Master Thesis

Role of Seladin-1 and Sirtuin-1 in Cyanate-Induced ICAM-1 Expression

Submitted by

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Summary

Chronic inflammation upon endothelial dysfunction, caused by hypertension, smoking, hyperlipidemia or diabetes, can lead to atherosclerosis. Transendothelial trafficking of leukocytes is the initial step of the inflammatory process. Intercellular adhesion molecule-1 (ICAM-1) expressed on endothelial cells has been shown to play a crucial role in leukocyte-endothelial interactions. Cyanate, a reactive molecule, generated by breakdown of urea in the human body and by combustion of biomass, coal and tobacco, is pro-inflammatory. Cyanate is sought to have a causal role in vascular inflammation, but the underlying mechanisms remain elusive. Recent evidence indicated that cyanate induces endothelial ICAM-1 expression via p38 MAPK and NF-κB–signaling pathways and enhances neutrophil adhesion in endothelial cells.

Putative underlying mechanisms of cyanate-induced ICAM-1 expression were further investigated in this thesis. In an attempt to find a possible contribution of seladin-1 in ICAM-1 expression, siRNA knock down approach was used.

Seladin-1, an enzyme of the human cholesterol biosynthesis, is known to have anti-inflammatory effects and was shown to down regulate adhesion molecule expression in endothelial cells. In the present study we observed that upon cyanate treatment, expression of seladin-1 in endothelial cells was markedly decreased in a concentration-dependent manner. Surprisingly knock down of seladin-1 elicited no increase in ICAM-1 expression, which only became significant upon cyanate treatment, indicating that reduction of seladin-1 protein expression is not involved in cyanate-induced ICAM-1 expression.

In the second part of the thesis, we investigated the role of sirtuin-1 (Sirt1) in cyanate-induced ICAM-1 expression. Sirt1 is a histone deacetylase that inhibits NF-κB expression and is thought to have anti-inflammatory properties. Sirt1 was shown to recognize carbamyllysine residues which are reaction products of reactive cyanate with lysine groups of proteins, but in contrast to the structurally related acetyllysine residues, carbamyllysine residues are weak substrates an may therefore reduce Sirt1 activity. Upon pharmacological inhibition of Sirt1, ICAM-1 expression was significantly increased in endothelial cells. In combination with cyanate an additional additive

increase of ICAM-1 expression was observed, indicating that cyanate-induced ICAM-1 expression is not mediated by Sirt1.

In summary, we observed that neither seladin-1 nor Sirt1 mediate cyanate-induced ICAM-1 expression. Further studies are required to identify the underlying mechanisms for cyanate-induced endothelial activation.

Zusammenfassung

Endotheliale Dysfunktion, bedingt durch Hypertonie, Rauchen, Hyperlipidämie oder Diabetes, kann zu chronischen Entzündungen und Arteriosklerose führen. Durch transendotheliale Migration von Leukozyten wird der Entzündungsprozess initiiert. Intrazelluläres Adhäsions-Molekül 1 (ICAM-1) spielt dabei eine zentrale Rolle. Bei Nierenerkrankungen führen gesteigerten Harnstoff Spiegel zu einer hohen Konzentration von reaktivem Zyanat, das spontan aus Harnstoff gebildet wird. Neuere Studien haben gezeigt, dass Zyanat auch bei Verbrennung von Biomasse, Kohle und Tabak entsteht. Zyanat ist ein Nukleophil, das irreversibel mit Aminosäuren oder mit Lysinresten innerhalb eines Proteins reagiert und zu Proteincarbamylierungen führt. Zyanat kann aber auch über einen anderen Mechanismus direkt bei entzündlichen Erkrankungen gebildet werden. Dabei wird durch das Enzym Myeloperoxidase (MPO) aus Thiozyanat und H_2O_2 Zyanat gebildet, was zu Proteincarbamylierungen im entzündlichen Gewebe führt. Vorangegangene Arbeiten haben gezeigt, dass Zyanat Endothelzellen aktivieren kann. Zyanat induziert die Expression des Ahäsions-Moleküles ICAM-1 über die p38 MAPK und NF-κB Signaltransduktion.

In dieser Arbeit wurden grundlegende Mechanismen untersucht, warum Zyanat eine deutlich gesteigerten ICAM-1 Expression in Endothelzellen induziert. In dieser Arbeit wurde untersucht, ob Zyanat zu einer Abnahme von Seladin-1 Expression in endothelialen Zellen führt. Seladin-1 ist ein Enzym der humanen Cholesterol-Biosynthese und hat antiinflammatorische Eigenschaften. In einigen Studien wurde gezeigt, dass eine erhöhte Expression von Seladin-1 die Expression von endothelialen Adhäsions-Molekülen deutlich reduzieren kann. In dieser Arbeit wurde zum ersten Mal gezeigt, dass Zyanat zu einer Abnahme von Seladin-1 Expression in endothelialen Zellen führt. Um die Rolle von Seladin-1 in der ICAM-1 Expression zu untersuchen, wurde die Seladin-1 Expression mittels siRNA- reduziert.

Interessanterweise führte der Seladin-1 Knockdown zu keinem Anstieg der endothelialen ICAM-1 Expression. Dies deutet darauf hin, dass Seladin-1 in der Zyanat-induzierten ICAM-1 Expression keine Rolle spielt.

Im zweiten Teil der vorliegenden Arbeit wurde untersucht, ob Zyanat Sirtuin-1 (Sirt-1), eine Histon Deacetylase, inaktiviert. Sirt-1 ist ein anti-inflammatorisches Enzym,

das die intrazellulären NF-κB Spiegel reduziert. Das eigentliche Substrat Acetyllysin weist eine starke strukturelle Ähnlichkeit mit Carbamyllysin auf, dass bei Reaktion von Zyanat mit Protein-Lysin Gruppen entsteht. Sirtuine binden an Carbamyllysin mit hoher Affinität, können es aber im Vergleich zu Acetyllysin nur sehr langsam umsetzen. Nun reduzieren Sirtuine NF-κB und senken dadurch möglicherweise die Adhäsionsmolekül Expression – ein potentieller Mechanismus, der erklären könnte, warum Zyanat (und durch Zyanat induzierte intrazellulare Carbamyllysinbildung) NF-κB Spiegel erhöht und zur Adhäsionsmolekülexpression führt. Wie erwartet, führte eine pharmakologische Inhibierung von Sirt1 zu einer deutlich erhöhten Expression von ICAM-1. Eine zusätzliche Behandlung der Zellen mit Zyanat erhöhte unabhängig davon die endotheliale ICAM-1 Expression. Woraus der Schluß gezogen werden kann, dass Sirt1 keine bedeutende Rolle bei der Zyanat-induzierte ICAM-1 Expression spielt

Zusammengefasst spielen weder Seladin-1 noch Sirt1 eine Rolle in der Zyanat-induzierter ICAM-1 Expression. Weitere Studien sind nötig, um die zugrundeliegenden Mechanismen zu identifizieren.

Index

1 Introduction

1.1 Endothelium

Endothelium is defined as the monolayer of endothelial cells at the inner surface of blood and lymphatic vessels. Endothelial cells can't be easily defined due to their heterogeneity in structure and function [1]. The adult endothelial cell surface accounts for approximately 1 to $6x10^{13}$ cells, covers 1 to 7 m^2 and weighs approximately 1 kg [2]. In every organ system the endothelium contributes to important physiological functions like blood cell trafficking, vessel permeability and hemostasis [1].

1.1.1 Endothelial Function

Many functions like cell permeability, hemostasis, angiogenesis, vascular tone, innate and acquired immunity and leukocyte trafficking are regulated by endothelial cells. Angiogenesis and leukocyte trafficking are processes that need activation of the endothelial layer, which includes the expression of certain endothelial adhesion molecules [3].

The endothelium regulates vasomotor tone via vasodilatator and vasoconstrictor substances [4]. Vasoactive substance, released from the endothelium upon humoral and mechanical stimuli include nitric oxide (NO), prostacyclin, endothelin and endothelin-derived hyperpolarizing factor. Those substances can affect function and structure of the underlying smooth muscle [5]. NO is released by endothelial NO-synthase (eNOS) and acts as a vasodilator. It also contributes to the inhibition of growth, inflammation and platelet-aggregation [6]. Endothelin acts as a vasoconstrictor [7] and stimulates cell proliferation, increasing expression of collagenase, prostaglandin endoperoxidase and platelet-derived growth factor. Vasoactive substances are regulating each other [8].

Endothelial cells and smooth muscle cells (SMC) express proteins, which are contributing to hemostasis. Those coagulation-related receptors, like tissue factor (TF) and circulating coagulation proteins, like thrombin, are strictly controlled, initiating coagulation response to vascular injury [9].

Angiogenesis, which is the development of new blood vessels from endothelial cells, is regulated by vascular endothlial growth factor (VEGF) [10]. VEGF is produced by endothelial cells upon activation of endothelium via tumor necrosis factor-α (TNF-α), angiogenin, interleukin 8 (IL-8) and the angiopoietins [11,12]. VEGF also plays a role in inflammation by stimulating the release of adhesion molecules [9].

The endothelium has important transport functions. It serves as a barrier for free passage of molecules and cells from the blood to the underlying interstitium and cells. For transport of essential circulating blood macromolecules through the endothelium into the subendothelium, specific mechanisms have been established. Additionally, the “tight” junctions between endothelial cells act as selective barriers for egressing molecules [13].

Leukocyte trafficking from blood to underlying tissue is mediated by a complex adhesion cascade, which includes initial attachment, rolling, arrest and transmigration through endothelial cells [1]. These steps are mediated by adhesion molecules, chemokines and integrins, as depicted in Figure 1 [14].

Transendothelial trafficking of leukocytes starts with the activation of endothelial cells by release of cytokines like TNF-α and interleukin 1 from macrophages [15]. Due to the release of these cytokines, nuclear factor-κB (NF-κB) transcription of E-selectin on endothelial surface is induced. Leukocytes interact with E-selectin via sialyl lewis^x like carbohydratic structures. L-Selectin is expressed on leukocytes and binds to endothelial CD34, P-selection glycoprotein ligand 1 (PSGL-1) and sialyl lewis^x. This process prolongs leukocyte-blood-vessel contact and increases contact of leukocytes with chemokines as monocyte chemotactic protein-1 (MCP-1), IL-8, RANTES and macrophage inflammatory protein1α/β (MIP-1α/β) (Step 1 in Figure 1) [16]. These chemokines induce activation of integrins on the leukocyte cell surface (lymphocyte function-associated antigen 1 “LFA-1” and very late antigen 4 “VLA-4”) and hence direct their migration along endothelial surface [17]. Activated integrins increase leukocyte adhesiveness to the endothelium and lead to an arrest (Step 2 in Figure 1). Allowing leukocytes to spread and slowly migrate over endothelial surface. Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) are expressed on the endothelium and generate firm adhesion of leukocytes via LFA-1/ ICAM-1 and VLA-4/ VCAM-1 onto the endothelium (Step 3 in Figure 1) [18–20]. This strong binding of leukocytes via ICAM-1 causes an increase of

intracellular Ca^{2+}, activation of p38 and Rho, while binding via VCAM-1 induces activation of rac1 [21]. These signaling molecules are believed to ease transmigration over contracting endothelial cells and deteriorating bonds of junctional adhesion molecules, between endothelial cells [22]. Studies demonstrated that ICAM-1 plays a crucial role in transendothelial leukocyte migration [18–20]. Leukocytes migrate through the endothelium into the subendothelial space in a paracellular or transcellular way, which is not completely understood yet (Step 4 in Figure 1) [21]. In paracellular transmigration, leukocytes squeeze through intercellular gaps between endothelial cells [23], whereas transcellular migration of leukocytes might be possible due to migratory cups on endothelial cells, rich in ICAM-1, VCAM-1 and cytoskeletal proteins [24]. It was thought that these projections lead to firm leukocyte arrest onto the endothelium and is now believed to instigate transcellular leukocyte trafficking [25,26].

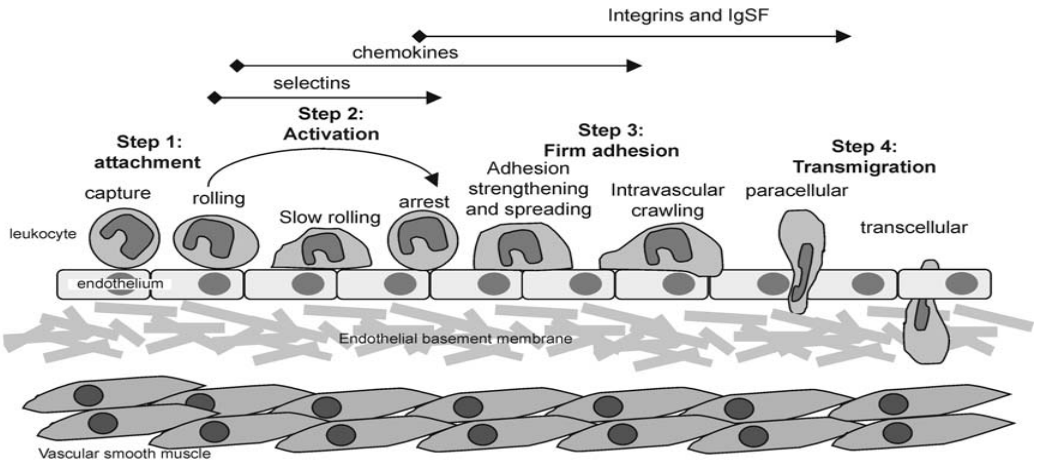

Figure 1: Leukocyte trafficking through the Endothelium. Illustrating the four major steps in the process of transendothelial leukocyte migration [14]

Within the subendothelial space leukocytes respond towards sources of inflammation, as the complement system is activated, and invaders are eliminated by granulocytes. Inflammation is critical to eliminate intruders and to renew tissue. Permanent stimuli via cytokines and chemokines are needed to sustain inflammation. As the half-life of these inflammatory mediators is limited, inflammatory response ends once stimuli have been removed [27].

1.1.2 Endothelial Dysfunction

Endothelial dysfunction is characterized by reduced vasodilatation, a proinflammatory and prothrombic state [28]. Multiple mechanisms, including decreased NO-bioavailability and upregulation of chemokines and adhesion molecules, are involved in the complex pathophysiology of endothelial dysfunction and can lead to an unregulated form of inflammation [27].

Endothelial dysfunction is thought to be a key event in the development of atherosclerosis.

1.2 Atherosclerosis

Atherosclerosis is defined as local thickenings of arteries intima or subendothelium. The thickening develops over time and might be asymptomatic until occlusion of the vessel or plaque rupture occurs, which can lead to cardiovascular diseases or sudden death. The progress of atherosclerosis is described as follows [29,30].

Upon endothelium dysfunction, ongoing release of pro-inflammatory cytokines leads to adhesion and transendothelial trafficking of leukocytes [31]. Monocytes, recruited by the chemoattractant monocyte chemotactic protein 1 (MCP-1), enter the subendothelium. Upon stimulation of macrophage colony stimulating factor (M-CSF), monocytes mature into macrophages [32,33]. Macrophages proliferate, sustain and amplify inflammatory responses by release of cytokines and growth factors. MCSF induces expression of scavenger receptors in macrophages, which mediate engulfing of modified (carbamylated or oxidized) lipoproteins, resulting in foam cells formation, which can rupture and lead to plaque formation. Foam cells release metalloproteinases (MMPs) and procoagulant tissue factor (TF) [34–36], as depicted in Figure 2, and migration of smooth muscle cells (SMCs) into the subendothelium. SMCs are producing collagen and hence stabilize plaque fibrous cap [37]. Additionally to monocytes, T-lymphocytes play critically role in inflammation leading to atherosclerosis and further on thrombosis. T-lymphocytes, present in the subendothlium, are activated via interferon-γ (IFN- γ)-inducible chemokines [38–40]. Upon activation, T-lymphocytes produce pro-inflammatory cytokines, which induce foam cells to produce MMP and TF [41,42]. Collagen is the key extracellular matrix molecule providing stability of the fibrous cap. Collagen production by the SMCs is

inhibited by release of IFN-γ from T-lymphocytes and indirectly via the induction of MMP, which can destroy arterial extracellular matrix [41,43]. TF leads to coagulation of platelets, which boosts the thrombogenicity of the plaque's lipid core [31].

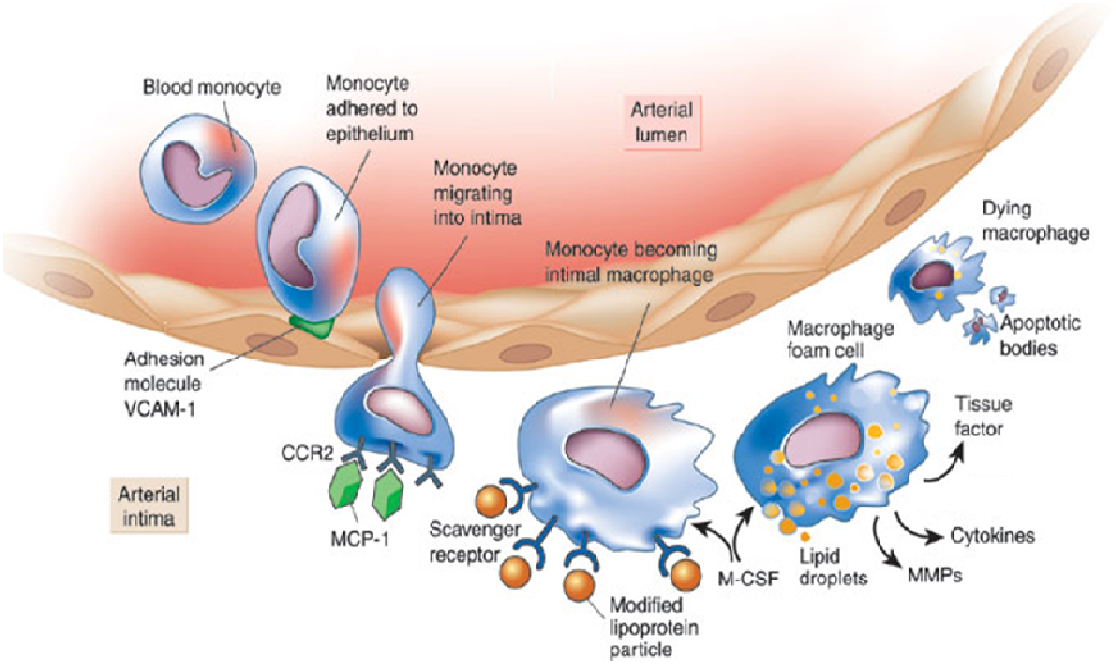

Figure 2: Mononuclear Phagocytes in Atherogenesis. Figure 2 shows initiating steps of atherosclerosis, including adhesion of monocytes onto activated/ dysfunctional endothelium, transendothelial migration and differentiation into macrophages. Macrophages express scavenger receptors and engulf modified lipoproteins, differentiating them into foam cells, which can release cytokines, MMPs and tissue factor. Foam cells might die and rupture. VCAM-1: vascular cell adhesion molecule 1, CCR2: chemokine receptor 2, MCP-1: monocyte chemotactic protein 1, M-CSF: macrophage colony stimulating factor, MMP: metalloproteinase. Figure was taken and modified from Libby, P. (2002) [34]

In the development of atherosclerosis, inflammation is involved throughout the whole process, from initiation to final complication of thrombosis.

Atherosclerosis is the underlying process of cardiovascular diseases (coronary heart disease and cerebrovascular stroke) [43], which is the leading cause death worldwide [44].

1.3 Renal Disease and Cyanate

Renal diseases are characterized by impaired kidney function, in which the kidney fails to filter toxins and waste products from the blood. Retention of toxic substances can lead to increased oxidative stress; wherein uremic toxins (as urea, indole-3-acetic acid, erythreitol …… etc.) might harm endothelial cells [45–48].

In chronic renal disease patients on hemodialysis, cardiovascular disease is the principal cause for morbidity and death due to heart attack which is 100-fold increased in patients aged 45 or younger compared to the general population [49–51].

Cyanate is a reactive molecule which converts lysine irreversibly to ε-carbamyllysine, also known as homocitrulline (HCit) and therefore leads to post-translational carbamylation of proteins [52,53]. Functional impairment of (lipo)proteins through cyanate-induced carbamylation is also a source for vascular endothelial dysfunction and hence a critical factor for atherosclerosis [52], which is prevalent in renal disease [54].

Carbamylation and its modifying role on proteins have been extensively assessed within the field of uremia research. Carbamylation has been identified as a side-effect during denaturation-renaturation studies of proteins with urea, where changes in protein properties as weight, iso-electric point and activity was noted [55–57]. In chronic renal patients, urea levels reach up to 110 mM, resulting in cyanate concentration of about 1 mM [58,59] since 0.8 % of urea decomposes to cyanate *in vivo* [60].

Recent findings show that cyanate is also a major product of phagocyte protein myeloperoxidase (MPO), which catalyzes oxidation of SCN^- in the presence of hydrogen peroxide into cyanate. SCN^- levels are elevated in the plasma of smokers [52] and MPO activity is increased in hemodialysis patients [61]. MPO selectively carbamylates high-density lipoprotein (HDL) rendering it dysfunctional [62]. Uremic low-density lipoprotein (LDL), carbamylated LDL and carbamylated HDL have pro-atherosclerotic effects and were shown to induce endothelial cell apoptosis [52,55]. In atherosclerotic lesions a correlation between high carbamylated HDL and the MPO-specific oxidation product 3-chlorotyrosine was shown. Therefore it can be assumed that MPO is generating high amounts of cyanate sites of inflammation [62]. Furthermore, it was shown that MPO, released from activated neutrophils, associates

with endothelial cells and accumulates in subendothelial matrix of vascular tissue [63]. This leads to the hypothesis that local high concentrations of cyanate are present and may have an impact on vascular endothelial cells.

An important exogenous source for isocyanic acid/ cyanate is smoke, generated from combustion of coal, biomass or tobacco. Those sources lead to protein carbamylation at physiologically relevant levels [64].

A recent study has demonstrated that cyanate induces endothelial ICAM-1 expression via p38 mitogen-activated protein kinase (MAPK) - and NF-κB – signaling pathways (Figure 3) promoting increased adhesion of neutrophils. Neutrophils contain large amounts of MPO that may induce further cyanate production generating a vicious circle [65].

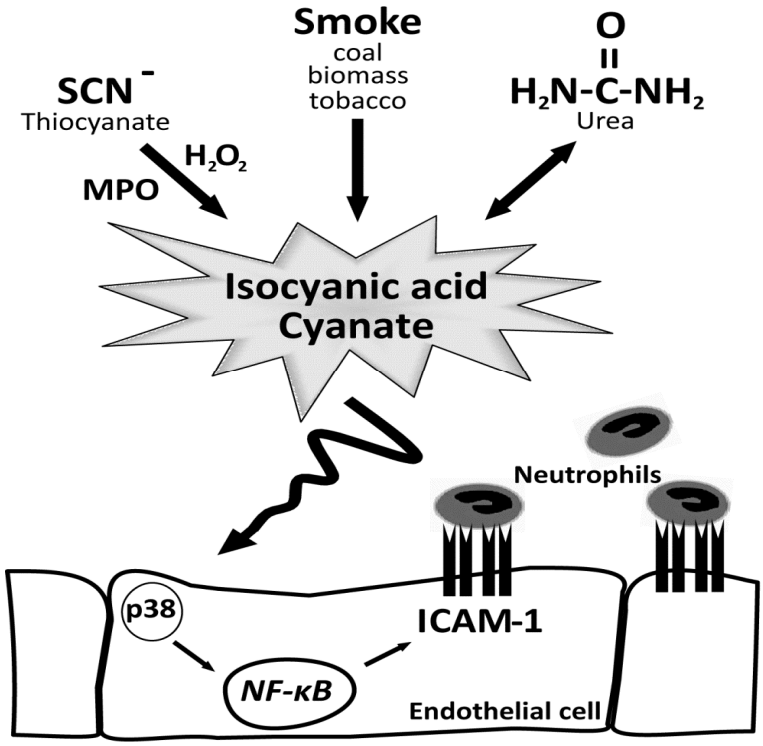

Figure 3: Generation of cyanate and its impact on endothelia ICAM-1 expression. kindly provided by Dalia El-Gamal, MSc (Institute of Experimental and Clinical Pharmacology, Medical Uinversity of Graz)

These latest findings may provide more knowledge of the underlying mechanisms contributing to prevalent cardiovascular disease associated with smoking, inflammation and renal disease.

1.4 Seladin-1

3β-hydroxysteroid-24 reductase is also known as seladin-1. Seladin-1, encoded by the *Dhcr*24 gene, is a flavine adenine dinucleotide-dependent oxidoreductase [66], which catalyzes the last step in the human cholesterol-biosynthesis from desmosterol to cholesterol [67].

Seladin-1 protects cells from apoptosis and controls cell growth and senescence via p53-interaction [68,69]. This anti-apoptotic effect of seladin-1 might be due to its scavanger capacity for hydrogen peroxide [70].

Seladin-1 levels are decreased in Alzheimer's disease [71] and adrenal cancer [72].

Latest research indicated that seladin-1 has anti-inflammatory activities. Knock down of seladin-1 in human coronary artery endothelial cells (HCAEC) was shown to induce adhesion molecule expression [73].

1.5 Sirtuin-1

Sirtuins have been identified to be associated with cell-responses regarding, aging, senescence and apoptosis [74] and are thought to contribute to longevity [75].

Human silent information regulator-two 1 (Sirt1) is the closest orthologue for yeast Sir2, which is an NAD^+-dependent class III histone deacetylase [76–78]. It was shown that Sirt1 regulates transcription of eNOS and p65 subunit of NF-κB. Subsequently, Sirt1 is playing an important role in cellular processes responding to oxidative stress, cell senescence and mitochondrial function [78-80]. Overexpression of Sirt1 in rodent endothelial cells reduces atherosclerotic plaque formation [82] and pharmacological activation of Sirt1 progresses glucose homeostasis in both human and mice [83].

Sirt1 levels are decreased in patient with chronic inflammatory diseases and post-translational modifications render Sirt1 dysfunctional. Lung epithelial cells, which were exposed to hydrogen peroxide and cigarette smoke extract, showed a dose-dependent and time-dependent decrease in protein expression and enzymatic activity of Sirt1 [84]. Since cyanate is a constituent of smoke [64], it is likely that cyanate contributes to inhibitory activity of smoke extracts.

Sirt1 was shown to recognize carbamyllysine residues which are reaction products of reactive cyanate with lysine groups of proteins, but in contrast to the structurally related acetyllysine residues, carbamyllysine residues are weak substrates an may therefore reduce Sirt1 activity [85].

2 Aim

As described above endothelial dysfunction, induced by smoking and renal disorder, can lead to chronic inflammation and may result in cardiovascular disease.

Our group previously showed that cyanate induces endothelial ICAM-1 expression, which is a critical step in leukocyte trafficking, via the p38-MAPK and NF-κB-pathway. The underlying mechanisms remain unclear.

The known anti-inflammatory effects of the enzymes seladin-1 and Sirt1 led us to the hypothesis that cyanate-induced ICAM-1 expression may be linked to a cyanate-induced inactivation of seladin-1 and/or Sirt1. The aim of this master thesis was to investigate the putative role of seladin-1 and Sirt1 in cyanate-induced ICAM-1 expression.

3 Materials

3.1 Equipment

Cell Culture Flask 250 ml, 75 cm^2	PAA Laboratories GmbH, Linz, Austria
Criterion Blotter	Bio-Rad, Wien, Austria
Criterion Blotten Filter Paper	Bio-Rad, Wien, Austria
FACS Calibur	BD Biosciences, Bredford, MA, USA
MTS4 Shaker	IKA Labortechnik, Staufen, Germany
Magnetic Stirrer	IKA Labortechnik, Staufen, Germany
Novex 4-20% Tris-Glycine Gel	Invitrogen, Karlsruhe, Germany
pH Meter Orion 3 Star	ThermoFisherScientific,Waltham, MA,USA
PROTRAN Nitrocellulose Transfer Membrane	Whatman GmbH, Dassel, Germany
Thermomixer comfort	Eppendorf Austria GmbH,Wien,Austria
Tissue Culture Flask 250 ml, 75 cm^2	Greiner, Frickenhausen, Germany
Vacuum Pump Unit	Vacuubrandt GMBH & Co KG
Water Jacketed CO2 Incubator	ThermoFisherScientific, Waltham, MA,USA
XCell SureLock™ Mini-Cell Electrophoresis System	Invitrogen, Karlsruhe, Germany
xMark Microplate Spectrophotometer	Bio-Rad, Wien, Austria
X-ray film D19 Devloper	Kodak, Rochester, NY, USA
48-well plates	Greiner, Frickenhausen, Germany
6-well plates	Greiner, Frickenhausen, Germany

3.2 Cell culture

EA.hy926	ATCC, Manassas, VA, USA
Human Coronary Artery Endothelial Cells (HCAEC)	Lonza, Verviers, Belgium

3.3 Chemicals

Antibody diluent	DAKO, Vancouver, Canada
Amphotericin B	PAA Laboratories GmbH, Linz, Austria
BD Cell Fix	BD Biosciences, San Jose, CA, USA
BD FACS Flow	BD Biosciences, San Jose, CA, USA
Bovine Serum Albumin (BSA)	Sigma-Aldrich, St. Louis, MO, USA
Bromophenol Blue	Sigma-Aldrich, St. Louis, MO, USA
$CaCl_2$	Merck, Darmstadt, Germany
Dimethylsulfoxid (DMSO)	Carl Roth GMBH & Co KG,Karlsruhe, Germany
DMEM Dulbecco's Modified Eagles's Medium	PAA Laboratories GmbH, Linz, Austria
Dulbecco´s PBS 1x liquid - $CaCl_2$, Mg_2	PAA Laboratories GmbH, Linz, Austria
EBM2 Endothelial Basal Medium-2	Lonza, Verviers, Belgium
EDTA	Carl Roth GMBH & Co KG, Karlsruhe, Germany
EGM2 Bullet Kit	Lonza, Verviers, Belgium
Fetal Bovine Serum (FBS)	PAA Laboratories GmbH, Linz, Austria
Fetal Calf Serum (FCS)	Lonza, Verviers, Belgium
Formaldehyde 37%	Carl Roth GMBH & Co KG, Karlsruhe, Germany
Glycine	Carl Roth GMBH & Co KG, Karlsruhe, Germany

Glycerol	Sigma-Aldrich, St. Louis, MO, USA
Acetic Acid (HAc)	Carl Roth GMBH & Co KG, Karlsruhe, Germany
HAT	Sigma-Aldrich, St. Louis, MO, USA
Hank's Buffered Saline Solution (HBSS)	Lonza, Verviers, Belgium
HCl	Carl Roth GMBH & Co KG, Karlsruhe, Germany
Isopropanol	Sigma-Aldrich, St. Louis, MO, USA
HEPES Buffered Saline Solution	Lonza Verviers, Belgium
KCl	Merck, Darmstadt, Germany
Liopolysaccharide (LPS)	Sigma-Aldrich, St. Louis, MO, USA
Methanol	Sigma-Aldrich, St. Louis, MO, USA
Mercaptoethanol	Sigma-Aldrich, St. Louis, MO, USA
NaCl	Carl Roth GMBH & Co KG, Karlsruhe, Germany
NaN_3	Carl Roth GMBH & Co KG, Karlsruhe, Germany
$NaHCO_3$	Merck, (Darmstadt, Germany
NF-κB Activation Inhibitor IV	Merck, Darmstadt, Germany
Non-fat dried milk	Fixmilch Instant. Wien, Austria
Phosphate-buffered Saline (PBS)	PAA Laboratories GmbH
Penicillin/ Streptomycin	PAA Laboratories GmbH, Linz, Austria
Ponceau Red S	Sigma-Aldrich, St. Louis, MO, USA
Recombinant Human TNF-α	Peprotech, Wien, Austria
Sodiumdodecylsulfate (SDS)	Sigma-Aldrich, St. Louis, MO, USA
Sirtinol	Sigma-Aldrich, St. Louis, MO, USA
Sodium Cyanate	Sigma-Aldrich, St. Louis, MO, USA

Splitomicin	Sigma-Aldrich, St. Louis, MO, USA
Trypsin Neutralizing Solution (TNS)	Lonza, Verviers, Belgium
TRIS	Carl Roth GMBH & Co KG, Karlsruhe, Germany
Triton-X 100	Sigma-Aldrich, St. Louis, MO, USA
Trypsin/EDTA	PAA Laboratories GmbH, Linz, Austria
Tween-20	Sigma-Aldrich, St. Louis, MO, USA

3.4 Kits and Reagents

MTT Kit	Sigma-Aldrich, St. Louis, MO, USA
Developer &Replenisher (5x) Kodak GBX	Rochester, NY, USA
Fixer & Replenisher (5x) Kodak GBX	Rochester, NY, USA
Immobilon Western HRP Substrate Peroxide Solution	Millipore, Billerica, MA, USA
Immobilon Western HRP Substrate Luminol Reagent	Millipore, Billerica, MA, USA
PrimeFect™ siRNA Transfection Diluent	Lonza, Verviers, Belgium
PrimeFect™ siRNA Transfection Reagent	Lonza, Verviers, Belgium
Transfast™ Transfection Reagent	Promega, Madison, WI, USA
siDHCR24-447	Microsynth, Balgach, Switzerland
siDHCR24-1054	Microsynth, Balgach, Switzerland
control siRNA	Microsynth, Balgach, Switzerland

3.5 Antibodies

Alexa Fluor® 488 goat-anti-rabbit IgG	Invitrogen, Lofer, Austria
B-actin mouse mAb	Sigma-Aldrich, St. Louis, MO, USA
DHCR24/ Seladin-1 (C59D8)Rabbit mAb	New England Biolabs GmbH, Frankfurt, Germany

Goat-anti-mouse IgG	Thermo Fisher Scientific, Waltham, MA, USA
PE- Cy 5 Mouse Anti-Human CD54	BD Pharmingen, San Jose, CA, USA
PE Mouse Anti-Human CD54	BD Pharmingen, San Jose, CA, USA

4 Methods

4.1 Cultivation of Endothelial Cells

4.1.1 EA.hy926

The human umbilical cell line EA.hy926 is a fusion of primary human umbilical vein cells with a thioguanine-resistant clone of A549 (human lung carcinoma epithelial cells). Hybrid clones are selected in HAT medium which contains 100 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine. To obtain a stable cell growth, EA.hy926 cells are cultivated with complete DMEM in surface treated cell culture flasks (PAA Laboratories GmbH). Complete DMEM contains of Dulbecco's Modified Eagles's Medium with high glucose (4.5g/L, 25mM), sodium pyruvate (110 mg/L, 1 mM), and L-glutamine (584 mg/L, 4 mM) to which fetal bovine serum (FBS, 10 %), 1 % penicillin/ streptomycin, 0,5 % amphotericin and HAT (1x) were added. All listed chemicals, except HAT (Sigma-Aldrich), were purchased from PAA Laboratories GmbH.

Every two days medium was changed and cells were harvested after reaching 90 % confluence. The cells were used between passage 74 and 80.

For harvesting and re-culturing, the cells ware washed with warm phosphate-buffered saline (PBS, PAA Laboratories GmbH), trypsinized, collected and spinned down (250xg, 7 min). Resulting cell-pellet was resuspended in complete DMEM fur further use.

4.1.2 Human Coronary Artery Endothelial Cells

HCAECs were cultured in surface treated cell culture flasks (Greiner) with complete medium, consisting of EBM-2 medium to which EGM-2 MV Bullet kit (Lonza), which contained hEGF, hydrocortisone, GA-1000 (gentamicin, amphotericin-B), 25ml FBS, hFGF-B, R3-IGF-1 and ascorbic acid, was added.

Every two days medium was changed and cells were harvested after reaching 90 % confluence. The cells were used between passage four and nine.

For harvesting and re-culturing, the cells were washed with warm Hank's Buffered Saline Solution (HBSS, Lonza), followed by trypsinization. For neutralizing pre-

warmed Trypsin Neutralizing Solution (TNS, Lonza) was added. The cells were collected and spinned down (250xg, 7min). The resulting pellet was resuspended in complete medium for further use.

4.1.3 Storage

Into 1ml of ice-cold freezing solution (8 ml of complete medium, 1 ml FBS, 1 ml DMSO), $1x10^6$ cells were added and transferred into a cryovial. The cryovial was stored in ethanol at -70℃ for 24 h, next day transferred into liquid nitrogen tank for further storage.

For thawing up cells, complete media were pre-warmed in cell culture flask for 30 min in the incubator before cells from cryovial were seeded in.

4.2 Cyanate Treatment of Endothelial Cells to Induce ICAM-1 Expression

Endothelial cells were seeded into 48-well plate and treated with 1, 2, 5 or 10 mM sodium cyanate (Sigma-Aldrich) for 12 h or 24 h. Cyanate was dissolved in pre-warmed PBS (PAA Laboratories GmbH), and diluted in complete DMEM for EA.hy926 cells and EGM2-MV for HCAEC to reach the desired concentration. Cyanate-containing media were then added to the cells for 12 h or 24 h.

4.3 Effect of Pharmacological Inhibition or Activation of Sirt1 on ICAM-1 Expression

HCAECs were seeded into 48-well plate and pre-treated with 100 or 200 µM Sirt1-inhibitor (splitomicin) and 2 or 4 µM Sirt1-activator NF-κB activation inhibitor IV, which is a resveratrol derivative. Splitomicin and resveratrol-derivate were diluted in complete medium to reach desired concentration and added to HCAEC. An hour later, 2 mM cyanate was added for 24 h.

4.4 Flow Cytometric Detection of ICAM-1 Expression

Surface expression of ICAM-1 on endothelial cells was assessed using flow cytometry (FACS Calibur, BD Biosciences). Endothelial cells were washed with PBS for EA.hy926 or HBSS for HCAEC and then harvested using detachment buffer (100 ml PBS, 10 mM EDTA, 25 mM HEPES) collected in FACS tubes, spinned down (400xg, 5 min) and stained with 50 µl anti-CD54 PE or anti-CD54 PE-Cy5 from BD Pharmingen in a dilution of 1:40 at 4℃ for 30 min. After staining, cells were washed with 250 µl PBS, spinned down (400xg, 5 min) and fixed with 150 µl fix solution (30 ml FACS flow (BD Biosciences), 10 ml distilled water, 1 ml cell fix (BD Biosciences)). Fluorophor was excited using the blue laser (488 nm). PE-staining was assessed using FL2-channel (585/42 nm) and PE-Cy5-staining using FL3-channel (670 LP).

4.5 MTT Viability Assay

After EA.hy926 cells were treated with different concentrations of cyanate (5 mM and 10 mM) overnight or for 24 h, a MTT (3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) reduction assay was performed to asses viability of EA.hy926 cells. Cyanate-treated cells were incubated with MTT (5 mg/ml dissolved in complete DMEM) for 4 h at 37℃. Yellow MTT is reduced to a purple formazan only in the mitochondria of living cells. Quantification of formazan is therefore directly related to the number of viable cells. Cells were washed with PBS and lysed with lysis solution (25 ml Isopropanol, 0.04 M HCl) on a shaker MTS4 (IKA Labortechnik) for 10 min at 1400 RPM to dissolve the generated formazan. The absorbance of formazan was quantified at 560 nm using xMark Microplate Spectrophotometer (Bio-Rad).

4.6 Seladin-1 Silencing in EA.hy926 cells

To silence seladin-1 siDHCR24-447, siDHCR24-1054 and scrambled siRNA, serving as siControl, were purchased from Microsynth and used as described [86]. Transfection reagent from Promega was prepared 1 day prior to transfection according to manufactors protocol. Transfection mixture was prepared as follows: to 500 µL of transfection medium (incomplete DMEM + HAT) 1.5 µL siRNA (to reach end concentration of 60 pM) and 4 µl transfection reagent was added.

EA.hy926 cells were cultivated in 6-well plates until they reached 70 % confluence. This transfection mixture was vortexed and allowed to rest for 15 min to induce liposome formation. Liposomes were then added drop-wise to cells and the plate gently moved. After one hour of incubation at 37℃, an additional 500 µl of transfection medium was added to the cells, followed by an overnight incubation at 37℃. On the next morning, transfection medium was removed and replaced with fresh complete DMEM. Subsequently, the cells were harvested to assess silencing efficiency after 48 h and 72 h.

4.7 Seladin-1 Silencing in HCAEC

To silence Seladin-1 siDHCR24-447 and scrambled siRNA were used, as it has been described [86]. HCAECs were grown on 6-well plates or 48-well plates until they reached 50 % confluence.

Transfection mixture was prepared by mixing PrimeFect siRNA transfection diluent and PrimeFect siRNA transfection reagent (Lonza) in the ratio 1:50, flicked and incubated for 15 min at room temperature. Furthermore, siDHCR24-447 or scrambled siRNA was added to reach a final concentration of 50 nM and allowed to rest for another 15 min at room temperature. Meanwhile the cells were starved with EBM-2 medium in the absence of serum at 37℃. The transfe ction mixture was then added drop-wise to the cells. After 3 h of incubation at 37℃ EGM2-MV medium was added. Transfected cells were harvested 48 h and 72 h post-transfection to detect silencing efficiency and impact on ICAM-1 expression.

4.8 Cyanate Treatment of Seladin-1 Silenced Endothelial Cells

Seladin-1 silenced cells were treated with cyanate (5 mM for EA.hy926, 1 mM or 2 mM for HCAEC) for 24 h to induce ICAM-1 expression. 72h post-transfection cells were harvested using detachment buffer and analyzed by flow cytometry, as described above.

4.9 Western Blotting

EA.hy926 cells and HCAECs were harvested 48h or 72h post-transfection using a cell extraction buffer (0,1 % Triton-X 100, 150 mM NaCl, 1 mM $CaCl_2$, 25 mM KCl, 10 mM TRIS), to which protease inhibitor cocktail (Sigma-Aldrich) was freshly added. Sample buffer (0.1 M TRIS/HCl pH 6.8, 4 % SDS, 15 % glycerol, bromophenol blue, 5 % mercaptoethanol) was added in the ratio 1:2 to cell extraction buffer. Cell-suspension lysates were denaturated (5 min, 95℃) and either directly used or stored at -20℃. The Western blot was performed as described previously [87].

Preparation of buffers used during Western Blot is found at the end of Western blot description.

Cell lysates were separated via gel-electrophoresis in 4-20 %-Tris/Glycine Gels (Invitrogen). The gels were run constantly at 20 mA for 3 h to guarantee distinct separation of proteins. Whole proteins of the gel were transferred onto a nitrocellulose transfer membrane (Whatman GmbH) in a Criterion Blotter (Bio-Rad) using blot buffer with 200 mA for 3 h at 4℃. To ve rify successful blotting, proteins on the membrane were visualized with Ponceau red (0.1 % Ponceau Red S from Sigma-Aldrich and 1 % HAc from Carl Roth GmbH & Co KG). The membranes were rinsed with washing buffer and blocked with blocking buffer (5 % milk in washing buffer) for 30 min at room temperature. Subsequently, blots were incubated overnight with primary Seladin-1 (C59D8) rabbit mAb (1:500 in blocking buffer) or β-actin mouse mAb (1:4000 in blocking buffer) at 4℃. Following, secondary Ab-staining was performed for 2 h at room temperature using horseradish peroxidase conjugated goat-anti-rabbit IgG (1:10000 in blocking buffer) or goat-anti-mouse IgG (1:1000 in blocking buffer). Signals were detected using Immobilon Western HRP Substrate Peroxide Solution and Immobilon Western HRP Substrate Luminol Reagent (Millipore) in a 1:1 ratio and visualized by exposing the membrane to X-Ray film. The X-Ray film was scanned and analyzed using ImageJ software. The band-densities of seladin-1 were normalized to β-actin (loading control). Expression of seladin-1 in transfected cells was calculated relatively to the expression in non-transfected cells.

For stripping off bound antibodies, membranes were washed 5 times (10 min per wash) in 20 mM $NaHCO_3$ (pH 7.4), followed by 4 times washing in 100 mM glycine (pH 2.4), then twice in 20 mM $NaHCO_3$ (pH 7.4) and finally once in washing buffer.

The membrane was then incubated in blocking buffer for 30 min followed by incubation with primary antibody and secondary antibody as described above.

4.9.1 Western Blot Buffers

All buffers used during Western Blot were prepared in 10-fold concentration.

To prepare blot buffer 12.1 g TRIS, 30 g glycin, 1 g EDTA and 1 g NaN_3 was weighed in and dissolved in 1 L of distilled water. For use 200 ml blot buffer and 400 ml methanol were completed to 2 L with water.

SDS run buffer is composed of 30.3 g TRIS, 150.1 g glycine, 10 g SDS, which are dissolved in 1 L of distilled water. For use it has to be diluted 1:10.

Washing buffer contains 5 g Tween 20, 90 g NaCl, 100 ml 1M TRIs (pH 7.4) completed to 1 L with distilled water For use it has to be diluted 1:10.

4.10 Statistical analysis

Data are shown as mean of ± SEM for n observations unless stated otherwise. Statistical analysis was performed with Graph Pad Prism Version 4 using One-Way ANOVA with Newman-Keuls Multiple Comparison post-hoc test. Significance was accepted at $P<0.05$.

5 Results

5.1 Cyanate Induces ICAM-1 Expression and Decreases Seladin-1 Expression in EA.hy926 Cells

EA-hy926 cells were treated with increasing concentrations of cyanate for 24 h.

Flow cytometric analysis revealed that cyanate treatment leads to a dose-dependent increase in ICAM-1 surface expression (Figure 4A).

Western blot was used to detect seladin-1 protein expression. Densitometric quantification of bands revealed a cyanate-induced dose-dependent decrease in seladin-1 protein (Figure 4B, 4C).

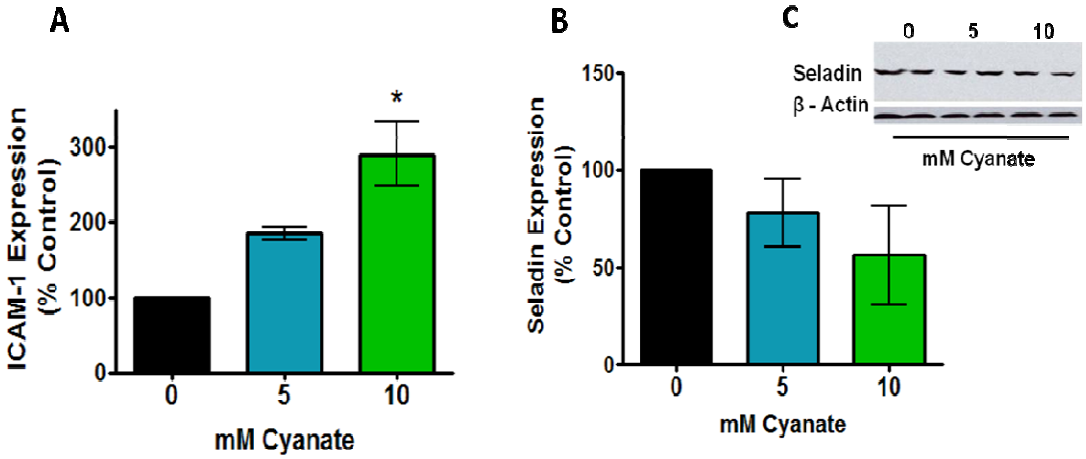

Figure 4: Cyanate Treatment Induces ICAM-1 Expression and Decreases Seladin-1 Expression in EA.hy926 Cells. Cells were cultured until confluence was reached, treated with 5 mM or 10 mM cyanate for 24 h and harvested for analysis. **(A)** Flow cytometric analysis of ICAM-1 expression in cyanate-treated cells, stained for ICAM-1 with mouse anti-human CD54. n=3, *P<0.05 compared to untreated cells. **(B)** Seladin-1 expression in cyanate-treated cells was quantified by Western blot (insert **C**), stained with primary rabbit anti-human C59D8 or primary mouse anti-human β-actin. Densitometric quantification was performed with ImageJ software. n=3.

5.2 Effect of Cyanate on Cell Viability of EA.hy926 cells

A MTT viability assay was performed to assess cell viability of cyanate-treated endothelial cells.

As shown in Figure 5, 12 to 24 h treatment with 5 mM cyanate did not affect the viability of EA.hy926 cells. Cells treated with 10 mM cyanate overnight showed a significant decrease in viability compared with untreated cells. Moreover, 24 h treatment with 10 mM of cyanate decreased viability to less than 75 %.

Accordingly, all following experiments in EA.hy926 cells were performed with a concentration of 5 mM cyanate.

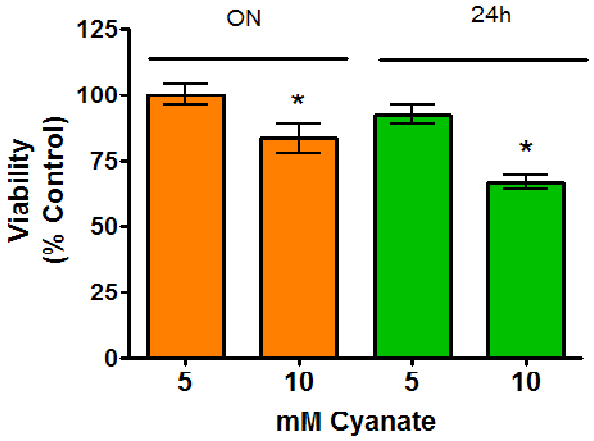

Figure 5: Effect of Cyanate on Cell Viability of EA.hy926. Cells were cultured in 48-well plates until confluence was reached and treated with 5 mM or 10 mM cyanate overnight or for 24 h followed by an incubation with MTT for 4 h at 37℃. n=4, *P<0.05 compared to untreated cells.

5.3 Seladin-1 Silencing in EA.hy926 Cells

Seladin-1 is known to play an anti-inflammatory role in endothelial cells [73]. As prior experiments indicate that cyanate leads to a decrease in seladin-1 expression, we hypothesized that seladin-1 plays a role in cyanate-induced ICAM-1 expression. To address this, siRNA approach was used to silence seladin-1 in EA.hy926 cells.

Figure 6 shows successful silencing of seladin-1 using a siRNA approach. Silencing seladin-1 with siDHCR24-447, siDHCR24-1054 was significant at both time points, but even more effective at 72 h. As siDHCR24-1054 showed an even stronger

decrease in seladin-1 expression, this siRNA was chosen to perform following experiments and will be further be referred to as siDHCR24.

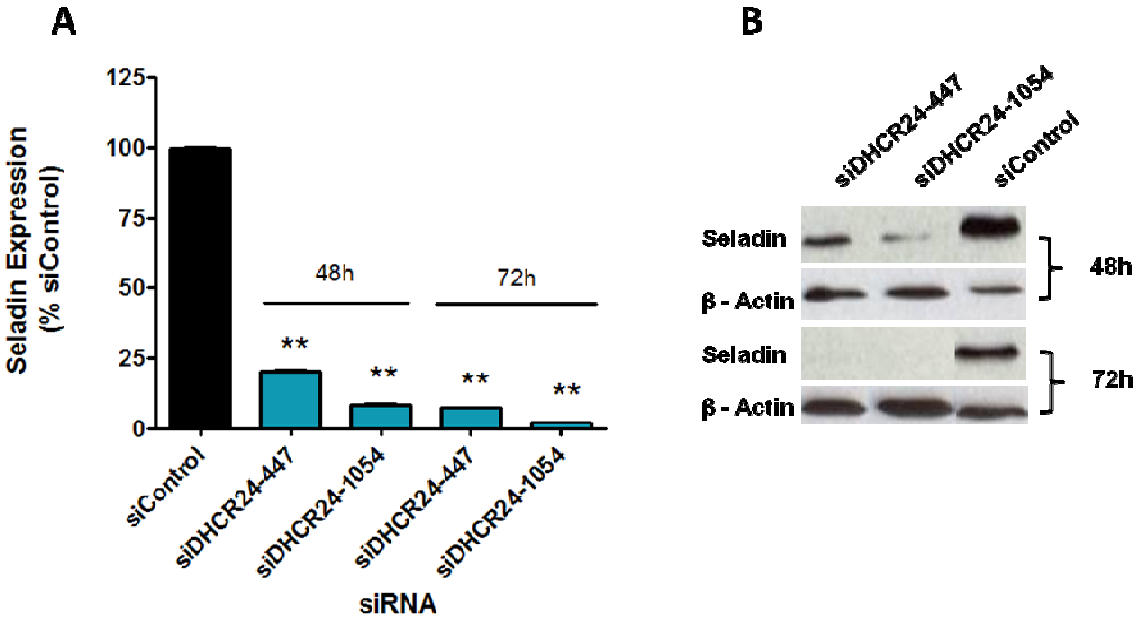

Figure 6: Down-regulation of Seladin-1 Expression in EA.hy926 Cells by siRNA Approach. EA.hy926 cells were cultured in 6-well plates until 70% confluence was reached and transfected with two different siRNAs specific for seladin-1 and scrambled siRNA as siControl for 48 h and 72 h. Cells were harvested for gel electrophoresis and Western blotting. Proteins were detected by staining with primary rabbit anti-human C59D8 or primary mouse anti-human β-actin, which served as a loading control. **(A)** Quantification of seladin-1 expression of **(B)** Western blot to detect seladin-1 expression in transfected cells. **P<0.001 compared to siControl.

5.4 Cyanate Treatment in Seladin-1 Silenced EA.hy926 Cells

To investigate the role of seladin-1 in cyanate induced ICAM-1 expression, EA.hy926 cells were transfected to silence seladin-1 and additionally treated with cyanate.

Figure 7A depicts that the knock down of seladin-1 does not significantly induce an increase in ICAM-1 expression compared to the siControl. In Figure 7B, it is seen that cyanate treatment leads to a significant increase of ICAM-1 expression in EA.hy926 cells in comparison to its untreated siControl. The increase of ICAM-1 expression in seladin-1 silenced EA.hy926 cells is significant upon cyanate treatment compared to untreated cells.

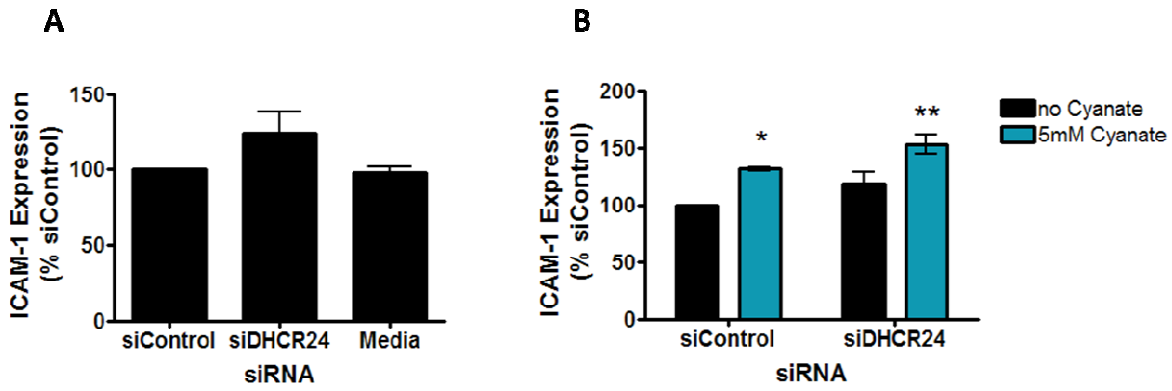

Figure 7: Effect of Cyanate on ICAM-1 Expression in Seladin-1 Silenced EA.hy926 Cells. Cells were cultured in 6-well plates till 70 % confluence was reached and transfected with siDHCR24. 48 h post-transfection 5 mM cyanate was added to the cells for 24 h, after which the cells were harvested and stained for ICAM-1 using mouse anti-human CD54. Flow cytometric analysis of **(A)** ICAM-1 expression in seladin-1 silenced cells. n=2, and of **(B)** ICAM-1 expression in seladin-1 silenced cells, treated with cyanate. n=3, *P<0.05 compared to untreated siControl, **P<0.05 compared to untreated siDHCR24.

The impact of cyanate on seladin-1 expression in seladin-1 silenced EA.hy926 cells was also investigated.

Figure 8 represents successful silencing of seladin-1 with siRNA approach. Silencing seladin-1 leads to a significant decrease in protein expression compared to its siControl (Figure 8A). Figures 8B and 8C show that cyanate treatment results in a significant decrease of seladin-1 expression in EA.hy926 cells compared to its untreated siControl. A slight decrease in seladin-1 expression is seen in cyanate-treated and seladin-1 silenced EA.hy926 cells compared to untreated equivalents.

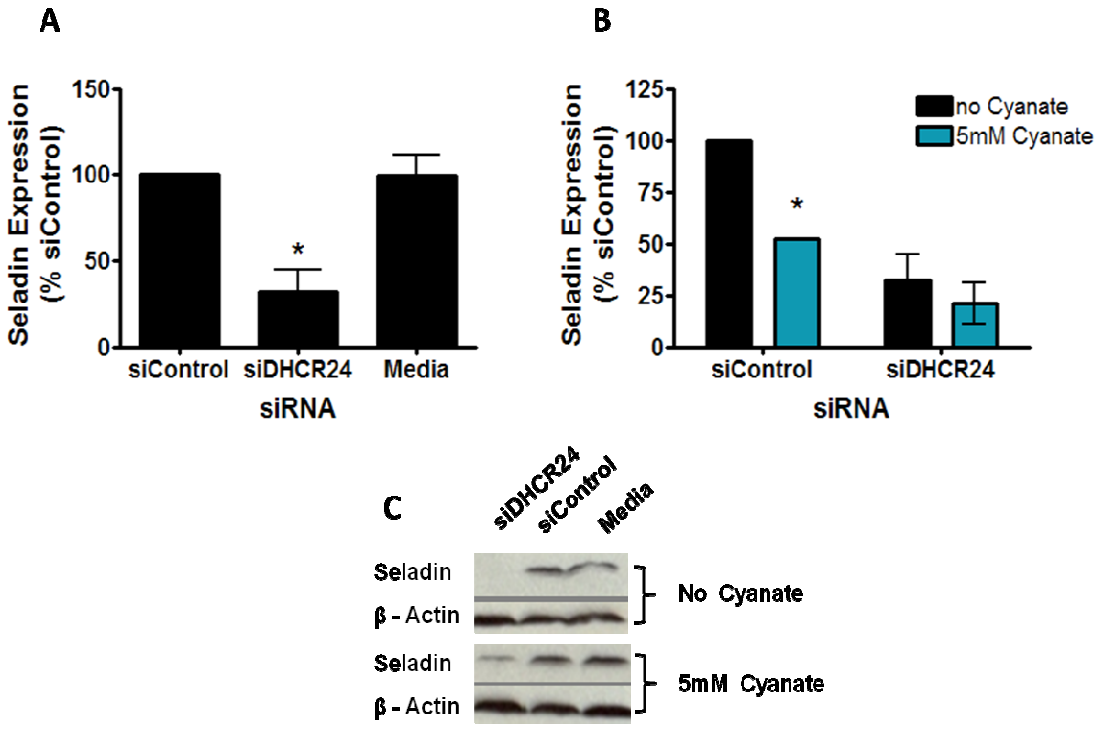

Figure 8: Effect of Cyanate on Seladin-1 Expression in Seladin-1 Silenced EA.hy926 Cells. Cells were cultured in 6-well plates until 70 % confluence was reached and transfected with siDHCR24. 48 h post-transfection 5 mM cyanate was added to the cells for 24 h, after which the cells were harvested and stained with primary rabbit anti-human C59D8 or primary mouse anti-human β-actin, which served as a loading control. Quantification for **(A)** seladin-1 expression of seladin-1 silenced EA.hy926. n=3, *P<0.05 compared to siControl, and for **(B)** seladin-1 expression in seladin-1 silenced cells, treated with cyanate. n=1-3, *P<0.05 compared to untreated siControl, of **(C)** Western Blot to detect seladin-1 expression in transfected cells.

5.5 Cyanate Induces ICAM-1 Expression and Decreases Seladin-1 Expression in Human Coronary Artery Endothelial Cells

Next we were interested whether similar results were obtained using primary human coronary aortic endothelial cells (HCAEC). HCAEC cells were cultured in 48-well plates and 6-well plates until confluence was reached and a 24 h treatment with increasing concentrations of cyanate was performed.

Flow cytometric detection showed that cyanate treatment lead to significant increase of ICAM-1 expression in 2 mM cyanate-treated cells (Figure 9A), indicating that primary endothelial cells are much more sensitive to cyanate treatment.

In line with results obtained with the EA-hy.926 cell line, cyanate treatment significantly decreased seladin-1 expression in a dose-dependent manner (Figure 9B, 9C).

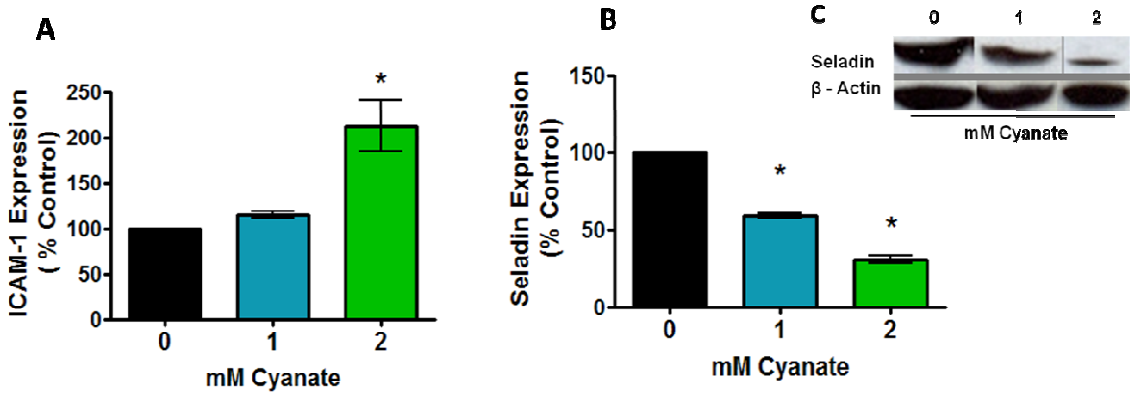

Figure 9: Cyanate Treatment Induces ICAM-1 Expression and Decreases Seladin-1 Expression in HCAEC. Cells were cultured in until confluence was reached, treated with 1 mM or 2 mM cyanate for 24 h and harvested. **(A)** Flow cytometric analysis of ICAM-1 expression in cyanate-treated cells, **(B)** Quantification of seladin-1 expression by **(C)** Western Blot. Picture quantification was performed using ImageJ software. n=2, *P<0.05 compared to untreated cells.

5.6 Seladin-1 Silencing in Human Coronary Artery Endothelial Cells

Figure 10 represents successful seladin-1 silencing using a siRNA approach. Silencing seladin-1 with siDHCR24 is significant at both time points. For following experiments 72 h was chosen.

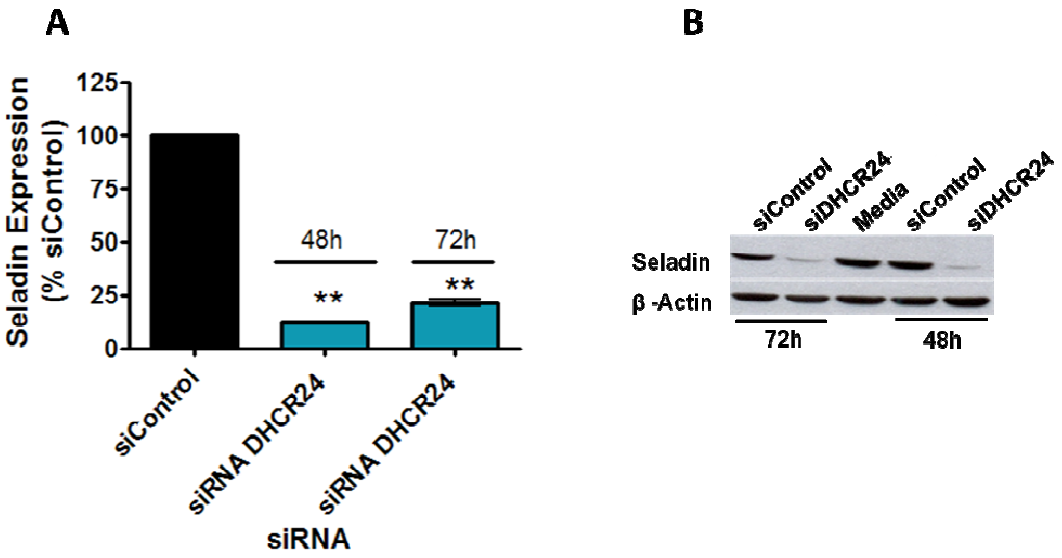

Figure 10: Down-Regulation of Seladin-1 Expression in HCAEC using siRNA Approach. HCAEC cells were cultured in 6-well plates until 50 % confluence was reached and transfected with siRNA specific for seladin-1 and scrambled siRNA, serving as siControl, for 48 h and 72 h. Cells were harvested for gel electrophoresis and Western blotting. Proteins were detected by staining with primary rabbit anti-human C59D8 or primary mouse anti-human β-actin. **(A)** Quantification of seladin-1 expression of **(B)** Western blot to detect seladin-1 expression in transfected cells. **P<0.001 compared to siControl.

5.7 Cyanate Treatment of Seladin-1 Silenced Human Coronary Artery Endothelial Cells

To investigate whether seladin-1 contributes to cyanate-induced ICAM-1 expression in primary cells (in contrast to the EA.hy926 cell line), HCAECs were transfected to silence seladin-1 and additionally treated with cyanate.

Figure 11A represents a significant increase in ICAM-1 expression in seladin-1 knock down compared to the siControl. Figure 11B indicates that cyanate treatment leads to a significant increase of ICAM-1 expression in HCAEC compared to its untreated siControl. The increase of ICAM-1 expression in seladin-1 silenced HCAEC upon 1 mM or 2 mM cyanate treatment is significant in comparison to untreated cells.

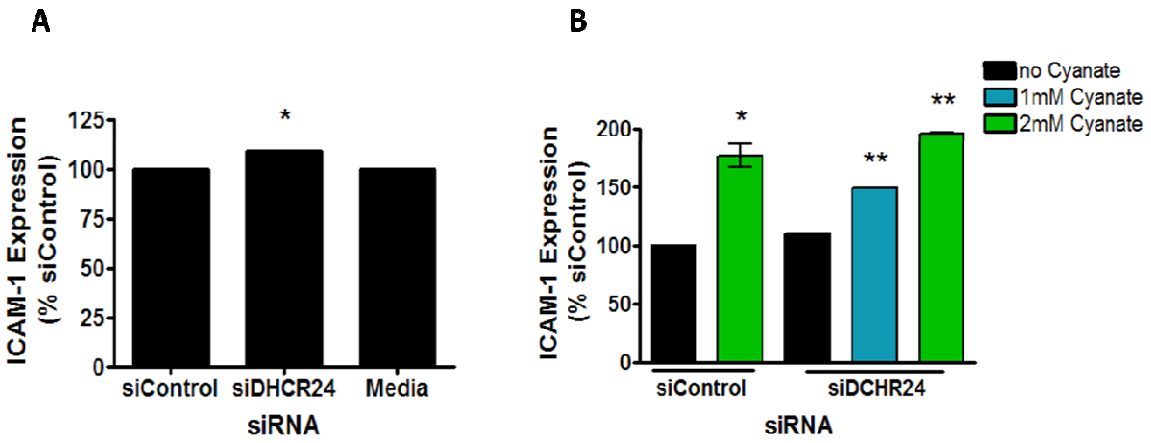

Figure 11: Effect of Cyanate on ICAM-1 Expression in Seladin-1 Silenced HCAEC. Cells were cultured in 48-well plates till 50 % confluence was reached and transfected with siDHCR24. 48h post-transfection cells were treated with 1 mM and 2 mM cyanate for 24 h. After which cells were harvested and stained for ICAM-1 with mouse anti-human CD54. Flow cytometric analysis of **(A)** ICAM-1 expression in seladin-1 silenced cells and of **(B)** ICAM-1 expression in seladin-1 silenced cells, treated with 1 mM or 2 mM cyanate. n=2-3, *P<0.05 compared to untreated siControl, **P<0.05 compared to untreated siDHCR24.

The impact of cyanate on seladin-1 expression in seladin-1 silenced HCAECs was also investigated.

Figure 12 represents successful seladin-1 silencing with siRNA approach. Silencing seladin-1 leads to a decrease in protein expression compared to its siControl (Figure 12A). Figures 12B and 12C show that cyanate treatment decreases seladin-1 expression in HCAEC compared to its untreated siControl. There is also a decrease in seladin-1 expression in seladin-1 silenced HCAEC upon cyanate treatment compared to untreated cells.

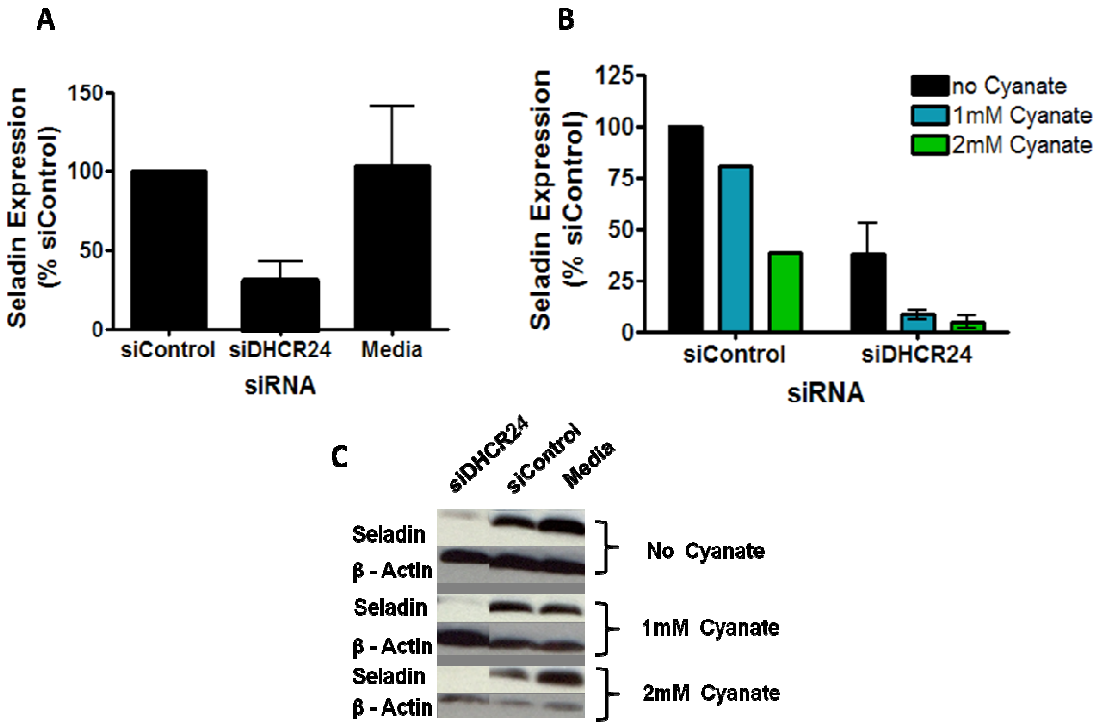

Figure 12: Effect of Cyanate on Seladin-1 Expression in Seladin-1 Silenced HCAEC. Cells were cultured in 6-well plates until 50 % confluence was reached and transfected with siDHCR24. 48 h post-transfection cells were treated with 1 mM or 2 mM cyanate for 24 h, after which cells were harvested for gel electrophoresis and blotting. Proteins were detected using primary rabbit anti-human C59D8 or primary mouse anti-human β-actin, which served as a loading control. Quantification of **(A)** seladin-1 expression in seladin-1 silenced cells. n=2, and of **(B)** seladin-1 expression in seladin-1 silenced cells, treated with 1mM or 2mM cyanate. n=1-2, of **(C)** Western blot to detect seladin-1 expression in transfected cells.

5.8 Pharmacological Activation or Inhibition of Sirtuin-1 on ICAM-1 Expression of Cyanate-Treated Human Coronary Artery Endothelial Cells

In the second part of the thesis, we investigated the role of sirtuin-1 (Sirt1) in cyanate-induced ICAM-1 expression. Sirt1 is a histone deacetylase that inhibits NF-κB expression and is thought to have anti-inflammatory properties. We wanted to investigate if inhibition or activation of Sirt1 influences cyanate-induced ICAM-1 expression.

Figure 13A indicates that Sirt1-inhibition with 100 µM and 200 µM splitomicin induces ICAM-1 expression similar to cyanate treatment alone. Additional 2 mM cyanate treatment to Sirt1-inhibition shows an additive induction of ICAM-1 expression. Sirt1-activation with 2 µM and 4 µM of resveratrol-derivative did not impact ICAM-1 expression. Resveratrol-derivative did not decrease cyanate-induced ICAM-1 expression (Figure 13B).

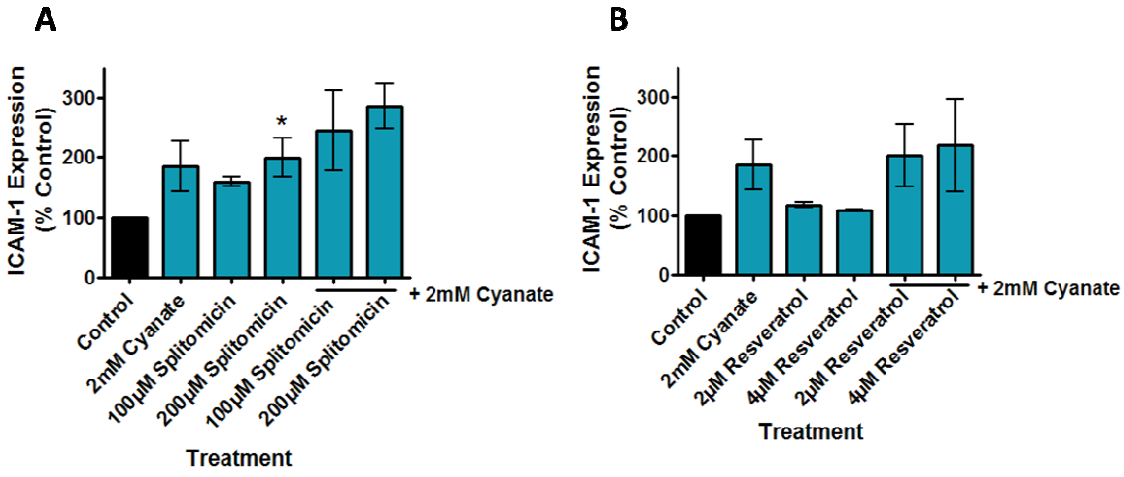

Figure 13: ICAM-1 Expression in Cyanate-Treated HCAEC upon Pharmacological Activation or Inhibition of Sirt1. Cells, cultured in 48-well plates, were treated with **(A)** Sirt1-inhibitor splitomicin (100 µM, 200 µM) and **(B)** Sirt1-activator resveratrol-derivative (2 µM, 4 µM) 1 h prior to cyanate treatment (2 mM for 24 h). Cells were harvested and stained with mouse anti-human CD54 for 30 min at 4℃. ICAM-1 expression was analysed using flow cytometry. n=2-3, * P<0.05 compared to control.

6 Discussion

In this present study we found that cyanate leads to a significant dose-dependent increase of ICAM-1 expression in EA.hy926 cells and HCAECs, as depicted in Figure 4A and Figure 9A. These results are consistent with prior findings of our group, which previously showed that cyanate induces endothelial ICAM-1 expression via p38 MAPK and NF-κB - signaling pathways and increases neutrophil adhesion [65]. To achieve deeper knowledge of underlying mechanism of cyanate-induced ICAM-1 expression the role of seladin-1 and Sirt1 was investigated.

Seladin-1, an enzyme of human cholesterol-biosynthesis, is known to protect cells from apoptosis and control cell growth and senescence via p53 [68,69]. This anti-apoptotic and protective effect might be due to its scavenger capacity for hydrogen peroxide [70]. We hypothesized that seladin-1 plays a regulating role in cyanate-induced ICAM-1 expression. Therefore seladin-1 expression in endothelial cells was investigated upon cyanate-treatment and siRNA approach was established to knock down seladin-1.

In our experiments seladin-1 expression was decreased in EA.hy926 cells and HCAECs upon cyanate treatment, as indicated in Figure 4B, 4C and Figure 9B, 9C respectively. A previous study showed a decrease in seladin-1 expression upon methylation of *Dhcr*24-promotor impaired seladin-1 transcription [72]. Cyanate targets lysine-residues in protein structures resulting in ε-carbamyllysine. The post-translational carbamylation of proteins may render them dysfunctional [52,53]. Carbamylation of *Dhcr*24-promotor or another functional site in seladin-1 transcription may be a possible explanation for the decrease in seladin-1 expression in cyanate-treated cells.

Adhesion molecule expression is induced by NF-κB transcription, which is redox sensitive. Anti-oxidant potential of seladin-1 might inhibit NF-κB activity as well as adhesion molecule expression. One study showed that knock down of seladin-1 in HCAECs, resulted in impaired anti-oxidant potential and increased NF-κB activity, leading to an induction of VCAM-1 expression [73]. However, we observed no significant increase in ICAM-1 expression in EA.hy926 cells and HCAEC, as shown

in Figure 7A and Figure 11A respectively. This may suggest that seladin-1 only regulates VCAM-1 expression.

In seladin-1 silenced endothelial cells, which were treated with cyanate, a significant increase in ICAM-1 expression was observed compared to untreated cells (Figure 7B and Figure 11B); leading to the conclusion, that cyanate-induced ICAM-1 expression is independent of seladin-1.

It is worth noting that cyanate treatment leads to an even further decrease of seladin-1 expression in the seladin-1 silenced endothelial cells, compared to untreated cells (Figure 8B, 8C and Figure 12B, 12C). Thus it is tempting to speculate that cyanate induced carbamylation of free amino acids results in further decreased protein synthesis.

Sirt1 is a NAD^+-dependent class III histone deacetylase [76–78] , which plays a critical role in cellular responses regarding stress, senescence and mitochondrial function [79–81]. Sirt1 levels are decreased in patients with chronic inflammatory diseases. The same study also showed decreased Sirt1 expression in lung epithelial cells, treated with hydrogen peroxide or cigarette smoke [84] indicating that Sirt1 may regulate inflammatory process behind cyanate-induced ICAM-1 expression. For this purpose a pharmacological Sirt1-inhibitor (splitomicin) and a Sirt1-activator (resveratrol-derivative) were used in the presence of cyanate.

Splitomicin, induced ICAM-1 expression significantly in endothelial cells, as represented in Figure 13A. This result, together with the fact of decreased Sirt1 levels in patients, suffering from chronic inflammation [84], suggests that Sirt1 inhibition leads to inflammatory processes. This is supported by the fact, that ICAM-1 needs active NF-κB to be expressed. NF-κB lysines have to be acetylated to allow NF-κB to translocate into nucleus and mediate gene expression, otherwhise NF-κB is retained in the cytoplasm [88]. Active Sirt1 leads to deacetylation of NF-κB lysines, resulting in cytoplasm-retained NF-κB. Inactive Sirt1 cannot deacetylse lysines, hence NF-κB translocates into nucleus and may mediate ICAM-1 expression [88,89]. Endothelial cells, which were treated with cyanate, exhibited even in the presence of an Sirt1 inhibitor an additive effect in ICAM-1 expression (Figure 13A), indicating that Sirt1 does not significantly contribute to cyanate-induced ICAM-1 expression.

Sirt1-activation with a resveratrol-derivative did not impact ICAM-1 expression and additional treatment of cyanate-treated cells with reservatrol-derivative promoted an increase of ICAM-1 expression similar to cyanate treatment alone (Figure 13B), thus confirming that Sirt1 is not significantly involved in cyanate-induced ICAM-1 expression under our experimental conditions.

In conclusion, increased ICAM-1 expression in endothelial cells upon cyanate treatment suggests that cyanate may potentiate vascular inflammation, linking inflammation, smoking and uremia [65]. Decreased levels of seladin-1 and Sirt1 upon inflammation, smoking, Alzheimer disease, cancer and resulting modifications have been elucidated [71,72,84]. This indicates that seladin-1 and Sirt1 play regulatory roles in inflammatory processes. Our present findings revealed that Sirt1, but not seladin-1, regulates ICAM-1 expression. However, our hypothesis that cyanate-induced ICAM-1 expression is mediated by inactivation of seladin-1 and/or Sirt1 could not be verified. Thus, further studies are required to investigate the mechanisms behind cyanate-induced ICAM-1 expression.

7 Abbreviations

BSA	bovine serum albumin
DMSO	dimethylsulfoxid
eNOS	endothelial NO-Synthase
FBS	fetal bovine serum
FCS	fetal calf serum
HAc	acetic acid
HBSS	Hank’s buffered saline solution
HCAEC	human coronary artery endothelial cell
HDL	high-density lipoprotein
ICAM-1	intercellular adhesion molecule
IFN-γ	interferon-γ
IL-8	interleukin-8
LDL	low-density lipoprotein
LFA-1	lymphocyte function-associated antigen 1
LPS	lipolysaccharide
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
M-CSF	macrophage colony stimulating factor
MIP-1α/β	macrophage inflammatory protein1α/β
MMP	metalloproteinase
MPO	myeloperoxidase
NO	nitric oxide
PBS	phosphate buffered saline solution
SDS	sodiumdodecylsulfate
Sirt1	human silent information regulator-two 1

SMC	smooth muscle cell
TF	tissue factor
TNS	Trypsin Neutralizing Solution
TNF-α	tumor necrosis factor-α
VCAM-1	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLA-4	very late antigen-4

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