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Henna-Kaisa Jyrkkänen

Transcription Factor Nrf2 Mediated Gene Regulation and Signaling in the Endothelium

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HENNA-KAISA JYRKKÄNEN

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ABSTRACT

Endothelial dysfunction plays a significant role in the development of atherosclerosis. Production of reactive oxygen species (ROS) is increased upon pathological conditions in the vascular wall and leads to the endothelial dysfunction. ROS are metabolized by antioxidant and detoxification enzymes regulated by Nuclear factor E2- Related Factor 2 (Nrf2). Nrf2 regulates gene expression via an antioxidant response element (ARE) that is conserved in the regulatory regions of target genes. Under physiological conditions, Nrf2 is regulated by Kelch-like ECH-associated protein 1 (Keap1), which mediates its degradation. Upon exposure to ROS, Nrf2 translocates to the nucleus and drives target gene expression. The aim of this thesis was to study regulatory mechanisms by which Nrf2 is activated in endothelial cells. Also, the mechanism by which Nrf2 mediated expression is silenced after induction and the potential role of Nrf2 as a negative regulator of gene expression were studied.

Oxidized 1-palmitoyl-2-arachidonyl-*sn*-glycero3-phosphocholine (oxPAPC) is a phospholipid present in atherosclerotic lesions. In this thesis we show that oxPAPC activates Nrf2 and target gene expression in human endothelial cells and murine carotid arteries, contributing to the anti-inflammatory effects of oxPAPC. Nrf2 activation is also induced by nitro-oleic acid (OA-NO_2) that is endogenously present in the circulation and its production is increased during cardiac ischemia-reperfusion injury. Here it is also shown that OA-NO_2 activates another protective signaling pathway, heat shock response. BTB and CNC homology 1 (BACH1) is a repressive transcription factor that binds to the same ARE sequence as Nrf2. The functional ARE was identified from the BACH1 promoter as it mediates the inducible expression of the BACH1 gene in an Nrf2 dependent manner. These findings suggest a novel negative feedback mechanism by which Nrf2 reduces its own activity. To determine the endothelial specific target genes of Nrf2, we studied gene expression genome-wide and found that Nrf2 over-expression leads to the down-regulation of thioredoxin interacting protein (TXNIP), an inhibitor of thioredoxin. We also showed that Nrf2 reduces TXNIP promoter activity and expression of both TXNIP mRNA and protein levels.

In conclusion, Nrf2 is activated by endogenous activators leading to the increased expression of the protective genes and inhibition of inflammatory pathway. These results support the role of Nrf2 in the protection of the endothelium due to its antiatherogenic features.

National Library of Medical Classification: QS 532.5.E7, QT 55.6, QU 93, QU 475, QV 312, WG 510, WG 550
Medical Subject Headings: Atherosclerosis; Basic –Leucine Zipper Transcription Factors; Endothelium, Vascular/pathology; Endothelial cells; Gene expression; Gene regulation; Heat Shock Response; NF-E2-Related Factor 2; Oleic Acids; Phospholipids; Phosphatidylcholines; Reactive Oxygen Species; Regulatory Sequences; Thioredoxin; Transcription factor

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TIIVISTELMÄ

Endoteelin toimintahäiriöllä on merkittävä rooli ateroskleroosin kehittymisessä. Endoteelin toimintahäiriössä, kuten useissa muissa patologisissa tiloissa, hapen reaktiivisten yhdisteiden esiintyminen lisääntyy. Hapen reaktiivisia yhdisteitä metaboloi antioksidantti- ja detoksifikaatioentsyymit, joiden ilmentymistä säätelee Nuclear factor E2-Related Factor 2 (Nrf2). Nrf2 säätelee geenien ilmentymistä antioksidanttivaste-elementin (ARE) välityksellä, joka on konservoitunut säätelyalue-elementti kohdegeenien säätelyalueilla. Fysiologisissa olosuhteissa Nrf2:n hajottaa inhiboiva proteiini, Kelch-like ECH-associated protein 1 (Keap1). Aktivoivissa olosuhteissa Nrf2 siirtyy tumaan, jossa se sitoutuu kohdegeenien säätelyalueille lisäten kohdegeenien luentaa. Tämän väitöskirjan tarkoituksena oli tutkia Nrf2:n aktivaatiomekanismeja verisuonen endoteelisoluissa, selvittää Nrf2-välitteisen geenien ilmentymisen hiljentymistä aktivaation jälkeen sekä Nrf2:n potentiaalista roolia geenien alassäätelyssä.

Hapettunut 1-palmityyli-2-arakinoni-*sn*-glysero-3-fosfokoliini (OxPAPC) on ateroskleroottisissa leesioissa esiintyvä fosfolipidi. Tässä työssä osoitimme oxPAPC:n aktivoivan Nrf2:n ja lisäävän kohdegeenien ilmentymistä ihmisen verisuonen endoteelisoluissa ja hiiren kaulavaltimossa välittäen oxPAPC:n tulehdusta vähentäviä vaikutuksia. Nrf2 aktivoitui myös nitratun öljyhapon vaikutuksesta, jota esiintyy verenkierrossa ja kudoksissa sydän- ja verisuonitapahtumien yhteydessä. Osoitimme nitratun öljyhapon aktivoivan myös lämpöshokkivasteen, joka Nrf2:n tavoin aktivoi soluja suojaavia signalointiteitä. BTB and CNC homology 1 (BACH1) on säätelytekijä, joka kilpailee Nrf2:n kanssa ARE-elementistä kohdegeenien säätelyalueilla. BACH1-geenin säätelyalueelta löytyi toiminnallinen ARE-elementti, jonka välityksellä Nrf2 aktivoi BACH1-geenin luennan. Havainnon perusteella voidaan ajatella Nrf2:n vaikuttavan negatiivisesti omien kohdegeeniensä ilmentymiseen BACH1:n ilmentymisen lisääntymisen kautta. Havaitsimme myös Nrf2:n vähentävän tioredoksiinia inhiboivan proteiinin (TXNIP) ilmentymistä lähetti-RNA- ja proteiinitasoilla sekä vähentävän TXNIP:n säätelyalueen aktiivisuutta. TXNIP estää tioredoksiinin toimintaa soluissa, mahdollistaen tulehdusta edistävien signaaliteiden aktivoitumisen.

Yhteenvetona voidaan todeta Nrf2:n aktivoituvan verisuonen endoteelisoluissa endogeenisesti esiintyvien aineiden vaikutuksesta johtaen soluja suojaavien geenien ilmentymisen lisääntymiseen ja tulehdusta edistävien tapahtumien vähentymiseen. Tämä vahvistaa käsitystä Nrf2:n roolista tulehdusta vähentävänä ja aterogeneesiä hidastavana tekijänä verisuonen endoteelisoluissa.

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“Me jäätiin kirjaan, virkkoi Myy,
niin käy, kun liiaks kehittyy”

-Tove Jansson-

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List of the original publications

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*equal contribution

II Kansanen E, Jyrkkänen H-K, Volger O L, Leinonen H, Kivelä A M, Häkkinen S-K, Woodcock S R, Schopfer F J, Horrevoets A J, Ylä-Herttuala S, Freeman B A, Levonen A-L. Nrf2-dependent and -independent responses to nitro-fatty acids in human endothelial cells: Identification of heat shock response as the major pathway activated by nitro-oleic acid. The *Journal of biological chemistry: 285:33233-41; 2009*

III Jyrkkänen H-K, Kuosmanen S, Heinäniemi M, Laitinen H, Kansanen E, Mella-Aho E, Leinonen H, Ylä-Herttuala S, Levonen A-L. Novel insights into the regulation of the antioxidant response element mediated gene expression by electrophiles: induction of the transcriptional repressor BACH1 by NRF2. *The Biochemical journal: Epub Aug 3; 2011*

IV Jyrkkänen H-K, Laitinen H, Heinäniemi M, Leinonen H, Ylä-Herttuala S, Levonen A-L. Sulforaphane and Nrf2 over-expression Reduce Thioredoxin Interacting Protein (TXNIP) expression in endothelial cells. *Manuscript 2011*

Contents

Abbreviations

AP-1	Activator protein-1
ApoB100	Apolipoprotein B100
ApoE	Apolipoprotein E
ARE	Antioxidant response element
ASK-1	Apoptosis signaling kinase 1
BACH1	BTB and CNC homology 1
B_2M	β_2-microglobulin
BTB	Broad complex, tramtrack and bric a brac
bZip	Basic leucine zipper domain
CBP	CREB binding protein
ChIP	Chromatin immunoprecipitation
CNC	Cap'n'collar
CO	Carbon monoxide
COX-2	Cyclooxygenase-2
CP	Cysteine-proline
CREB	cAMP responsive element binding protein 1
CRM1	Chromosome region maintenance 1
CUL3	Cullin 3
Cys	Cysteine
DBD	DNA-binding domain
15d-PGJ_2	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
EHR	Extended homology region
EMSA	Electromobility shift assay
ENCODE	Encyclopedia of DNA Elements
GCL	Glutamate-cysteine ligase
GCLC	Glutamate-cysteine ligase catalytic subunit
GCLM	Glutamate-cysteine ligase modifier subunit
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HEK293T	Human embryonic kidney 293T cells
HO-1	Heme oxygenase-1
HSE	Heat shock response element
HSF	Heat shock factor
HSP	Heat shock protein
HSR	Heat shock response
HUVEC	Human umbilical vein endothelial cell
I/R	Ischemia reperfusion injury
IVR	Intervening region
JNK	Jun-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
LDL	Low density lipoprotein
LNO_2	Nitrated linoleic acid
MARE	Maf recognition element
MCP1	Monocyte chemoattractant protein 1
miRNA	Micro-RNA
mmLDL	Minimally modified LDL
MOI	Multiplicity of infection
Neh	Nrf2-ECH homology domain
NES	Nuclear export signal
NFE2L2	Nuclear factor E2- Related Factor 2 (gene)

NF-kB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NLS	Nuclear localization signal
$^{\cdot}NO$	Nitric oxide
NO_2^-	Nitrite
NO_2-FA	Nitrated fatty acids
NOS	Nitric oxide synthase
Nox	NAD(P)H oxidase
NQO1	NAD(P)H quinone oxidoreductase 1
Nrf2	Nuclear factor E2- Related Factor 2 (protein)
$O_2^{\cdot-}$	Superoxide
OA	Oleic acid
OA-NO_2	Nitrated oleic acid
$OH^{\cdot}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
oxLDL	Oxidized LDL
oxPAPC	Oxidized PAPC
oxPL	Oxidized phospholipid
PAPC	1-palmitoyl-2-arachidonoyl-*sn*-glycero-phosphocholine
PEIPC	1-palmitoyl-2-(5,6 epoxyisoprostanoyl)-*sn*-glycero-3-phosphocholine
PERK	PKR-like endoplasmic reticulum kinase
PGPC	1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine
POVPC	1-palmitoyl-2-oxovaleryl-*sn*-glycero-3-phosphorylcholine
POZ	Poxvirus and zinc finger
PPAR	Peroxisome proliferator activated receptor
PWM	Position weight matrix
RBX1	Ring box 1
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SFN	Sulforaphane
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
tBHQ	tert-Butylhydroquinone
TNF-α	Tumor necrosis factor- α
TRX-1	Thioredoxin 1 (protein)
TSS	Transcription start site
TXN	Thioredoxin 1 (gene)
TXNIP	Thioredoxin interacting protein
TXNRD1	Thioredoxin reductase 1
UPR	Unfolded protein response
UTR	Untranslated region
VCAM1	Vascular cell adhesion molecule 1
SMC	Smooth muscle cell

1 Introduction

Atherosclerosis is a leading cause of morbidity and mortality in Western countries despite progress in its prevention and treatment. The development of atherosclerosis, called atherogenesis, is a progressive process (Libby, Ridker & Hansson 2011). At the early phase, endothelial dysfunction plays a significant role. The endothelium functions as a barrier between blood and the vascular wall, maintaining vascular homeostasis. The force caused by circulating blood (shear stress) also has a remarkable role in endothelial functions and homeostasis. In endothelial dysfunction, the normal actions of the endothelium are disrupted and its ability to maintain its integrity is reduced, leading to the increased expression of proatherogenic genes (Chiu, Chien 2011).

The production of reactive oxygen species (ROS) is increased during ageing and pathological conditions in the vascular wall, which mediates many proinflammatory and proatherogenic processes. When low density lipoprotein (LDL) is present at high concentrations in plasma, it accumulates in the intima and is oxidized by ROS (Stocker, Keaney 2004). ROS and secondary products of lipid peroxidation are metabolized by antioxidant and detoxification enzymes that protect cells against the harmful effects. Nuclear factor E2- Related Factor 2 (Nrf2) is a transcription factor that regulates the expression of antioxidant and detoxification enzymes on exposure to ROS or other electrophiles. Nrf2 regulates gene expression via the antioxidant response element (ARE) present in the regulatory regions of target genes. Under physiological conditions, Nrf2 is regulated by Kelch-like associated protein 1 (Keap1) that mediates rapid proteasomal degradation of Nrf2. In oxidative or electrophilic stress, Nrf2 escapes degradation, translocates to the nucleus and drives target gene expression (Taguchi, Motohashi & Yamamoto 2011).

Since increased ROS production has an emerging role in the disruption of vascular homeostasis, the factors that are capable of protection against the ROS may serve as targets for prevention or therapy of endothelial dysfunction and atherosclerosis. Nrf2 and its target genes have been postulated to have a protective role against endothelial dysfunction and the development of atherosclerosis (Cheng, Siow & Mann 2011). However, the mechanisms by which Nrf2 is activated in endothelial cells in the vasculature are largely unknown and more knowledge is needed.

This thesis describes the mechanisms by which two endogenous lipid peroxidation products, oxidized 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (oxPAPC) and nitro-oleic acid (OA-NO_2) activate Nrf2 in endothelial cells and murine arteries. A novel negative feedback mechanism, by which Nrf2 signaling is silenced after activation, is also suggested. BTB and CNC homology 1 (BACH1) is a repressive transcription factor that competes with Nrf2 for binding to the same consensus sequence (Igarashi, Sun 2006), and it is shown here that BACH1 is induced by Nrf2. Further, the role of Nrf2 in the down-regulation of genes is clarified by demonstrating the effect of Nrf2 on the expression of thioredoxin interacting protein (TXNIP). The suppression of the expression of proinflammatory TXNIP supports the role of Nrf2 as an antiatherogenic protector of the endothelium.

2 Review of the literature

2.1 OXIDATIVE STRESS

Oxygen is a very common molecule in living organisms. It is essential in mammalian cells and has roles in cell respiration, metabolism, and signaling. The oxygen molecule (O_2) has two unpaired electrons in different orbitals. Those electrons can be reduced by enzymes or reactions with redox active compounds by forming superoxide ($O_2^{\cdot-}$). $O_2^{\cdot-}$ is formed by the reactions of NAD(P)H oxidase (Nox) and Xantine oxidase or in the mitochondrial electron transport chain. $O_2^{\cdot-}$ is a highly reactive molecule. It reacts with other molecules or is dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutases (SODs). H_2O_2 can form a highly reactive hydroxyl radical ($OH^{\cdot}$). $OH^{\cdot}$ is also generated in the myeloperoxidase mediated reaction from hydrogen chloride and $O_2^{\cdot-}$. Free radicals and other reactive oxygen molecules; $O_2^{\cdot-}$, $OH^{\cdot}$, H_2O_2, and hydrogen chloride, are collectively termed as ROS (Droge 2002).

ROS are naturally formed during physiological processes such as cell respiration and during normal metabolic events. In addition, ROS have a role in cell signaling. Cells have effective enzymatic and nonenzymatic protection systems against ROS (Droge 2002). ROS activate the expression of antioxidant and detoxification enzymes that metabolize and detoxify ROS (Stocker, Keaney 2004). In many diseases the balance between ROS production and disposal is disrupted leading to the accumulation of ROS, termed oxidative stress. Oxidative stress can occur for two reasons; increased production of ROS or defective cellular defense systems. During oxidative stress, antioxidative and detoxification enzymes cannot protect cells against increased ROS production, leading to oxidative damage in lipids, proteins and DNA. ROS production is increased during ageing and is involved in the pathogenesis of many diseases, such as cardiovascular diseases, diabetes, cancer and neurodegenerative diseases (Stocker, Keaney 2004, Droge 2002).

In endothelial cells, oxidative stress leads to endothelial dysfunction and activates other pathological processes such as apoptosis, monocyte aggregation and migration. These processes advance vascular inflammatory processes, eventually leading to atherosclerosis (Stocker, Keaney 2004, Madamanchi, Vendrov & Runge 2005, Yokoyama 2004).

2.2 ROLE OF THE ENDOTHELIUM IN ATHEROPROTECTION

The artery wall is formed from four layers: the endothelium, intima, media and adventitia, each having specific roles in vessel function. The inner layer of the vessel wall, the endothelium, functions as a physical barrier, regulates hemodynamics and angiogenesis, synthesizes nitric oxide ($^{\cdot}NO$), and it inhibits inflammation and thrombosis. During ageing and in the context of many clinical conditions, such as hypercholesterolemia, hypertension, and diabetes, the endothelium is damaged and ROS production is increased. Rapidly increased ROS production and decreased capacity of protective mechanisms leads to endothelial dysfunction (Chiu, Chien 2011).

Dysfunctional endothelium cannot inhibit blood coagulation and vascular inflammation, and the regulation of cell growth, proliferation and organization is disrupted. Endothelial dysfunction also leads to increased vascular contraction, leukocyte adhesion, platelet aggregation and thrombosis, and smooth muscle cell (SMC) proliferation

(Figure 1). These effects are largely due to decreased bioactivity of ·NO in the vessel wall (Chiu, Chien 2011).

At the molecular level, decreased ·NO bioactivity is caused by repressed expression of endothelial specific nitric oxide synthase (eNOS), ROS production by eNOS, or reduction of cofactors or increased reactions of ·NO with ROS. The $O_2^{\cdot-}$ reaction with ·NO is three times faster than the reaction with SOD (Stocker, Keaney 2004). The main reaction product of ·NO and $O_2^{\cdot-}$ is peroxynitrite ($ONOO^-$) that forms other radicals and maintains oxidative stress (Stocker, Keaney 2004, Carr, McCall & Frei 2000). In addition to $ONOO^-$ formation, ·NO can be degraded to nitrite (NO_2^-) that functions as a substrate for myeloperoxide to form nitrogen dioxide. Nitrogen dioxide can oxidize LDL, nitrate lipoproteins (Carr, McCall & Frei 2000) or react with unsaturated lipids to form nitrated fatty acids (O'Donnell et al. 1999). Nitrated fatty acids (NO_2-FAs) activate antioxidative and anti-inflammatory signaling to provide atheroprotective effects (Coles et al. 2002, Rudolph et al. 2010a). Molecules derived from ·NO are collectively termed as reactive nitrogen species (RNS).

2.2.1 Shear stress

The force caused by unilateral blood flow (shear stress or laminar flow) is one of the main factors maintaining normal endothelial functions. Shear stress protects the endothelium from apoptosis and prevents atherogenesis, since areas where flow is turbulent are more prone to lesion formation than areas subjected to laminar flow. Branches of the blood vessels and atherosclerotic lesions change the flow to turbulent, thus modifying the biomechanical forces acting on the endothelium. Altered flow conditions affect the expression profile of endothelial cells leading to the activation of adhesion and inflammatory genes as well as the inhibition of protective genes. (Chiu, Chien 2011, Gimbrone et al. 2000, Ross 1999, Traub, Berk 1998)

2.2.2 Endothelial dysfunction in atherogenesis

Atherosclerosis is a disease of medium and large arteries leading to the formation of fatty streaks and finally fibrous plaques in the arterial wall. Oxidative stress has a role in all stages of atherogenesis. The first manifestation of altered vascular homeostasis is endothelial dysfunction, in which oxidative stress plays a significant role. The presence of a high concentration of LDL in the circulation leads to the accumulation of LDL in the intima. In the intima LDL is oxidized via enzymatic and nonenzymatic reactions forming minimally modified LDL (mmLDL) and oxidized (oxLDL). The outer layer of the LDL molecule is formed from phospholipids, cholesterol and apolipoprotein B100 (apoB100) with triglycerides and cholesterol esters inside. In mmLDL phospholipids of LDL are mildly oxidized and proteins are intact or have minor modifications. Oxidation leads to formation of oxLDL whereby in addition of highly oxidized phospholipids, apoB100 is degraded and modified (Parthasarathy et al. 1999). Endothelial dysfunction increases the expression of adhesion molecules on the surface of endothelial cells, which increases their capacity to bind monocytes. Monocytes bind to endothelial adhesion molecules and migrate into the intima where they are transformed to macrophages. Scavenger receptors of macrophages recognize oxLDL, which is internalized and accumulates in cells, forming foam cells. Foam cells form a fatty streak in the intima and secrete proinflammatory cytokines that maintain chronic inflammation. Inflammation activates SMCs that migrate to the intima from the media. In the intima, SMCs start to rapidly proliferate and produce extracellular matrix molecules, such as collagen and elastin that cover the atheroma forming a fibrotic (or advanced) plaque. Inside the plaque SMCs and lipid rich foam cells can die, for example, via apoptosis. This leads to the release of the lipids into the extracellular space and formation of the necrotic or lipid core. Over time, the fibrotic plaque grows and can obstruct the artery, thus limiting blood flow. Plaque rupture can evoke

thrombus formation that can disrupt blood flow locally or create emboli that may occlude distal arteries (Libby, Ridker & Hansson 2011, Glass, Witztum 2001).

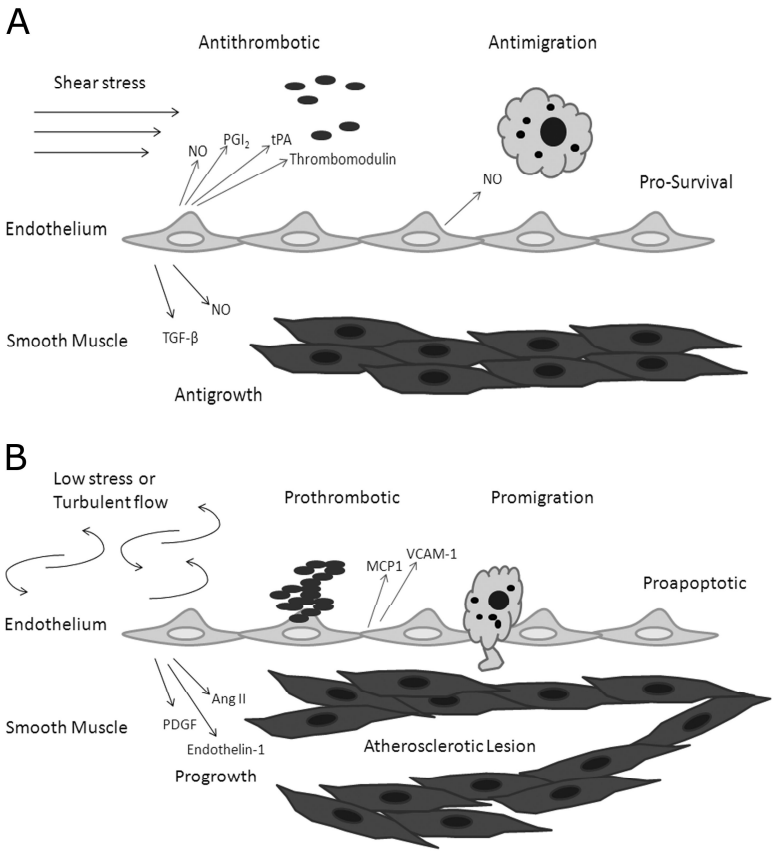

Figure 1. Shear stress and the role of endothelial dysfunction in atherogenesis. A, During vascular homeostasis and shear stress, endothelial cells produce ·NO, PGI_2 (prostacyclin), tPA (tissue plasminogen activator), thrombomodulin and TGF-β (transforming growth factor-β), that mediate antithrombotic, antimigratory, growth inhibitory and prosurvival effects. B, Low shear stress, ageing, and vascular diseases disrupt normal functions of endothelial cells leading to changes in gene expression of the cells and endothelial dysfunction. The expression of antithrombotic and antiapoptotic genes decreases, the migration of monocytes is increased via induction of MCP-1 and VCAM1, and the growth and migration of the SMCs advances via induction of PDGF (platelet-derived growth factor), ANGII (angiotensin II), and endothelin-1. These changes promote atherogenesis. Modified from (Traub, Berk 1998).

2.3 STRESS ACTIVATED SIGNALING PATHWAYS

ROS are generated during normal physiological functions, degenerative diseases and ageing. Cells are adapted to the presence of ROS and multiple signaling pathways are activated in response to ROS. Herein, the two important cytoprotective pathways activated by ROS are discussed, namely the Keap1-Nrf2 pathway and the heat shock response (HSR).

2.3.1 Keap1-Nrf2 pathway

The Keap1-Nrf2 pathway is a protective signaling pathway, which is activated by oxidative and electrophilic stress. The main players are transcription factor Nrf2 and Keap1, which mediate proteasomal degradation of Nrf2. When activated, Nrf2 translocates to the nucleus and binds to the ARE of its target genes.

2.3.1.1Nuclear factor E2-related factor 2

Nrf2 (*NFE2L2*) is a transcription factor activated by multiple stimuli, such as ROS, electrophiles, xenobiotics, heavy metals, UV-radiation, and shear stress. Nrf2 belongs to the cap'n'collar (CNC) family of transcription factors that have a common CNC-like basic leucine zipper (bZip) domain. Other known members of the CNC family are Nrf1, Nrf3, p45, BACH1, and BACH2. Via their CNC-domain, these factors dimerize with small Maf proteins and bind to DNA. Complex formation with small Mafs is essential for the regulation of transcription. Nrf2 regulates the expression of antioxidant and phase II detoxification enzymes (Zhang 2006).

The regulation of the *NFE2L2* gene itself is poorly understood. Nrf2 protein is expressed ubiquitously in all tissues and $Nrf2^{-/-}$ mice are viable (Chan et al. 1996, Itoh et al. 1997). It has recently been suggested that NFE2L2 expression in cancer cell lines is regulated by Jun and Myc transcription factors (DeNicola et al. 2011). The translation of NFE2L2 has been reported to be inhibited by microRNAs (miRNA) miRNA-28 and miRNA-144 (Yang et al. 2011, Sangokoya, Telen & Chi 2010).

The structure and regulation of the Nrf2 protein has been extensively investigated. Nrf2 contains six NRF2-ECH homology domains (Neh1-Neh6, Figure 2) that all have specific roles in protein function (Itoh et al. 1999). Neh1 contains CNC basic and leucine zipper domains, which are critical for binding with small Maf proteins and the ARE in the regulatory regions of target genes (Itoh et al. 1997). Lysine residues of Neh1 are also acetylated by a co-transactivator, histone acetyltransferase p300/CREB-binding protein (CBP) that regulates DNA binding (Sun, Chin & Zhang 2009). Protein stability is partially regulated via Neh1 by ubiquitin-conjugating enzyme UbcM2 that binds to the cysteine (Cys) 136 in the Neh1 domain. UbcM2 stabilizes Nrf2, enhances transcriptional activity and has a cysteine residue that functions as a redox sensor (Plafker et al. 2010). Neh2 contains ETGE and DLG motifs that serve as binding sites for the Nrf2 inhibitor protein Keap1 (Katoh et al. 2005, McMahon et al. 2006). Both domains are required for maximal destabilization of Nrf2. Neh domains 3 to 5 are involved in Nrf2-mediated transcriptional regulation. Neh3 contains the C-terminal end of the protein and takes part in the initiation of transcription (Nioi et al. 2005). Neh4 and Neh5 bind CBP that further binds CREB (cAMP responsive element binding protein I) to regulate the start of transcription (Katoh et al. 2001). Neh6 has a role in Keap1-independent degradation of Nrf2 during oxidative or electrophilic stress (McMahon et al. 2004). Glycogen synthase kinase 3 phosphorylates the serine residues of Neh6, leading to the SKP1-Cul1-F-box protein mediated ubiquitination of Nrf2 (Rada et al. 2011). It has been proposed that these foregoing mechanisms mediate Nrf2 degradation when activated in strong stress conditions and when modified Keap1 is incapable of degrading Nrf2, thus this may serve as an additional degradation mechanism.

Cellular localization of Nrf2 is regulated by nuclear export and localization signals (NES and NLS) (Jain, Bloom & Jaiswal 2005, Li et al. 2005). NES and NLS direct localization and nuclear export of Nrf2 in response to redox balance. Mutation studies have shown that both localization signals are essential for the functions of Nrf2 protein. Deletion of NLS inhibits nuclear translocation of Nrf2 and expression of target genes. Deficiency of NES leads to the nuclear accumulation of Nrf2 and increased expression of target genes (Jain, Bloom & Jaiswal 2005). NES and NLS sequences are recognized by chromosome region maintenance (CRM1) cargo protein. CRM1 binds the protein and transports it

through the membrane. Both localization signals are in the leucine zipper domain and are also found in other CNC members (Suzuki et al. 2004).

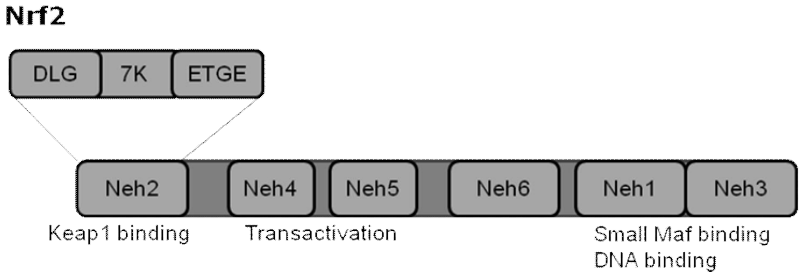

Figure 2. Structure of Nrf2. Nrf2 contains six Neh domains, each with specific roles in protein function. Neh2 contains DLG and ETGE motifs that bind with Keap1. Neh4 and 5 correspond to transactivation, Neh6 has role in degradation, and Neh1 and 3 bind small Mafs and DNA. Modified from (Kansanen et al. 2011).

2.3.1.2 Kelch-like ECH- associated protein 1

Nrf2 activation is mainly regulated by Keap1. Keap1 functions as a substrate adapter protein between Nrf2 and Cullin 3 (CUL3)/Ring box 1 (RBX1)-dependent ubiquitin ligase complex that mediates Nrf2 degradation (Figure 4) (Zhang et al. 2004, Kobayashi et al. 2004). The Keap1 structure contains four functional domains (Figure 3); the Broad complex, Tramtrack and Bric a brac/ the poxvirus and zinc finger-domain (BTB/POZ), the intervening region (IVR), the Kelch domain or double glycine repeat,and the C-terminal region. BTB/POZ -domain is characteristic for BTB-protein family members as it enable them to form protein-protein interactions (Zipper, Mulcahy 2002). The Kelch domain mediates binding with Nrf2 (Kang et al. 2004). The Kelch domain contains six conserved β-propeller structures that were found from structural analysis of another Kelch-protein, galactose oxidase, and two glycine repeats. Adjacent to the Kelch domain is an IVR and BTB that house three cysteine residues (Cys151, Cys273, and Cys288) that are important for the regulation of Nrf2 activity. The IVR- and BTB-domains also serve as a binding site for CUL3-ubiquitin ligase that mediates the ubiquitination and proteosomal degradation of Nrf2 (Kobayashi et al. 2004). Human Keap1 contains 27 and mouse Keap1 contains 25 cysteines whose alkylation or oxidation plays a major role in Keap1 mediated Nrf2 regulation (Dinkova-Kostova et al. 2002).

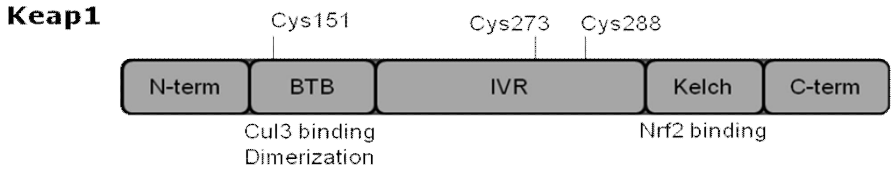

Figure 3. Structure of Keap1. Functional domains of Keap1 are: BTB, IVR, Kelch and the C-terminal region. BTB and IVR mediate complex formation with CUL3 and the Kelch domain binds with Nrf2. Modifications of Cys151, Cys273, and Cys288 are important in Nrf2 activation. Modified from (Kansanen et al. 2011).

2.3.1.3 Keap1 mediated Nrf2 regulation

In basal conditions, Keap1 binds Nrf2 and functions as a substrate adaptor between Nrf2 and CUL3-ubiquitin ligase complex to mediate ubiquitination and proteosomal degradation (Figure 4) (Zhang et al. 2004). Keap1 forms a homodimeric structure in the cytosol that is essential for binding the Nrf2 and CUL3-complex (McMahon et al. 2006, Furukawa, Xiong 2005). Keap1 molecules are bound to each other from BTB domains by

forming a cherry-bob-like structure. A DLG motif in the Neh2 domain in Nrf2 binds one Kelch domain with low affinity and the ETGE motif by another Kelch domain with high affinity, demonstrating that the DLG motif is an essential motif that provides the latch to lock and unlock Nrf2 for ubiquitination (Tong et al. 2006, Tong et al. 2007). The configuration of this complex activates ubiquitination of lysine residues located in the Neh2 domain. Ubiquitinated Nrf2 is rapidly degraded by the 26S-proteasome.

During oxidative or electrophilic stress, the cysteine residues of Keap1 are modified via two alternative mechanisms. Mass spectrometry studies have revealed that several Nrf2 activating agents bind to Keap1 cysteines. Different Nrf2 activating agents affect different cysteines and there is no single cysteine that mediates the effects (Kansanen et al. 2011, Kansanen, Kivela & Levonen 2009). The "cysteine code" hypothesis proposes that structurally different Nrf2 activating agents affect different Keap1 cysteines. A modification of Keap1 cysteines leads to the inhibition of Nrf2 ubiquitination via conformational changes in the Keap1-Nrf2-CUL3 complex and translocation of Nrf2 to the nucleus as presented in Figure 4 (Kobayashi et al. 2009). Modifications of Cys273 and 288 lead to a conformational change of Keap1 that disturbs the interaction between the DLG of Nrf2 and the Kelch domain of Keap1 (Kobayashi et al. 2006). Lysine residues of Nrf2 are thus no longer aligned for ubiquitination. Alternatively, when Cys151 located in the BTB domain of Keap1 is modified, this modification leads to disruption between Keap1 and CUL3 binding and hence, the Keap1-Nrf2-CUL3 complex itself (Rachakonda et al. 2008, Eggler et al. 2009). Summarizing several mass spectrometry studies, it is well documented that most of the Keap1 cysteines are modified by various electrophiles. However, contrary to mass spectrometry, functional studies suggest that only Cys151, Cys273, and Cys288 are important for regulation of Nrf2 (Kansanen, Kivela & Levonen 2009).

Nrf2 activation is regulated by a positive feedback loop via the expression of the Nrf2 target gene p62/Sequestome 1. P62 is a protein that binds to multiple signaling mediators upon a stress stimulus, and it is involved in autophagosome formation and ubiquitination. It has been shown that p62 expression is Nrf2-dependent (Jain et al. 2010). P62 can compete for the binding to the Kelch domain of Keap1 with the DLG motif of Nrf2, thus leading to nuclear accumulation of Nrf2 and target gene expression (Komatsu et al. 2010). The DLG motif also binds p21, which is a regulator of cellular processes such as cell cycle arrest, DNA replication and repair, and cell differentiation. P21 stabilizes Nrf2 and p21 deficient mice have decreased expression of Nrf2-dependent genes (Chen et al. 2009). Taken together, Nrf2 activation is stabilized by compensatory binding of p62 with Keap1 and binding of Nrf2 with p21. These results suggest that in addition to Keap1, there are other players modulating Nrf2 degradation and activation.

2.3.1.4 Nrf2 activating agents

2.4.1.4.1 Classic Nrf2 activating agents (SFN, tBHQ, 15d-PGJ$_2$)

Nrf2 is activated by multiple agents, such as phenol antioxidants, electrophilic compounds, and heavy metals (Figure 5). Sulforaphane (1-isothiocyanato-4-methylsulfinylbutane, SFN, Figure 5A) is a naturally occurring isothiocyanate presented in cruciferous vegetables, such as broccoli, having multiple anti-inflammatory and anti-oxidative effects. The effects of SFN are mediated via regulation of transcription factors, for example by activation of Nrf2 and inhibition of nuclear factor-κB (NF-κB) (Thimmulappa et al. 2002, Kivela et al. 2010). SFN induces a large battery of antioxidant and detoxification genes (Thimmulappa et al. 2002). In the endothelium, SFN induces protective genes during injury and inhibits inflammatory genes via Nrf2 in vivo as well (Zakkar et al. 2009, Zhao et al. 2007). NF-κB activation is inhibited by SFN through several mechanisms such as via inhibition of tumor necrosis factor-α (TNF-α) or lipopolysaccharide mediated phosphorylation of downstream signaling mediators in the NF-κB pathway (Kivela et al. 2010).

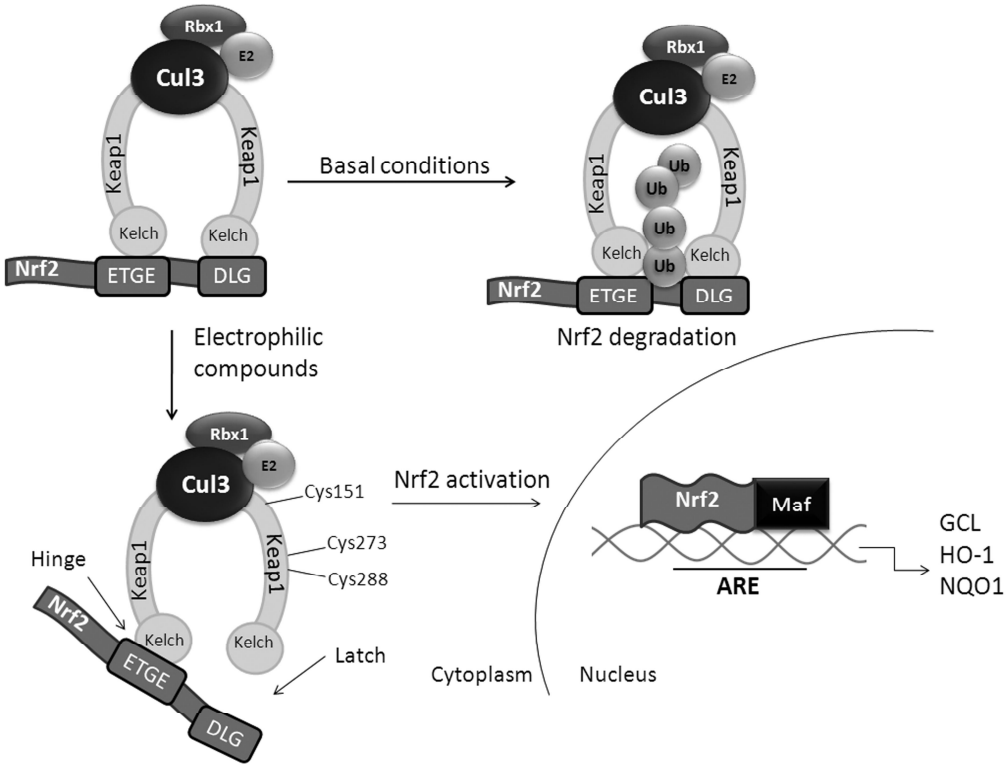

Figure 4. The Keap1-Nrf2 pathway. Nrf2 is bound by two Keap1 proteins that function as adapters for the CUL3-ubiquitin ligase complex. In basal conditions, Nrf2 is rapidly ubiquitinated and degraded. During electrophilic or oxidative stress, cysteine residues of Keap1 are modified leading to the release of the Nrf2 DLG motif from Keap1. Nrf2 escapes degradation, translocates to the nucleus and drives target gene expression via the ARE. Modified from (Kansanen et al. 2011).

Tert-butylhydroquinone (tBHQ, Figure 5B) is a phenol antioxidant that auto-oxidizes and forms a semiquinone antioxidant radical. TBHQ activates Nrf2 dependent gene expression to provide a protective role in many tissues, including endothelial cells (Lee et al. 2003, Li et al. 2007). Both SFN and tBHQ activate Nrf2 via modification of the Keap1 Cys151 (Zhang, Hannink 2003, Wang et al. 2008).

15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2, Figure 5C) is a J series prostaglandin produced by the cyclooxygenase (COX) pathway from prostaglandin D_2 in a nonenzymatic dehydration reaction. 15d-PGJ_2 forms covalent adducts with thiol residues of proteins, thus it has a role in multiple signaling pathways. One of the targets of 15d-PGJ_2 is Keap1 Cys273 and probably also Cys288 (Kobayashi et al. 2009, Levonen et al. 2004). Adduction of 15d-PGJ_2 to the Keap1 Cys273 leads to Nrf2 activation. Unlike with SFN and tBHQ, the effect of 15d-PGJ_2 is Cys151-independent and do not involve Keap1-CUL3-dissociation (Kansanen et al. 2011). Via activation of Nrf2-Keap1 signaling, 15d-PGJ_2 has cytoprotective features (Kansanen, Kivela & Levonen 2009).

2.3.1.4.2 Oxidized phospholipids

Phospholipids are structural components of lipid layers in cell membranes and lipoproteins and can be oxidized through the lipoxygenase and myeloperoxidase pathways to form biologically active oxidized phospholipids (oxPLs). Phospholipids are hetoregenous group with variations in sn2- and sn3-residue fatty acids, so therefore during oxidation and fragmentation, numerous different lipid molecules are formed. A lipid layer derived of

oxPLs can be formed inside of cells and has local effects such as activation of the endoplasmic reticulum stress response (Gargalovic et al. 2006a). A large amount of oxPLs are formed via oxidation of LDL. OxPLs can be cleaved out from mmLDL or oxLDL molecules, or oxPLs can be recognized as a part of the molecule. It has been demonstrated that oxPLs go into the endothelial cells, but the precise mechanisms of this are unclear. It has been suggested that oxPLs as small, lipophilic molecules can go straight through membranes. Receptors such as the scavenger receptor CD36 can recognize and bind to oxPLs (Bochkov et al. 2010, Gao et al. 2010). OxPLs are present at high concentrations in atherosclerotic vessels and lesions, and it has been suggested that oxPLs are involve in all stages of atherogenesis (Bochkov et al. 2010). It has been estimated that 10 to 20% of total lipids of the atheroma are different species of phospholipids (Ravandi et al. 2004).

One of the oxPLs in mmLDL is oxPAPC. The most abundant and well characterized oxidation products of PAPC are 1-palmitoyl-2-oxovaleryl-sn-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC), and 1-palmitoyl-2-(5, 6 epoxyisoprostanoyl)-*sn*-glycero-3-phosphocholine (PEIPC) (Figure 6) (Navab et al. 2004).

OxPAPC activates protective, inflammatory and apoptotic pathways. In the vascular endothelium it induces the expression of inflammatory molecules and adhesion molecules to increase the binding of monocytes in murine arteries (Furnkranz et al. 2005). It also induces oxidative stress by Nox mediated $O_2^{\cdot-}$ production in endothelial cells (Rouhanizadeh et al. 2005). The unfolded protein response (UPR) is a signaling pathway activated by stress via the endoplasmic reticulum or by oxPAPC (Gargalovic et al. 2006a). The UPR response can activate three adaptive or proapoptotic signaling pathways regulated by immunoglobulin binding protein/glucose regulated protein 78 that is an endoplasmic reticulum resident chaperone locking downstream signaling mediators such as activating transcription factor-6, inositol requiring protein 1, and double-stranded RNA-dependent protein kinase (PKR)-like endoplasmic reticulum kinase (PERK). UPR activation leads to chaperone activation, release of signaling factors and regulation of UPR target genes (Ron, Walter 2007). OxPAPC activates all branches of UPR in human aortic endothelial cells, mediating angiogenesis and vascular inflammation by activating transcription factor-4 and X-box binding protein-1 signaling pathways, which are downstream factors of the PERK and inositol requiring protein 1 pathways respectively (Gargalovic et al. 2006a, Bochkov et al. 2006, Gargalovic et al. 2006b, Oskolkova et al. 2008). In the vascular endothelium it has been demonstrated that oxPAPC induces the expression of ARE regulated protective genes (Li et al. 2007, Gargalovic et al. 2006a, Gargalovic et al. 2006b). OxPAPC induces HO-1 and GCL in human endothelial cells (Gargalovic et al. 2006b). Nrf2 has been shown to be activated by oxPAPC and to induce the expression of the ARE-mediated gene oxidative stress induced growth inhibitor 1 (Li et al. 2007).

Different fractions of oxPAPC have different biological effects and they can also modulate the functions of other oxPL species. When the effects of oxPLs on the activation of the UPR were studied, results showed that the oxidized sn2-residue has a critical role (Oskolkova et al. 2008, Subbanagounder et al. 2000). It has also been demonstrated that high density lipoprotein modifies the effects of oxPAPC and PEIPC by inhibiting proinflammatory signaling and maintaining antioxidant activities (Gharavi et al. 2007).

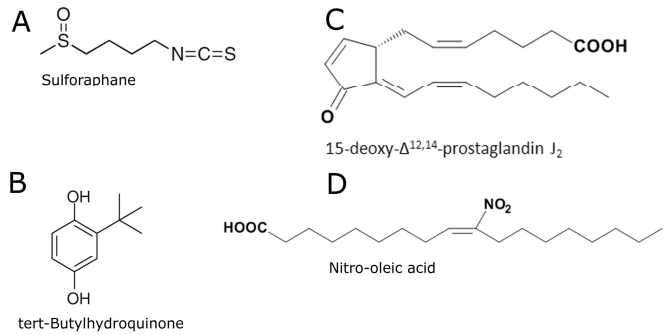

Figure 5. Structures of selected Nrf2 activating agents. A, sulforaphane (SFN); B, tert-butylhydroquinone (tBHQ); C, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2); D, nitrated oleic acid (OA-NO_2).

2.3.1.4.1 Nitrated lipids

Nitrated fatty acids (NO_2-FAs) are electrophilic fatty acid derivates that are formed via reactions of RNS, such as an $^{\cdot}NO$, NO_2^-, or $ONOO^-$ with unsaturated fatty acids. Nitrated linoleic acid (LNO_2) and oleic acid (OA-NO_2, Figure 5D) have a role as signaling mediators by the induction of protective signaling, such as Keap1-Nrf2-, and peroxisome proliferator activated receptor (PPARγ)- pathways, and on the other hand, by inhibiting the NF-κB pathway (Koenitzer, Freeman 2010). LNO_2 and OA-NO_2 modify proteins post-translationally to form covalent adducts with thiol residues (Baker et al. 2007). NO_2-FAs activate the Nrf2 pathway independently of Keap1 Cys151; instead Cys273 and Cys288 seem to be functionally important for this Nrf2 activation and Nrf2-dependent gene expression (Kansanen et al. 2011, Tsujita et al. 2011). The formation of LNO_2 and OA-NO_2 has been demonstrated also in vivo after ischemia-reperfusion injury (I/R) (Rudolph et al. 2010b). Administration of NO_2-FA protects against I/R, inhibits neointima formation and NF-κB mediated inflammation, showing a protective and antiatherogenic role for NO_2-FAs in the cardiovascular system (Rudolph et al. 2010a, Cole et al. 2009).

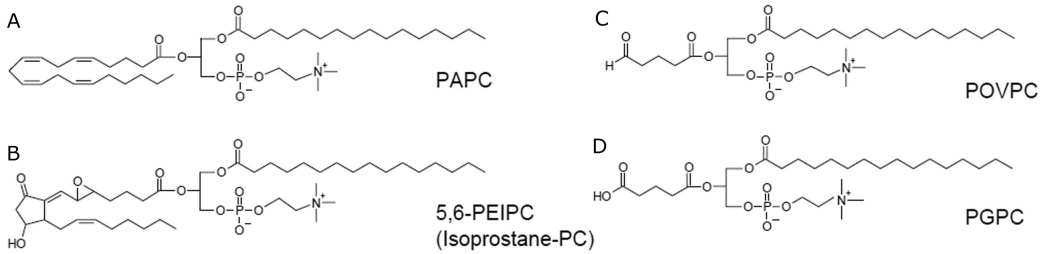

Figure 6. Structures of the oxidation products of PAPC. A, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-phosphocholine (PAPC), B, 1-palmitoyl-2-(5,6 epoxyisoprostanoyl)-*sn*-glycero-3-phosphocholine (PEIPC), C, 1-palmitoyl-2-oxovaleryl-sn-glycero-3-phosphocholine (POVPC), D, 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC).

2.3.1.4 Posttranslational modifications of Nrf2

Phosphorylation has a general role in the activity and stability of proteins. Nrf2 has been demonstrated to be phosphorylated by protein kinase C, extracellular signaling-regulated kinase, p38 mitogen-activated kinase and during endoplasmic reticulum stress, by PERK. Protein kinase C phosphorylates the serine 40 residue in the Neh2 domain of Nrf2 but this is not necessary for nuclear translocation and target gene expression (Bloom, Jaiswal 2003, Huang, Nguyen & Pickett 2000). Nevertheless, it seems to disturb Keap1 mediated ubiquitination (Huang, Nguyen & Pickett 2002). PERK mediates phosphorylation and activation of Nrf2 during endoplasmic reticulum stress, contributing to cell survival

(Cullinan et al. 2003, Cullinan, Diehl 2004). However, the studies that examine the roles of different kinase pathways on Nrf2 activation have somewhat controversial results. The role of kinases seems to be dependent on cell types and target genes. Phosphorylation of dimerization partners and co-activators may have a role in the transcriptional regulation of ARE regulated genes (Kensler, Wakabayashi & Biswal 2007).

Nrf2 has been demonstrated to be acetylated by p300/CBP via lysine residues located in Neh1 (Sun, Chin & Zhang 2009). P300/CBP is known to be a co-transactivator of Nrf2 and essential for DNA binding and transactivation. Sun and colleagues (2009) showed that p300/CBP has a function as a histone acetyltransferase and it stabilizes Nrf2 via acetylation of lysine residues. Acetylation increases and stabilizes transactivation, but it is not obligatory. Acetylation does not have any effect on the ubiquitination or degradation of Nrf2.

2.3.2 Heat shock response

HSR is a common cellular defense mechanism turned on by a wide array of acute and chronic stress conditions. HSR is highly conserved and it has been found from yeast to mammalian cells. HSR is regulated by heat shock factors (HSFs) that bind to the heat shock response element (HSE) on the promoters of target genes upon activation. Mammalian cells express three HSFs; 1, 2, and 4. HSF3 is avian-specific. HSF1 is the first factor identified and is also the best studied. It is activated by elevated temperatures, exposure to oxidants and heavy metals, or by bacterial or viral infection. HSF1 is a transactivator and other HSFs cannot compensate for its functions. HSF2 is involved in development and differentiation processes, and HSF4 is recently characterized and expressed mainly in the lens of the eye and in the brain (Akerfelt et al. 2007).

HSF1 activation is a multistep process where it trimerizes, translocates to the nucleus, and binds to the HSEs on target genes. Post-translational modifications have an important role in the regulation of HSF1. Under basal conditions, HSF1 is a monomer that is phosphorylated via multiple serine and lysine residues. Upon activation, HSF1 undergoes a transition from a monomer to a trimer and binds to the regulatory regions of target genes. For DNA binding, HSFs contain DNA-binding domains. HSFs bind to the DNA in sites where each of the DNA-binding domains faces the HSE. HSEs are highly conserved and contain multiple repeats of the pentameric sequence nGAAn. Target genes often have multiple HSEs that are able to bind several HSFs at the same time (Akerfelt, Morimoto & Sistonen 2010). HSR activated genes and heat shock proteins (HSPs) function as chaperones, proteases, or essential protectors against proteotoxic stress (Akerfelt et al. 2007).

Post-translational modifications regulate HSF1 functions and activity. Phosphorylation and sumoylation positively regulate activity (Akerfelt et al. 2007, Akerfelt, Morimoto & Sistonen 2010). It has also been proposed that HSF2 has a function as a modulator of HSF1 (Ostling et al. 2007). The activation of HSF1 is negatively regulated by feedback mediated by HSPs and by acetylation of a lysine residue located in the DNA binding area (Akerfelt, Morimoto & Sistonen 2010).

HSP90 has been demonstrated to play role in protective signaling via inhibition of Keap1. Casein kinase 2 phosphorylates threonine residue of Keap1 serving a binding site for HSP90. Keap1-HSP90 interaction leads to the disruption of the Keap1-CUL3-ubiquitine ligase complex, release of Nrf2 for degradation and target gene expression (Niture, Jaiswal 2010).

2.4 NRF2 MEDIATED GENE REGULATION

2.4.1 Small Maf proteins-dimerization partners of Nrf2

Upon oxidative or electrophilic exposure, Nrf2 translocates to the nucleus and heterodimerizes with small Maf protein family members (Itoh et al. 1995). The Maf protein family is named according to the first member found, v-Maf oncogene (Musculo Aponeurotic Fibrosarcoma virus), and contains either large or small Mafs. All Mafs contain a bZip domain and an extended homology region that mediates DNA binding. Large Mafs are transcription factors that regulate tissue-specific gene expression and cellular differentiation in mammals. Small Mafs; MafG, MafK, and MafF, are highly homologous, small proteins (18 kDa), lacking the transactivation domain (Blank 2008). Several CNC family transcription factors heterodimerize with small Mafs and bind to the Maf recognition element (MARE) or ARE (Itoh et al. 1997, Itoh et al. 1995). Even though small Mafs cannot work as independent transactivators; they have a remarkable role as modulators of gene regulation. Small Mafs modulate gene expression via the CNC-family transcription factors and by inhibiting expression by binding to the target regulatory regions as homodimers (Kataoka et al. 1995a). The effects of small Mafs on gene expression have been studied by using small Maf knockout mice and cells. Knocking down an individual small Maf has no effect on the viability of mice. Deficiency of MafG and MafK together leads to severe defects in erythroid cell development and the defective capability of antioxidant and detoxification gene induction. The life span of those mice was short. Deficiency of all three small Mafs is lethal during embryogenesis (Blank 2008).

The regulation of small Maf genes is complex and has both temporal and tissue-specific variation. The MafG gene is regulated by electrophilic insults via Nrf2, serving as an autoregulatory feedback loop (Katsuoka et al. 2005). The MafK gene contains two brain tissue-specific promoters and specific enhancer areas for expression in hematopoietic and cardiac tissues. The promoter of MafF contains three promoter areas with unknown functions (Blank 2008). In addition to the regulation of gene expression, MafG is also post-translationally modified by acetylation and sumoylation. Acetylation is mediated by CBP and it increases the DNA binding activity of MafG (Hung et al. 2001). Extended homology region is a site for sumoylation and it is suggested that sumoylation plays a role in MafG mediated gene repression (Motohashi et al. 2006). Post-translational modifications are not essential but enhance MafG function and it is suggested that such modifications modulate functions of all Mafs (Blank 2008).

2.4.2 Antioxidant response element

In the nucleus, the Nrf2-small Maf heterodimer binds to the ARE (also called the electrophile response element, EpRE) in the regulatory region of target genes (Itoh et al. 1997, Itoh et al. 1997). Rushmore et al (1991) made the first identification of the ARE core as 5′-RGTGACnnnGC-3′ (n = A, C, G, or T) (Rushmore, Morton & Pickett 1991). They also identified cis-acting features and the response to oxidative stress by studying the ARE of the NAD(P)H: quinone oxidoreductase 1 (NQO1) gene. Wasserman & Fahl, by studying the ARE of the glutathione S-transferase gene, concluded that nucleotides outside of the previously identified core sequence are essential for inducible expression, and suggested a longer core sequence, 5′-TMAnn<u>RTGAY</u>nnn<u>GC</u>Rwwww-3′ (M = A or C, R = A or G, Y = C or T, w = A or T, core sequence underlined) (Wasserman, Fahl 1997). Erickson et al further studied the ARE of the glutamate-cysteine ligase modifier subunit (GCLM) and noticed that there is more variation than in the ARE core identified previously. The core sequence was modified to 5′-RTKAYnnnGCR-3′ (K = G or T, Y = C or T) (Erickson et al. 2002). Later on, Nioi et al reported that a G at Wassermans position 14 is not essential (Nioi et al. 2003). It was concluded that there is no universal ARE core sequence and that the ARE sequence varies among genes and species.

Single nucleotide polymorphisms (SNPs) in transcription factor binding sites may affect the binding of transcription factors and gene regulation. Human ARE SNPs were identified in a study where a position weight matrix (PWM, Figure 7) for the identification of polymorphic AREs was generated. PWM identified SNPs that may be important in the regulation of an Nrf2-dependent response. PWM can be used as a computational tool for searching and estimating functionality of putative AREs in the promoters of novel ARE regulated genes (Wang et al. 2007). SNPs in AREs may also play a role in susceptibility and pathogenesis of diseases.

The ARE consensus sequence has an internal activator protein-1 (AP-1) - binding site (5'-TGAC-3'), challenging the identification of ARE target genes. However AP-1 binding factors are not capable of binding ARE with high enough affinity that would promote transcription. Also, the reporter assay studies have demonstrated that the elements are activated by different stimuli (Nguyen, Sherratt & Pickett 2003).

An ARE serves as a binding site for multiple CNC-family transcription factors that regulate both basal and inducible expression of target genes. The same target gene can be regulated via an ARE by different transcription factors due to various stimuli and may be either transactivated or repressed (Kim et al. 2001).

Figure 7. Antioxidant response element position weight matrix. The size of the letter describes the importance of the nucleotide, generated in WebLogo (http://weblogo.berkeley.edu/logo.cgi).

2.4.3 BTB and CNC homology 1-a negative regulator of ARE

The activation of an ARE is known to be repressed by the CNC-family transcription factors, BACH1 and BACH2, and by small Maf protein homodimers. BACH1 and BACH2 are CNC transcription factor family members that have a repressive function. Via a CNC/bZip-domain, the BACH proteins dimerize with small Mafs and bind to DNA. At the N-terminal end, BACH proteins have a BTB-domain that mediates homodimerization. The function of homodimerization is unknown (Ito et al. 2009). BACH proteins form heterodimers with small Maf proteins, mainly with MafK, and regulate gene expression via competitive binding to the MARE. MARE is a regulatory element closely related to an ARE, with a main function of regulating proliferation and differentiation (Oyake et al. 1996). The functions of BACH2 are less studied. BACH2 is expressed in developing neuronal cells and B cells and it has a role in antibody class switching and antibody response. In oxidative stress, BACH2 counteracts Nrf2 and induces apoptosis (Igarashi, Sun 2006).

Transcriptional regulation of the BACH1 gene is poorly understood. There are three transcript variants (1, 2 and 3) of the BACH1 gene. Isoform A is a functional protein coded by transcript variants 1 and 2, which have different 5' untranslated regions (UTRs). Isoform B, translated from transcript variant 3, differs from the other two by having a distinct 5' UTR, 3' coding region and 3' UTR, resulting in a shorter protein that lacks DNA binding properties (Kanezaki et al. 2001). It has been suggested that the shorter isoform recruits the longer active isoform to the nucleus through the interaction with the BTB domain.

The mouse Bach1 gene contains two guanine-cysteine boxes in the promoter which bind Sp1. Sp1 regulates basal transcription of mouse Bach1 (Sun et al. 2001). The same study reported a putative ARE-like element downstream of the transcription start site (TSS). However, the functionality of this element was not fully addressed. In human and

mouse hepatic cell lines, BACH1 expression has been shown to be induced by an Nrf2 activating agent, tBHQ, both at the mRNA and protein levels. This suggests that BACH1 might be an Nrf2 target gene (Kaspar, Jaiswal 2010). The human BACH1 gene has also been suggested to contain a hypoxia response element based on the observations that Bach1 mRNA is induced in hypoxic conditions (Kitamuro et al. 2003). Translation of BACH1 is reported to be negatively regulated by miRNA-196 and miRNA-155, leading to the up-regulation of hemi oxygenase-1 (HO-1) (Hou et al. 2010, Yin et al. 2008).

BACH1 protein is regulated by phenolic antioxidants, oxidative stress and heme. BACH1 contains five cysteine residues near the CNC/bZip domain (Figure 8). Cys574 is modified by oxidative stress, leading to the nuclear export and release of the ARE. It has been proposed that the redox state of cysteine residues is critical for DNA binding and oxidation can trigger dissociation of BACH1 from its target sequence (Ishikawa, Numazawa & Yoshida 2005). Increased phosphorylation of tyrosine 486 caused by phenolic antioxidants leads to the nuclear export of BACH1, allowing Nrf2 to bind to the target gene AREs. Subsequently, BACH1 is induced and de novo synthesized BACH1 translocates to the nucleus (Kaspar, Jaiswal 2010). Heme binds to the C-terminal region of BACH1 where it has four dipeptide cysteine-proline (CP) motifs (Figure 8). Binding of heme to BACH1 inhibits DNA binding (Ogawa et al. 2001). Heme also triggers nuclear export of BACH1, mediated by NES and CRM1 (Suzuki et al. 2004), leading to the ubiquitination and degradation of BACH1 (Zenke-Kawasaki et al. 2007). BACH1 also regulates cellular senescence via negative regulation of p53. BACH1 forms a complex with p53 and histone deacetylase 1 that leads to the repression of p53 target genes (Dohi et al. 2008). Cellular senescence caused by oxidative stress is inhibited by BACH1 mediated modulation of the p53 pathway (Ota et al. 2011).

Relatively little is known about the target genes of BACH1. BACH1 lacks a transactivation domain and it is therefore unable to support transcription (Igarashi et al. 1998, Igarashi et al. 1998). The expression of β-globin is regulated via a locus control region during differentiation, when BACH1 is one of the players (Igarashi, Sun 2006). Under low cellular heme concentration or unstressed conditions, BACH1 binds to the β-globin ARE sequence, thereby repressing gene expression. BACH1 competes with Nrf2 for the ARE binding site. One of the main targets of BACH1 is HO-1, which is tightly regulated by BACH1 under physiological conditions (Sun et al. 2002, Reichard, Motz & Puga 2007, Reichard, Sartor & Puga 2008). BACH1 plays a role in turning off HO-1 expression after the induction. In hepatocytes, BACH1 regulates the expression of NQO1 and thioredoxin reductase 1 (TXNRD1) in response to tBHQ (Dhakshinamoorthy et al. 2005, Hintze et al. 2007). However, there are controversial results regarding TXNRD1 as a putative BACH1 target gene (Reichard, Motz & Puga 2007). A high-throughput sequencing study with BACH-chromatin immunoprecipitation (ChIP) combined with microarrays suggests that BACH1 has a more significant role in the regulation of metabolism and signal transduction than was previously thought. The study identified 59 genes that were bound by BACH1 and up-regulated by the siRNA against BACH1. The new target genes are involved in cell proliferation and neurodegenerative processes (Warnatz et al. 2011). The importance of BACH1 in proliferation is also evident in vivo. Neointima formation in BACH1 deficient mice was significantly less than in wild type mice in the vascular cuff model (Omura et al. 2005). Deficiency of BACH1 in atherosclerosis-prone apolipoprotein E deficient mice ($ApoE^{-/-}$) mice demonstrated that BACH1 has a role in atherosclerotic lesion formation via inhibition of HO-1 (Watari et al. 2008). However, BACH1 may not be a universal negative regulator of the ARE-dependent genes and there may be cell type specific differences in the regulation (Warnatz et al. 2011).

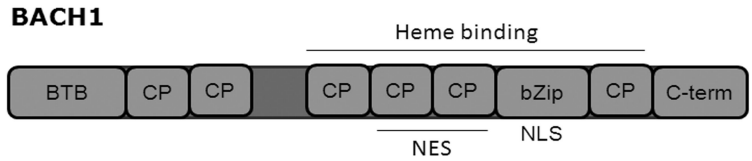

Fig 8. Structure of the BACH1 protein. BACH1 domains include BTB, bZip, a C-terminal domain and six CP motifs. BTB mediates homodimerization, bZip helps homodimerization with small Mafs, and four C-terminal CPs form a binding site for heme. NLS is localized to the bZip domain and heme regulated NES in CP motifs. Modified from (Igarashi, Sun 2006).

2.5 VASCULAR EFFECTS OF NRF2 AND ITS TARGET GENES

2.5.1. Role of Nrf2 in shear stress

In arteries, Nrf2 is activated by shear stress, the force caused by normal blood flow. This leads to ARE activation, induction of target genes, and Nrf2-dependent inhibition of vascular cell adhesion molecule 1 (VCAM1) (Chen et al. 2003). Instead, in endothelial cells of areas with low shear stress, Nrf2 is not activated and cells are susceptible for atherogenic changes (Zakkar et al. 2009).

The mechanisms by which Nrf2 is activated by shear stress have been explored to some degree. One possible mechanism is COX-2 mediated production of the Nrf2 activating agent, 15d-PGJ_2, which is increased by shear stress (Hosoya et al. 2005). It has been proposed that Nrf2 activation during shear stress protects against ROS and RNS mediated inflammation. Production of ROS and RNS is increased in low shear areas, where Nrf2 expression is decreased (Takabe, Warabi & Noguchi 2011). Upon shear stress ROS and RNS are produced at low levels, thus maintaining Nrf2 activation directly or via initiation of lipid peroxidation (Takabe, Warabi & Noguchi 2011). Nrf2 activation inhibits TNF-α mediated induction of VCAM1 and monocyte chemoattractant protein 1 (MCP1) (Chen et al. 2003, Hosoya et al. 2005). SFN mediated Nrf2 activation inhibits VCAM1 expression via suppression of p38-signaling both in endothelial cell cultures as well as in murine arteries at low shear stress areas (Zakkar et al. 2009). Shear stress also induces the expression of Krüppel-like factor 2 that enhances Nrf2 activation (Fledderus et al. 2008). In summary, the activation of Nrf2 by shear stress plays an anti-atheroprotective role in the vascular endothelium by maintaining cellular redox homeostasis.

2.5.2 Role of Nrf2 in atherogenesis

The role of Nrf2 in atherogenesis is not fully established. The protective effects of Nrf2 are largely mediated by its target genes, HO-1 and glutamate-cysteine ligase (GCL), the rate-limiting enzyme of glutathione (GSH) synthesis (Ishii et al. 2004, Bea et al. 2003). In endothelial cells, Nrf2 has role in the protection and maintenance of physiological homeostasis induced by shear stress. In macrophages, Nrf2 dependent HO-1 activation inhibits lipopolysaccharide mediated inducible NOS expression (Kuhn et al. 2011). Nrf2 also plays a role in macrophage phenotype transformation in the intima. OxPAPC has been proposed to modify macrophage phenotype via Nrf2. This novel phenotype is specific for atherosclerotic lesions (Kadl et al. 2010). On the other hand, it has been shown that Nrf2 increases the expression of the CD36 scavenger receptor of macrophages that recognizes oxLDL leading to increased foam cell formation (Ishii et al. 2004).

In SMCs, Nrf2 inhibits proliferation in addition to the protective functions mediated by target genes (Anwar et al. 2005). Nrf2 dependent inhibition of proliferation has also been shown in vivo in the rabbit aortic balloon injury model. Local adenoviral Nrf2 over-expression inhibits SMC proliferation, oxidative stress, and inflammation. However, Nrf2 over-expression does not affect neointima hyperplasia. The reason for this might be

that the survival of cells is improved even if the proliferation rate is decreased (Levonen et al. 2007).

In an atherosclerotic mouse model ,ApoE$^{-/-}$, instead, the lack of Nrf2 protects against atherosclerosis. It has been suggested that this is because of Nrf2-dependent induction of CD36, which leads to increased oxLDL uptake and accelerated atherogenesis (Barajas et al. 2011, Sussan et al. 2008). However, there are no reports from other physiological models of atherosclerosis.

2.5.3 General role and vascular features of Nrf2 target genes

Nrf2 is a strong transactivator that regulates basal and inducible expression of antioxidant and detoxification enzymes. Nrf2 target genes are involved in glutathione synthesis, ROS and RNS elimination, xenobiotic metabolism, and drug transport. The development of new sequencing techniques has offered a new platform to study the targets of transcription factors. The Nrf2 dependent gene expression battery has been studied by microarray based expression profiling and high-throughput sequencing in combination with Nrf2-ChIP. Studies confirm the role of Nrf2 as a protector against toxic conditions and also highlight the less studied role of Nrf2 in proliferation control (Malhotra et al. 2010). Nrf2 is ubiquitously expressed in the vasculature. Herein, the functions of selected target genes and their roles in vascular biology and pathogenesis are reviewed.

2.5.3.1 Glutathione system

GSH is one of the most important antioxidants in human cells. The intracellular concentration of GSH is between 1-5 mM depending on the cell type. GSH functions as a cofactor for an H_2O_2 metabolizing enzyme, glutathione peroxidase, and as an electron transporter in Glutathione-S-transferase catalyzed detoxification reactions, for neutralizing xenobiotics and their metabolites. The rate-limiting enzyme of GSH synthesis is GCL that has two subunits; catalytic and modifier subunits (GCLC, GCLM). GCL catalyzes the reaction of glutamate and cysteine. The cellular level of GSH regulates the enzymatic activity of GCLC, thus this provides a negative feedback system (Huang et al. 1993). GCLM regulates the kinetic features of GCLC catalyzed reactions by decreasing the saturation constant of glutamate and by elevating the GSH inhibition constant. The role of GCLM becomes important when concentrations of glutamate and cysteine decrease below the physiological rate levels (Huang, Anderson & Meister 1993). In the next step, glycine is added to the γ-glutamylcysteine in a reaction catalyzed by glutathione synthetase, producing the end product GSH.

Genes coding GCLC and GCLM are regulated by multiple transcription factors and promoter elements, such as Nrf2 and AP-1 (Dickinson et al. 2004). Functional transcription factor and response elements are dependent on activating conditions and cell types. The Nrf2-ARE -signaling is one of the main regulatory pathways. Both GCLC and GCLM contain AREs (Ericson a 2002). The levels of GCLC and GCLM are decreased in Nrf2 deficient tissues and cells.

GCL deficiency or poor functionality leads to the decrease or even loss of GSH synthesis. GCLC$^{-/-}$ mice completely lack GSH synthesis, which is lethal during embryogenesis. GCLM$^{-/-}$ mice do not have a clear phenotype, but their GSH and cysteine levels are reduced compared to wild type mice (Yang et al. 2002). GCLM$^{-/-}$ mice are also more sensitive to the effects of oxidative stress. GCLC and GCLM promoter polymorphisms have been identified (Koide et al. 2003, Nakamura et al. 2002). Polymorphisms decrease inducible GSH synthesis, which impairs cellular defense against oxidative stress. Deficiency of GSH also decreases the sensitivity of endothelial cells to $^{\cdot}NO$ and promotes endothelial dysfunction. Such polymorphisms are also associated with a higher risk of myocardial infarction in humans (Nakamura et al. 2003).

2.5.3.2 Heme oxygenase-1

HO-1 is an enzyme involved in heme degradation that has multiple protective effects. HO-1 is activated by a host of different activators and stress conditions. The basal activity of HO-1 is repressed during cellular homeostasis by BACH1 and the inducible activity is highly regulated by Nrf2. Both the repression in basal conditions and inducible expression during cellular stress are mediated by the ARE (Alam et al. 1999). The HO-1 promoter carries two ARE regions, the proximal and distal enhancers. BACH1 and Nrf2 bind both AREs of HO-1, but bind the distal ARE with higher affinity and in a reciprocal manner (Reichard, Motz & Puga 2007). In addition to the Nrf2-ARE pathway, other transcription factors and regulatory elements take part in the inducible expression of HO-1 (Alam et al. 2004). The effects of HO-1 are mediated via a reaction in which heme is metabolized to biliverdin and further to bilirubin by biliverdin reductase. The reaction also produces carbon monoxide (CO) and iron. CO plays a role as a signaling molecule especially in vascular cells and it has an important role in cardiovascular diseases. CO is anti-inflammatory, it prevents platelet aggregation, and it inhibits apoptosis of endothelial cells and fibroblasts. Thus CO is an anti-inflammatory and antiapoptotic molecule in the vasculature. The free iron released is stored as ferritin. Bilirubin is an important antioxidant that has a role as a scavenger of lipid peroxides (Otterbein et al. 2003).

Polymorphisms of human HO-1 promoter guanine-thymine-repeats are associated with cardiovascular diseases. Studies in the Japanese population have suggested that the lengthening of guanine-thymine-repeats in the presence of other risk factors associates with increased prevalence of atherosclerosis (Kaneda et al. 2002). In human atherosclerotic lesions, HO-1 expression is increased and the expression level correlates with the increased expression of proinflammatory genes. In a mouse study, HO-1 promoted progression of the plaque by increasing the thickness of the fibrous cap and by enhancing SMC accumulation. In contrast, the necrotic core area and lipid deposition inside of the plaque was reduced. These effects were due to HO-1 mediated protection of SMC survival that leads to increased plaque stability (Cheng et al. 2009).

2.5.3.3 NAD(P)H: quinone oxidoreductase-1

NQO1 is quinone and quinoneimide detoxifying enzyme, and its expression is regulated via ARE both in basal conditions and during oxidative stress. Nrf2 deficiency impairs the constitutive expression of NQO1 and inhibits its induction, and NQO1 is often referred to a classical Nrf2-dependent gene (Nioi, Hayes 2004).

NQO1 also has a function in recycling cellular antioxidants, vitamin E and ubiquinone, after radical attacks. Quinones are reactive molecules found in exhaust fumes and cigarette smoke that cause DNA damage leading to cancer and neurodegenerative diseases. Partially reduced quinones produce O_2^- and H_2O_2 in a redox cycle. The injurious effects of quinones are a consequence of their metabolism in one electron chain reaction, catalyzed by p450 enzymes. The reaction produces a molecules containing oxygen with a highly reactive unpaired electron that leads to the formation of new radicals. In reactions catalyzed by NQO1, two electrons of the target molecule are reduced, and then a stabile molecule is formed and conjugated to the secretory product (Nioi, Hayes 2004). Ubiquinone and vitamin E are important liposoluble antioxidants that protect cells against lipid peroxides. NQO1 reduces ubiquinone to ubiquinolol (Nioi, Hayes 2004). Vitamin E is converted to α-tokoferolquinone during a radical attack and further to α-tokoferolhydroquinone in an NQO1 catalyzed reaction. α-tokoferolhydroquinone is an antioxidant that protects cell membranes during injury (Siegel et al. 1997). NQO1 also has a role in stabilizing p53 by inhibiting its degradation via an unknown mechanism. As well, $NQO1^{-/-}$ mice express lower levels of p53 compared with controls (Long et al. 2002). These studies demonstrate the important role of NQO1 in cancer prevention (Nioi, Hayes 2004).

Recently, the role of NQO1 in vascular diseases has been studied and it has been proposed that NQO1 plays a role in the development and progression of atherosclerosis via regulation of SMC proliferation. Pharmacological induction of NQO1 has also been shown to prevent arterial restenosis via inhibition of SMC proliferation (Kim et al. 2009). It has also been demonstrated that single nucleotide polymorphisms in NQO1 lead to a decreased level of functional protein and are associated with carotid artery plaques in type two diabetic patients (Han et al. 2009).

2.5.3.4 Thioredoxin system

2.5.3.4.1 Thioredoxin 1 - Thioredoxin Reductase 1

Expression of thioredoxin (TXN/TRX-1) and TXNRD1 genes are both regulated via ARE and Nrf2 at the transcriptional level (Kim et al. 2001, Sakurai et al. 2005). TRXs are ubiquitously expressed and highly conserved antioxidant enzymes that reduces oxidized thiol groups in proteins. The TXN family contains two known members; cytoplasmic and nuclear TRX-1, and mitochondrial TRX-2. Both forms have a critical -Cys-Gly-Pro-Cys- site, which is essential for reduction-oxidation function (Cys32 and Cys35 in TRX-1). During the reduction of proteins, thioredoxin itself oxidizes. TXNRD1 is an enzyme that reduces TRXs in an NAD(P)H-dependent reaction (Holmgren, Lu 2010). Cytosolic TRX-1 regulates cell death and survival signaling pathways by controlling interactions of TRX-binding proteins (Saitoh et al. 1998). Under oxidative stress, TRX-1 translocates to the nucleus where it modulates the DNA-binding activity of transcription factors (Altschmied, Haendeler 2009). In healthy vessels, TRX-1 is mainly expressed in SMCs but in vessels containing atherosclerotic lesions TRX-1 is also expressed in the endothelium and in macrophages (Okuda et al. 2001). Thioredoxin is an important inhibitor of apoptosis signaling kinase 1 (ASK-1, Figure 9B). TRX-1 binds to the N-terminus of ASK-1 both in vitro and in vivo, thus inhibiting its downstream effects (Saitoh et al. 1998). If the activity of TRX-1 is inhibited, TNF-α phosphorylates ASK-1, leading to the activation of downstream p38 and Jun-terminal kinase (JNK) (Ichijo et al. 1997). Downstream kinases mediate the expression of adhesion and inflammatory genes, such as VCAM1. TRX-1 also has a role in signaling pathways mediated by physiological concentrations of ROS in endothelial cells. One mechanism is the modification of Cys69 by ·NO that leads to increased TRX-1 redox-regulatory activity, reduction of intracellular ROS, and inhibition of apoptosis (Haendeler et al. 2004, Haendeler et al. 2002). The activity of TRX-1 is increased in shear stress. Increased S-nitrosylation of TRX-1 and decreased S-nitrosylation of TXNIP might be the main mechanisms (Hoffmann, Dimmeler & Haendeler 2003, Yamawaki et al. 2005). Recently, it has been proposed that mitochondrial TRX-2 also plays a role in cardiovascular diseases because its function is similar to that of TRX-1 (Dai et al. 2009).

2.5.3.4.2 Thioredoxin Interacting Protein

TXNIP (also termed vitamin D-upregulated protein 1, VDUP1) inhibits TRX-1 activity in a redox-dependent manner by binding reduced TRX-1 but not oxidized TRX-1 (Nishiyama et al. 1999). The oxidation status of TXNIP is also important. TXNIP has two important cysteines; Cys63 and Cys247 that mediate TRX-1 binding through disulfide complex formation (Patwari et al. 2006). In the endothelium, shear stress down-regulates TXNIP expression to support the cytoprotective effects of TRX-1. In contrast, in low or turbulent flow TXNIP expression is induced and this leads to the formation of TRX-1-TXNIP complexes. This results in a release of ASK-1 from TRX-1 and the activation of the ASK-1 - TNF-α mediated pro-inflammatory pathway (Yamawaki et al. 2005). In SMCs, ·NO reduces TXNIP expression and enhances TRX-1 activity without affecting the TXR-1-TXNIP complex formation, supporting the protective role of ·NO in the vascular wall (Schulze et al. 2006). ·NO also induces protein S-nitrosylation via transcriptional repression of TXNIP, thus

releasing TRX-1, which then acts in the denitrosylation pathway, protecting cells against nitrosative stress (Forrester et al. 2009). The TRX-1-TXNIP-complex also has a role in redox-dependent regulation of vascular endothelial growth factor receptor-2 signaling that supports survival in endothelial cells (World, Spindel & Berk 2011).

The transcriptional regulation of TXNIP is mainly studied in pancreatic and cancer cell lines, where it is regulated via multiple transcription factors and stimuli. Only a few studies demonstrate the transcriptional regulation of TXNIP in vascular cells. In human aortic endothelial cells TXNIP expression is induced in glucose mediated manner by the forkhead box O1 transcription factor, which leads to increased ROS production (Li et al. 2009). In macrophages, TXNIP is transcriptionally regulated via an AP-1 site. The binding of c-Jun and c-Fos to the TXNIP AP-1 element is inhibited in the presence of PPARα or PPARα agonist GW647. However, even though the regulatory region of TXNIP contains a putative PPAR binding site it does not bind it directly (Billiet et al. 2008).

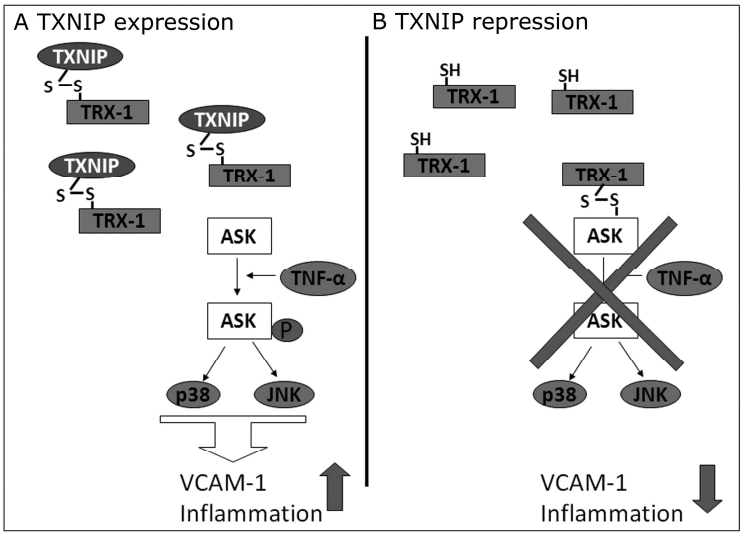

Figure 9. The TRX-1-TXNIP system. A, When TXNIP is expressed it binds to TRX-1, leading to the activation of the ASK-1 pathway and increased expression of adhesion molecules and proinflammatory genes. B, During repression of TXNIP, TRX-1 binds ASK-1 to inhibit its activity and decrease inflammation. Modified from (Harrison 2005).

3 Aims of the study

The aim of this thesis research was to explore the activation mechanisms of Nrf2 by agents relevant to cardiovascular diseases, profiling new target genes and finding regulatory mechanisms in human and mouse vascular endothelial cells.

I To investigate whether oxPLs evoke nuclear translocation of Nrf2 and activation of target gene expression in human endothelial cells and mouse carotid arteries, and to identify the specific phospholipids that are responsible for Nrf2 activation.

II To elucidate the role of OA-NO_2 as an Nrf2 activating agent and explore OA-NO_2 inducible, Nrf2-dependent and -independent genes to through a genome-wide approach.

III To investigate whether transcription factor BACH1 is a novel Nrf2 target gene and identify the genomic region responsible for the Nrf2 mediated effect.

IV To demonstrate the role of Nrf2 in down-regulation of TXNIP and explore the mechanisms by which Nrf2 plays a role in negative gene regulation.

4 Materials and methods

4.1 NRF2 ACTIVATING AGENTS AND CELL CULTURE (I-IV)

Phospholipids were purchased from Avanti Polar Lipids or Sigma or synthesized as described (Watson et al. 1999). OA-NO_2 was synthesized as previously described (Woodcock S R et al. 2006, studies II, III, IV). SFN (Sigma-Aldrich, studies III, IV), and tBHQ (Sigma-Aldrich, study III) are commercially available. Human umbilical vein endothelial cells (HUVECs, studies I-IV) were isolated from umbilical cords obtained from the maternity ward of Kuopio University Hospital, by the approval of the Kuopio University Hospital Ethics Committee, and cultivated as previously described (Levonen et al. 2004). Human embryonic kidney 293T cells (HEK293T, studies III, IV) were purchased from ATCC. HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich), supplemented with 10% (v/v) fetal bovine serum (HyClone) and 1% penicillin/streptomycin (Invitrogen-Gibco).

4.2 CLONING OF PLASMIDS (I-IV)

For luciferase reporter assays, wild type or mutated AREs of NQO1 or BACH1 were cloned into the pGL3-promoter vector (studies I, II, and IV) or pGL4-promoter vector (study III, Promega) containing the minimal thymidine kinase promoter (Turunen et al. 2007). For study IV, the full length TXNIP promoter region was PCR amplified from DNA isolated from human blood cells and cloned into the pGL3-backbone. Specific oligonucleotides containing the required restriction sites were acquired from TAG Copenhagen. Complementary oligonucleotides were annealed and ligated into their respective restriction sites of the pGL3- or pGL4-promoter vector (Promega) and verified by sequencing. Details of the primers and cloning are described in studies I-IV.

4.3 CLONING OF ADENOVIRUSES (III, IV)

The cloning of AdNrf2 has been previously described (Levonen et al. 2007). The pAd-Keap1 was cloned by excising the BamHI-XhoI fragment containing the human Keap1 cDNA from pCDNA3 and ligating the fragment into pAdCMV. The pAd-Keap1 was then used to generate the recombinant adenoviruses using standard techniques (Kozarsky, Wilson 1993). AdNrf2 was used in studies III and IV, AdKeap1 in study IV.

4.4 SIRNA TRANSFECTIONS (I-IV)

Small interfering RNA (siRNA) oligonucleotides targeting Nrf2 (studies I-IV), HSF1 (study II), Nrf1 (study III), and a non-specific RNA control were obtained from Invitrogen. HUVECs were transfected with 50 nM siRNA oligonucleotides using Oligofectamine (Invitrogen).

4.5 RNA ISOLATION AND QUANTITATIVE REAL-TIME PCR (I-IV)

Messenger RNA was extracted with TRI-reagent (Invitrogen) and 0.5-1 μg of total-RNA was used for the cDNA synthesis using random hexamer primers (Promega) and ReverAid

M-MuLV reverse transcriptase (Fermentas Life Sciences). The relative expression levels of target mRNA were measured according to the manufacturer's protocol by quantitative RT-PCR (StepOnePlus™ Real-Time PCR systems, Applied Biosystems), using specific Assays-on-Demand (Applied Biosystems). The expression levels were normalized to the level of ribosomal RNA or β_2-microglobulin (Applied Biosystems). The target mixes used are presented in Table 1.

Table 1. Analyzed genes and used assays for quantitative RT-PCR

Gene	Assay ID	Study
Nuclear factor-E2 Related Factor 2, *NFE2L2*	Hs00232352_1	I, II, III, IV
Nuclear factor-E2 Related Factor 1, *NFE2L1*	Hs00231457_m1	III
Heme Oxygenese-1, *HO-1*	Hs00157965_m1	I, II
NAD(P)H; quinone oxidoreductase 1, *NQO1*	Hs00168547_m1	I, II, III
Glutamate-cysteine ligase modifier subunit, *GCLM*	Hs00157694_m1	I, II
BACH1 transcript variant 1 and 2	Hs00230917_m1	III
BACH1 transcript variant 1	Custom assay	III
BACH1 transcript variant 3	Hs01110003_s1	III
BACH1 all transcript variants	Hs00895421_m1	III
β_2-microglobulin, *B_2M*	Hs00187842_m1	II, III
Heat shock 70 kDa protein 1A, *HSPA1A*	Hs00359147_s1	II
DNAJ (Hsp40) homolog A4, *DNAJA4*	Hs00388055_m1	II
Heat shock 22 kDa protein 8, *HSPB8*	Hs00205056_m1	II
Heat shock 70 kDa protein 6, *HSPA6*	Hs00275682_m1	II
Heat shock factor 1, *HSF1*	Hs00232134_m1	II
Thioredoxin Interacting protein 1, *TXNIP*	Hs00197750_m1	IV
Thioredoxin, *TXN*	Hs00828652_m1	IV
Thioredoxin reductase 1, *TXNRD1*	Hs00182418_m1	IV
Heme Oxygenase-1, *Ho-1*	Mm00516004_m1	I
NAD(P)H; quinone oxidoreductase 1, *Nqo1*	Mm00500821_m1	I
Glutamate-cysteine ligase modifier subunit, *Gclm*	Mm00514996_m1	I

4.6 WESTERN BLOTTING (I, II, IV)

Cells were lysed and the total protein concentration was measured with a BCA-assay (Pierce). Ten to 20 μg of protein were used for electrophoresis. The proteins were transferred to a nitrocellulose membrane, blocked, and incubated with primary antibodies. The primary antibodies used were rabbit polyclonal anti-Nrf2 (sc-722, Santa Cruz Biotechnologies, studies I and II), rabbit polyclonal anti-HO-1 (Stressgen, studies I and II), rabbit monoclonal anti-GCLM (a gift from Dr. Terrance Kavanagh, University of Washington, Seattle, WA, studies I and II), mouse monoclonal anti-NQO1 (Clones A180 and B771, gifts from Dr. David Ross, University of Colorado, Denver, CO, studies I and II), rabbit polyclonal β-actin (Cell Signaling, studies I, II and IV), rabbit polyclonal anti-Lamin B1 (Abcam, studies I and II), mouse monoclonal anti-HSP70/72 (Assay Designs, study II), rabbit polyclonal anti-HSF1 (Cell Signaling, study II), and rabbit polyclonal anti-VDUP1 (C-terminal, 403700, Invitrogen). Blots were visualized using HRP-conjugated secondary antibodies and Supersignal™ chemiluminescence substrate (Pierce, Rockford, IL).

4.7 LUCIFERASE REPORTER GENE ASSAY (I-IV)

Activation of the ARE by Nrf2 activating agents or Nrf2 co-transfection was measured using the luciferase Reporter Assay (Promega) with HUVECs (studies I and II) or HEK293T cells (studies III and IV). In studies I and II, HUVECs were transfected with an empty pGL3-promoter vector, NQO1-ARE or NQO1-AREmut luciferase reporter plasmids as in ((Levonen et al. 2004)). Sixteen hours after transfection, the cells were treated with 50 μg/ml of oxPAPC (study I) or increasing concentrations of OA-NO_2 (study II supplement). After 16 h, the cells were collected and analyzed as described. In study III, HEK293T cells were transfected with wild type or mutated NQO1-, BACH1-ARE1 or BACH1-ARE2 and 16 h after transfection treated with SFN, tBHQ or OA-NO_2 for 24 h. In addition, HEK293T cells were co-transfected with BACH1-ARE1 or NQO1-ARE and Nrf2 with or without Keap1. In study IV, HEK293T cells were transfected with the TXNIP promoter construct with or without Nrf2 and Keap1.

4.8 CHROMATIN IMMUNOPECIPITATION (CHIP, I-IV)

ChIP was performed as described in study I with some modifications. HUVECs or HEK293T cells were treated with Nrf2 activating agents for 2 to 3 h. DNA-protein complexes were cross-linked with 1% formaldehyde and stopped with 0.125 mol/l glycine. Nuclear pellets were extracted and chromatin was sonicated to 200 to 1000 bp fragments by a Bioruptor UCD-200 (Diagenode). Cellular debris was removed by centrifugation and samples were prepared for immunoprecipitation. The following antibodies were used in immunoprecipitations; anti-Nrf2 (sc-722, Santa Cruz Biotechnologies, studies I, II, III), anti-Maf F/G/K (sc-22831, Santa Cruz Biotechnologies, study III), anti-HSF1 (SPA901, Stressgene, study II), anti-BACH1 (sc-14700, Santa Cruz Biotechnologies, study III) or nonspecific IgG (anti-rabbit IgG, Upstate Biotechnology, all studies). Immunocomplexes were collected and washed, reverse cross-linked and then DNA was extracted for PCRs. Immunoprecipitated chromatin DNA was then used as a template for reverse trasnscriptase-PCR or quantitative real-time PCR. ChIP protocols, PCR primers and amplification protocols are described in detail in their respective studies.

4.9 APPLICATION OF OXPAPC TO THE CAROTID ARTERIES AND IMMUNOHISTOCHEMISTRY (I)

$Nrf2^{-/-}$ mice were a kind gift from Dr. Masayuki Yamamoto, University of Tsukuba, Japan, and the mouse creation approach has been previously described (Chan et al. 1996, Itoh et al. 1997). Wild type C57Bl/6 were originally obtained from the Jackson laboratory. Mice were housed in groups and maintained in controlled conditions in the National Laboratory Animal Center in Kuopio. Experiments were approved by the Experimental Animal Committee of the University of Kuopio and the National Experimental Animal Board. The application of oxPAPC, dissolved in pluronic gel, to mouse carotid arteries was performed as previously described (Furnkranz et al. 2005). Both genders of wild type or $Nrf2^{-/-}$ mice (6 mo of age) were used for the study. The animals were anesthetized using ketamine (Ketalar, Pfizer), xylazin (Rompun Vet, Bayer) and Lidocaine (Lidocain Orion Pharma). The left carotid artery was exposed through a midline cervical incision and 60 μl of 30% pluronic gel with or without 50 μg/ml oxPAPC was applied on the carotid artery. The wound was closed, animals were sacrificed with carbon dioxide 6 h after the surgery and carotid arteries were collected for mRNA analysis with quantitative PCR, or 24 h after for immunohistochemistry. For immunohistochemistry, carotid arteries were cast in paraffin and stained against rabbit polyclonal anti-HO-1 (Stressgene) and anti-NQO1 (Abcam), the

avidin-biotin-horseradish peroxidase system (Vector laboratories) and DAB-plus kit (Zymed laboratories) were used for signal detection.

4.10 MICROARRAY ANALYSIS (II)

For microarray analysis, HUVECs from three separate donors were pooled and transfected with 50 nM Nrf2 or non-specific control siRNA using Oligofectamine (Invitrogen). Twenty-four hours after transfection, cells were treated with methanol (vehicle), 3 μM OA, or 3 μM OA-NO_2 for 8 h. The mRNA was extracted with TRI-reagent (Sigma) according to the manufacturer's protocol and probes for the expression analysis were made according the Affymetrix protocol. Complementary RNA was hybridized to Human Genome U133 Plus 2.0 GeneChips (Affymetrix) and chips were stained, washed (Affymetrix Fluidics Station 400) and scanned (Affymetrix GeneChip Scanner 3000) according to the manufacturer's protocol. Microarray data were extracted from scanned images by using MAS 5.1 software (Affymetrix) and GeneChip Operating Software (GCOS, Affymetrix) was used to generate CEL files. The details of data handling and statistical analysis are discussed in study II.

4.11 ELECTROMOBILITY SHIFT ASSAY (III)

For the electromobility shift assay (EMSA), the DNA binding region of Nrf2 (amino acids 327-605 (Wild, Moinova & Mulcahy 1999)) and MafG cDNA (German Research Center for Genome Research, Germany) were amplified by PCR and cloned into the pcDNA3 vector with a Kozac sequence. Nrf2 and MafG proteins were generated by coupled in vitro transcription/translation using a TNT Quick Coupled Transcription/Translation kit as recommended by the supplier (Promega, Madison, WI, USA). The proteins were incubated with [^{32}P]-labeled double-stranded oligonucleotides containing one copy of the respective response elements. Protein-DNA complexes were resolved by electrophoresis and quantified on an FLA-3000 reader (Fuji, Tokyo, Japan) using ScienceLab99 software (Fuji). Further details are described in study III.

4.12 STATISTICAL ANALYSIS (I-IV)

Statistical analyses were performed with GraphPad Prism Software (GraphPad Software, Inc.). Statistical significance was evaluated by unpaired t-test or one-way ANOVA using Bonferroni's post hoc comparisons. Results are expressed as mean±SEM and differences depicted as *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5 Results

5.1 OXPAPC AND OA-NO_2 ACTIVATES NRF2 DEPENDENT TARGET GENE EXPRESSION (I-II)

Nrf2 has been shown to be activated by multiple agents. Herein we studied the effect of two agents relevant to cardiovascular disease, oxPAPC and OA-NO_2, on the activation of Nrf2 and the induction of target genes HO-1, GCLM, and NQO1. Both oxPAPC and OA-NO_2 induce Nrf2 target genes in concentration and time dependent ways in HUVECs (studies I and II). The nuclear accumulation of Nrf2 increased upon oxPAPC treatment time-dependently (Figure 10A) and the translocation is reduced when siRNA against Nrf2 was used (Figure 11A).

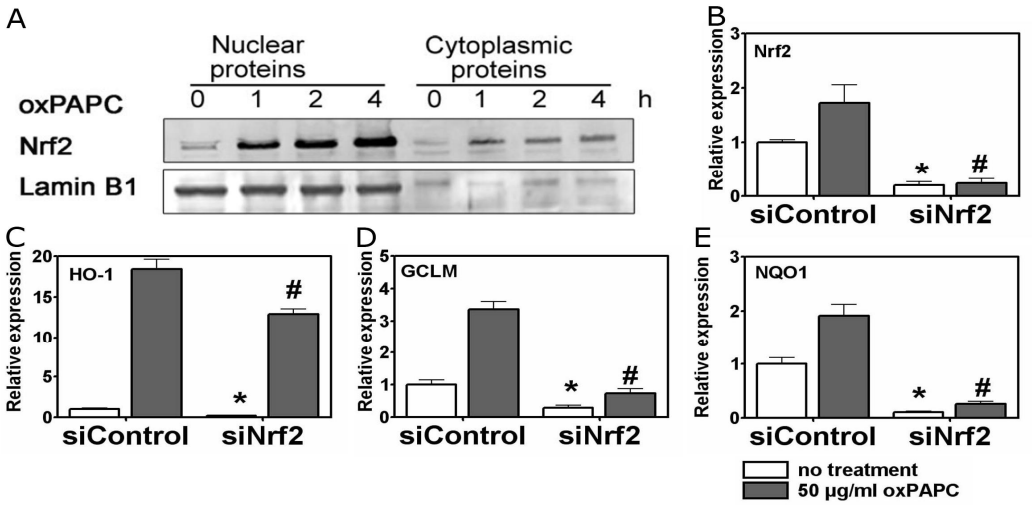

Figure 10. OxPAPC induce nuclear accumulation of Nrf2, and the expression of Nrf2 target genes Nrf2-dependently. A, Nuclear and cytoplasmic Nrf2 from HUVECs treated with 50 µg/ml oxPAPC 0 to 4 h detected by Western blotting. Lamin B1 was used as a nuclear protein control. B-E, HUVECs were transfected with 50 nM siControl or siNrf2 and cells were treated with 50 µg/ml oxPAPC 24 h after transduction for 8 h. The expression of Nrf2 (B), HO-1 (C), GCLM (D), and NQO1 (E) were detected by quantitative RT-PCR. The expression was normalized to the ribosomal RNA, and presented as fold change compared to untreated siControl. Values are presented as mean±SEM, (n=3), *$p<0.05$ vs. untreated siControl, #$p<0.05$ vs. oxPAPC treated siControl. Modified from study I.

OxPAPC and OA-NO_2 induce the activation of Nrf2 and the increase in target gene expression was reduced with siNrf2 (Figures 10B-E, and Figures 11B-E). Induction of GCLM (Figure 10D, and Figure 11D) and NQO1 (Figure 10E, and Figure 11E) mRNA and protein expression (studies I and II) was blocked when siNrf2 was used. HO-1 (Figure 10C, and Figure 11C) is not blocked as effectively as other Nrf2 targets with siNrf2, suggesting that there are redundant pathways taking part in the induction.

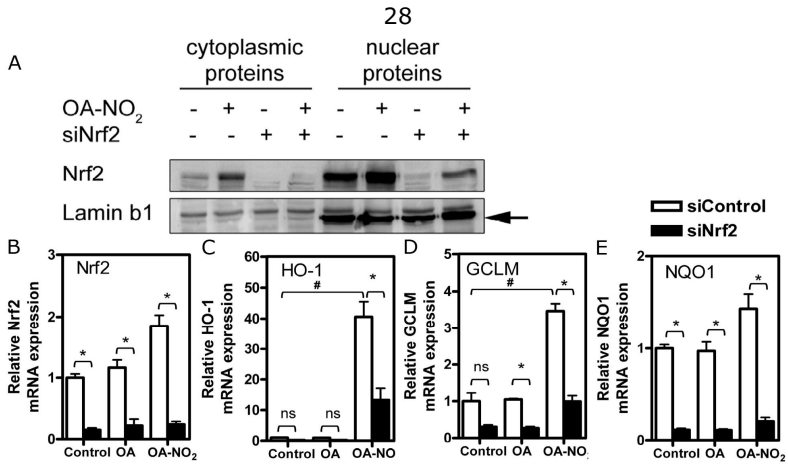

Figure 11. OA-NO_2 induce nuclear accumulation of Nrf2, and the expression of Nrf2 target genes Nrf2-dependently. HUVECs were transfected with 50 nM siControl or siNrf2 and cells were treated with 3 μM OA-NO_2 24 h after transduction for 8 h. A, Nuclear and cytoplasmic Nrf2 were detected by Western blotting. Lamin B1 was used as a nuclear protein control. B-E, The expression of Nrf2 (B), HO-1 (C), GCLM (D), and NQO1 (E) was detected by quantitative RT-PCR, normalized to the ribosomal RNA, and presented as fold change compared to the untreated siControl. Values are presented as mean±SEM, (n=3), *p<0.05 vs. relative siControl. Modified from study II.

The activation of ARE-dependent expression was studied by the luciferase reporter assay. Both oxPAPC and OA-NO_2 activate NQO1-ARE and the activation was inhibited by point mutation of the ARE (Figures 12A, and B). Binding of Nrf2 to NQO1 and HO-1 AREs was verified by ChIP (Figures 12C, and D).

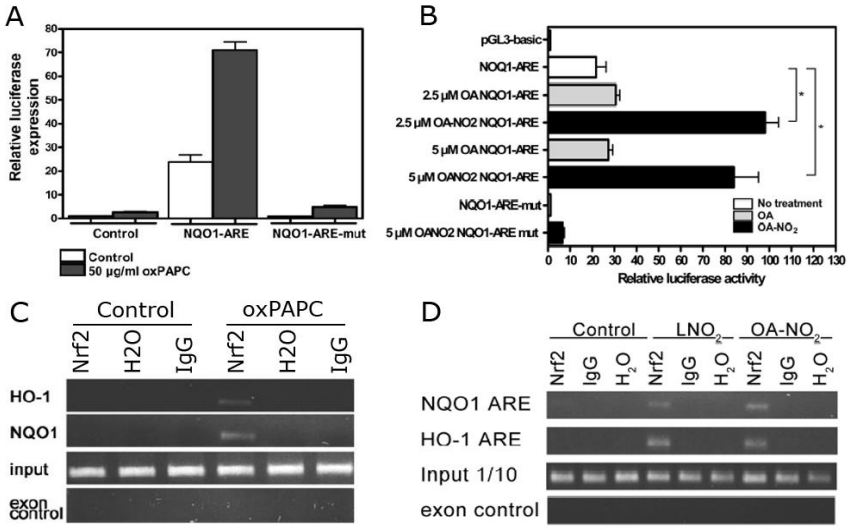

Figure 12. OxPAPC and nitrolipids activate AREs and induce the binding of Nrf2 to the ARE of HO-1 and NQO1. A-B, For the luciferase assay, HUVECs were transfected with empty pGL3 - promoter vector as a control, or vector containing wild type or mutated NQO1-ARE. Twenty-four hours after transfection, cells were treated with 50 mg/ml oxPAPC, 2.5 or 5 μM OA, or 2.5 or 5 μM OA-NO_2 for 16 h. Luciferase activity was normalized to total protein or β-galactosidase and presented relative to empty vector control. Values are presented as mean±SEM, (n=3-4), B, *p<0.05 vs. relative control. C-D, HUVECs were treated with 50 μg/ml oxPAPC for 3 h, 3 μM LNO_2 or OA-NO_2 for 4 h. Binding of Nrf2 to the ARE of the HO-1 or NQO1 was analyzed by ChIP. Rabbit IgG and H_2O were used as an immunoprecipitation control, and a nonspecific exon area from the HO-1 gene was used as a negative PCR control. Results are representative of 3 independent experiments. Modified from studies I and II.

5.2 IDENTIFICATION OF CRITICAL STRUCTURAL CHARACTERISTICS OF OXPAPC MEDIATING NRF2 ACTIVATION (I)

Next the structural requirements of oxPAPC for Nrf2 activation were studied. First the role of the polar head group in the sn-3 position was examined. HUVECs were treated with native or oxPLs having palmitoyl and arachidonoyl in positions sn-1 and sn-2 respectively, but phosphocholine in the sn-3 position was replaced with phosphatidylglycerol, phosphatidic acid, phosphatidylethanolamine, or phosphatidylserine.

The mRNA expression of NQO1 and GCLM was increased by all studied oxPLs suggesting that the polar head group in the sn-3 position does not play a significant role in the activation (Figure 13A). Native PLs did not induce the expression. During PAPC oxidation three oxPLs differing from the sn-2 position are formed; PGPC, POVPC and PEIPC (isoprostane-PC). When HUVECs were treated with: PGPC, POVPC, PEIPC (Figure 13B) or LysoPC (1-palmitoyl-2-hydroxyl-sn-3-glycero-phosphocholine, study I) that contains a hydroxyl group in the sn-2 position, the expression of GCLM and NQO1 were induced only by PEIPC. For further verification of the importance of the isoprostane group, cells were treated with native or oxidized palmitoyl-linoleoyl-phosphatidylcholine (PLPC) that cannot form prostanoid structures. Nevertheless, oxPLPC activated target gene expression, indicating that the isoprostane group is not the only important structure for activation (Fig 13C). GCLM was also induced by PAPC hydroperoxide (PAPCOOH) but not by the reduced hydroxide form (PAPCOH, Figure 13D).

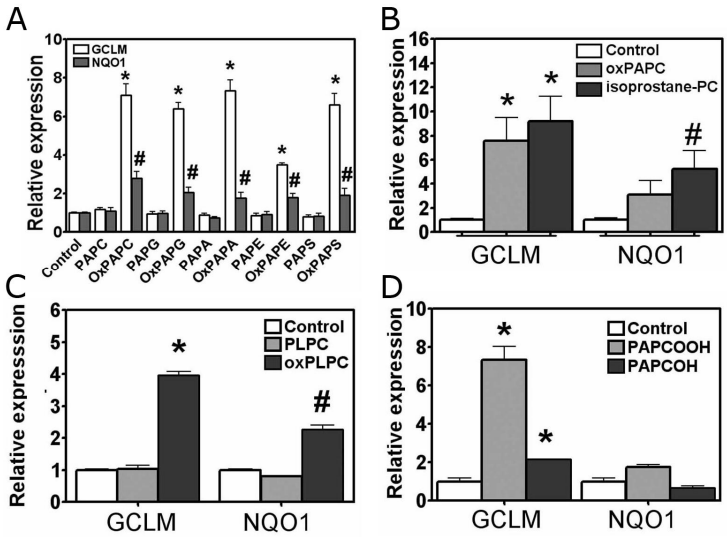

Figure 13. The induction of Nrf2 target genes by different classes of phospholipids. A, HUVECs were treated with 130 μmol/l native or oxidized PAPC, phosphatidylglycerol (PAPG), phosphatidic acid (PAPA), phosphatidylethanolamine (PAPE), or phosphatidylserine (PAPS) for 6 h. B, HUVECs were treated with 130 μmol/l oxPAPC or isoprostane-PC for 6 h. C, HUVECs were treated with 130 μmol/l native or oxidized PLPC. D, HUVECs were treated with 130 μmol/l PAPCOOH or PAPCOH for 6 h. The expression of GCLM and NQO1 mRNA was analyzed with quantitative RT-PCR and normalized to B_2M. The expression was presented as fold change compared to the untreated siControl. Values are presented as mean±SEM, (n=3), *$p<0.05$ vs. siControl. Modified from study I.

Next the role of the electrophilic groups in oxPAPC was examined. By reducing electrophilic groups of oxPAPC with sodium borohydride, or incubating with small molecular weight thiols N-acetylcysteine or GSH, the expression of GCLM, NQO1 and HO-

1 was investigated (study I). In summary, these results show that oxPLs with an oxidized group in the sn-2 position (an isoprostane or hydroperoxide residue) are capable of activating Nrf2 mediated gene expression. In addition, the electrophilic character of oxPAPC and its active components have a significant role in the activation.

5.3 OXPAPC INDUCES NRF2 TARGET GENES IN VIVO (I)

To explore the effect of oxPAPC on Nrf2 dependent target gene induction in vivo, surgically exposed carotid arteries of wild type and Nrf2$^{-/-}$ mice were covered with pluronic gel with or without 50 µg oxPAPC. The expression of HO-1, GCLM and NQO1 mRNA was significantly induced by oxPAPC in wild type mice arteries and Nrf2 deficiency inhibited the induction (Figures 14A, B, and C). Similar effects were seen in immunohistochemical analysis where samples were stained against HO-1 or NQO1. Again oxPAPC treatment increased protein levels in wild type mice whereas the induction was prevented in Nrf2$^{-/-}$ arteries (Figures 14D, and E). To summarize, Nrf2 is activated and induces target gene expression by oxPAPC both in vitro and in vivo.

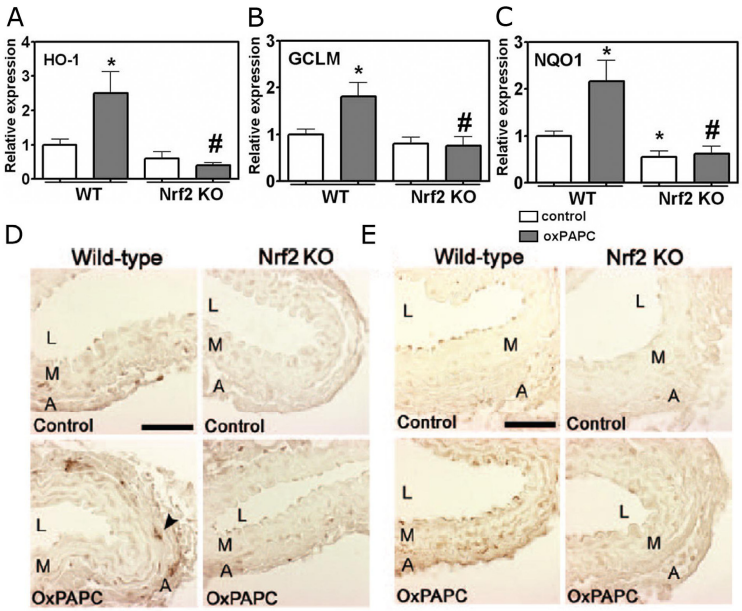

Figure 14. OxPAPC induces the expression of target genes in murine carotid arteries. The carotid arteries of wt or Nrf2$^{-/-}$(KO) mice were surgically exposed and covered with 30% pluronic gel with or without 50 µg oxPAPC. A-C, Animals were sacrificed after 6 h treatment and mRNA expressions of HO-1 (A), GCLM (B), and NQO1 (C) were measured with quantitative RT-PCR. The expression was normalized to ribosomal RNA, and presented as fold change compared to the untreated wt control. Values are presented as mean±SEM, (n=5-11), *p<0.05 vs. relative control. D and E, Animals were sacrificed after 24 h, and the protein expression of HO-1 (D) and NQO1 (E) was measured by immunostaining. The arrowhead indicates HO-1 positivity. Original magnification x400, scale bars=50 µm, L=lumen, M=media, A=adventitia. Modified from study I.

5.4 NRF2 DEPENDENT AND INDEPENDENT EFFECTS OF OA-NO_2 ON THE MRNA EXPRESSION OF HUVECS (II)

The effects of OA-NO_2 on the expression profile of HUVECs and the identification of Nrf2-dependent and -independent target genes responsive to OA-NO_2 were studied by a microarray analysis. HUVECs were transfected with nonspecific siRNA or siRNA against Nrf2 for 24 h and treated with OA-NO_2 or OA for 8 h. Untreated transfected cells were used as a control. Genome-wide transcriptional profiling comparing untreated and OA treated cells did not shown any significant difference revealing that the effects of OA-NO_2 are specific. OA-NO_2 treatment leads to the up-regulation of 363 and down-regulation of 103 genes. SiNrf2 specifically down-regulated 150 and up-regulated 34 genes. The number of genes that were regulated by both OA-NO_2 and siNrf2 were 43, where 31 genes were up-regulated by OA-NO_2 and down-regulated by siNrf2. Most of those genes were known Nrf2 targets. These results indicate that the majority of the effects of OA-NO_2 are mediated by other mechanisms and transcription factors other than Nrf2. Using the geneset enrichment analysis, it was shown that one of the major pathways activated by OA-NO_2 was the HSR. Multiple genes known to be involved in HSR were significantly up-regulated after OA-NO_2 treatment. The expression of the selected HSR targets was verified by RNA and protein expression analysis, siRNA experiments using siRNA against HSF1 and binding of HSF1 to the heat shock response element of the target genes after OA-NO_2 treatment (study II).

5.5 BACH1 IS AN NRF2 INDUCIBLE GENE (III)

Previous microarray studies with Nrf2 over-expressing adenovirus (Jyrkkänen et al. 2006) and our previous study (II) suggest that BACH1 might be an Nrf2 inducible gene. Kaspar and Jaiswal have also shown that BACH1 is induced by tBHQ treatment in human and mice hepatic cells (Kaspar, Jaiswal 2010). Thus we hypothesized that BACH1 is a new Nrf2 target gene. HUVECs were transduced with Nrf2 over-expressing adenovirus with MOI 10 to 100. The expression of BACH1 transcript variants was induced by AdNrf2 and by treating HUVECs with Nrf2 activating agents; SFN, tBHQ or OA-NO_2 (Figures 15A, and B, study III). The induction was inhibited by using actinomycin D, showing that the induction is regulated at the transcriptional level (study III). Nrf2 dependency of BACH1 induction was studied by siNrf2 that inhibits SFN activated induction of BACH1 expression (Figure 15C), indicating that Nrf2 mediates BACH1 induction by SFN.

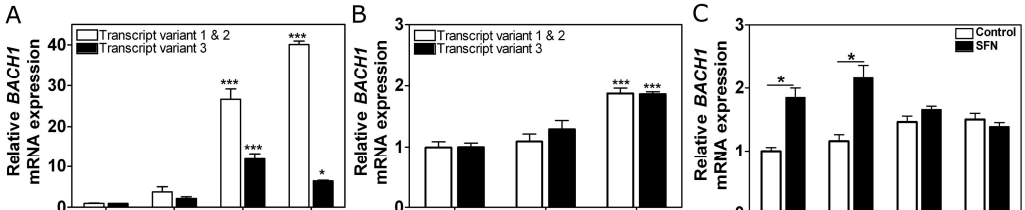

Figure 15. Nrf2 induces mRNA expression of human BACH1. A, HUVECs were transduced with Nrf2 over-expressing adenovirus using MOI 10 to 100 for 48 h. B, HUVECs were treated with 0 to 10 μM SFN for 6 h. C, HUVECs were transfected with siRNA against Nrf1, Nrf2 or control siRNA for 48 h and treated with 10 μM SFN for 6 h. The expression of BACH1 was measured with quantitative RT-PCR and normalized to B_2M. Values are presented as mean±SEM, (n=3). A and B; vs. control, C; untreated vs. treated cells, *$p<0.05$. Modified from study III.

The promoter of the BACH1 gene was studied with in silico analysis using the Genomatix software. Two putative core ARE sequences (TGAC/GnnnGC, (Erickson et al. 2002)) were found in the BACH1 gene. The elements were located on the forward strand 1270 (BACH1-ARE2) and reverse strand 1411 (BACH1-ARE1) downstream from the TSS of transcript variant 2 (study III).

The functionality of the elements was tested by EMSA and luciferase reporter assays. In EMSA analysis, Nrf2 and small Mafs form a strong complex with BACH1-ARE1, whereas BACH1-ARE2 does not show any binding (study III). Binding of Nrf2 to BACH1-ARE1 was decreased with a specific NQO1-ARE but not with a nonspecific NF-κB element, confirming the specificity of binding. In the luciferase assay, BACH1-ARE1 was strongly activated by Nrf2 activating agents (Figure 16A).

The activation of BACH1-ARE1 was further studied by co-transfection with Nrf2 and Keap1, demonstrating Nrf2 mediated activation. NQO1-ARE was used as a control in the experiments. Binding of Nrf2 was further confirmed by ChIP. Results show that Nrf2 binds to the BACH1 ARE after SFN treatment (Figure 16B). Taken together, these results indicate that the inducible expression of BACH1 is regulated by Nrf2 via an intronic ARE.

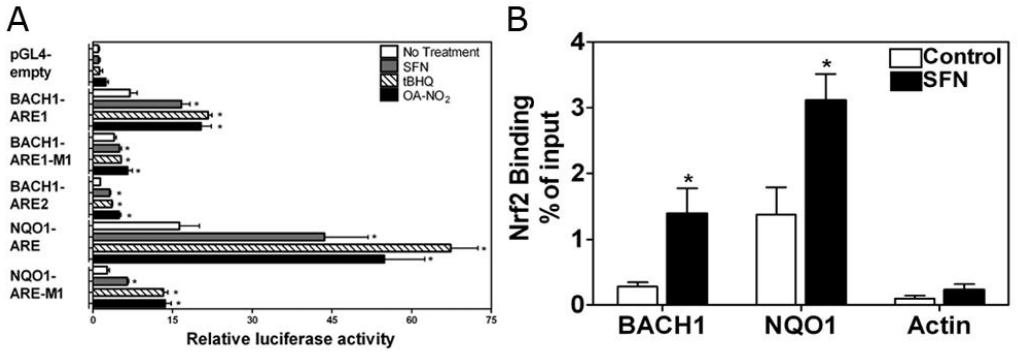

Figure 16. The binding of Nrf2 to the AREs of BACH1 or NQO1. A, For the luciferase assay, HEK293T cells were transfected with empty pGL-promoter vector as a control, or vectors containing wild type or mutated BACH1 or NQO1 AREs. Twenty-four hours after transfection, cells were treated with 10 μM SFN, 30 μM tBHQ, or 5 μM OA-NO_2 for 16 h. Luciferase activity was normalized to β-galactosidase and presented relative to an empty vector control. B, HEK293T cells were treated with 10 μM SFN for 2 h and ChIP was performed using an antibody against Nrf2. Quantitative RT-PCR against BACH1, or NQO1 ARE or against actin as a specific control was performed. Binding is presented as % of input values from three independent experiments. Values are presented as mean±SEM, (n=3), *$p<0.05$ vs. control treatment. Modified from study III.

5.6 NRF2 NEGATIVELY REGULATES THE EXPRESSION OF TXNIP (IV)

The thioredoxin system is one of the most important reductive pathways in mammalian cells. TXNIP inhibits the thioredoxin system leading to the activation of inflammatory pathways and increased ROS production. It is known that TXNIP is down-regulated by laminar shear stress, which also activates Nrf2. TXNIP expression is down-regulated by adenoviral Nrf2 over-expression. TXNIP expression is also decreased in OA-NO_2 treated siControl cells by 1.8 fold (study II). We therefore first examined the effect of adenoviral Nrf2 over-expression on TXNIP mRNA. The TXNIP mRNA expression was reduced by 86.6 ± 3.7% with AdNrf2 MOI 50 and 91.7 ± 1.5% with MOI 100 (Figure 17A) comparing to control virus. The highest MOI used also reduced TXNIP protein levels (study IV). The effect of the Nrf2 inhibitor, Keap1, was also tested by transducing HUVECs with AdKeap1.

Keap1 over-expression increased TXNIP mRNA expression approximately 1.5 fold. Treatment with Nrf2 activating agent SFN, lead to the reduction of both TXNIP mRNA and protein expression dose- and time-dependently (study IV). Transfection of HUVECs with specific siRNA against Nrf2 induced TXNIP expression 1.9 fold (Figure 17B). However, SFN treatment significantly repressed TXNIP expression also when siNrf2 was used, suggesting an Nrf2-independent component is involved in its effect (Figure 17B). We also examined mRNA expression of TXNRD1 and TXN as these are important components of the TXN system. TXNRD1 is tightly regulated by the Nrf2-ARE and its expression is clearly Nrf2 dependent, as shown by Nrf2 over-expression, SFN treatment or siNrf2 (study IV). In contrast, TXN expression was not affected by these treatments.

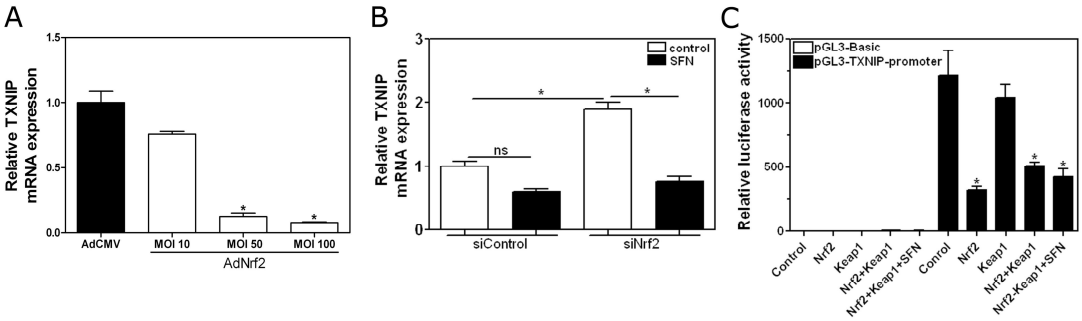

Figure 17. TXNIP expression and promoter activity is reduced by Nrf2. A, HUVECs were transduced with Nrf2 over-expressing adenovirus using MOI 10 to 100 for 48 h. B, HUVECs were transfected with siRNA against Nrf2 or control siRNA for 48 h and treated with 10 μM SFN for 6 h. The expression of TXNIP was measured with quantitative RT-PCR and normalized to B_2M. Values are presented as mean±SEM, (n=3), *p<0.05. C, For the luciferase assay, HUVECs were transfected with the construct containing 3224 nucleotides upstream from the TXNIP TSS, or empty pGL3 -promoter vector as a control with Nrf2- and Keap1-expressing plasmids. Twenty-four hours after transfection, cells were treated with 10 μM SFN for 16 h. Luciferase activity was normalized to β-galactosidase and presented relative to the empty vector control. Modified from study IV.

To study the effect of Nrf2 on the TXNIP transcription, a 3224 nucleotides regulatory region upstream of the TXNIP TSS was cloned into a pGL3-vector and the promoter activity was studied with the luciferase reporter assay. The HEK293T cells were transfected with the pGL3-TXNIP-promoter construct along with Nrf2- and Keap1-expressing plasmids. Nrf2 reduced the activity of the TXNIP-promoter (Figure 17C). Keap1 alone has no effect and it was not able to inhibit the repressive activity of Nrf2. The SFN treatment did not increase the repressive effect of Nrf2 (Figure 17C). However, in HUVECs transfected with the pGL3-TXNIP-promoter without Nrf2 co-transfection, SFN repressed the promoter activity of TXNIP (study IV).

In silico analysis of the TXNIP regulatory region revealed two putative AREs (Figure 18A) 3544 and 2453 nucleotides upstream from the TXNIP TSS. The data, from the Encyclopedia of DNA Elements project (ENCODE), reveals the presence of markers for active promoter (H3K4Me3) and active enhancer regions (H3K4Me1 and H2K27A) as open chromatin in that area (Figure 18B). The Blat sequences display the location of putative AREs. Only the proximal element was included in the promoter construct used, necessitating the validation of the distal element.

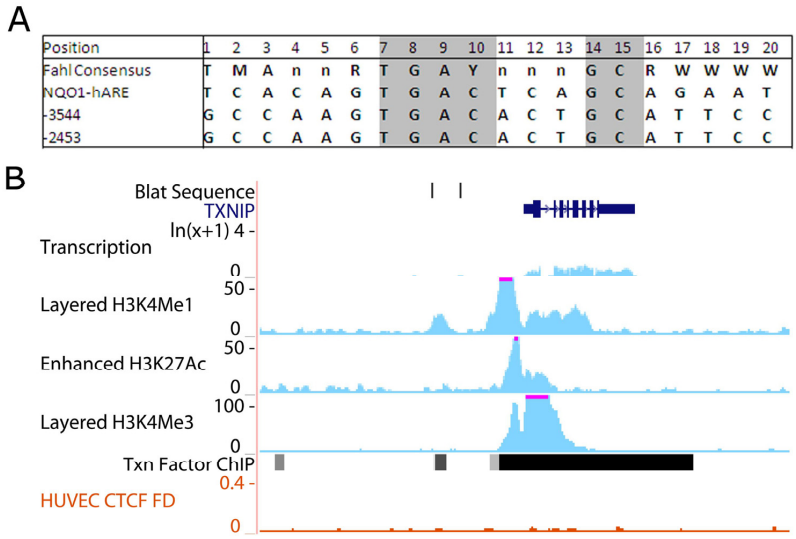

Figure 18. Putative AREs of theTXNIP gene and the ENCODE data set displays the TXNIP region. A, The sequences of putative TXNIP AREs are located at -3544 and -2453 from the TSS, aligned to the ARE consensus and NQO1 ARE sequences (Wasserman, Fahl 1997). B, ENCODE data from the TXNIP region displaying putative AREs (Blat sequences), active promoter (H3K4Me3) and enhancer regions (H3K4Me1 and H2K27A), and TF ChIPseq data. Modified from study IV.

6 Discussion

6.1 ACTIVATION OF NRF2 BY AGENTS RELEVANT TO CARDIOVASCULAR DISEASES

Lipids and inflammation play a central role in atherogenesis. Different classes of modified lipids are formed in plasma and in the cells of the vascular wall during atherogenesis. OxPAPC is produced during oxidation of LDL in cell membranes and has been shown to be localized in human atherosclerotic lesions and plays a role in all stages of atherogenesis (Gargalovic et al. 2006a, Bochkov et al. 2010). Endogenous OA-NO_2 is found in cardiac mitochondria, cardiac tissue and plasma, and its production is increased during inflammation (Rudolph et al. 2010b, Nadtochiy et al. 2009, Tsikas et al. 2009). In this thesis, it is shown that both oxPAPC and OA-NO_2 activate nuclear localization of Nrf2 and the target gene expression in human endothelial cells and murine carotid arteries (studies I and II).

Nrf2 activation is mediated via Keap1 modifications. OA-NO_2 seems to bind the Cys273 and Cys288 of Keap1, leading to a conformational change in the Keap1-Nrf2-CUL3 complex (Kansanen et al. 2011, Tsujita et al. 2011). In contrast, the exact activation mechanism of Nrf2 by oxPAPC is not known. OxPAPC that contains esterified cyclopentenone isoprostane, PEIPC (Watson et al. 1999), which is important in the activation of adaptive branches of the UPR (Oskolkova et al. 2008). In this thesis it was shown that isoprostane phospholipids is a favorable Nrf2 activator. PEIPC contains an isoprostane group that is highly similar to the cyclopentenone group of 15d-PGJ_2 that modifies Keap1 cysteines (Levonen et al. 2004). OxPLs and specifically PEIPC are capable of modifying intracellular proteins (Gugiu et al. 2008, Jung et al. 2008). Therefore, it can be suggested that PEIPC induced Nrf2 activation is mediated via modification of Keap1 Cys residues. Otherwise, oxPAPC increases Nox mediated production of $O_2^{\cdot-}$ and this has been proposed as one of the mechanisms for Nrf2 activation (Li et al. 2007, Rouhanizadeh et al. 2005). Nrf2 dependent activation of oxidative stress induced growth inhibitor 1 is dependent on Nox mediated ROS production and induction is inhibited by using Nox inhibitors. As discussed in study I, we examined the effect of Nox4 silencing on the expression of Nrf2 target genes using siRNA or apocynin treatment without seeing any effect, supporting the hypothesis that oxPAPC directly actives the Keap1-Nrf2 pathway.

NO_2-FAs activate many anti-inflammatory signaling pathways in endothelial cells. Since NO_2-FAs can act on multiple signaling pathways, we wanted to identify Nrf2-dependent and -independent OA-NO_2 targets by microarray analysis. Several genes up-regulated by OA-NO_2 treatment and down-regulated with siNrf2 were previously identified Nrf2 targets, thus confirming that the approach was feasible. However, the number of genes was quite small and for methodological reasons some Nrf2 targets may have been excluded. Most of the OA-NO_2 induced genes were not Nrf2 dependent. Using gene enrichment analysis, the predominant pathway was HSR. HSR is activated by e.g. elevated temperatures and by electrophilic compounds. In endothelial cells, expression of HSPs such as HSP70 has been shown to inhibit inflammation via inhibition of NF-κB and to display cytoprotective effects via protection against H_2O_2 (Westerheide, Morimoto 2005, Ran et al. 2004, Gill et al. 1998). Thus the activation of HSR supports the anti-inflammatory feature of OA-NO_2.

The activation of cytoprotective signaling pathways is a physiological response to the generation of ROS and ageing. These pathways are the key defense against harmful effects of ROS. In addition to their independent effects, these protective pathways have a

certain degree of cross-talk between each other. Signaling by Keap1-Nrf2 pathway and HSR is mediated by different transcription factors, but both are activated by electrophilic lipids, such as OA-NO_2 (study II) and 15d-PGJ_2 (Zingarelli et al. 2007). Genome-wide analysis of Nrf2 and HSF1 target genes indicates that these do not overlap, supporting the notion that responses are largely distinct (study II, (Trinklein et al. 2004)). However, there is evidence that Nrf2 and HSF1 share common signaling proteins, which modulate their activation and DNA binding (Niture, Jaiswal 2010, Kaitsuka, Tomizawa & Matsushita 2011). It has been demonstrated in hepatoma cells that the Keap1-CUL3-complex is disrupted by HSP90, leading to Nrf2 activation upon heat shock (Niture, Jaiswal 2010). Our studies (study II, supplement) do not support this observation since heat shock does not activate expression of the Nrf2 target gene HO-1 in HUVECs. As discussed with phosphorylation, the effects are cell type and target gene dependent (Kensler, Wakabayashi & Biswal 2007), suggesting that the effect of heat shock leading to Nrf2 activation might also be cell type or target gene specific.

In summary, oxPLs and NO-FAs are Nrf2 activator molecules present in the vasculature during stress conditions. They activate several signaling pathways, mediating both anti- and pro-atherogenic effects. Protective signaling pathways cross-talk with each other to fine-tune the signaling effects.

6.2 REGULATION OF NOVEL NRF2 TARGET GENES

Nrf2 is demonstrated to be a transactivator that regulates gene expression via an ARE. The number of experimentally confirmed Nrf2 target genes is limited and new targets are yet to be found. The expression of BACH1 mRNA induced by electrophiles was Nrf2 dependent and mediated by an intronic ARE. Nrf2 has no effect on the basal expression of BACH1, verified by the siRNA experiment (study III). This indicates that the basal expression is supported by other transcription factors. The ChIP results indicate that the same amount of small Mafs is bound to the BACH1 ARE in basal conditions and upon SFN treatment. The basal expression of the TXN-1 gene is regulated by an NF-E2-small Maf complex and heme induced expression is regulated by an Nrf2-small Maf complex via an ARE (Kim et al. 2001). It could be suggested that the basal expression of human BACH1 might be mediated by the ARE, but by a transcription factor other than Nrf2. The role of Nrf1 in compensating for Nrf2 deficiency through partially shared targets was researched in study III. However, the use of siRNA against Nrf1 had no effect on BACH1 expression. Furthermore, BACH1 itself does not regulate its own activity via an ARE, as confirmed by ChIP. It is possible that small Mafs act as homodimers to repress the basal expression, similar to what has been demonstrated with the MafG gene (Kataoka et al. 1995b). BACH1 is also regulated post-translationally by miRNAs, which provides another layer of regulation to its expression (Hou et al. 2010, Yin et al. 2008).

Nrf2 is a highly inducible transcription factor and it drives target gene expression effectively. It has recently been suggested that hyperactivated Nrf2 can mediate liver disease (Komatsu et al. 2010). The mechanisms by which Nrf2 activation is turned off are not known. Our results suggest that Nrf2 negatively regulates its own activity via BACH1. BACH1 competes with Nrf2 for the same binding sites and by inducing BACH1 expression, Nrf2 negatively regulates its own target genes. This is particularly true with HO-1 as the role of BACH1 in its expression is firmly established. However, the battery of genes regulated by both Nrf2 and BACH1 is unclear. Recently ChIP combined with a high-throughput sequencing technique was used for identifying targets of either Nrf2 or BACH1 (Warnatz et al. 2011, Malhotra et al. 2010). However, the overlap of genes regulated by both of these two transcription factors at the same time has not yet been explored across the genome.

Herein, is demonstrated that TXNIP expression is down-regulated by Nrf2. In silico analysis demonstrated that the regulatory area of TXNIP contains putative AREs, but whether these are functional remains to be investigated (study IV). Nrf2 has earlier been shown to down-regulate the expression of vascular inflammatory genes via the p38 kinase (Zakkar et al. 2009, Chen et al. 2003). In human aortic endothelial cells, TXNIP is up-regulated by forkhead box O1 transcription factor via p38 (Li et al. 2009). Therefore, it remains to be clarified whether Nrf2 affects the TXNIP expression via the same mechanism. However, as demonstrated in this thesis, SFN also has an Nrf2-independent repressive role in TXNIP regulation. We have also seen that other electrophilic Nrf2 activators, such as OA-NO_2, functions the same way. These findings suggest that the down-regulation of TXNIP may be affected by cellular redox balance. That hypothesis is supported by Schulze et al, who studied the role of TXNIP in SMC proliferation. The authors demonstrated that H_2O_2 treatment decreased TXNIP protein expression over short time points and the reduction was inhibited with antioxidants (Schulze et al. 2002). In addition, it has been shown that the oxidative status of TXNIP regulates its cellular localization (Saxena, Chen & Shalev 2010) and its expression further induces ROS production (Junn et al. 2000). However, the role of redox balance in transcriptional and translational regulation of TXNIP needs to be clarified further. In macrophages, TXNIP is regulated via an AP-1 region by the transcription factors c-Jun and c-Fos (Billiet et al. 2008). These factors are known to bind to the internal AP-1 within the ARE and they have a role in the regulation of ARE target genes. For example, TXR-1 is regulated by these factors as well as by ARE binding factors such as Nrf2, in different conditions (Kim et al. 2001). Also, it is known that Nrf2 and Jun proteins together regulate TXR-1 expression via an ARE or the internal AP-1 element (Venugopal, Jaiswal 1998).

6.3 ROLE OF NRF2 IN VASCULAR DISEASES

Nrf2 has been reported to have conflicting roles in atherogenesis. Via induction of protective target genes, Nrf2 is potentially antiatherogenic. In the endothelium, Nrf2 maintains cellular homeostasis and the antiatherogenic role of Nrf2 has been demonstrated in vivo (Zakkar et al. 2009). This is also supported by studies within this thesis, since Nrf2 is demonstrated to be activated by endogenously presented lipids, activation of protective genes, and inhibition of inflammation in endothelial cells. In macrophages, Nrf2 has important protective features via Nrf2 target genes and at the same time it potentiates foam cell formation via increased expression of CD36 (Ishii et al. 2004). However, as a main function of macrophages is to scavenge xenobiotics in cells, it should be considered that (via induction of CD36) Nrf2 may protect cells from modified lipids and upon atherogenesis these pious mechanisms become reversed. The role of Nrf2 in atherogenesis has been studied in $ApoE^{-/-}$ mice. $ApoE^{-/-}$ mouse provide a commonly used model of atherosclerosis, where the deficiency of ApoE causes an increase in plasma cholesterol that leads to the development of advanced lesions, even for mice on a low fat diet (Veniant et al. 2008). In an $ApoE^{-/-}$ background, Nrf2 increases lesion size (Barajas et al. 2011, Sussan et al. 2008). Barajas et al noted that the lesion size in male mice correlated with total and non-HDL plasma cholesterols, which were decreased in $Nrf2^{-/-}$ males (Barajas et al. 2011). Increased CD36 expression was demonstrated in Nrf2 expressing mice in both studies, which has been postulated to increase foam cell formation in an Nrf2-dependent fashion. However, Barajas et al discussed that there are multiple mechanisms by which Nrf2 accelerates atherogenesis. They noticed that Nrf2 has multiple effects on lipid metabolism that are partially sex dependent. The severe atherosclerotic model together with a high-fat-diet may confuse the progression of the atherogenesis. It would therefore be valuable to study the role of Nrf2 in a less severe model, such as in LDL-receptor deficient mice expressing ApoB100 only. The ApoB100 only/$LDLR^{-/-}$ model mimics the lipid profile of

human disease better than ApoE$^{-/-}$ (Veniant et al. 1998). Our preliminary data with ApoB100 only/LDLR$^{-/-}$ mice on a normal diet suggests that Nrf2 is antiatherogenic (Ruotsalainen A-K et al. 2011). A study with ApoE$^{-/-}$ -BACH1$^{-/-}$ mice shows that the induction of Nrf2 inducible HO-1, due to BACH1 deficiency, inhibits lesion formation (Watari et al. 2008). However, HO-1 is expressed in advanced atherosclerotic lesions and induces thickening of the fibrous cap and SMC accumulation, but on the other hand it has been shown to decrease necrosis and inhibit plaque rupture (Cheng et al. 2009). To conclude, it seems that Nrf2 has both pro- and anti-inflammatory features. It has been demonstrated that a polymorphism of the Nrf2 gene associates with oxidative stress induced lung diseases (Marzec et al. 2007) and Nrf2 mutations are common in many cancer types, contributing to poorer prognosis of the disease (Taguchi, Motohashi & Yamamoto 2011). Whether these mutations are common in atherosclerosis is not known, but they may explain the dual role of Nrf2 in atherogenesis. In summary, Nrf2 plays a protective role in the endothelium but in the later phase of the disease, constitutive Nrf2 activation in macrophages promotes their survival and atherogenesis. Therefore, further studies are needed to explore the role of Nrf2 in different cell types and phases of atherogenesis.

7 Conclusions

I OxPAPC induced the expression of target genes HO-1, NQO1 and GCLM in human endothelial cells and in murine carotid arteries in an Nrf2 dependent manner. The characterization of lipid species that are critical for gene activation showed that the oxidation of the sn-2 group, isoprostane, hydroxyl- or electrophilic groups all play a role.

II Nrf2 is activated and its target genes are induced by OA-NO_2. Most of the OA-NO_2 induced genes are Nrf2 independent, the heat shock response being one of the main activated pathways.

III The repressive transcription factor BACH1 is a novel Nrf2 target gene. Nrf2 regulates inducible expression of BACH1 via an intronic ARE. This may serve as a novel mechanism by which Nrf2 regulates its own activity by negative feedback.

IV Nrf2 downregulates the expression of TXNIP in endothelial cells and can thus act as a negative regulator of gene expression.

To conclude, Nrf2 is activated by endogenous agents relevant to cardiovascular diseases and it has a multifunctional role in gene regulation in endothelial cells. The role of Nrf2 in atherogenesis is controversial, but the results shown in this thesis support the action of Nrf2 as an endothelial protector. However, future studies are needed to establish the role of Nrf2 in other vascular cell types and at different stages of atherogenesis. According to the knowledge available, it can be hypothesized that endothelium specific activation of Nrf2 may prevent endothelial dysfunction and the progression of atherogenesis.

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Transcription Factor Nrf2 Mediated Gene Regulation and Signaling in the Endothelium

Increased production of reactive oxygen species (ROS) disrupts vascular homeostasis leading to the development of endothelial dysfunction and atherosclerosis. Transcription factor Nrf2 protects cells against ROS via regulation of antioxidant and cytoprotective genes. In this thesis, the mechanisms by which endogenous molecules activate Nrf2 in the vascular endothelium are described. In addition, novel mechanisms that inhibit the Nrf2 activity and the role of Nrf2 as a factor decreasing the vascular inflammation are suggested.

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