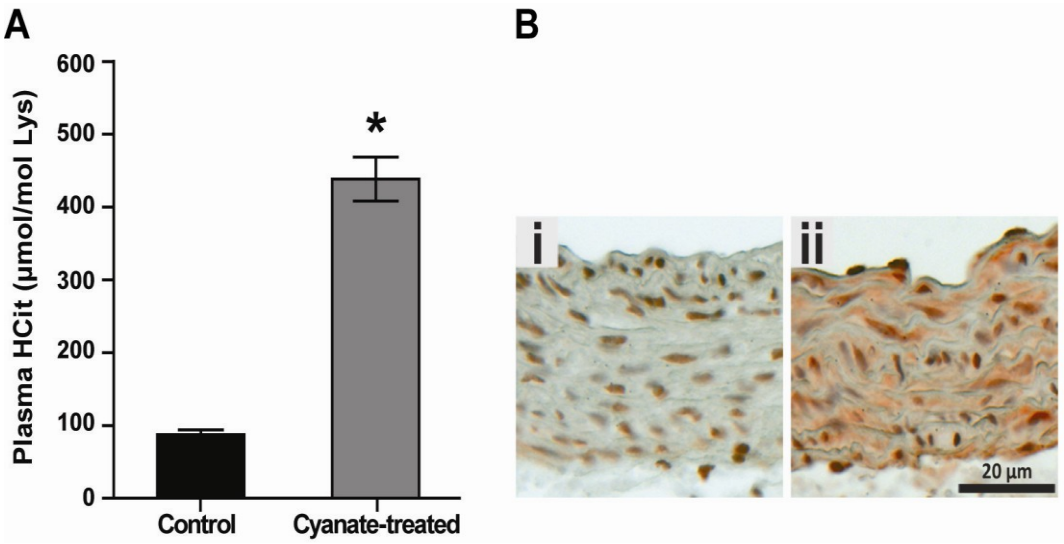

Figure 10. Inhalation of cyanate induces protein carbamylation in mice. (A) *Increased plasma levels of HCit in cyanate-treated mice. Plasma levels of HCit in control and cyanate-treated mice were quantified by LC-MS/MS and expressed as µmol HCit per mol lysine (Lys). Results are shown as mean ± SEM (n = 8 mice/group). * p<0.001 versus control.* ***(B)*** *Aortas of cyanate-treated mice show increased cyanate-induced protein carbamylation. Sections of paraffin-embedded aortas*

were stained with an anti-HCit antibody using immunohistochemistry. In contrast to control mice (i), mice receiving cyanate (ii) showed marked staining for carbamylated epitopes in the aorta. Positive immunohistochemical staining is indicated by a red immunoreaction product. Images are representatives of the treatment groups (n=5 mice/group). Scale bar indicates 20 µm.

Being the chief regulator of vascular homeostasis, the endothelium exerts a number of vasoprotective effects which are often regulated by nitric oxide (NO). In this respect, impaired endothelium-dependent vasodilation is considered a hallmark of endothelial dysfunction (Davignon, Ganz 2004). Therefore, I was further interested in investigating the effects of cyanate on endothelial function with a particular focus on eNOS.

Effect of cyanate on endothelium-dependent vasorelaxation in mice

To assess the impact of chronic cyanate exposure on vascular reactivity, aortic rings from cyanate-treated and control mice were pre-constricted with norepinephrine, and were exposed to increasing concentrations of acetylcholine (ACh). Compared with control aortas, ACh-induced vasorelaxation was markedly attenuated in aortic rings from cyanate-treated mice at all applied ACh concentrations ***(Figure 11A)***. In contrast, there was no significant difference in relaxation in response to the NO-donor sodium nitroprusside (SNP) ***(Figure 11B)***. These findings indicate that not an impaired responsiveness of aortic smooth muscles, but rather decreased production of endothelium-derived vasodilator substance(s) is responsible for the impaired vasorelaxation upon cyanate treatment.

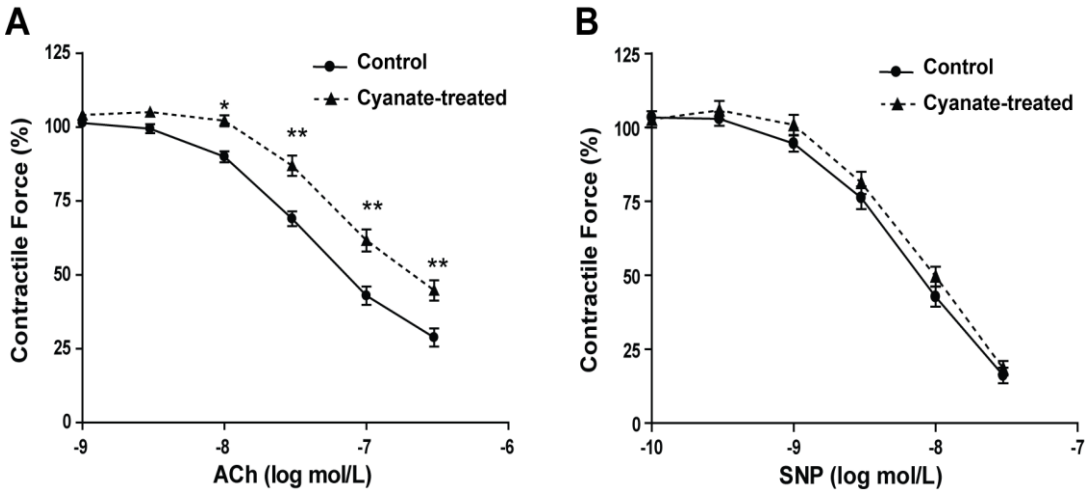

Figure 11. Cyanate-induced impairment of vascular function in mouse aorta. *Aortic rings were pre-constricted with norepinephrine, and relaxant responses to ACh and the NO donor sodium nitroprusside (SNP) were measured.* ***(A)*** *Vasorelaxation in response to ACh was markedly*

attenuated in cyanate-treated mice. ***(B)*** *No significant difference in arterial relaxation in response to SNP was observed between treatment groups. Data are expressed as mean ± SEM (n=9 mice/group). * p< 0.01, ** p<0.001 versus control.*

Indeed, the impaired vasorelaxation of cyanate-treated animals was accompanied with markedly lower nitrite levels (indicative of NO production) released from aortic rings of cyanate-treated mice compared with control mice ***(Figure 12A)***.

Furthermore, the pre-incubation of aortic rings of control mice with an eNOS inhibitor (L-NNA; 200 µM) abolished the vasorelaxation in response to ACh ***(Figure 12B)***, suggesting that NO is the predominant mediator of ACh-induced relaxation in mouse aortic rings.

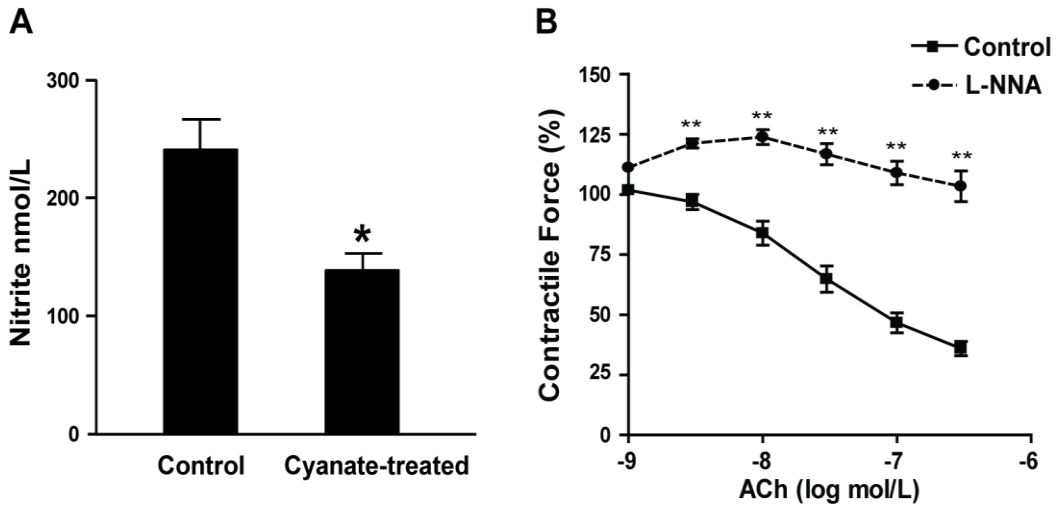

Figure 12. Cyanate decreases production of nitric oxide, the predominant mediator of ACh-induced relaxation in mouse aortic rings. ***(A)*** *Total nitrite (indicative of NO production) was determined by a fluorometric HPLC method in aliquots of the bath solution taken after ACh-induced relaxation of mouse aortic rings (n=8 mice/group). Fluorescence was monitored at an excitation wavelength of 375 nm and an emission wavelength of 415 nm.* ***(B)*** *Abolished ACh-mediated vasorelaxation in mouse aortic rings in the presence of the eNOS inhibitor N5-[imino(nitroamino)methyl]-L-ornithine (L-NNA; 200 µM), indicating the principal role of NO in aortic vasorelaxation (n=7 mice/group). Data are expressed as mean ± SEM. * p<0.01, ** p<0.001.*

eNOS protein expression in aorta of cyanate-treated mice

Based on the decreased nitrite release from cyanate-treated rings ***(Figure 12A)*** and the principal role of NO in aortic vasorelaxation ***(Figure 12B)***, it was tempting to assume that the detrimental effect of cyanate on eNOS protein accounts for the impaired vasorelaxation

in cyanate-treated mice described above in Figure 11A. In fact, immunoblot analysis revealed decreased total- and phospho-eNOS (S1177) protein levels in aortas of cyanate-treated mice when compared to control ***(Figure 13A)***. However, the ratio of phospho-eNOS/eNOS remained unaltered indicating that a decreased protein expression may account for the decreased NO production in aortas of cyanate-treated animals.

Previous studies have shown that the formation of eNOS homodimers is necessary for proper eNOS activity (Forstermann, Munzel 2006). Interestingly, reduced eNOS dimer levels were observed in cyanate-treated mice with a decrease in dimer/monomer ratio ***(Figure 13B)***.

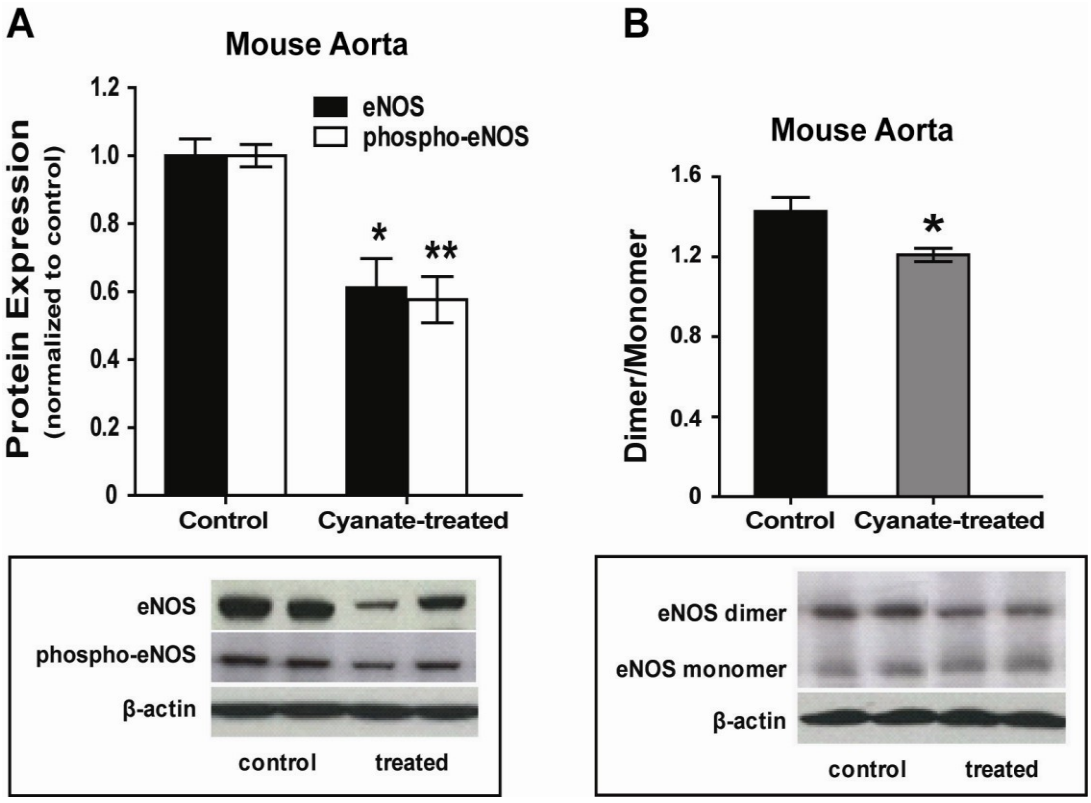

Figure 13. Effect of cyanate on endothelial nitric oxide synthase (eNOS) in mice. (A) *Representative Western blots of protein from mouse aorta probed with antibodies recognizing serine phosphorylation of residue 1177 of eNOS protein and total eNOS protein with quantification.* ***(B)*** *Representative Western blot showing eNOS dimers and monomers in mouse aorta with quantification of eNOS dimer/monomer levels. In order to detect eNOS dimers and monomers, low-temperature SDS-PAGE (4°C) was performed. β-Actin was used to normalize the data, and quantification of bands was done using ImageJ software. Data are expressed as mean ± SEM (n=5-7 mice/group). * p<0.05 versus control.*

Cyanate decreases eNOS protein in cultured endothelial cells

Next it was interesting to assess whether direct addition of cyanate to cell culture medium could alter eNOS protein expression in HCAEC.

Treatment of HCAEC for up to 48 hours with increasing concentrations of cyanate decreased eNOS protein expression in a concentration-dependent ***(Figure 14A)*** and time-dependent manner ***(Figure 14B)***.

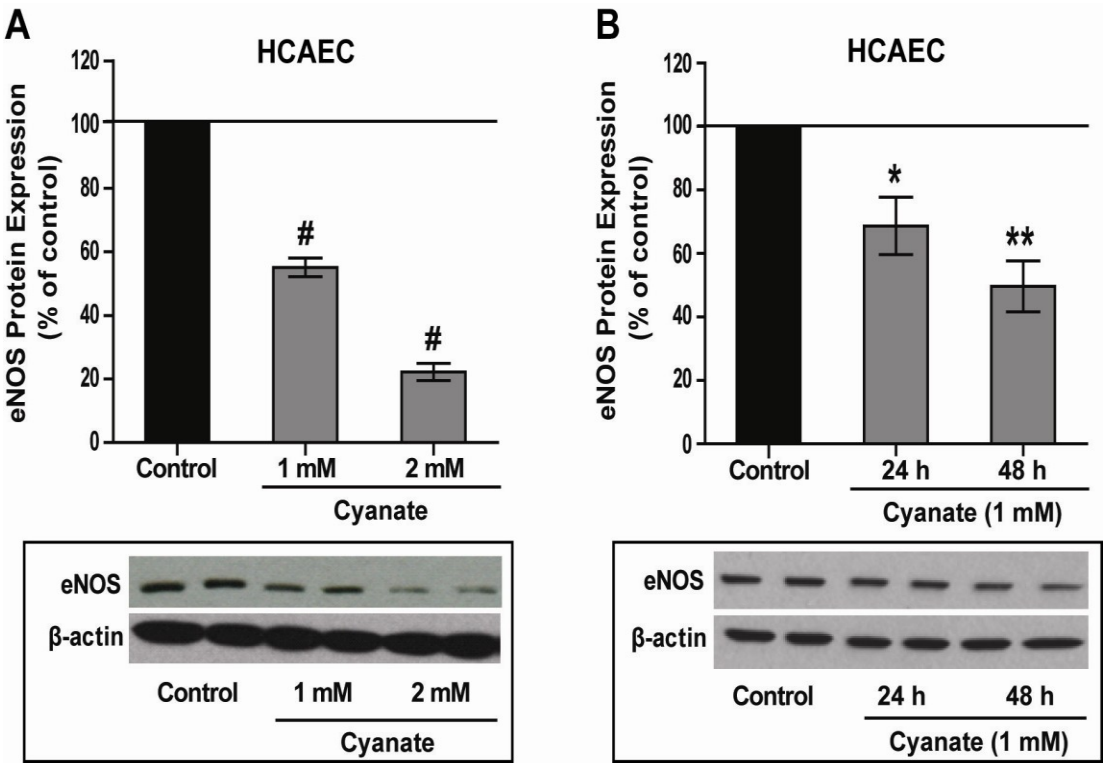

Figure 14. Cyanate alters eNOS protein expression in HCAEC. *Representative Western blots from HCAEC treated with sodium cyanate (1 or 2 mM) for up to 48 h. Cyanate elicits a concentration-dependent* ***(A)*** *and time-dependent* ***(B)*** *decrease in eNOS protein expression. β-Actin was used to normalize data, and quantification of bands was done using ImageJ software. Data are expressed as mean ± SEM (n=4). * $p < 0.05$, ** $p < 0.01$, # $p < 0.001$ versus control.*

This observed decrease in total eNOS protein was paralleled by a decrease in phospho-eNOS (S1177) levels ***(Figure 15)***. However, cyanate treatment (1 mM; 48 hours), had no impact on eNOS dimer/monomer ratio in HCAEC ***(Figure 16)***.

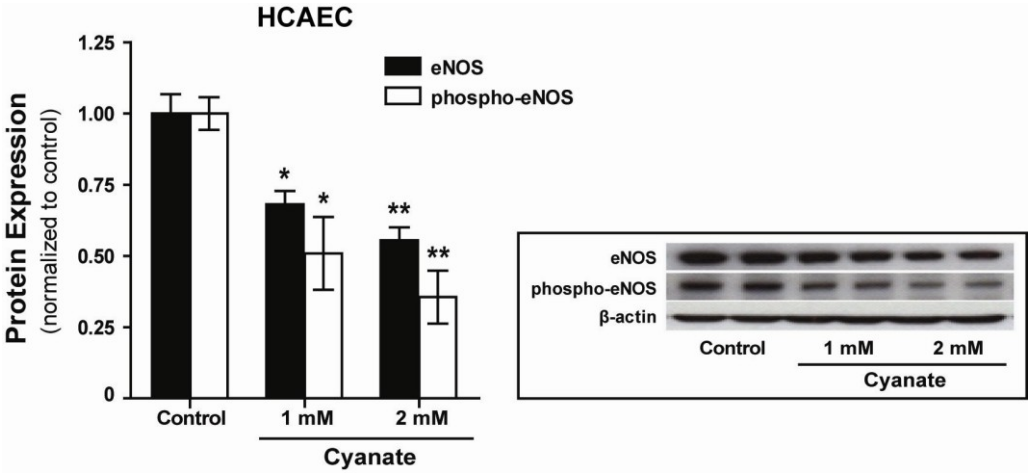

Figure 15. Cyanate decreases eNOS phosphorylation in HCAEC. *Representative Western blot with quantification for eNOS and phospho-eNOS (S1177) in lysates from HCAEC treated with sodium cyanate (1 and 2 mM) for 48 h. β-Actin was used to normalize the data, and quantification of bands was done using ImageJ software. Data are expressed as mean ± SEM (n=3). * p<0.05, ** p<0.01 versus control.*

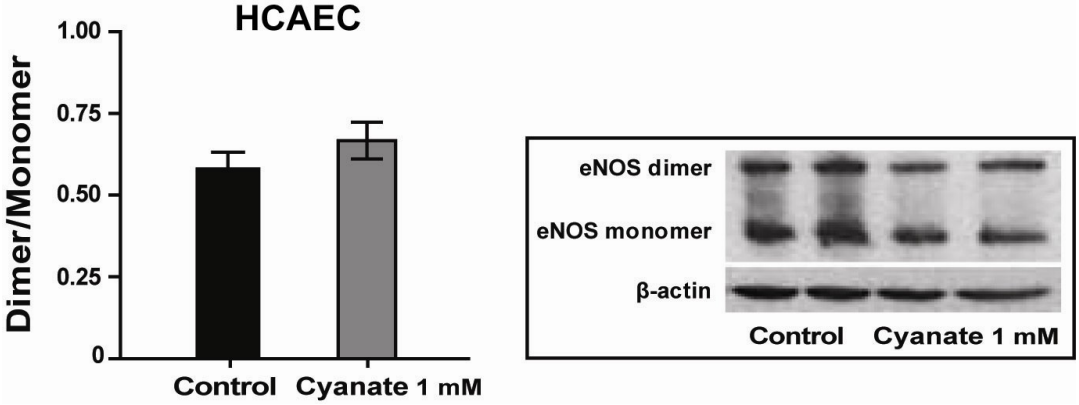

Figure 16. Effect of cyanate on eNOS dimerization in HCAEC. *Representative Western blot for eNOS dimerization with quantification of eNOS dimer/monomer levels in lysates from HCAEC treated with 1 mM sodium cyanate for 48 h. β-Actin was used to normalize the data, and quantification of bands was done using ImageJ software. Data are expressed as mean ± SEM (n=3).*

Effect of cyanate-driven protein carbamylation on eNOS expression

All previous experiments were performed in the presence of serum; therefore, cyanate-driven carbamylation of (lipo)proteins may have contributed to reduced eNOS expression in HCAEC.

To investigate the contribution of cyanate-driven protein carbamylation to eNOS protein expression, serum-containing cell culture medium was incubated for 48 hours with cyanate (1 mM) or vehicle in the absence of cells to induce protein carbamylation (preconditioned medium). To remove low molecular weight substances (i.e. cyanate), the preconditioned medium was gel-filtered on Sephadex PD-10 columns. When cells were treated with the filtered preconditioned medium for 48 hours, no decrease in eNOS protein expression was observed in comparison to control ***(Figure 17)***.

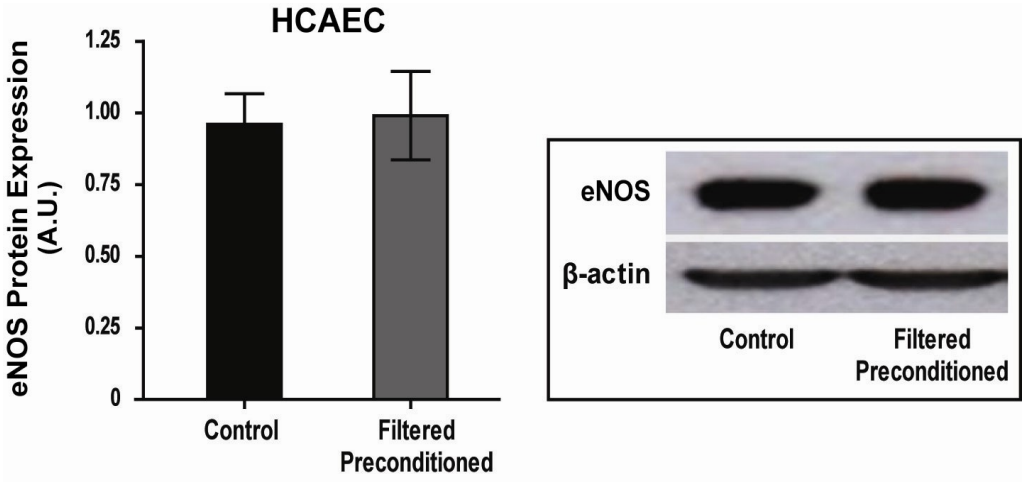

Figure 17. Carbamylated (lipo)proteins do not alter eNOS protein expression in HCAEC. *Cell culture medium was pre-incubated with 1 mM sodium cyanate (preconditioned medium) and filtered on Sephadex PD-10 columns to remove low molecular weight substances (i.e. cyanate). HCAEC were then treated for 48 h with filtered preconditioned medium (cyanate-free) or control medium. eNOS protein expression was determined by Western blot. β-Actin was used to normalize the data, and quantification of bands was done using ImageJ software. Data are expressed as mean ± SEM (n = 3). * p<0.05 versus control.*

Finally to sum up the observed effects of cyanate on vascular endothelial cells, ***Figure 18*** illustrates the potential harmful effects of cyanate on endothelial cells. In conclusion, the findings of this study are compatible with the overall reduction in eNOS-derived NO bioavailability observed in smokers as well as in uremic patients, suggesting cyanate as a common factor underlying endothelial dysfunction, thereby linking inflammation, uremia and smoking.

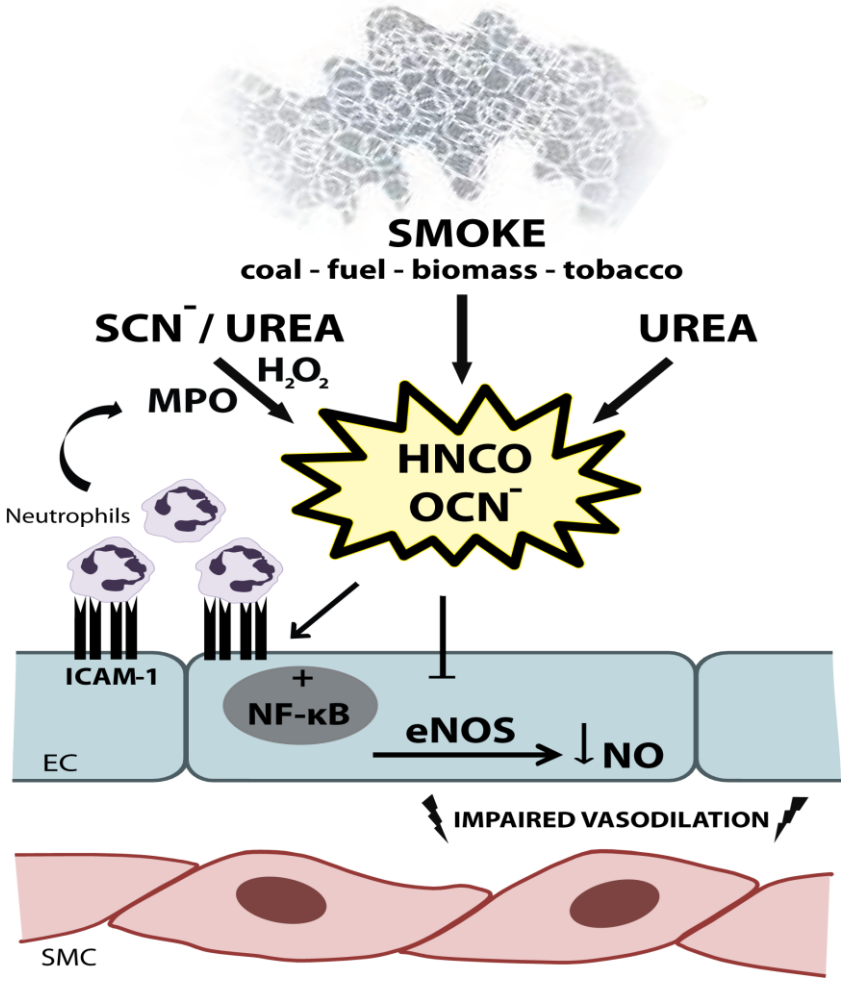

Figure 18. Cyanate and endothelial dysfunction. *A schematic illustration summarizing the potential harmful effects of isocyanic acid/cyanate ($HNCO/OCN^-$) on endothelial cells (EC). Humans are exposed to cyanate from various sources: exogenous sources include smoke resulting from burning of coal, fuel, biomass and tobacco, whereas endogenous sources include in vivo generation of cyanate from either oxidation of thiocyanate (SCN^-) or urea by myeloperoxidase (MPO) in the presence of hydrogen peroxide (H_2O_2) or from the breakdown of urea. Cyanate induces endothelial ICAM-1 expression (via NK-κB stimulation) thus enabling the firm adhesion of neutrophils (Nu), which being rich in MPO can contribute to more cyanate formation at sites of inflammation, creating a vicious circle. In the underlying smooth muscle cells (SMC), endothelium-dependent vasodilation is impaired due to the negative effect of cyanate on endothelial nitric oxide synthase (eNOS), thereby reducing the supply of nitric oxide (NO) to the vasculature.*

IV. DISCUSSION

Discussion of PART I

In PART I, I demonstrate that cyanate activates vascular endothelial cells without altering cell viability. Cyanate induced a time- and concentration-dependent up-regulation of endothelial ICAM-1 expression and subsequently increased neutrophil adhesion in HCAEC. Similarly, oral administration of cyanate dose-dependently increased ICAM-1 expression in vascular endothelial cells in mice aorta. Importantly, plasma levels of carbamyllysine in mice of the low-cyanate group reached levels observed in hemodialysis patients (276 ± 31 vs. 290 ± 30 µmol HCit/mol Lys, respectively), indicating that cyanate concentrations used in my mice experiments are of biological relevance.

Several lines of evidence indicate a role for various cell adhesion molecules in atherogenesis. Increased expression of ICAM-1, VCAM-1, PECAM, and E-selectin has been consistently observed in human atherosclerosis (Davies et al. 1993, O'Brien et al. 1996, DeGraba et al. 1998). Furthermore, inflammatory cytokines such as IL-1 and TNF-α (Takahashi et al. 2001), modified LDL (Apostolov et al. 2007, Weber et al. 1999), and low shear stress (Warboys et al. 2011) contribute to ICAM-1 expression, a common finding in atherosclerotic plaques.

Data obtained in this study indicate that cyanate-induced ICAM-1 expression in HCAEC is mediated through activation of the stress-activated p38 MAPK and NF-κB signaling pathways. Many studies have implicated a role of p38 MAPK and NF-κB in the regulation of ICAM-1 expression by various inducers, such as pro-inflammatory cytokines lipopolysaccharide, bile acids or sphingosine 1-phosphate (Takahashi et al. 2001, Rahman, Fazal 2009). Recently it was shown that ketoaldehyde-modified phosphatidylethanolamine induces p38 MAPK and adhesion molecule expression in endothelial cells (Guo et al. 2011). Of particular interest, in a previous study it was shown that cyanate targeted phosphatidylethanolamine on human HDL particals (Holzer et al. 2011b). Thus it is tempting to speculate whether phosphatidylethanolamine modified by cyanate could activate endothelial cells.

A significant body of literature has demonstrated the multiple pro-atherosclerotic effects of carbamylated LDL such as monocyte adhesion, over-expression of adhesion molecules, proliferation of vascular smooth muscle cells, and endothelial cell apoptosis (Apostolov et al. 2007, Ok et al. 2005, Asci et al. 2008). On the contrary, under our experimental

conditions, the carbamylated LDL preparations used failed to induce ICAM-1 expression, however; one cannot rule out that more extensively carbamylated LDL may trigger adhesion molecule expression (Apostolov et al. 2007).

Secretion of chemokines by endothelial cells has been shown to mediate leukocyte recruitment to the artery wall during atherosclerotic lesion formation. Recently, IL-8 and MCP-1, together with their receptors CCR2 and CXCR2, respectively, have been implicated in the pathogenesis of atherosclerosis by promoting monocyte-endothelial adhesion to human atherosclerotic plaques (Papadopoulou et al. 2008).

Interestingly, in the current study, cyanate-stimulated HCAEC demonstrated increased concentrations of MCP-1 and IL-8 in cell-culture supernatants. Indeed, several diverse stimuli relevant to CVD have been shown to promote chemokine secretion by vascular endothelial cells. Inflammatory stimuli including bacterial lipopolysaccharide and pro-inflammatory cytokines (e.g. IL-1β and TNF-α) have been shown to induce MCP-1 expression in endothelial cells (Rollins et al. 1990, Sica et al. 1990, Liu et al. 2009). Similarly, IL-8 production is induced by various factors such as advanced glycation end products, lipopolysaccharide, oxidized LDL, and low shear stress (Liu et al. 2009, Yang et al. 2005).

Previous studies evaluated the impact of protein carbamylation on structure and/or function of proteins, enzymes and hormones (Kraus, Kraus 2001, Ganea, Harding 1996, Kraus et al. 2001, Mun, Golper 2000, Jaisson et al. 2007). It was long thought that formation of cyanate occurs to a significant extent only during renal dysfunction. In chronic kidney disease patients, urea levels reach up to 110 mM (Bell et al. 1991, Blackmore, Elder & Bowden 1963), wherein about 0.8% spontaneously decomposes into cyanate *in vivo* (Dirnhuber, Schutz 1948), hence cyanate concentrations of about 1 mM may be formed. Notably, the time-average concentration of urea in plasma of renal patients, which correlates with plasma levels of protein-bound carbamyllysine, is associated with an increased odds ratio for death (Owen et al. 1993).

Recent studies have unambiguously demonstrated that humans are exposed to significant amounts of isocyanic acid/cyanate formed by pyrolysis/combustion of coal, fuel, biomass or tobacco (Roberts et al. 2011) and that cyanate formation is also catalyzed by the leukocyte heme peroxidase MPO (Wang et al. 2007, Arlandson et al. 2001). Importantly,

in hemodialysis patients with high-grade persistent inflammation, significantly elevated MPO activity was recently demonstrated supporting the association between inflammation and cumulative oxidative stress (Rodriguez-Ayala et al. 2005). It was recently shown that the high carbamyllysine content of HDL in human atherosclerotic lesions correlated with the MPO-specific oxidation product 3-chlorotyrosine, which strongly supports the notion that macrophage-associated MPO generates significant amounts of cyanate (Holzer et al. 2011b). Hence, inflammation-driven formation of cyanate is a quantitatively important mechanism for cyanate formation and protein carbamylation *in vivo*.

Levels of soluble adhesion molecules have been shown to correlate to various cardiovascular risk factors such as smoking (Blann, Steele & McCollum 1997, Mazzone et al. 2001, Takeuchi et al. 2002), hypertension (Blann et al. 1994, DeSouza et al. 1997, Preston et al. 2002), dyslipidemia (Abe et al. 1998, Hackman et al. 1996, Hackman et al. 1996) and low HDL-cholesterol (Calabresi et al. 2002). Furthermore, levels of soluble adhesion molecules have been postulated to be useful risk predictors of cardiovascular events in healthy populations and various diseases (Mulvihill et al. 2002, Ridker, Buring & Rifai 2001).

Circulating adhesion molecules were found to be increased in a variety of inflammatory disorders (Gearing, Newman 1993), indicating endothelial activation and enhanced endothelial-leukocyte interaction. In patients with chronic renal failure, increased levels of soluble adhesion molecules have been reported, yet little is known about their clearance and catabolism (Jacobson et al. 2002, Rabb et al. 1996, Bonomini et al. 1998). Recent data from renal patients strongly suggest that high serum levels of soluble adhesion molecules may predict future cardiovascular events (Suliman et al. 2006, Rabb et al. 1996, Stenvinkel et al. 2000). Serum concentrations of adhesion molecules may also increase with the progression of renal dysfunction, suggesting that inadequate clearance contributes to elevated serum levels of adhesion molecules in chronic renal failure (Bonomini et al. 1998). However, no significant relationship between creatinine levels (residual renal function) and plasma levels of sICAM-1 was observed in our study, which is in agreement with a previous study (Stenvinkel et al. 2000).

A most important finding of the present study is that sICAM-1 plasma levels in hemodialysis patients significantly correlate with plasma concentrations of carbamyllysine. Therefore, monitoring of plasma carbamyllysine levels may offer a novel basis for

identification of humans at increased risk of CVD. In addition, anti-ICAM-1 antibodies and/or interventions aimed at reducing levels of cyanate are potential promising approaches in reducing vascular disease in renal patients.

Of note, plasma concentrations of the advanced glycation end product carboxymethyl-lysine (CML), the formation of which is closely linked to oxidative stress in hemodialysis patients, showed no correlation with sICAM levels (Suzuki et al. 1999). Accordingly, it needs to be noted that high serum levels of advanced glycation end products, as measured by CML, may not be linked to increased mortality in hemodialysis patients (Schwedler et al. 2002).

The localization of phagocytes in the immediate vicinity of endothelial cells at sites of inflammation may contribute to cyanate-induced endothelial activation, since it was previously shown that MPO-containing neutrophils are markedly enriched with carbamylated proteins (Kraus et al. 1994). Therefore, cyanate-induced expression of endothelial ICAM-1 and the subsequently enhanced endothelial-neutrophil interaction might form a vicious circle inducing endothelial dysfunction. In this respect, it was shown that serum MPO levels correlate with levels of inflammatory markers and mortality in renal disease patients on hemodialysis (Kalantar-Zadeh, Brennan & Hazen 2006).

Hence, the findings of our study provide further insight into the underlying mechanisms that contribute to the enhanced cardiovascular risk associated with smoking and chronic renal failure.

Discussion of PART II

As endothelial dysfunction is not only characterized by increased expression of cell adhesion molecules and leukocyte recruitment but also by decreased eNOS activity and NO bioavailability, I sought to explore the effect(s) of cyanate on eNOS to gain further insight into cyanate-induced endothelial dysfunction.

In PART II, I demonstrate that cyanate promotes endothelial dysfunction through alterations in eNOS protein expression and post-translational modifications pivotal for enzymatic activity.

In the second part of this study, endothelium-dependent vasodilation was markedly attenuated in cyanate-treated mice. This impaired vessel reactivity was accompanied by diminished NO production. These findings are in agreement with previous studies demonstrating blunt endothelium-dependent vasodilatations with active and passive smoking (Argacha et al. 2008, Celermajer, Ng 2008, de Sousa et al. 2005, Heiss et al. 2008, Sumida et al. 1998, Celermajer et al. 1996). Moreover, another study showed that endothelial cells exposed to cigarette smoke extract (CSE) exhibited less eNOS activity and NO production (Zhang et al. 2006). Given the complex nature of CSE, the compound responsible for this action was not specified. Thus, our findings strongly suggest that cyanate promotes or at least may contribute to such an effect.

Decreased production or activity of NO, manifested as impaired endothelium-dependent vasodilation, is one of the earliest signs of atherosclerosis (Ludmer et al. 1986, Davignon, Ganz 2004). Impaired production or activity of NO has been implicated in various events that promote atherosclerosis, such as vasoconstriction, platelet aggregation, proliferation and migration of smooth muscle cells, and leukocyte adhesion (Palmer, Ferrige & Moncada 1987, Endres et al. 1998).

Cyanate treatment markedly decreased eNOS protein expression in aortas of mice and HCAEC, which was paralleled by a decrease in eNOS phosphorylation at S1177, a crucial post-translational modification regulating eNOS activity (Huang 2009). Additionally, a decrease in eNOS dimer levels was observed which may also contribute to the reduced availability of NO, since eNOS requires proper dimerization for full enzymatic activity (Forstermann, Munzel 2006). It has been shown that at the dimer interface of the enzyme, within the zinc-thiolate center, zinc ion is tetrahedrally coordinated to pairs of

symmetrically oriented and phylogenetically conserved cysteine residues Cys($_{\times 4}$)Cys (Raman et al. 1998), which may pose as possible targets for cyanate. Mutation within the Cys($_{\times 4}$)Cys motif has been shown to disrupt eNOS dimers, consequently eliminating eNOS activity, suggesting that stabilization of the dimer interface by the zinc-thiolate center is key for enzymatic activity (Scheele et al. 2001).

My results indicate that cyanate, as a major constituent of tobacco smoke, contributes to the impaired endothelium-dependent vasodilation and decreased NO bioavailability in smokers. This is supported by the fact that smokers have higher blood leukocyte count than nonsmokers (Nieto et al. 1992, Schwartz, Weiss 1991) in particular granulocytes (Smith et al. 2003). Moreover, neutrophils from smokers express more MPO than those of non-smokers (van Eeden, Hogg 2000). Therefore, it can be concluded that MPO-catalyzed oxidation of thiocyanate in smokers may augment the cyanate pool *in vivo* (Holzer et al. 2012, Wang et al. 2007, Arlandson et al. 2001). This may be of particular interest as thiocyanate levels are significantly elevated in smokers (Husgafvel-Pursiainen et al. 1987, Morgan et al. 2011). In this respect, a recent study suggested a predictive value of MPO levels for major adverse cardiovascular events in smoking patients with a history of peripheral artery disease (Haslacher et al. 2012).

In the context of uremia, endogenously formed cyanate may be of particular relevance. Cyanate is a decomposition product of urea, and renal patients have been reported to have high urea levels (up to 110 mM) (Bell et al. 1991, Blackmore, Elder & Bowden 1963). In hemodialysis patients with high-grade persistent inflammation, increased MPO activity was demonstrated (Rodriguez-Ayala et al. 2005) and most recently MPO-derived chlorinating species have been demonstrated to rapidly decompose urea resulting in cyanate formation (Holzer et al. 2012). Chronic renal disease dramatically increases cardiovascular risk, and causes endothelial dysfunction as reflected by altered endothelium-dependent vasodilation resulting from reduced vascular bioavailability of NO (Passauer et al. 2005).

It is noteworthy to signify that the localization of phagocytes in close vicinity to endothelial cells at sites of inflammation may contribute to cyanate-induced endothelial dysfunction, as it was previously shown that MPO-containing neutrophils are markedly enriched with carbamylated proteins (Kraus et al. 1994). In addition, the high carbamyllysine content of HDL in human atherosclerotic lesions correlated with the MPO-

specific oxidation product 3-chlorotyrosine, which strongly supports the notion that macrophage-associated MPO generates cyanate in significant amounts (Holzer et al. 2011b). Moreover, as demonstrated in PART I; cyanate - through inducing endothelial ICAM-1 expression - enhances endothelial-leukocyte interaction (El-Gamal et al. 2012), thus a vicious cycle may be formed resulting in locally higher cyanate levels generated by leukocyte-derived MPO. NO itself was shown to inhibit the expression of ICAM-1, thereby reducing leukocyte adhesion and transmigration (Biffl et al. 1996). Another study reported eNOS gene deficiency to result in increased leukocyte-endothelial interactions (Lefer et al. 1999). This strongly links the detrimental negative effects of cyanate at amplifying endothelial dysfunction (Karlsson et al. 2005, Kamath et al. 1999).

The impact of protein carbamylation on structure and/or function of proteins, enzymes, and hormones has been evaluated in numerous studies (Kraus, Kraus 2001, Mun, Golper 2000, Jaisson et al. 2007). In my experiments with cultured endothelial cells, cyanate induced significant alterations in eNOS protein expression. Interestingly, this reduction was not observed when low molecular weight substances (i.e. cyanate) were removed, thereby indicating that the decrease in eNOS is exclusively owed to cyanate, independent from carbamylated proteins.

It is tempting to speculate how cyanate exerts such a negative effect on eNOS. A possible mechanism may be through cyanate-induced modifications of phosphatidylethanolamine, a phospholipid which has been suggested to play an important role in modulating eNOS activity (Ohashi et al. 1993). This might be of significance, as it was previously shown that cyanate targets phosphatidylethanolamine (Holzer et al. 2011b). Furthermore, it was shown that cyanate reacts with α-amino groups of free amino acids, hence interfering with protein synthesis (Kraus, Jones & Kraus 1998), wherein the derivatized α-amino group blocks peptide bond formation. This could explain the reduction in eNOS protein observed with cyanate exposure.

Intriguingly, it has been recently shown that carbon monoxide in combustion smoke acts via hypoxia to alter energy metabolism and induce cell damage (Lee et al. 2010). This is of particular interest as cyanate has been long known to target hemoglobin by irreversibly carbamylating the amino terminal of valine consequently increasing hemoglobin's affinity to oxygen (CERAMI et al. 1973). In spite of this enhanced oxygen uptake, oxygen release at tissues was reported to be limited due to a reduction in the capillary-to-tissue partial

oxygen pressure required to unload oxygen. This resulted in “hypoxia-like” effects as erythrocytosis and pulmonary hypertension in rats upon chronic cyanate administration (McCanse et al. 1999, Teisseire et al. 1986). Additionally, hypoxia and erythropoietin have been shown to depress eNOS expression in human endothelial cells (McQuillan et al. 1994). Such findings may provide another putative mechanism behind the impairment of endothelium-dependent vasodilatation upon cyanate exposure.

In conclusion, the cyanate-induced effects observed throughout the course of my study are compatible with the overall reduction of eNOS-derived NO bioavailability and amplified inflammation observed in both smokers and uremic patients. These novel findings provide further insight into the underlying mechanisms contributing to the enhanced cardiovascular risk associated with smoking and uremia. Hence, cyanate may be an important factor promoting endothelial dysfunction, thereby linking inflammation, uremia and smoking.

All in all, our findings highlight the profound negative effect(s) of cyanate on endothelial function recommending that it be recognized as a potential harmful substance. This may be of-particular importance, as it has not yet been included in the FDA‘s *“Established List of Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke”* (U.S., Food and Drug Administration (FDA) 2012).

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PUBLICATIONS

ANTIOXIDANTS & REDOX SIGNALING
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ORIGINAL RESEARCH COMMUNICATION

Cyanate Is a Novel Inducer of Endothelial ICAM-1 Expression

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Abstract

Aim: Recent work has shown that humans are significantly exposed to isocyanic acid/cyanate, which is generated when coal, biomass, or tobacco is burned. *In vivo*, cyanate is formed by the phagocyte protein myeloperoxidase and by breakdown of urea. Carbamylation of proteins through cyanate has been demonstrated to predict cardiovascular risk and is thought to promote vascular dysfunction; however, the underlying mechanisms remain unclear. ***Results:*** Here, we show that cyanate induces intercellular cell adhesion molecule-1 (ICAM-1) expression with subsequently enhanced neutrophil adhesion in human coronary artery endothelial cells. Cyanate triggers ICAM-1 expression through a mechanism depending on activation of the mitogen-activated protein kinase p38 and nuclear factor-kappaB. Endothelial ICAM-1 expression was not induced when low-molecular-weight substances were removed from cell culture medium, thus ruling out a role of carbamylated (lipo)proteins in ICAM-1 induction. In mice, oral administration of cyanate induced marked endothelial ICAM-1 expression in the aorta. Moreover, in patients with end-stage renal disease, the extent of plasma protein carbamylation (a marker for cyanate exposure) significantly correlated with plasma levels of soluble ICAM-1. ***Innovation:*** Here, we demonstrate for the first time that cyanate, rather than carbamylated lipoproteins, induces vascular ICAM-1 expression *in vivo*. ***Conclusion:*** Collectively, our data raise the possibility that cyanate amplifies vascular inflammation, linking inflammation, smoking, and uremia. *Antioxid. Redox Signal*. 16, 129–137.

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ORIGINAL RESEARCH COMMUNICATION

Myeloperoxidase-Derived Chlorinating Species Induce Protein Carbamylation Through Decomposition of Thiocyanate and Urea: Novel Pathways Generating Dysfunctional High-Density Lipoprotein

Michael Holzer,[1] Klaus Zangger,[2] Dalia El-Gamal,[1] Veronika Binder,[1] Sanja Curcic,[1] Viktoria Konya,[1] Rufina Schuligoi,[1] Akos Heinemann,[1] and Gunther Marsche[1]

BASIC RESEARCH www.jasn.org

Uremia Alters HDL Composition and Function

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Psoriasis alters HDL composition and cholesterol efflux capacity

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Running title: Psoriasis alters HDL composition and function

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2010	Poster Prize at the 2nd Joint ZMF and Doctoral Days Medical University of Graz, Austria
2009	Best assistant lecturer, Cairo University, Egypt
2007	Best teaching assistant, Cairo University, Egypt
2002	Dean's award for top 5 distinguished honor students Faculty of Pharmacy, Cairo University
2002	Amoun Pharmaceuticals' award for top 5 distinguished honor pharmacy graduate students, Egypt
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Publications

Holzer M, Wolf P, Curcic S, Birner-Gruenberger R, Weger W, Inzinger M, El-Gamal D, Wadsack C, Heinemann A, Marsche G. Psoriasis alters HDL composition and cholesterol efflux capacity. J Lipid Res. 2012 Aug; 53(8):1618-24. PMID: 22649206.

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El-Gamal D, Holzer M, Gauster M, Schicho R, Binder V, Konya V, Wadsack C, Schuligoi R, Heinemann A, Marsche G. Cyanate is a novel inducer of endothelial intercellular cell adhesion molecule-1 (ICAM-1) expression. Presented at the 3rd Doctoral day; Nov, 2011; Graz, Austria. 2011. [Poster]

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Conferences

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17th Scientific Symposium of the Austrian Pharmacological Society (APHAR). Joint meeting with the Hungarian Society for Experimental and Clinical Pharmacology (MFT). Innsbruck, Austria. 29 – 30 Sept., 2011. [Oral Communication]

16th Scientific Symposium of APHAR. Vienna, Austria. 26-27 Nov., 2010.

2nd "Joint ZMF and Doctoral Days" Medical University of Graz 'PhD Program Molecular Medicine'. Graz, Austria. 5 – 6 Nov., 2010. [Poster]

15th Scientific Symposium of APHAR. Joint meeting with the Hungarian Society for Experimental and Clinical Pharmacology (MFT) and the Slovenian Pharmacological Society (SDF). Graz, Austria. 19 – 21 Nov., 2009

1st "Joint ZMF & Doctoral Days" Medical University of Graz 'PhD Program Molecular Medicine'. Graz, Austria. 5 - 6 Nov., 2009

Annual Scientific Conference of the Egyptian Society of Pharmacology and Experimental Therapeutics. Egypt. 2 – 3 May, 2007

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