"Never let the fear of striking out keep you from playing the game" -Babe Ruth-

UNIVERSITEIT GENT

FACULTEIT WETENSCHAPPEN

Modulation of Nrf2-KEAP1 signaling by the natural compound Withaferin A: potential therapeutic effect in cardiovascular diseases?

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Dankwoord

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* , al een flinke tijd weg uit het labo, * al een tijdje weg uit het labo en * nog maar kersvers uit het labo vertrokken. Zij zijn het bewijs dat collega´s soms echt vrienden kunnen worden. * ,hoewel we zo goed als niets schelen in leeftijd, ben je van tijd tot tijd een beetje mijn mentor geweest. Praktische tips voor het wetenschappelijk gebeuren, diplomatische tips als ik me van tijd tot tijd druk maakte in bepaalde onrechtvaardigheden, maar vooral veeeeeel leute en vriendschap in en buiten de ledeganck! * , het aantal jaar dat wij van elkaar schelen kan je nog net op 2 handen tellen ☺. Dit merken we dan ook enkel als we eens aan het tellen zijn, want verder voel ik helemaal geen leeftijdsbarrière bij jou! Al van dag 1 vond ik je de max en met de jaren ben je steeds belangrijker voor mij gaan worden!! Van gezellige tante, waar je altijd plezier mee kon maken ben je veranderd tot een vriendin waar ik hartsgeheimen aan toevertrouw, diegene die een shit-load aan lief en leed van Phd gerelateerde zaken tot liefdesperikelen van me heeft mogen aanhoren ☺. Je bent m'n maatje, zonder jou was het niet half zo leuk geweest! *, de enige die ik in mijn leven al ben tegengekomen die vernuftig slim met een ietsiepietsie johnny-look weet te combineren ☺. Voor raad en daad kon ik bij jou altijd terecht, zelfs tijdens het schrijven van je PhD en je laatste loodjes. Wat me dan zo evident leek, krijgt nu steeds meer een waaaw, echt chapeau effect. Jij en * hebben ook de tijd en energie gevonden om jullie kritische geest even los te laten op mijn doctoraat voor ik het indiende in de FR, zomaar tussen de soep en de patatten door! Je kunt niet geloven hoe dankbaar ik jullie hiervoor ben! "Ach, een vriendendienstje" zouden jullie zeggen, wat nog maar eens blijkt dat jullie meer dan ex-collega's zijn!

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is, heeft me alles weer in perspectief laten zien. Het motto werd plots: shiiiiiit, ik ga zeker vragen krijgen op mn interne verdediging waar ik niet alles over weet …..pfffff who cares mn zussie is zwanger ☺ ☺ Hophop vreugde dansje in het rond! En ook, toen de fundamenten een beetje begonnen te wankelen en de twijfel begon te groeien of ik misschien toch echt niet beter een bezoekje aan K12F kon brengen, stond jij er Hercules-like stevig om me op te vangen! Lof'ya sissie! *, smaartiebol (of basmatibol volgens PJ ☺). Van alle bijnamen de meest toepasselijk van allemaal me dunkt! Smart as hell ☺ en die hersenmassa heb je dan ook een paar keertjes met veel broederliefde ingezet voor mijn doctoraat! Of je ook zo lekker bent als een smartie laat ik aan helena over, maar kleurrijk ben je alvast (en dan heb ik het niet enkel over oranje ☺). Ego Amo te broere!

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,,*,*, en *,*,*,*,* en *,*,*,* en niet te vergeten *, en *,*, en *,*,*,*, en ook nog *,* en iedereen die me dierbaar is die nu even uit mijn geheugen glipt!

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Contents

Part I. Introduction

The obligation of a deadline, the responsibility to pick up the kids, the expectations to cook a Christmas dinner for 20 people… Everybody would agree, from time to time we experience more stress than we prefer. When we think about stress, we often think about emotional stress as the examples above. However, we can also experience physical stress. This is a physical reaction of the body to various triggers. So is pain experienced after surgery an example of physical stress. Those two types of stresses are inseparable of each other. Physical stress often leads to emotional stress, and emotional stress often occurs in the form of physical stress (e.g. stomach cramps). But what doesn´t kill you, makes you stronger one would say, however sometimes it can go wrong! A human being is built up from a large amount of cells, and all those individual cells also cope with various types of stress. The dysregulation of the biological systems associated with injuries due to excessive stress can impair biological structures, which in turn are associated with several types of diseases, including cardiovascular disease or carcinogenesis. Therefore, stress can eventually kill you! Nevertheless, cells are in a way adapted to stressful insults as they are constantly exposed to various stress conditions. They do not worry about picking up the kids of course, but they need to deal with endogenous or exogenous challenges such as DNA-damage, metabolic, proteolytic, mitotic or oxidative stress. Cells are all the time busy with maintaining cellular balance, fighting those stresses. I will further explain by the example of oxidative stresses where cells try to maintain redox homeostasis. In our daily lives, we are constantly exposed to an array of chemical and physical insults that can lead to the disturbance of the redox balance. Those oxidative stresses can be contributed by food additives, ultraviolet light, environmental pollutants, ionizing radiation etc. Next to those extrinsic stresses, that we best try to avoid as much as possible, we also have to deal with intrinsic stresses that are inherent to living, breathing organisms like we are. As we live, we continuously produce intrinsic toxicants during normal physiological metabolism or during pathological processes. One of the main products leading to oxidative stress are reactive oxygen species (ROS). They

are chemically reactive molecules that contain oxygen. Under normal physiological conditions ROS are formed as natural byproducts of normal metabolism of oxygen. However, one cannot see ROS only as waste products, because they have several physiological functions. Thus, ROS in general act as a double-edged sword as they can be detrimental and beneficial. For example, they are used as signaling molecules or used in the immune system where they are produced by immune cells to attack and kill pathogens. During evolution cells became adapted to deal with this intrinsic production of ROS and with everyday extrinsic stresses. Unfortunately, a dramatic increase in ROS levels will result in significant cellular damage with detrimental effects to human health. This can happen when the exogenous or endogenous stresses are too extreme or if the cell fails to trigger an appropriate cellular response to increased ROS concentrations. Stricto sensu, we speak only at this point about oxidative stress. Literally, oxidative stress is an overall negative manifestation, where an imbalance occurs between production of ROS and the ability of the cell to detoxify reactive intermediates. However, the term oxidative stress is frequently used if changes occur that are only small perturbations which the cell is able to overcome to regain its original redox state. Thus, there is some confusion concerning the term oxidative stress and it is rather a matter of size to determine whether it is a negative manifestation or not. Oxidative stress damage is involved in an array of diseases, such as cancer, Alzheimer´s disease, Parkinson disease, atherosclerosis and other cardiovascular-related diseases, sickle cell disease and chronic fatigue syndrome.

To conclude, health can be considered as the ability of an individual to adapt to his environment containing all sorts of stresses. Those stresses can change during evolution consequently, the adaptation can be seen as the ability to adjust to shifting forces that shape the well-being of an individual or population. Thus, priming a cell or organism with limited amount of stress can be health beneficial, whereas excessive stress exposure is considered to be detrimental.

In this PhD thesis, we will investigate how Withaferin A derived from *Withania Somnifera* can induce potential health beneficial effects via cellular adaptive stress reponses in the context of cardiovascular diseases. Briefly, in the thesis introduction, the transcription factor Nrf2 will be described as an important player in stress management linked to cardiovascular health. Next, a possible crosstalk of NF-κB, an important transcription factor in inflammation and cardiovascular disease, and Nrf2 pathways will be discussed. Finally, an overview of the withanolide Withaferin A, isolated from *Withania Somnifera* will be described.

I.1 Nrf2 pathway

I.1.1 Important players of the Nrf2 activation pathway

I.1.1.1 The transcription factor Nrf2

A key transcription factor involved in managing environmental and endogenous oxidative and electrophilic stresses is the nuclear factor erythroid-related factor-2 (Nrf2). This pathway is the primary cellular defense against cytotoxic effects of oxidative and electrophilic stress. Activation of the pathway renders animals resistant to different forms of toxicity. Nrf2 is a cap´n´collar (CNC) basic leucine zipper (bzip) belonging to a group of xenobiotic-activated receptors. Those receptors can sense chemical changes in the cell and coordinate transcriptional responses to maintain chemical homeostasis of the cell [1-5]. Within the CNC-bzip family of transcription factors one can find nuclear factor erythroid derived 2 (NFE2), Nrf1, Nrf2, Nrf3 and breakpoint cluster region/abelson murine leukemia viral oncogene homolog 1 and 2 (Bach1 and Bach2). There are 3 types of Nrf proteins, all ubiquitously expressed, having overlapping though distinct expression profiles [6]. Human and mouse Nrf2 proteins consist of 605 and 597 amino acids, respectively and have a predicted molecular mass of 66kDa [4], but Nrf2 is detected as a higher molecular weight protein. A molecular weight shift is possibly due to specific amino acid composition and post-transcriptional modifications. The Nrf2 protein contains, as its family name indicates, a bZip motif in the C-terminal half, where the basic region is responsible for DNA binding and where the leucine zipper mediates dimerization with a small musculoaponeurotic fibrosarcoma oncogene homolog (maf) protein. Small maf proteins are members of the maf protein family consisting of large and small maf proteins. MafF, mafG, mafK and mafT are identified as “small” ones. Maf proteins itself also contain a bzip domain for DNA binding and dimerization. Nrf2 contains 6 conserved domains, the Nrf2 erythroid-derived CNC homology protein homology domains (Neh). Neh1 is important for dimerization with other transcription factors and DNA binding. Neh3 recruits coactivators. Neh4 en Neh5 are important for transactivation activity which involves an interaction with cyclicAMP response element binding protein (CREB)-binding protein (CBP), that acts as a co-activator. Neh6 has a role in Kelch-like ECH-associated protein 1 (KEAP1)-independent degradation of Nrf2. Neh2, the most particular domain, binds a homodimer of the negative regulator of Nrf2, KEAP1 [6, 7]. Neh2 is highly conserved across species and a deletion of this domain results in a tremendous increase in Nrf2 transactivation activity [8]. Nrf2 is recognizing the antioxidant response element (ARE) core sequence 5’-TGACnnnGC-3’, *cis*-acting DNA sequences laying in the enhancer region 1 and 2 of more than 200 genes. The small maf protein is binding to the 3’ end GC dinucleotide of this ARE sequence [9].

I.1.1.2 The negative regulator KEAP1

KEAP1, binding as a homodimer to Neh2 domain of Nrf2, has a dual function: I) it senses disturbances in redox homeostasis and II) it regulates Nrf2 stability and switches the Nrf2-mediated response on or off. Both human and mouse KEAP1 protein contain 624 amino acids with a predicted weight of 68kDa. Similar to Nrf2, KEAP1 contains several conserved domains. There are two important protein-protein interaction motifs: the BTB (Bric-a-brac tramtrack broad complex) domain and DGR (double glycine repeat) domain. The latter is also called Kelch domain by some researchers as it contains 6 Kelch repeats [8]. In between those two regions there is an IVR (intervening region) also termed linker domain. The last two domains are the CTR and NTR (C-terminal and N-terminal region, respectively). The BTB domain and a portion of the IVR are responsible for homodimerization of two KEAP1 proteins as well as binding with Cullin3 (Cul3) [10]. The DGR and CTR together form a propeller structure what is defined as the DC domain that mediates the interaction with the Neh2 domain of Nrf2.

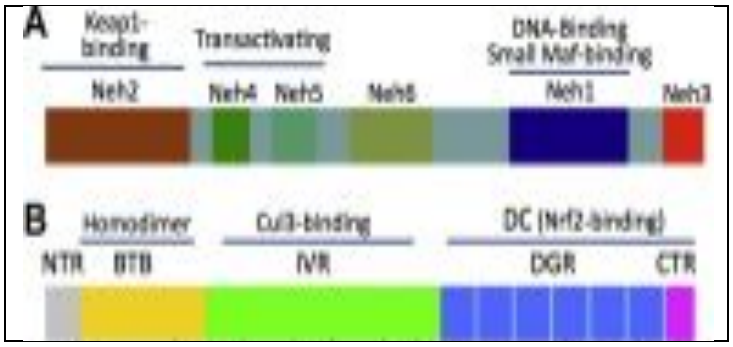

Fig 1.1 Nrf2 and KEAP1 domains. Nrf2 contains 6 conserved Neh domains. KEAP1 contains several conserved domains, including Bric-a-brac tramtrack broad complex (BTB), double glycine repeat (DGR), intervening region (IVR) and the C-terminal and N-terminal region (NTR and CTR, respectively). [11]

I.1.2 KEAP1-dependent regulation of Nrf2 activity

I.1.2.1 The KEAP1/Cul3/Rbx1-E3 complex: Ubiquitination and proteasomal degradation

Under homeostatic conditions, Nrf2 is associated with a KEAP1/Cul3/Rbx1-E3 complex in the cytoplasm. This association is leading to constant ubiquitination and degradation, regulated by the 26S proteasome, of this transcription factor. The proteasome is also called the protease complex of the ubiquitin and proteasome dependent proteolytic sytem (UPS). It is the major eukaryotic pathway for regulated protein degradation. Proteins are marked for degradation by attachment of multiple ubiquitin parts. This is mediated through a coordinated reaction of three enzymes. The first step involves E1, a ubiquitin-activating enzyme, that is forming an adenylylated ubiquitin. This ubiquitin is passed on to the second enzyme E2, the ubiquitin-conjugating enzyme. In the last step, the ubiquitin ligase E3

recognizes the specific protein and catalyzes transfer of the ubiquitin moiety from E2 to the target protein, in this case Nrf2. Due to this constant degradation half-life of the Nrf2 protein is estimated to be less than 20 minutes [12-17]. The KEAP1 dimer is controlling ubiquitination of Nrf2 as it acts as an adaptor protein to bring Nrf2 into a Cul3-dependent ubiquitin ligase (E3) complex. In this E3 complex, Cul3 functions as a scaffold protein, where its N-terminal domain binds with BTB of KEAP1 and its C-terminal domain binds with RING box protein 1 (Rbx1). The latter can recruit E2, the catalytic function of the ubiquitin-conjugating enzyme to the complex. It appears that KEAP1 is immobilizing Nrf2 and giving the Rbx1 bound E2 of the Cul3 ubiquitin ligase complex the possibility to add ubiquitin groups on lysines of Nrf2 in the Neh2 domain. After this polyubiquitination Nrf2 is degraded by the 26S proteasome. KEAP1 is not only tightly regulating ARE-dependent gene expression through degradation of Nrf2, it appears also to be bound to the actin cytoskeleton which prevents the complex to enter the nucleus [18]. The DGR domain is important for this binding with the actin cytoskeleton. Activation of Nrf2 involves inhibition of this KEAP1-mediated ubiquitination and following proteasomal degradation of Nrf2. Thus, when Nrf2 inducers are present, this constant ubiquitination and degradation is interrupted. As a consequence, Nrf2 levels are increasing and Nrf2 translocates to the nucleus to activate ARE-dependent genes. However, the exact molecular changes occurring within the KEAP1/Cul3/Rbx1-E3/Nrf2 complex are not completely understood.

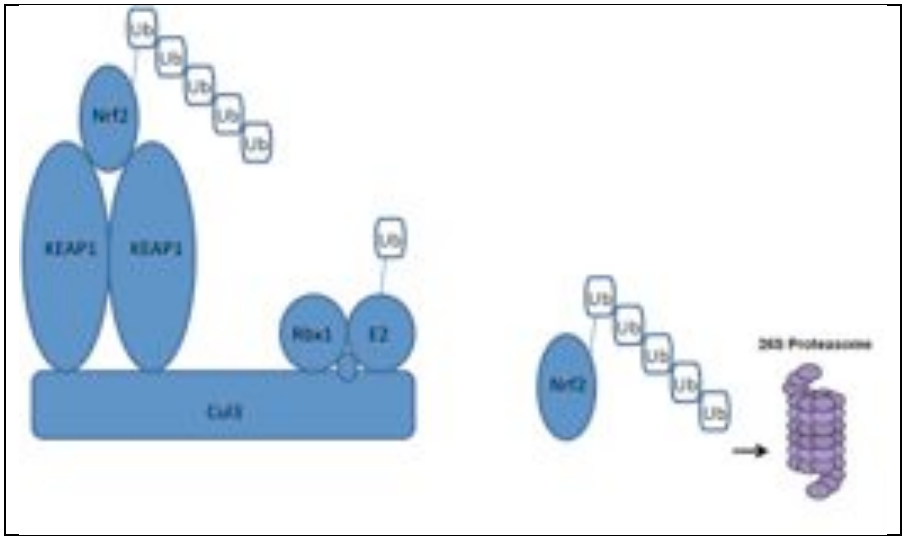

Fig 1.2 Binding of Nrf2 to KEAP1/Cul3/Rbx1-E3 complex lead to constant ubiquitination of Nrf2 followed by degradation of Nrf2 through proteasomal degradation. The KEAP1 dimer is controlling ubiquitination of Nrf2 as it acts as an adaptor protein to bring Nrf2 into a Cul3-dependent ubiquitin ligase (E3) complex.

I.1.2.2 The two-site binding model

Concerning the binding of KEAP1 and Nrf2, a “two-side binding model” has been proposed involving a two-site substrate recognition. Baird *et al*. recently developed an assay to study the dynamic interaction between Nrf2 and KEAP1 in single live cells. They showed that under basal conditions the complex exists in two conformations: one closed formation where the

Nrf2 protein is bound to two KEAP1 proteins of the dimer, and an open conformation where Nrf2 only interacts with one KEAP1 protein. Interestingly, they noticed that the complex does not dissociate when exposed to a range of inducers but that the dynamics is modulated. This observation can be explained by a hinge and latch system. Two distinct sites in the Neh2 domain of Nrf2, the high affinity ETGE motif and low affinity DLG motif, are both able to associate with one DC domain (containing DGR and CTR) of each KEAP1 protein in the KEAP1 homodimer. The binding affinity of ETGE is two orders of magnitude higher that the affinity of DLG leading to a hinge and latch binding. Because of its higher affinity ETGE binds to one of the dimeric KEAP1 globular subunits at the DC domain as the first step. This binding of ETGE promotes the binding of DLG to the other KEAP1 protein, leading to a closed conformation of the two-side binding model. DLG will bind KEAP1 only when ETGE is already bound to the other KEAP1 of the homodimer. Thus, the high affinity binding site (hinge) allows Nrf2 to bind KEAP1, yet it is still able to move freely, whilst the low affinity binding site acts as a latch that impedes movement of Nrf2. When bound at both sites, Nrf2 is perfectly positioned to undergo ubiquitination [10, 19, 20]

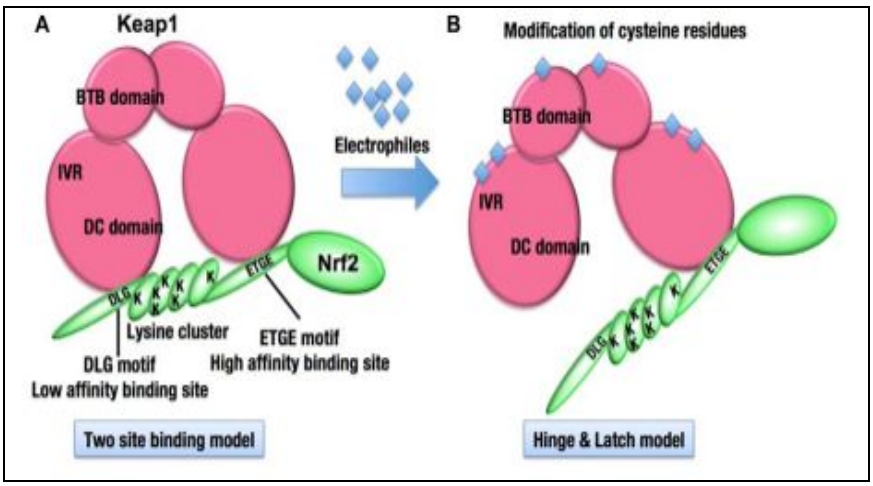

Fig 1.3 The two side binding model. Two DC domains of KEAP1 are binding a high affinity site ETGE (hinge) and low affinity binding site DLG (latch) within Nrf2. In this conformation, 7 lysine residues (K) within Nrf2 are perfectly positioned to become ubiquitinated following proteasomal degradation of Nrf2. When Nrf2 inducers are present modifications of cysteine residues within KEAP1 occur leading to loss of the latch binding and interruption of ubiquitination of the Nrf2 lysine residues [21] .

I.1.2.3 KEAP1 as a sensor to stresses: role of cysteines

In addition to deciphering the complex wherein Nrf2 resides and the main mechanism of degradation, we should also question how the system senses the wide variety of stresses that are activating the Nrf2 transcription factor. KEAP1 is not only the inhibitor of Nrf2 and responsible for its proteasomal degradation, it is apparently also able to act as a sensor to stress. The main chemical mechanism how KEAP1 is sensing stresses has been proposed for the first time by Dinkova-kostova *et al*. in 2001. A whole range of different kinds of chemicals within the Nrf2 inducers, that differ considerably in structure, have a common feature: their reactivity with sulfhydryl groups [22]. Bearing this in mind, they compared the reactivity of these compounds for cysteine thiols with their rate of potency for induction of NAD(P)H

dehydrogenase quinone 1 (NQO1), a typical ARE-dependent gene. Interestingly, close relationship was observed between this cysteine reactivity and induction of NQO1. Thereby, they concluded that cysteines are playing an important role in the chemical mechanism of activation of Nrf2 in response to several inducers. As human KEAP1 has 27 cysteines and mouse KEAP1 25 cysteines, this protein is, with its relatively high cysteine content, an excellent sensor for those inducers, giving KEAP1 the ability to sense chemical stresses. The majority of KEAP1 cysteines are flanked by basic amino acids which increases the reactivity of cysteine residues by lowering the predicted pKa value [23]. When recombinant KEAP1 and inducers were mixed, more than half of the cysteines can be modified. However, it was shown from molecular studies that modification of only a few cysteine residues (e.g. cysteine 151, cysteine 273, and cysteine 288) had functional consequences on Nrf2 regulation [16, 24-26]. In 2003 Zhang and Hannink published a breakthrough in the understanding of the mechanism of the Nrf2 pathway. Their data contributed especially to the knowledge concerning the importance of cysteine residues of KEAP1 within regulation of Nrf2. First of all, they demonstrated that KEAP1 does not passively sequester Nrf2 in the cytoplasm but actively targets the transcription factor for ubiquitination and degradation by the proteasome. Secondly, they proposed KEAP1 as a component of a novel E3 ubiquitin ligase complex. And thirdly, they identified cysteine 273 and 288 within KEAP1 as being required for KEAP1-dependent ubiquitination of Nrf2 and cysteine 151 for inhibition of KEAP1-dependent degradation of Nrf2 by oxidative stress [16]. The finding that transgenic expression of cysteine 273 and cysteine 288 mutant KEAP1 into KEAP1 knockout mice is not leading to inhibition of constitutive activation of Nrf2 confirms the necessity of these residues in KEAP1 function. On the other hand, cysteine 151 mutant retained the ability to suppress Nrf2 activation but it had a decreased activation after electrophilic and oxidative insults [24]. It is also noteworthy that loss of cysteine 151 residue may cause compensatory enhancement of other sensing activity of KEAP1. Cysteine 151-dependent inducers are not restricted exclusively to the cysteine 151-dependent pathway. Takaya *et al.* described that also cysteine 151 targeting compounds still activate Nrf2 and induce gene expression in C151S-MEFs and C151S-macrophages although in a moderate way. It is possible that in absence of cysteine 151, cysteine 151-targeting inducers attack other cysteines to transduce their signal [27]. Moreover, more evidence is obtained that there is not a single cysteine or group of cysteines that are reactive to all Nrf2 inducers. Nonetheless, there is the opinion that there are some residues more active than others [26]. Thus, the sensor function of KEAP1 to respond to Nrf2 inducers leading to activation of Nrf2 is highly complex. Although a vast number of publications concerning the importance of KEAP1 cysteines in response to stress inducers is available, still more research is needed to define the reactivity and sensitivity of each of the cysteines. Moreover, there is more insight needed in the collaboration and/or precise contribution of each reactive cysteine in response to all different Nrf2 inducers.

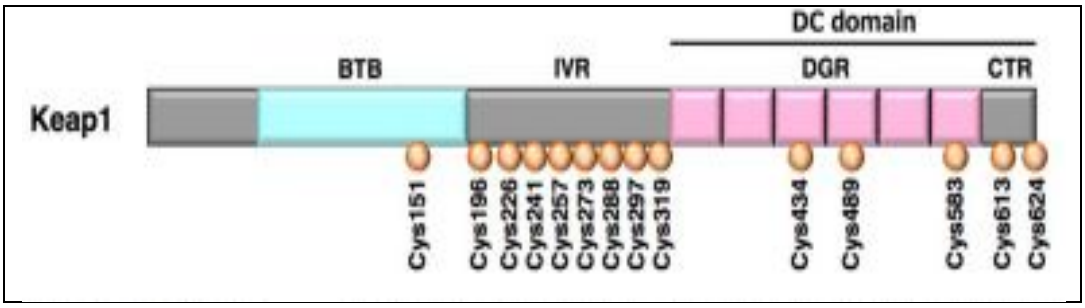

Fig 1.4 KEAP1 is a thiol-rich protein that is sensitive to electrophilic covalent modification**.** Domain structure of KEAP1 and some reactive cysteine residues out of 27 cysteine residues are shown. Direct modification of those cysteine residues using various electrophiles was demonstrated by other researchers. Each electrophile attacks a unique set of cysteines, rather than one cysteine residue [21].

It is clear that in the presence of Nrf2 inducers, Nrf2 can no longer be ubiquitinated and escapes from subsequent degradation. Accumulation of the transcription factor occurs, leading to increased transcription of Nrf2 target genes. It is also clear that KEAP1 is involved in proteasomal degradation of Nrf2 and that it can serve as a sensor to stresses in which its cysteines are involved. However, the molecular mode of action is still not completely understood and several mechanisms are proposed. Whether Nrf2 releases immediately from the KEAP1 dimer and translocates to the nucleus or whether its accumulation is due to the inability of KEAP1, still in a complex with Nrf2, to bind newly translated Nrf2 proteins is not straightforward.

Via single particle electron microscopy it was shown that the structure of the KEAP1 dimer resembles a cherry-bob structure where two globular structures are connected with a stem-like structure. In the globular structure the IVR and DC domain are in close proximity of each other. Important to note is that cysteine 273 and cysteine 288 are located in the IVR. Bearing the two-side binding model in mind, it is comprehensible that modifications of those critical cysteine thiols in the IVR can lead to conformational distortion in the binding between Nrf2 and the KEAP1 dimer. As expected, modification of cysteine 273 or cysteine 288 in the IVR can lead to dissociation of DLG in Nrf2 from the DC domain in KEAP1. Since the ETGE-DC binding is stronger, this binding remains. Consequently, the Nrf2 protein will still remain in the complex but due to conformational changes in the complex ubiquitination of Nrf2 will no longer occur. In contrast, based on co-immunoprecipitation studies it was demonstrated that some inducers, including metal inducers As(3+), Cd(2+) or Cr(6+) are fully capable of disrupting the binding between Nrf2 and KEAP1. A third molecular mode of action that is proposed is disruption of the binding between KEAP1 and Cul3, thereby preventing further ubiquitination of Nrf2. Such a disruption occurs when certain KEAP1 cysteines close to the interface between KEAP1 and Cul3 such as cysteine 151 are modified. In analogy with the first model proposed, Nrf2 will not dissociate from KEAP1 but Nrf2 will no longer be ubiquitinated. Eggler and colleagues suggested that mutation of cysteine 151 to a bulky residue is sufficient to downregulate Nrf2 ubiquitination and significantly activate ARE gene expression. The effect appeared to be mediated by a degreased KEAP1-Cul3 interaction.

Partial molar volume of the residue at position 151, and no other physico-chemical properties, correlated with the degree of Nrf2 activity [28]. The disruption of the KEAP1-Cul3 interaction as a way of Nrf2 activation by cysteine 151 preferable compounds however, still needs to be clarified. Substitution of cysteine 151 with the small hydrophobic amino acid alanine never disrupts the KEAP1-Cul3 interaction but it inhibits degradation of Nrf2 [29]. Moreover, investigating the diffusion dynamics of the KEAP1-Cul3 interaction led to the conclusion that exposure for 1 hour to inducers of 4 different types, the oleanane triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO), the isothiocyanate sulforaphane (SFN), the sulfoxythiocarbamate alkyne (STCA), and the oxidant hydrogen peroxide does not dissociate the KEAP1-Cul3 complex. This lead to the conclusion that changes in conformation rather than dissociation from Cul3 inactivate the repressor function of KEAP1 leading to Nrf2 stabilization [19]. Thus, there are until now 3 main models proposed: one where binding between Nrf2 and KEAP1 is disrupted at the DLG binding site, one where a complete disruption between Nrf2 and the complex is seen and a last model where the binding between KEAP1 and Cul3 is modified. Moreover, it has been suggested that different inducers can lead to different molecular models of action. Now, there is a need to further clarify the exact mode of the different molecular models of action and to define the preference of different Nrf2 inducers.

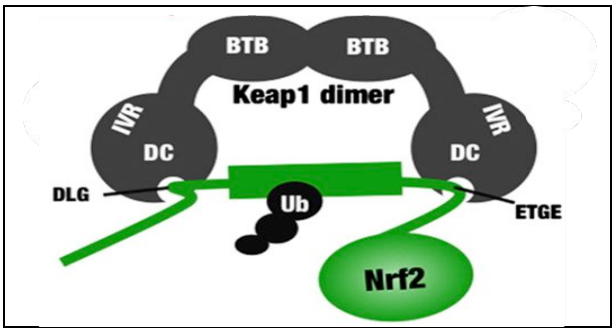

Fig 1.5 Cherry bob structure of KEAP1 homodimer built up by two globular structures connected with a stem-like structure. In the globular structure the IVR and the DC domain are in close proximity of each other. BTB domains are responsible for homodimerization and binding with Cul3 [27].

I.1.2.4 Other KEAP1-dependent mechanisms of regulation

Proteasomal degradation via KEAP1-dependent ubiquitination is generally considered as the main mechanism of regulation of Nrf2 under homeostatic conditions. In addition, activation of Nrf2 upon exposure to stresses via regulation of cysteine residues within KEAP1 is generally considered as the main regulatory system to activate Nrf2. However, also other KEAP1-dependent mechanisms are proposed that contribute to regulation of Nrf2.

Ubiquitination switch from Nrf2 to KEAP1

As an additional regulatory step in the Nrf2 pathway, a switch from Nrf2 to KEAP1 ubiquitination is suggested. Through this mechanism steady-state levels of both KEAP1 and Nrf2 would be controlled. Zhang and Hannink reported that KEAP1 and three other BTB-Kelch proteins are ubiquitinated by a Cul3-dependent complex. Ubiquitination of KEAP1 was strongly increased when cells were exposed to oxidative stress concomitant with inhibition

of KEAP1-dependent ubiquitination of Nrf2. The oxidative stress finally led to a decrease of steady-state levels of KEAP1-independent of the 26S proteasome [30]. The observation that KEAP1 could hardly be degraded by the proteasome, suggesting involvement of other degradation mechanisms, was also demonstrated by several other researchers [27, 31, 32]. As a regulatory mechanism, autophagic degradation of KEAP1 is proposed. Previously, autophagy was thought to be a bulk degrading pathway. Nowadays, it is believed to be able to degrade specific target proteins. p62, also called sequestosome 1 (SQSTM1), can act as a scaffold protein, binding to polyubiquitinated proteins and the autophagic machinery [33, 34]. Deregulation of autophagy causes upregulation of p62 and formation of p62-containing aggregates, which are associated with neurodegenerative diseases, cancer and cardiovascular diseases. One of p62s target proteins is KEAP1, for whom a physical and functional relationship was elucidated [35-40]. Thus, this is a possible mechanism of KEAP1 degradation following ubiquitination switch from Nrf2 to KEAP1. However, p62 and autophagy can also lead to activation of Nrf2 in other ways. An overproduction of p62 or a deficiency in autophagy led p62 to compete with Nrf2 for interaction with KEAP1. This competition results in stabilization of Nrf2 and transcriptional activation of Nrf2 target genes [35]. Moreover, overexpression of mutated p62, which lost its ability to interact with KEAP1, had no effect on Nrf2 stability. This suggests that p62-mediated Nrf2 upregulation is KEAP1-dependent [36]. P62 is competing with Nrf2 to bind KEAP1 as it can bind KEAP1 through its KIR motif, a motif similar to that of Nrf2-ETGE or Nrf2-DLG. Another type of disruptor is the cyclin-dependent protein kinase p21. This kinase is able to interact directly with the ETGE and DLG motifs of Nrf2, thereby competing directly with KEAP1 for binding with Nrf2 [41].

Nucleocytoplasmic shuttling of KEAP1

Furthermore, some researchers favor the hypothesis that Nrf2 is primarily located in the nucleus. They suggest that degradation of Nrf2 by KEAP1 is downstream of the transcriptional activity of Nrf2. Thereby, Nrf2 controls constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling of KEAP1. Shuttling of KEAP1 may repress Nrf2 activity by promoting its ubiquitination inside the nucleus [42, 43]. Moreover, the function of KEAP1 as an escort for nuclear export of Nrf2 was also suggested whereby KEAP1 translocates into the nucleus in order to dissociate Nrf2 from the ARE. The Nrf2-KEAP1 complex is then transported out of the nucleus by the Nuclear Export Signal in KEAP1 [44]. In another model it is suggested that prothymosin-α (ProTα), containing a nuclear localization signal, is required for nuclear import of the whole KEAP1/Cul3/Rbx1-E3 complex, as ProTα binds to the DGR of KEAP1. Once inside, the complex releases ProTα, binds Nrf2, ubiquitinates it and degrades Nrf2 inside the nucleus instead of exporting it to the cytoplasm. [45]. Controversially, many other researchers demonstrated that KEAP1 is localized primarily in the cytoplasm with minimal amounts in the nucleus under normal, homeostatic conditions. In addition, the nucleocytoplasmic shuttling mechanism is questionable as other researchers have already claimed not to observe this phenomenon [46]. Furthermore, KEAP1 was suggested to bind to the actin cytoskeleton which prevents the complex to enter the nucleus [18]. If the model in which

KEAP1 is entering the nucleus, removing Nrf2 from the ARE of downstream target genes and finally escorting Nrf2 back to the cytoplasm or degrading Nrf2 immediately in the nucleus would be correct, than KEAP1 is functioning also as a postinduction repressor. In this way, KEAP1 is not only an adaptor, but acts also as a guard.

Phosphorylation of tyrosine 141 of KEAP1

Besides reactive cysteine residues, tyrosine 141 is another proposed regulatory, highly conserved residue within KEAP1. Treatment of cells with hydrogen peroxide leads to a decrease in phosphorylation of tyrosine 141 followed by a rapid degradation of KEAP1. Phosphorylation and dephosphorylation of tyrosine 141 appears to plays a role in controlling the stability and degradation of KEAP1. Tyrosine 141 might also be involved in the ubiquitination switch from Nrf2 to KEAP1 [47].

We can conclude that the main regulation of the Nrf2 activation pathway by KEAP1 is mediated through proteasomal degradation. Furthermore, activation of Nrf2 is regulated mainly via cysteine residues of KEAP1. However, additional KEAP1-related functions are involved increasing the complexity of regulation of the Nrf2 pathway.

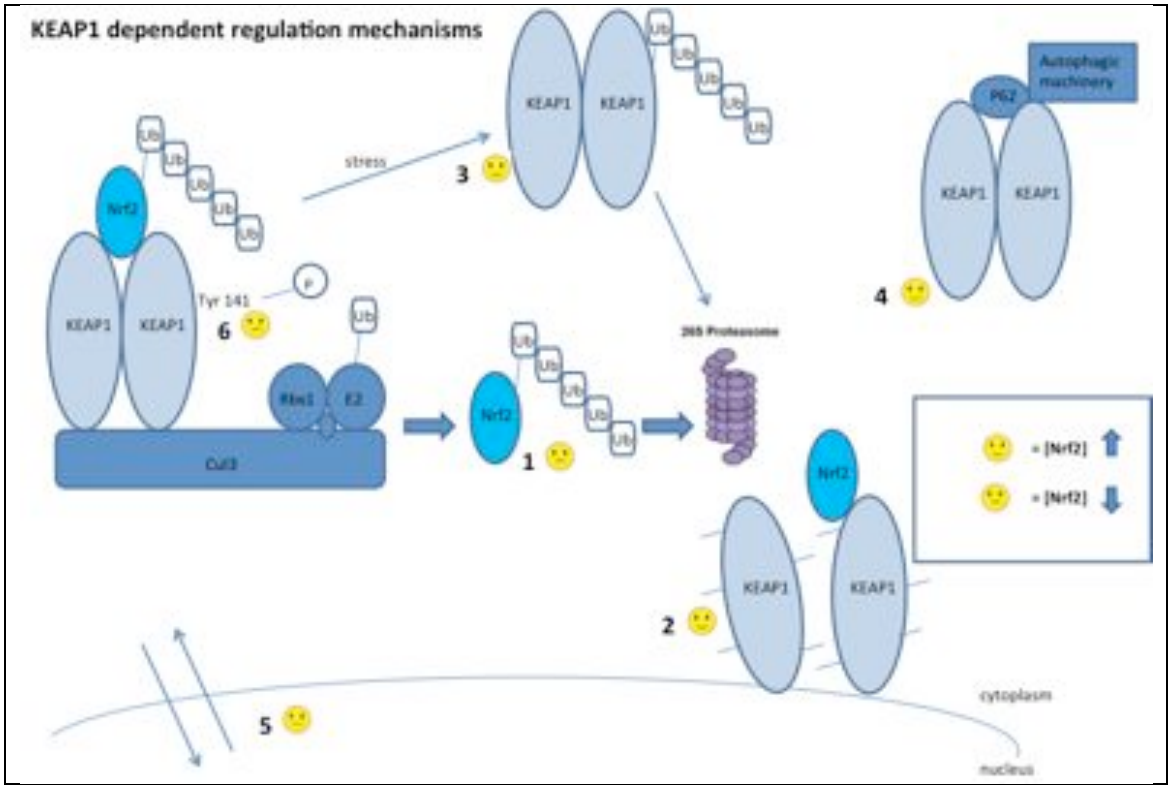

Fig 1.6 KEAP1-dependent regulation mechanisms of Nrf2 activity. 1) Main mechanism in cells under homeostatic condictions: proteasomal degradation of Nrf2 via KEAP1-dependent ubiquitination. 2) Main mechanism in the presence of stress: activation of Nrf2 via modification of KEAP1 cysteine residues. 3) Switch of ubiquitination from Nrf2 to KEAP1 lead to reduced KEAP1 levels. 4) Competition between p62 and Nrf2 to bind KEAP1. 5) Nucleocytoplasmic shuttling of KEAP1 or KEAP1/Cul3/Rbx1-E3 complex. 6) Phosphorylation of tyrosine 141 of KEAP1 lead to increased stability of KEAP1.

I.1.3 KEAP1-independent regulation of Nrf2 activity

In addition to different KEAP1-dependent mechanisms, as they are described above, there are also several different alternative regulatory mechanisms suggested, independent of the main regulator KEAP1.

I.1.3.1 Nrf2 cysteines

Apart from KEAP1 cysteines, cysteine residues in Nrf2 itself are proposed to have a regulatory role as well. Human and mouse Nrf2 contain 6 and 7 cysteines, respectively, that are highly conserved across species. It is shown that two arsenic-based Nrf2 inducers are able to directly bind Nrf2 cysteine residues. Further analyses revealed that also those cysteine residues are involved in Nrf2 signaling [48].

I.1.3.2 Post-transcriptional regulation: phosphorylation of Nrf2

Phosphorylation of Nrf2 has been proposed to be one of the main alternative mechanisms. Earlier it was believed that the action of Nrf2 activators was mediated solely via reactive cysteine residues of KEAP1. Nowadays, it seems more likely that kinase signaling pathways are nearly always involved [49]. Nrf2 contains many serine, threonine and tyrosine residues which may provide sites for phosphorylation by different kinases. Several protein kinase-dependent signal transduction pathways like casein kinase 2 (CK2) , glycogen synthase kinase 3β (GSK3β) , extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways, protein kinase C (PKC) and phosphatidylinositol 3-kinase-dependent pathway (PI3K)/Akt, are linked with regulation of the Nrf2 pathway.

CK2

The sequence of Nrf2 contains 13 potential CK2 phosphorylation target sites. In addition, Neh4 and Neh5 are identified especially as target regions for phosphorylation by CK2 [50, 51]. Nonetheless, there are only few publications concerning the effect of CK2 activity on the Nrf2 pathway. For example, tert-butylhydrochinon (tBHQ) induces two forms of Nrf2 protein in neuroblastoma cells, which migrate as distinctive bands on SDS-PAGE. The slower migrating form is identified as phosphorylated Nrf2 and the faster form as unphosphorylated Nrf2. Unphosphorylated Nrf2 predominates in the cytoplasm, whereas the phosphorylated form is preferentially localized in the nucleus [51]. Generally, other publications concerning this topic are in line with this observation. Moreover, it is even observed that inhibition of CK2 is leading to inhibition of Nrf2 phosphorylation and nuclear translocation [51-53]. As an exception, Pi *et al.* suggested that the two bands of Nrf2 observed after chemically-induced oxidative stress are both phosphorylated forms. Nrf2-98kDa seems to execute transcriptional activity, whereas Nrf2-118kDa is more susceptible to degradation. These data suggest that protein kinase CK2-mediated phosphorylation plays a potential role in Nrf2 activation and degradation [50].

MAPK

In comparison to CK2, kinases of the MAPK family are more intensively studied concerning their effect within the Nrf2 pathway. Several serine and threonine residues in Nrf2 can be phosphorylated by MAP kinases. In this way, JNK and ERK were demonstrated to be involved in the activation of Nrf2 [54-57]. Butylated hydroxyanisole (BHA) is able to activate both JNK and ERK MAPK thereby increasing the phosphorylation status of Nrf2 leading to translocation of the transcription factor to the nucleus. BHA-induced ARE transcriptional activity was attenuated by inhibition of ERK and JNK signaling using biochemical inhibitors and dominant-negative mutants [57]. Another MAPK, p38 is capable to achieve the opposite result. P38 phosphorylates Nrf2 and promotes its association with KEAP1, thereby preventing nuclear translocation of Nrf2 [58, 59]. Regulating the activity of p38 is a possible additional mechanism for induction of Nrf2 by the inducer SFN. The inhibiting effect of p38 on Nrf2 is reversed upon SFN treatment [60].

PKC

The involvement of PKC in phosphorylating Nrf2 and triggering its nuclear translocation in response to oxidative stress was for the first time suggested by Huang and colleagues [61]. They showed that Nrf2 bearing a serine to alanine mutation at amino acid 40 (S40A) could not be phosphorylated by PKC. Moreover, they found that phosphorylation of wild-type Nrf2 by PKC promoted its dissociation from KEAP1, whereas the Nrf2-S40A mutant remained associated. Thus, PKC is able to phosphorylate Nrf2 in the Neh2 domain at serine 40 leading to disruption of the binding between Nrf2 and KEAP1 [62]. Moreover, phosphorylation and nuclear translocation of Nrf2 was shown to be inhibited by PKC inhibitors [61-64]. However, whether phosphorylation of S40 in general is responsible for nuclear translocation or not, is still a matter of debate as some conflicting reports are published [49, 63].

GSK3β

Tyrosine kinase Fyn has been shown to phosphorylate tyrosine 568 in Nrf2. This kinase can act as a negative regulator, meaning that this type of phosphorylation will lead to nuclear export and degradation of the transcription factor. Mutation of tyrosine 568 resulted in loss of interaction of Nrf2 with chromosome region maintenance 1 (Crm1) and abrogation of nuclear export of Nrf2 [65]. Moreover, Fyn export out of the nucleus after an antioxidant treatment, allowing Nrf2 to bind the AREs. [66]. Interestingly, kinase GSK3β acts upstream of Fyn, activating its phosphorylation and resulting in its nuclear accumulation [67]. GSK3β was shown to phosphorylate not only Fyn but all Src kinases (Src, Yes, Fyn, Fgr) in general, leading to their nuclear import and accumulation. Studies on the physiological role of the Src family revealed that alterations in these kinases were inversely related with nuclear accumulation of Nrf2 [68]. Besides GSK3β´s effect on Nrf2 via Src kinases, GSK3β was also demonstrated to phosphorylate directly the Neh6 domain of Nrf2 leading to ubiquitination of the transcription factor [69]. Additionally, GSK3β is a downstream target of multiple kinase pathways like Akt and MAPK. Activation of these pathways inhibit GSK3β through phosphorylation, stabilizing Nrf2 by its reduced phosphorylation, ubiquitination, and nuclear

export. GSK3β is identified as a key regulator of Nrf2 stability and it may act as a "common" downstream effector of several Nrf2 inducers [70].

Despite a vast number of publications showing the effect of several Nrf2 activators on a multitude of kinases, the exact contribution of those kinases in the Nrf2 pathway is still largely unknown. Nevertheless, it is generally believed that all those kinases and phosphorylation linked signaling pathways are important in modulating Nrf2 activation steps, such as nuclear translocation and transcription activation. However, they do not replace the KEAP1-dependent ubiquitination and proteasomal degradation of Nrf2 as the primary mechanism of Nrf2 regulation.

I.1.3.3 Post-transcriptional regulation: Acetylation/deacetylation

In addition to ubiquitination that is leading to proteasomal degradation, phosphorylation is suggested to be the most important additional post-transcriptional way of Nrf2 regulation. However, other post-transcriptional modifications are proposed that might contribute to the regulation of Nrf2. CBP is acting as a co-activator of Nrf2 in the nucleus but it is also able to promote acetylation of Nrf2 leading to nuclear localization of the transcription factor and increased transcription of ARE-dependent genes. Sirtuin 1 (SIRT1) has an opposite function as it decreases acetylation of Nrf2 resulting in nuclear export and cytoplasmic localization of the transcription factor [71].

I.1.3.4 Post-transcriptional regulation: MicroRNAs

MircoRNAs can bind mRNA leading to mRNA degradation or to protein translation inhibition. They play an important role in fine-tuning the expression of various proteins and processes. Some microRNAs were demonstrated to be involved in Nrf2 regulation [72-76]. For example, an increase of miR-144 expression was linked with reduced Nrf2 protein levels leading to a reduced antioxidant capacity of erythroid cells [74]. MiR-28 could also regulate Nrf2 via facilitating degradation of Nrf2 mRNA and protein. Nevertheless, it had no effect on KEAP1 protein expression or KEAP1/Nrf2 binding [73]. On the other hand, miR-200 was able to target KEAP1 mRNA [75].

I.1.3.5 Transcriptional regulation: binding partners

Eventually, when Nrf2 is imported into the nucleus, it recruits transcriptional machinery to transactivate ARE-driven genes. As explained earlier, Nrf2 will heterodimerize with a small maf-F/G/K protein upon entering the nucleus. In addition, after Nrf2/maf heteromere binding to the ARE sequence, it will also recruit and bind other nuclear regulatory factors that can modulate gene transcription. This modulation however, can increase or decrease transcription of the ARE regulated gene. As previously described, CBP is binding to the Neh4 and Neh5 domain of Nrf2 leading to transactivation. This is by far not the only co-regulator as there are many other different binding partners modulating transcription of the ARE driven genes. Among them are co-activators, fulfilling their functions first of all through protein-protein interactions bridging Nrf2 to the basal transcription machinery. Secondly,

some of them can co-activate through their chromatin remodeling activities that facilitate assembly of a transcription initiation complex. For example, the co-activators CBP, co-activator arginine methyltransferase (CARM1) and protein arginine methyl-transferase (PRMT1), enhance the ability of receptor associated co-activator 3 (RAC3) to initiate the transactivation domain [77]. In contrast to the activators, caveolin-1 is described as a repressor of Nrf2. It inhibits expression of antioxidant enzymes through direct interaction with Nrf2 [78]. There are numerous other proteins described that are able to antagonize Nrf2 (e.g. Activating transcription factor 3 (ATF3), estrogen receptor alfa (ERα), peroxisome proliferator-activated receptor gamma (PPARγ), Retinoic acid receptor alfa (RARα), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), protein 53 or tumor protein 53 (p53) etc). Interestingly, also some bzip proteins like Nrf1, Bach proteins or activator protein 1 (AP-1) can directly interfere with Nrf2-dependent transcription. AP-1 can bind TPA response element (TRE) or cAMP response element (CRE) elements laying within ARE sequences. Nrf1 and Bach can heterodimerize small maf proteins and bind to enhancers [79, 80]. For example, Bach1 can heterodimerize mafK and bind to E1 and E2 enhancers of the human HO-1 gene promoter to repress transcription. On the other hand, it has been suggested that Nrf1, AP-1 and other bZip proteins can activate transcription of ARE-dependent genes in absence of Nrf2 since it has been noted that ARE-dependent genes are still induced in the intestine in Nrf2 knockout mice upon stimulation with BHA, coumarin and diterpenes [81-83]. This variability in Nrf2 dependence for upregulation of ARE-dependent genes in different organs suggests that there are also other factors besides Nrf2 that become activated by inducers of ARE-dependent expression. It is suggested that those factors might be other bZip proteins.

I.1.3.6 Autoregulation

An ARE binding site is found in the promoter region of Nrf2 suggesting a positive feedback loop, leading to enhanced cell defense. In support of this theory, Kwak *et al*. showed a direct binding of Nrf2 to its own promoter. Moreover, increase of Nrf2 protein and mRNA was induced by an ARE inducer. Also, Nrf2 promoter-luciferase reporter activity was enhanced by treatment with an Nrf2 activator and overexpression of Nrf2 increased activity of the Nrf2 promoter-luciferase reporter, while expression of mutant Nrf2 protein repressed activity. ARE-like sequences were demonstrated to be required for this activation [84]. However, also a regulatory feedback loop between Nrf2 and KEAP1 exists. When Nrf2 is activated, it induces promoter activity of KEAP1. Nrf2-induced KEAP1 protein will accelerate ubiquitination of Nrf2 for degradation. Thus, Nrf2 regulates KEAP1 by controlling its transcription, and KEAP1 controls Nrf2 by degrading it [85].

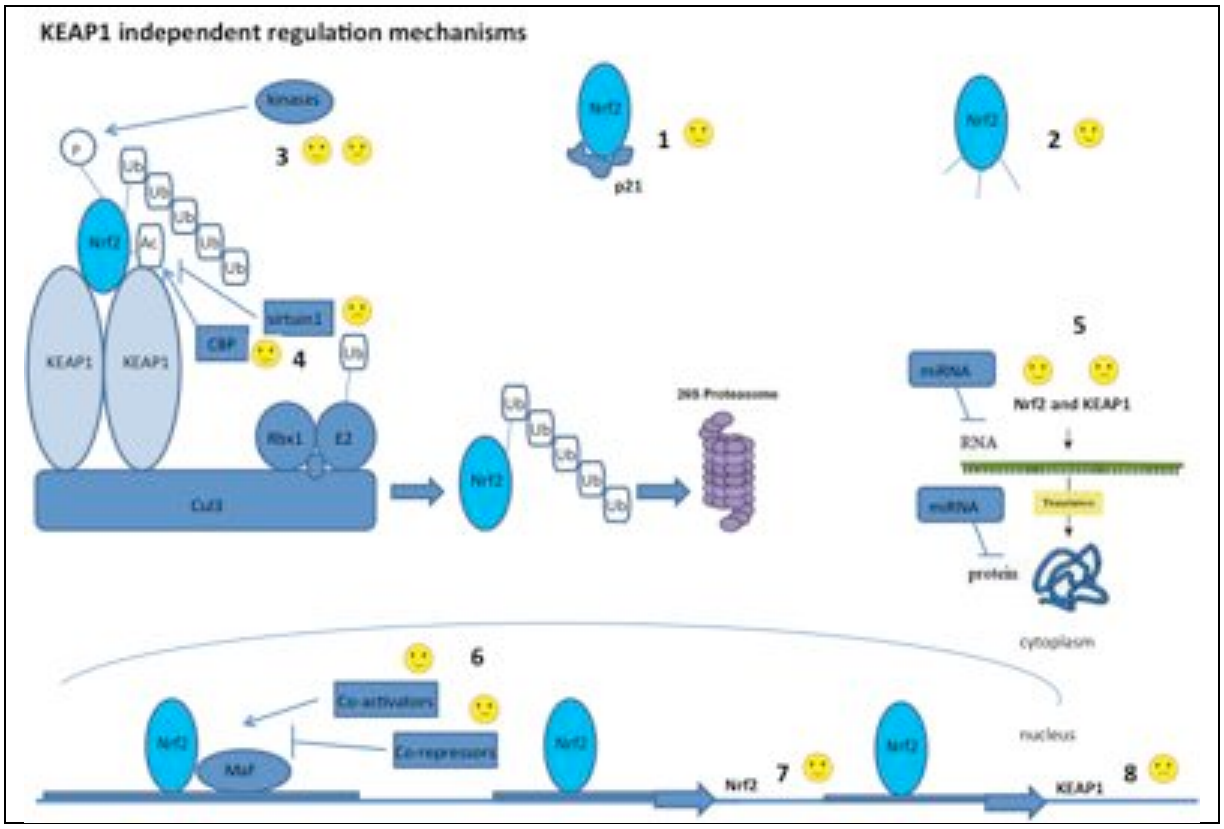

Fig 1.7 KEAP1-independent regulation mechanisms of Nrf2 activity. 1) Disruptor p21 binds ETGE and DLG motifs of Nrf2 inhibiting binding of KEAP1 with Nrf2. 2) Modification of cysteine residues in Nrf2 lead to activation of Nrf2. 3) Phosphorylation of Nrf2 by CK2, ERK, JNK, PKC lead to activation of Nrf2. Phosphorylation of Nrf2 by p38, GSK3β lead to repression of Nrf2 activity. 4) Acetylation by CBP promote activation whether deacetylation by sirtuin1 promote repression of Nrf2. 5) Specific mRNAs targeting Nrf2 reduce activation of Nrf2 whether other mRNAs targeting KEAP1 promote Nrf2 activation. 6) Binding of co-activators, including small maf-F/G/K, CBP, CARM1, RAC3, PRMT1 promote ARE-dependent gene transcription whether corepressors, including caveolin-1, ATF1, ERα, PPAR-γ, RARα, NF-κB, p53, Nrf1, Bach1, AP-1 reduce ARE-dependent gene transcription. 7) Positive feedbackloop via ARE binding site in Nrf2 promoter. 8) Negative feedbackloop via increased promoter activity of KEAP1 via Nrf2.

I.1.4 Conclusion concerning the molecular mechanisms of regulation

It is of great importance that Nrf2 is activated at the right moment in response to a stimulus. Nevertheless, it is as important that Nrf2 is properly switched off when the stimulus is removed. Thus, a vital factor in the functioning of Nrf2 is spatio-temporal regulation, as this is the case for many transcription factors. For this reason, there are a number of different mechanisms leading to tight regulation of Nrf2. Loss of this tight regulation will lead to aberrant activation or complete lack of Nrf2 activity both having malignant consequences. Those different kinds of KEAP1-dependent and -independent mechanisms explained earlier

highlight just how complicated and highly regulated this pathway is. In addition, Papp and colleagues defined an Nrf2-related interactome and regulome containing many multifunctional proteins and fine-tuned autoregulatory loops. At least 289 protein-protein, 7469 transcription factor-DNA and 85 miRNA interactions were included, emphasizing the complexity of regulation of the Nrf2 pathway [86]. Therefore, we have to bear in mind that the mechanisms explained in this work are probably only still the top of the iceberg. Under homeostatic conditions, it is clear that the main regulation of Nrf2 is driven by KEAP1 regulated proteasomal degradation. When stresses are present however, regulation of Nrf2 becomes complex and involves KEAP1-dependent and -independent mechanisms. Changes in intracellular localization of KEAP1 and Nrf2, post-transcriptional modifications, changes in mRNA levels etc. become involved in regulation of this transcription factor. Therefore, a better understanding in the molecular mechanism of Nrf2 regulation and activation under stressed conditions is needed. This understanding is certainly needed if we want to improve the current therapies or develop novel therapies targeting the Nrf2 pathway.

I.1.5 Transcriptional targets of Nrf2

This tight regulation of Nrf2 activity is of such great importance because Nrf2 contributes to a plethora of functions and regulation of an array of genes. Strong evolutionary conservation of Nrf2 for example is reflecting its importance. Nrf2 is a transcription factor that has evolved from primitive origins over millions of years, where homologues are traceable back to *Drosophila* species. The ancestry of Nrf2 has deep-seated roots in hematopoiesis. Nowadays, the transcription factor as it diversified, mediates a wide range of genes important in several functions [87]. The main function of Nrf2 is maintaining cellular homeostasis, especially upon exposure to stresses. This is through its ability to regulate expression of a multitude of antioxidant proteins, detoxification enzymes and xenobiotic transporters [88]. Nrf2 activation will lead to reduction of reactive compounds, such as free radicals and electrophiles to less toxic intermediates. Together with this response, an increasing ability to repair any damage ensued will be provided. It is shown that Nrf2 is not necessarily vital for survival in unstressed cells. Nevertheless, Nrf2 exerts more functions, as it contributes to diverse cellular functions, including inflammation, differentiation, proliferation, growth, apoptosis and lipid synthesis [88]. Genome wide search has led to the identification of a plethora of ARE-dependent Nrf2 regulated genes involved in a variety of cellular functions. Genes regulated by Nrf2 can be grouped into several categories, including antioxidant genes, phase II detoxifying enzymes, transporters, scavenger receptors, chaperone proteins, DNA repair enzymes, anti-inflammatory proteins and other transcription factors. Even though Nrf2 is constantly degraded there is still a small pool of Nrf2 that accumulates in the nucleus. This small pool mediates a low but detectable basal expression of some ARE-dependent genes. For example, some constitutive active processes

like lipid homeostasis are under control of Nrf2. However, the main function of Nrf2 lies in its inducibility when exposed to inducers such as oxidative stress or xenobiotics [7].

I.1.5.1 HMOX-1

One of the intensively studied Nrf2 target genes is the inducible form of heme oxygenase (HO-1). Three isoforms of heme oxygenase have been identified in humans, HO-1, HO-2 and HO-3, all products of different genes [89-91]. The HMOX-1 gene has evolved as an important antioxidant gene, encoding the HO-1 protein. HO-1 is composed of 288 amino acids with a molecular weight of 32kDa [92]. As the most important isoform of heme oxygenases, HO-1 is the inducible isoform of the enzyme regulated via transcriptional and post-transcriptional regulation mechanisms (e.g. increased HO-1 mRNA half-life in response to nitric oxide (NO)) [93]. It is involved in the first and rate-limiting step of heme degradation. During degradation of heme, catabolism takes place of free iron, carbon monoxide and biliverdin, which is subsequently converted to bilirubin by the enzyme biliverdin reductase [94]. In addition to degradation of heme, HO-1 provides protection by regulating and maintaining intracellular redox states. It has been reported that, compared to other ARE-dependent genes, HO-1 has the highest number of AREs in its promoter. Besides HO-1, one can find two other heme oxygenases. HO-2 is constitutively expressed in many organs throughout the body and especially in the brain and testes. It appears to be unresponsive to many HO-1 inducers. The last one is HO-3 that is almost completely devoid of any catalytic activity.

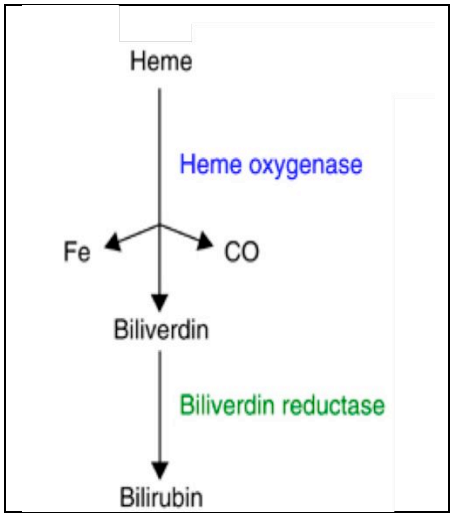

Fig 1.8 Degradation of heme by HO-1 in to free iron, carbon monoxide and biliverdin, which is subsequently converted to bilirubin by the enzyme biliverdin reductase [95].

Regulation of HO-1 gene expression is mediated by the “common” Nrf2 regulated antioxidant protective response (intensively explained earlier). However, it is presumed that there are mechanisms that could mediate induction of HO-1 independently of the general Nrf2 regulated antioxidant protective response. Thus, it concerns mechanisms that are particularly assigned to HO-1 that cannot be seen as general mechanisms of regulation for ARE-dependent gene expression. Brahma-related gene 1 (BRG1), a catalytic subunit of SWI2/SNF2-like chromatin-remodeling complexes, has been shown to be necessary for

specific recruitment of RNA polymerase II to the binding site in the promoter of HO-1. Interestingly, chromatin immunoprecipitation analysis revealed that Nrf2 recruits BRG1 to both HO-1 and NQO1 regulatory regions. However, BRG1 knockdown selectively decreased recruitment of RNA polymerase II to the HO-1 promoter but not to the NQO1 promoter. Thus, BRG1 interacts with Nrf2 to selectively mediate HO-1 induction in response to oxidative stress [96]. Mentioned earlier as an alternative regulatory mechanism, Bach1 can directly interfere with Nrf2-dependent transcription. Regulation by Bach1 has been suggested to be crucial, specifically for HO-1 regulation. Sodium arsenite has been shown to cause HO-1 induction via Bach1, independently from Nrf2. Surprisingly, no regulation of other ARE-dependent genes via this mechanism was seen [97]. The loss of Bach1 function in human keratinocytes results almost exclusively in HO-1 induction. This knockdown of Bach1 increased HO-1 gene expression 135-fold but induced other Nrf2-related genes to a maximum of only 2.7-fold [98]. Thus, inhibition of Bach1, without activation of Nrf2, is leading to HO-1 upregulation and not of other ARE-dependent genes. On the other hand, HO-1 regulation is particularly sensitive to the repressor effects of Bach1. Bach1 can be a mechanism to make sure that the activation of HO-1 is transient, because prolonged HO-1 overexpression is likely to be detrimental. Raval and colleagues showed that UV-light irradiation of cultured human skin fibroblasts enhances accumulation of Bach1 mRNA and protein level. Endogenous Bach1 protein accumulated in the nucleus after 8 hours and provided a compensatory mechanism to control HO-1 overexpression [99]. Nevertheless, the idea that Bach1 is specifically regulating HO-1 expression is still controversial since other investigators reported that Bach1 induced HO-1 gene expression together with some other Nrf2 regulated genes [100, 101]. The promoter region of HO-1 is characterized by the presence of different transcription factor binding motifs (e.g. AP-1, AP-2, NF-κB, and ARE) [102, 103]. The main transcription factor is undoubtedly Nrf2, binding to AREs in the promoter of HO-1. Besides this, presence of other transcription factor binding motifs suggests possible combinatorial control of these factors with Nrf2, though, the exact role of those other transcription factors in regulation of HO-1 still remains to be clarified. Regarding NF-κB, there are some reports describing functional binding sites of NF-κB in the promoter of HO-1 in rat and mouse. Naidu and colleagues have shown that activation of HO-1 by phorbol 12-myristate 13-acetate (PMA) was mediated via a κB element in the proximal rat HO-1 gene promoter region. Moreover, PMA-dependent induction of endogenous HO-1 gene expression and promoter activity was abrogated in cells from mice deficient for the NF-κB subunit p65 [104]. Several research groups have also reported a role of AP-1 in the induction of HO-1 [102, 105-109]. Yet, the molecular mechanism behind this effect is not completely understood. AP-1 is a transcription factor composed of functionally- and structurally-related members of the jun, fos and ATF protein families. Dimers of those family members are binding to the AP-1 sites or TRE. Of particular interest a consensus DNA binding sequence of AP-1 is located within the ARE sequence in the HO-1 promoter [110-113]. Similarly to NF-κB, AP-1 is upregulated by a wide variety of pro-oxidants and pro-inflammatory stimuli. Inducible gene expression regulated by AP-1 is involved in a range of immunological and

antioxidant stress responses [114, 115]. Altogether, further studies are required to dissect regulation by other transcription factors and combinatorial control of Nrf2 with other transcription factor families at the HO-1 gene promoter.

I.1.6 Nrf2 inducers

Nrf2 activation is generally linked to perturbation of cellular thiol status and/or oxidative and electrophilic stress. Thus, pending some exceptions, almost all currently known Nrf2 inducers are influencing thiol status, oxidative and electrophilic stress or both. Most ARE inducers are indirect inhibitors of KEAP1–Nrf2 interaction and they are believed to form covalent adducts with sulfhydryl groups of cysteines in KEAP1 by oxidation or alkylation.

Inducers of Nrf2-regulated genes can be categorized in different chemical classes, including 1) oxidizable diphenols, phenylenediamines and quinones; 2) Michael acceptors (olefins or acetylenes conjugated to electron-withdrawing groups); 3) isothiocyanates; 4) thiocarbamates; 5) trivalent arsenicals; 6) dithiolethiones; 7) hydroperoxides; 8) vicinal demercaptans; 9) heavy metals; 10) polyenes; 11) polyfenols; 12) triterpenoids; 13) particles and fibers and 14) protein factors. So this group includes a whole range of different types of chemicals that differ considerably in structure but having a common feature: their reactivity towards sulfhydryls [22]. As mentioned earlier, cysteine residues in the amino acid sequence of KEAP1 are detecting changes in cellular redox state. An increase in intracellular ROS or electrophiles leads to an increase in oxidation or conjugation of KEAP1 cysteines. Some inducers, such as Michael reaction acceptors induce ARE-dependent genes by direct binding to the cysteine thiols of KEAP1 and Nrf2. Polyphenols and triterpenoids are listed as other groups even though they also contain diphenol or Michael acceptor structures. Some inducers only acquire there thiol reactive properties after oxidative metabolism, like BHA that is turned into tBHQ via dealkylation. Besides their capacity to react directly with sulfhydryls, fibers and particles like diesel exhaust can also stimulate the cells to produce endogenous inducing agents of Nrf2 like ROS. Heavy metals, including cadmium, are also known to activate Nrf2, but the precise mechanism remains to be elucidated. $CdCl_2$ for example, has been shown to activate Nrf2 signaling by modulating the zinc binding capacity of KEAP1 [116]. However, there are also sulfhydryl-reactive heavy metals reported, for which a direct interaction of the metal ions with sulfhydryl groups was noted [117].

Different classes of inducers have different preferences for cysteine residues and they modify them via different chemical mechanisms like oxidation or alkylation. However, it is not known if those differences are leading to distinct gene expression patterns. As mentioned above, KEAP1 possesses a high number of cysteine residues, where some of them are highly reactive and are serving as sensors to those Nrf2 inducers. Numerous researchers have shown that the response of several electrophils is abrogated when a single point mutation of one cysteine in KEAP1 is introduced. However, this mutation does not

affect the responsiveness of all inducers suggesting that there are multiple sensor mechanisms within the cysteine residues of KEAP1 sensing the presence of Nrf2 inducers. Moreover, it is suggested that different chemicals that trigger the KEAP1-Nrf2 system are associated with distinct patterns of KEAP1 cysteine modifications, resulting in the concept of a “cysteine code”. Structurally dissimilar inducers reacting with similar cysteine residues would lead hereby to similar biological responses. Thus as stated previously, the precise contribution of each reactive cysteine in KEAP1 should be clarified for every Nrf2 inducer. Besides the categories of the different chemical classes, the Nrf2 inducers are often also categorized as cysteine 151-dependent or -independent, as cysteine 151 is a critical residue for a subset of Nrf2 inducers. With the use of a transgenic complementation rescue assay Takaya and colleagues contributed to a clear and trustworthy subdivision of cysteine 151-dependent and -independent inducers *in vivo.* Experiments with embryonic fibroblasts and primary macrophages yielded consistent results. They resolved a discrepancy concerning the cysteine 151 dependency of ebselen, CDDO-Im and SFN. They demonstrated that the first two are independent and the latter dependent of cysteine 151 in contrast to previous reports [27].

The different classes of ARE inducers can derive from a variety of endogenous or exogenous sources. As an example of exogenous inducers, many phytochemicals and there derivatives are potent inducers of ARE-dependent genes. They often contain a Michael acceptor function. One example is the acid natural triterpenoid used in Chinese herbal medicine. A synthetic derivative thereof, CDDO-Im , is the most potent Nrf2 inducer known until today. There is also a growing list of endogenous inducers, which are not as potent as synthetics, but their presence in target tissues at critical time points make them important regulators. For example, NO, Zn(2+), and alkenals are endogenously occurring chemicals whose concentrations increase during stress. KEAP1 directly recognizes NO, Zn(2+), and alkenals through three distinct sensors, others than cysteine 151 [29].

In contrast to Nrf2 inducers, the identification of Nrf2 inhibitors or inactivators has not gained that much attention. Nontheless, several small molecules have been found to suppress the Nrf2 pathway, including Ascorbic acid (Vitamin C) [118].

I.1.7 Nrf2 in disease and toxicity, therapeutic application of Nrf2 modifiers

Oxidative/electrophilic stress conditions have been linked to various diseases i.e. cancer, Alzheimer’s disease, Parkinson’s disease, atherosclerosis, sickle cell disease, chronic fatigue syndrome etc. An excessive number of endogenous and exogenous stressors can lead to an overload of ROS, electrophils and reactive intermediates exceeding the ability of the cells to detoxify. However, this stress is dependent on the cellular regulation of gene expression to

which the transcription factor Nrf2 largely contributes and is therefore referred to as the "master regulator" of cellular homeostasis. Besides, Nrf2 also regulates large numbers of genes that control seemingly disparate processes such as immune and inflammatory responses, tissue remodeling and fibrosis, carcinogenesis and metastasis, cognitive dysfunction and addictive behavior. As expected, inappropriate regulation of Nrf2, can have severe health consequences. Today, perhaps 200 human diseases have been linked to perturbed stress responses by Nrf2. From an “aging” perspective, Nrf2 activators could become attractive therapeutic drugs to prevent the so called “aging diseases ” (cancer, inflammatory and autoimmune diseases, neurodegenerative diseases, cardiovascular diseases,..) compensating for the decline in Nrf2 levels due to aging.

I.1.7.1 Cancer

Today, pro- and anticarcinogenic effects of Nrf2 activation have been reported in cancer, which paradoxically suggest that both Nrf2 inhibitors and Nrf2 activators could be applied as successful cancer therapeutic strategies. Activation of Nrf2 renders animals resistant to chemical carcinogens and other forms of toxicity that can lead to cancer. When Nrf2 is disrupted in those situations it exacerbates the outcome. Thus, detoxifying activity of Nrf2 is important in cancer prevention. On the other hand, Nrf2 regulation can be affected in cancer cells whereby it can lead to constitutive dominant hyperactivation of Nrf2. Such hyperactivation is responsible for preservation of a cancer phenotype rather than attenuation. As a central transcription factor responsible for many aspects of cytoprotection and detoxification, Nrf2 is expected to regulate genes that are beneficial from an evolutionary point of view. Unfortunately, certain types of cancers favor constitutive activation of Nrf2 just for the same reason. Thus, cancer cells also exploit Nrf2´s protective functions to thrive and resist chemotherapy.

In general, it is known that Nrf2 is a cytoprotective factor that is beneficial for health. This important role of Nrf2 in protection from oxidative stress and xenobiotics is shown in various analyses with Nrf2-deficient mice. It is shown that Nrf2-deficient mice have an increased sensitivity to carcinogenic chemicals. Moreover, incidence of cancer is not reduced by chemopreventive agents such as oltipraz, a 1,3-dithiolethione in Nrf2-deficient mice [119]. Oltipraz induces phase II detoxifying enzymes and reduces cancer incidents in Nrf2 wild type (WT) mice. However, it did not reduce cancer incidents in Nrf2 KO mice [120]. Similarly, other natural compounds and genetic models were shown to rely on Nrf2 for their protective activity [121-125]. Thus, chemopreventive agents such as oltipraz and other natural compounds exert their anticarcinogenic effects through Nrf2. Similarly, Fahey and colleagues showed that SFN, a natural Nrf2 inducer, is preventing against stomach tumors. SFN appeared to be bactericidal and was able to eliminate *Helicobacter Pylori*, a common infection that enhances the risk of gastric cancer. This protection resulted from induction of phase II detoxifying and antioxidant enzymes. Moreover, this protective effect was abrogated in mice lacking Nrf2 [121]. Furthermore, protection against UV-light-induced skin carcinogenesis in mice by SFN-containing broccoli sprout extracts has been suggested [122].

In addition, a topical application of SFN-containing broccoli sprouts extracts induced phase II response in mouse skin *in vivo*. WTX and PALB2, both tumor suppressor genes are shown to interact with the DC domain of KEAP1. This binding leads to inhibition of ubiquitination of Nrf2 and induction of Nrf2-dependent transcription. Mutations in WTX are often found in kidney tumors and mutations in PALB2 in breast and pancreatic cancers. Those mutations lead to decreased activition of Nrf2. Thus, those genes suppress carcinogenesis partly through maintaining the activity of Nrf2 [126]. These data together are leading to the notion that activation of Nrf2 is an attractive strategy to prevent cancer and reduce stress-related damage. Additionally, they show that Nrf2-dependent transcription is required for cancer treatment by several anticarcinogens.

In contrast to this, there are several data promoting the idea that Nrf2 activation is procarcinogenic. They suggest a dark side of Nrf2: its propensity to be hijacked by cancer cells and to facilitate a pro-survival phenotype. Highly activated Nrf2 genes, such as detoxifying and antioxidant enzymes, confer a great advantage for cancer cells for survival against anticancer drugs. Moreover, Nrf2 also promotes cell proliferation. Thus, cancer cells have benefits by promoting hyperactivation of Nrf2. The prognoses of cancer patients with high levels of Nrf2 are indeed significantly poor [127-129]. There are several mechanisms suggested for increased activity of Nrf2 in cancer cells. Various mutations in KEAP1 and Nrf2 have already been reported in human cancers, responsible for constitutive expression of pro-survival cytoprotective genes regulated by Nrf2 [130-137]. More than half of the mutations in KEAP1 are laying in the DC domain, suggesting interference with binding of KEAP1 to Nrf2 [138]. Concerning the mutations of Nrf2, most of the mutations are clustered in the DLG or ETGE domain. High and low affinity binding of Nrf2 with KEAP1 are in this way interrupted [127]. DNA hypermethylation of the promoter of the KEAP1 gene is suggested to be another mechanism for elevated levels of Nrf2 [139-142]. Those epigenetic alterations lead to an inhibition of KEAP1 expression resulting in Nrf2 accumulation. Epigenetic abnormalities in the promoter of KEAP1 in cancer patients are associated with poor clinical outcome. Another explanation for hyperactivation of Nrf2 can be found in an accumulation of proteins that are able to disrupt binding between KEAP1 and Nrf2, such as p62 which cumulates in several cancers [40, 143, 144]. In addition, activity of transcription of Nrf2 is influenced by oncogenes, like K-Ras, which activates Nrf2 through the ERK signaling pathway [145]. A last mechanism that has been suggested, is modification of KEAP1 through oncometabolites. The latter are metabolites, unique for cancer cells that are involved in initiation and progression of cancer. The oncometabolite fumarate can modify cysteines in KEAP1 resulting in accumulation of Nrf2 [146]. From these studies we can conclude that constitutive hyperactivation of Nrf2 in cancer cells evokes selective resistance to stresses, leading to preservation of the tumor rather than attenuation.

Nontheless, hyperactivation of Nrf2 due to global disruption of KEAP1 in mice led to postnatal death within 3 weeks after birth [147]. When this KEAP1 knockout was crossed with a Nrf2 mutant mouse, generating the Nrf2-KEAP1 double mutant mouse the phenotype

linked with this early death was reversed. However, the impact of altered regulation of the Nrf2 pathway can vary greatly from tissue to tissue. It has been shown that in certain mouse organs a constitutive activation of Nrf2 is not detrimental but rather cytoprotective [148, 149]. As such, to combat cancer with Nrf2 inhibitors, one preferably should deliver or target drug to cancer cells. Upon systemic inhibition of Nrf2, one may expect exacerbation of side effects of chemo- and radiotherapies. Another challenge is achieving drug specificity towards Nrf2 only. It is suggested that the bZip structure of Nrf2 shares many common properties with that of other Cap-N-Collar members. In this respect, Nrf2 inhibitors should preferably target outside the bZip motif.

To conclude the Nrf2 pathway is a critical master regulator of stress responses to preserve human health. As such, aberrant, constitutive (hyper)activation, as well as disruption of Nrf2 both have health detrimental effects. Since constitutive Nrf2 activation promotes cancer cell survival, Nrf2 inhibitors hold promise to suppress tumor cell proliferation and sensitize for apoptosis. Reciprocally, hypo-activation or complete disruption of the Nrf2 pathway sensitizes cells to carcinogenic chemicals and increases their susceptibility to develop cancer. Thus, further research is needed to understand the dual role of Nrf2 in various stages of cancer development.

I.1.7.2 Other diseases

In addition to cancer, there is an array of other diseases with deregulated Nrf2 responses, including various neurological disorders, cardiovascular diseases, chronic kidney diseases, diabetes, airway disorders, inflammatory bowel disease, autoimmune diseases etc. [150-168].

Nrf2-deficient mice suffer from various deficiencies and demonstrate the important role of Nrf2 in protection from exogenous or intrinsic stressors [169, 170]. As an example, Nrf2-deficient mice are more susceptible to cigarette smoke-induced emphysema, bleomycin-induced pulmonary fibrosis and hyperoxic lung injury [171-173]. Moreover, Nrf2-deficient mice also suffer from accumulation of intrinsic oxidative stress and can develop a lupus-like autoimmune disorder [174]. In general, Nrf2-deficient mice tend to develop various inflammatory disorders, including multi-organ autoimmune inflammation, glomerulonephritis and immune-mediated hemolytic anemia [174-176]. In conclusion, lack of Nrf2 is leading to malignancies due to ineffective protection against oxidative, xenobiotic, extrinsic and intrinsic insults. Besides a full disruption of Nrf2 in mouse knockout models, single-nucleotide promoter polymorphisms have been demonstrated which decrease Nrf2 transcription levels and cause lung damage due to hyperoxia. Along the same line, mutations have been reported in the human Nrf2 gene promoter leading to increased risk of acute lung injury [160, 177]. Reciprocally, increased levels of Nrf2 can have beneficial effects in the pathogenesis of different neurological disorders [178-180].

In conclusion, to consider Nrf2 as a good or bad factor per se is clearly an oversimplification and is context and concentration dependent. Certainly, a more detailed investigation of its

regulation and activity in different pathologies may unveil new strategies for prevention and therapy of diseases like cancer, chronic diseases and toxicity [7].

I.1.7.3 Therapeutic applications

Some plant derived compounds with chemopreventive properties like, broccoli derived SFN [181, 182] and curcumin isolated from Curcuma root [183] induce Nrf2 and are currently evaluated in clinical trials for treatment of a variety of cancers. Moreover, several other Nrf2 inducers such as bardoxolone methyl (CDDO-Me), NO-donating non-steroidal anti-inflammatory drugs, idarubicin and cytarabine are also studied for their anticancer properties [184-187]. While a number of Nrf2 inducers entered clinical trials, only a few Nrf2 inhibitors have been developed. One example is brusatol, which is purified from the plant *Brucea javanica.* A decrease in drug detoxification and impairment in drug removal may be the primary mechanisms by which brusatol enhances the efficacy of chemotherapeutic drugs [188].

Alternative to cancer treatment, various compounds such as allyl sulfides, dithiolethiones, flavonoids, isothiocyanates, polyphenols and triterpenoids are tested in animal studies or clinical trials for prevention of chronic oxidative stress-related diseases [189]. BG-12, dimethyl fumarate produced by Biogen Idec, passed placebo-controlled phase 3 trials, for treatment of relapsing-remitting multiple sclerosis [190-192]. Bardoxolone methyl, produced by Reata Pharmaceuticals is currently in phase 3 trials. A phase 2 human clinical trial with bardoxolone methyl in patients with moderate chronic kidney disease associated with type 2 diabetes mellitus showed a significant and sustained improvement in several measures of kidney function [184]. These observations suggest Nrf2 activation as a valid therapeutic approach for renal disease. Unfortunately, serious off-target side effects have recently been reported for several new drugs for diabetic kidney disease, including Bardoxolone methyl [193, 194]. Among the more interesting drugs related to our ongoing research is Protandim, a product from LifeVantage Corporation. This patented dietary supplement consists of five low-dose natural Nrf2 activators. The five herbal components are *silybum marianum, bacopa monniera, camellia sinensis, curcuma longa* and *withania somnifera* [195]. *Silybum marianum* or milk thistle contains a group of active constituents, the silmarin that exerts liver protective, regenerative and antioxidative properties. The second component is *bacopa monniera* or water hyssop used in Ayurvedic treatment for epilepsy and asthma. It contains many chemical constituents like alkaloids, saponins and flavanoids. *Camellia sinsensis* is well known as tea. This extract has been used in the traditional Chinese medicine to treat cardiovascular diseases, cancer, and bacterial infections. This compound was also demonstrated to affect cholesterol levels. These effects are mainly attributable to the presence of epigallocatechin gallate. *Curcuma longa* or turmeric is the plant of which curry powder is derived. It contains the phytochemical curcumin which has potential effects on many clinical disorders. The last extract is derived from *withania somnifera*. Since we used compounds of this plant in this study, this plant is more extensively described in the last chapter of the introduction. The company claims that Protandim can indirectly increase the

antioxidant activity via upregulation of endogenous antioxidant factors such as superoxide dismutase (SOD), glutathione peroxidase and catalase. It achieves its effect via a 9-fold synergy when all five components are present together [196]. It was brought on the market as an “anti-aging” supplement with the aim to allow people to live up to the age of 120. Oral administration of Protandim in humans showed significant elevation of SOD1 and catalase, whereby plasma markers for lipid peroxidation were decreased [197]. Yet, this was a non-randomized, non-controlled trial. Burnham and colleagues published another study, double-blinded, randomized and placebo controlled where Protandim had no significant effect on the pulmonary oxidative stress questioning the previous human study [198]. In a rat model for hypoxia-induced pulmonary hypertension, Protandim was able to induce Nrf2 and HO-1, leading to a better clinical outcome [199]. Mdx mice, a model for Duchenne muscular dystrophy, a disease that is leading to heart and diaphragm fibrosis, treated orally with Protandim demonstrated a decreased level of a marker of fibrosis [200]. Also in a mouse skin cancer model, a Protandim-diet could reduce the skin tumor incidence via the suppression of p53 and an increase of mitochondrial SOD [195, 201].

Interestingly, different Nrf2 activators do not produce identical gene expression patterns, reflecting compound-specific qualitative and quantitative differences in the mode of Nrf2 activation. Clearly, Nrf2 inducers do not work as a “one-size-fits-all” signal but instead may modulate parallel signaling pathways to finetune Nrf2 signaling dynamics.

I.1.7.4 Administration of Nrf2 modifiers

Several data indicate that Nrf2 is a promising therapeutic target for a variety of chronic diseases. However, one would suggest that therapeutic benefit of Nrf2 inhibitors/activators is rather limited within a particular concentration range (U shaped curve), as excessive inhibition or stimulation of Nrf2 activity may rather worsen disease states or have adverse therapeutic effects. Yet, regulating Nrf2 activity via pharmacological intervention does not appear to induce such excessive inhibition or stimulation as it occurs in knockout models or Nrf2-related pathologies. When comparing KEAP1 knockout mice to cells treated with Nrf2 activating compounds, both the magnitude and duration of activation of a battery of Nrf2 regulated genes were clearly different. Genes influenced, were overall similar but compounds had only a small dynamic range and magnitude of activating the genes. Thus, pharmacological interventions cause transient fluctuations in expression of Nrf2 target genes rather than chronic malignant activation caused by knockouts. Nonetheless, if there is a need for chronic elevated responses intermittent dosing of compounds is still possible. The pharmacokinetic half-lives of many inducers is measured in minutes but half-lives of most proteins that are induced are measured in hours and even days. Therefore, intermittent dosing could still lead to chronic presence of the proteins wanted, avoiding chronic malignant activation of Nrf2, as this is sometimes required in cancer treatment [202]. To avoid overstimulation of Nrf2 signaling, there are also negative feedback regulation mechanisms to dampen the Nrf2 response. For example, high amounts of Nrf2 activate deacetylases, which then reverse the Nrf2 activation process to restore homeostasis. SIRT1

deacetylases regulate Nrf2 activities via decrease of Nrf2 acetylation [71]. Finally, taking in account that cysteines in KEAP1 have an ultra-sensitive nature for electrophiles, low compound concentrations should be sufficient to regulate the Nrf2 pathway and to avoid further modifications of non-specific targets such as other proteins, nucleic acids or lipids. As such, when defining therapeutic concentration range of drugs, like triterpenoid CDDO-Im, dose-limiting toxicity is plausibly related to its off-target effects rather than through activation of the Nrf2 pathway [203-206]. Moreover, there is also no need to increase concentration of the compound in time to reach the same levels of Nrf2 activation as cells do not become refractory to repeated activation of the Nrf2 pathway [202].

I.1.8 Crosstalk between the Nrf2 and NF-κB pathway

Another transcription factor sensitive to electrophilic stress and redox perturbations is NF-κB. Those proteins belong to a family of transcription factors involved in several prominent processes, including inflammation, immune response, apoptosis, cell growth and development. NF-κB is found in almost all animal cell types [207-209]. The NF-κB family consists of 5 members: p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), p100/p52 (NF-κB2). Based on the presence of a transactivation domain in the C-terminus, NF-κB proteins can be divided in two groups. A first group, including p65, RelB and c-rel, all contain a transactivation domain involved in gene activation. The second group such as p50 and p52, lacking a transactivation domain, plays more critical roles in modulating the DNA-binding specificity of NF-κB. P105 and p100 are synthesized as inactive precursors for p50 and p52, respectively.

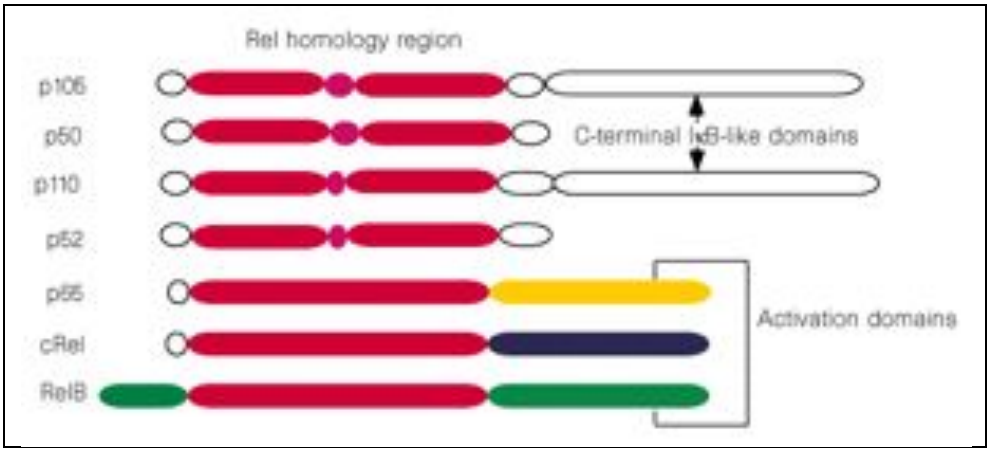

Fig 1.9 NF-κB family members consisting of p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), p100/p52 (NF-κB2). P65, RelB and c-rel all contain a transactivation domain, whether p50 and p52, synthesized from the inactive precursors P105 and p100, respectively, are lacking a transactivation domain. Source: sbi.imim.es

In unstimulated cells, inactive NF-κB is present in the cytoplasm bound to its inhibitor IκB. Via this binding NF-κB is sequestered into the cytoplasm as binding of IκB masks the nuclear

localization signal of NF-κB. The best-studied and major IκB protein is IκBα. There are two main signaling pathways leading to the activation of NF-κB known as the canonical pathway (or classical pathway) and the non-canonical pathway (or alternative pathway). The common regulatory step in both of these cascades is activation of an IκB kinase (IKK). This activated IKK phosphorylates serine residues located in an IκB regulatory domain. Through this phosphorylation IκB become targeted for ubiquitination, leading to proteasomal degradation. With this degradation of IκB, the NF-κB complex is freed to enter the nucleus where NF-κB dimers lead to expression of NF-κB-dependent genes [208-216]. The IKK complex within the canonical pathway is composed of two catalytic kinase subunits (IKKα, IKKβ) and the regulatory non-enzymatic scaffold protein IKKγ. The latter is also called NEMO and is the master regulator protein within the heterotrimer. There is a wide range of agents that are able to stimulate IKK to activate NF-κB, including tumor necrosis factor (TNF), hypoxia, oxidized low density protein (oxLDL), H_20_2, UV radiation, bacterial and viral antigens etc. [215].

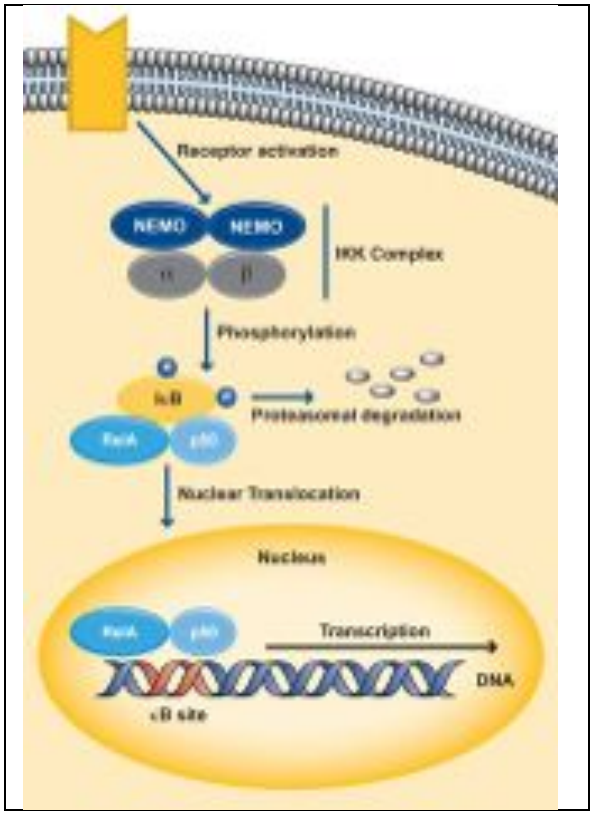

Fig 1.10 The canonical NF-κB pathway. Source: abcam.

Alterations of the NF-κB pathway have been described in more than 100 diseases, such as cancer, arthritis, Alzheimer's disease, autoimmunity, Crohn´s disease, inflammatory bowel disease, ulcerative colitis, aging, allergies, pain, diabetes, obesity, cardiovascular diseases etc. [217-228]. As NF-κB is critically involved in inflammation, it is not surprising that NF-κB is deregulated in many chronic inflammatory disorders.

An activation and interplay of Nrf2 and NF-κB cell defense pathways provides a coordinated response to electrophilic stress and redox perturbations to confer cell protection. However,

the interplay is often leading to an opposite effect where upregulation of one transcription factor is inhibiting the other. Several disease states are linked with impaired control of both transcription factors, including cardiovascular diseases, obesity, diabetes type 2 and inflammatory diseases, pathologies where Nrf2 is in general downregulated and NF-κB hyperactivated. There is much crosstalk between both pathways, going from direct effects on the transcription factors themselves, to protein-protein interaction as second messenger effects on common target genes [229]. For a full understanding, the interplay between both pathways still requires further investigation in various diseases.

I.1.8.1 Parallel effect

Identification of a NF-κB binding site in the promoter region of Nrf2 suggests that Nrf2 can be directly regulated by NF-κB [230]. For example, in human acute myeloid leukemia high Nrf2 expression levels depend on NF-κB expression levels. NF-κB subunits p50 and p65 induce transcription of Nrf2 in AML cells by binding to a specific promoter κB-site [231]. Also, interleukin-10 IL-10, an anti-inflammatory cytokine induced by NF-κB, can induce HO-1 *in vivo* [232]. Moreover, more NF-κB targets were positively influenced by increased Nrf2 levels. In human aortic endothelial cells an overexpression of Nrf2 was leading to an increase in TNF-induced Monocyte chemoattractant protein-1 (MCP-1) and vascular cell adhesion molecule 1 (VCAM-1), but no changes in NF-κB activity were observed [233]. These results are in sharp contradiction to the negatively regulating effects of HO-1 on those pro-atherogenic genes.

As NF-κB and Nrf2 are both sensitive to electrophilic stress and redox perturbation, activation of both cell defense pathways provides a coordinated response to such insults to confer cell protection. However, the effects on Nrf2 and NF-κB can differ depending on the type of induction of the cellular stress. Distinct effects on both transcription factors were seen when cellular stress was induced by the compounds N-acetyl-p-benzoquinineimine (NAPQI), the reactive metabolite of acetaminophen, dinitrochlorobenzene (DNCB), a model electrophile and buthionine (S,R)-sulfoximine (BSO), an inhibitor of glutamate-cysteine ligase. All three chemicals induced Nrf2, but NF-κB binding activity was only increased by BSO treatment. Remarkably, NF-κB binding activity was even decreased after exposure to NAPQI and DNCB. On the other hand, increased Nrf2 caused by KEAP1 depletion was reversed by co-depletion of NF-κB. Thus, it is clear that KEAP1/Nrf2 and NF-κB can respond differently to stresses and the crosstalk may enable NF-κB to partly influence Nrf2 expression during cellular stress [234].

Of particular interest, KEAP1 was found to bind IKKβ, promoting its ubiquitination and degradation. IKKβ contains an ETGE motif similar to that of Nrf2. Reduction of KEAP1 expression by small interfering RNA enhanced NF-κB activity, and up-regulated the expression of NF-κB target genes. Moreover, ectopic expression of KEAP1 decreased the expression of IKKβ, indicating negative crosstalk with the NF-κB pathway [235]. Moreover, a systematic analysis of the Cul3, KEAP1, and Rbx1 genomic loci revealed a high percentage of

genome loss and missense mutations in human cancers that failed to facilitate IKKβ degradation. These data demonstrate that dysregulation of KEAP1-mediated IKKβ ubiquitination can contribute to tumorigenesis [236].

I.1.8.2 Opposite effect

Contradictory to the previous described observations, an opposite regulating effect between both transcription factors has also been described. There are many chemopreventive or anti-inflammatory agents that can trigger Nrf2 activation with concomitant repression of NF-κB [237]. For example, SFN-dependent activation of Nrf2, corresponds with a reduction of DNA binding of NF-κB without affecting IκB degradation and nuclear translocation of NF-κB. In several pathologies, Nrf2 expression is influenced by regulation of NF-κB [238]. NF-κB can inhibit the expression of genes regulated by Nrf2 in several ways. NF-κB can reduce the availability of co-activators for Nrf2-dependent gene transcription. When p65 is activated it can bind CBP at the same binding site as Nrf2 does. Thereby, NF-κB competes with Nrf2 for binding of co-activator CBP preventing Nrf2-dependent transcription. Alternatively, NF-κB can promote recruitment of a co-repressor for Nrf2 [239]. Phosphorylated active p65 can recruit histon deacetylase 3 (HDAC3) to ARE sites where this histone deacetylase can bind and deacetylate CBP. This will lead to a reduced co-activation activity of CBP [240]. Thus, phosphorylation of p65 leading to its activation can reduce the levels of activated CBP, a co-activator of Nrf2, via direct competitive binding with CBP or via activation of HDAC3 which decreases CBP activity. Moreover, HDAC3 was also shown to bind mafK in the region responsible for dimerization with Nrf2. This is leading to steric hindrance for Nrf2 to bind but also results in deacetylation and inactivation of mafK. Moreover, absence of CBP in the Nrf2-mafK heterodimer causes destabilization and promotes the binding of HDAC3 [239].

Reciprocally, Nrf2 has also been implicated in the control of NF-κB. Nrf2 is suggested to control NF-κB levels via attenuation of phosphorylated IκB. Nrf2 knockout cells have higher levels of phosphorylated IκB compared to WT cells after stimulation with a pro-inflammatory stimulus [241]. Overall, there is an increased activation of NF-κB seen in Nrf2 knockout mice by pro-inflammatory stimuli [241-244]. Pro-inflammatory biomarkers such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), TNF, inducible nitric oxide (iNOS) and cyclo-oxygenase 2 (COX-2), which are all effector genes regulated by the NF-κB pathway, have higher induction level in Nrf2-deficient mice as compared with WT mice [245]. These data indicate that ablation of Nrf2 accelerates NF-κB-mediated pro-inflammatory reactions. Nonetheless, a direct role of Nrf2 in the activity of NF-κB is not yet proven by this. There is a possibility that increased ROS levels, as a result of the absence of Nrf2, are the reason of increased activation of NF-κB. Indeed, overexpression of Nrf2 did not lead to reduced NF-κB activity or reduced phosphorylation of IκB. Nonetheless, although no ARE binding site could be observed in the rat glutamate cysteine ligase catalytic subunit (GCLC) promoter, downregulation of Nrf2 led to reduced GCLC transcription after stimulation. Indeed Nrf1 and Nrf2 were shown to regulate rat GCLC mRNA levels indirectly via modulation of expression of NF-κB and AP-1 family members [246].

Moreover, there is also crosstalk between the two pathways by secondary processes involving increased expression of Nrf2 regulated proteins. A nice example is HO-1. It was suggested that one of the anti-atherogenic effects of HO-1 was through attenuation of NF-κB regulated expression of adhesion molecules, as demonstrated both in *in vitro* and *in vivo* studies [247-249]. One hypothesis could be that NF-κB phosphorylation is inhibited via HO-1-mediated production of free iron [250]. Moreover, HO-1 has also been demonstrated to inhibit degradation of IκB [251]. Besides adhesion molecules, regulation of many other NF-κB regulated genes via Nrf2-mediated HO-1 activation have been investigated [252, 253]. For example, HO-1 could inhibit NO production in Lipopolysaccharide (LPS)- stimulated macrophages, probably through degrading heme, the cofactor of iNOS, through the action of free iron [254] or carbon monoxide (CO) [255]. Since NO is inducing HO-1, this phenomenon can be seen as a negative feedback mechanism to protect from NO-dependent toxicity [256-260]. One of the NF-κB targets that can influence Nrf2 activity is COX-2 and its product prostaglandin J2 (PGJ2). Low shear stress is inducing COX-2 that can inhibit PI3-kinase leading to a reduced transcription of Nrf2 and HO-1 [261]. Links of ERK, JNK, PI3K/Akt, p38, PKC and others kinases are described with both transcription factor pathways. Thus, modulation of the kinases would almost certainly lead to an indirect interplay between the two pathways and modulation of the activity of the transcription factors.

Since several disease states are linked with impaired control of both transcription factors where Nrf2 is in general downregulated and NF-κB hyperactivated it is likely that opposite effects are of greater importance than parallel effects. All parallel and opposite effects described in this part are summarized in table 1.1.

Parallel effect	Ref	remarks
NF-κB -> Nrf2		
NF-κB binding site in Nrf2 promoter	[230] [231]	
IL-10 induce HO-1 transcription	[232]	Appears to be mediated via p38 activation however, p38 is known to inhibit Nrf2 activation.
Increased Nrf2 levels positively influence NF-κB target genes	[233]	In endothelial cells overexpression of Nrf2 led to increased VCAM-1 and MCP-1 however, HO-1 negatively regulated those genes.
Nrf2-> NF-κB		
KEAP1 bind IKKβ, promoting its ubiquitination and degradation	[235] [236]	
Opposite effect		
NF-κB -> Nrf2		
NF-κB reduce bioavailability of co-activators of Nrf2	[239]	
NF-κB promote recruitment of co-repressors of Nrf2	[239] [240]	
Nrf2-> NF-κB		
Nrf2 attenuates phosphorylation of IκB		Can be indirect via increased ROS
Via secondary processes		
Nrf2-> NF-κB		
HO-1 attenuates NF-κB regulated expression of proinflammatory proteins	[247-250, 252-254, 256-260]	
NF-κB -> Nrf2		
COX-2 lead to reduced transcription of Nrf2 and HO-1		

Table 1.1 Overview of parallel and opposite effects of the NF-κB and Nrf2 pathways.

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I.2 Cardiovascular disease

I.2.1 Cardiovascular disease and atherosclerosis

I.2.1.1 Prevalence of cardiovascular disease

Cardiovascular disease (CVD) is nowadays the most deadly disease in the world. So more people die annually from CVDs than from any other cause. Over 80% of CVD deaths take place in low- and middle-income countries. Overall, cardiovascular deaths and diseases have increased at a fast rate in those countries. This increasing burden on those low- and middle-income countries is likely due to global aging of the population and an increased exposure to detrimental lifestyle-related risks, i.e. lack of physical exercise and unhealthy food. On the other hand, cardiovascular mortality rates have declined in many high-income countries [1]. This is probably due to strong efforts that have been made to modify behavior that is related to four main risks: tobacco use, unhealthy diet, low physical activity and excessive alcohol consumption. However, despite all the efforts, in 2006 this disease was still responsible for more than 34% of all deaths in the US. Roughly calculated almost 2300 Americans died from cardiovascular diseases each day [2]. In Belgium, similar death rates are seen, in 2005 35% of the deaths were caused by CVD [3]. In line with the high contribution of deaths, this disease is also responsible for a high medical and social cost. In 2004, the total Belgian cost for CVD was €3.5billion [3]. Thus, cardiovascular diseases impose a significant economic burden on the Belgian society. To conclude, research concerning prevention or treatment of CVD is highly important.

I.2.1.2 What are cardiovascular diseases

To understand the origin of CVDs, one has to understand first the concept of the cardiovascular system. Blood from the heart travels from the left side of the heart and is rich in oxygen. It flows via arteries, which are decreasing in size, till it reaches the narrowest of arteries called capillaries in all organs and parts of the body. At this site the blood is delivering its oxygen and nutrients and collects waste products. Afterwards, this blood is brought back to the right side of the heart through a system of progressively enlarging veins. A closed loop is created, as this blood becomes rich of oxygen again and travels again from the heart to the organs. This is called the circulatory system or cardiovascular system which is vital for living. Cardiovascular system literally means “cardio” or heart and “vascular” or a system or network of blood vessels.

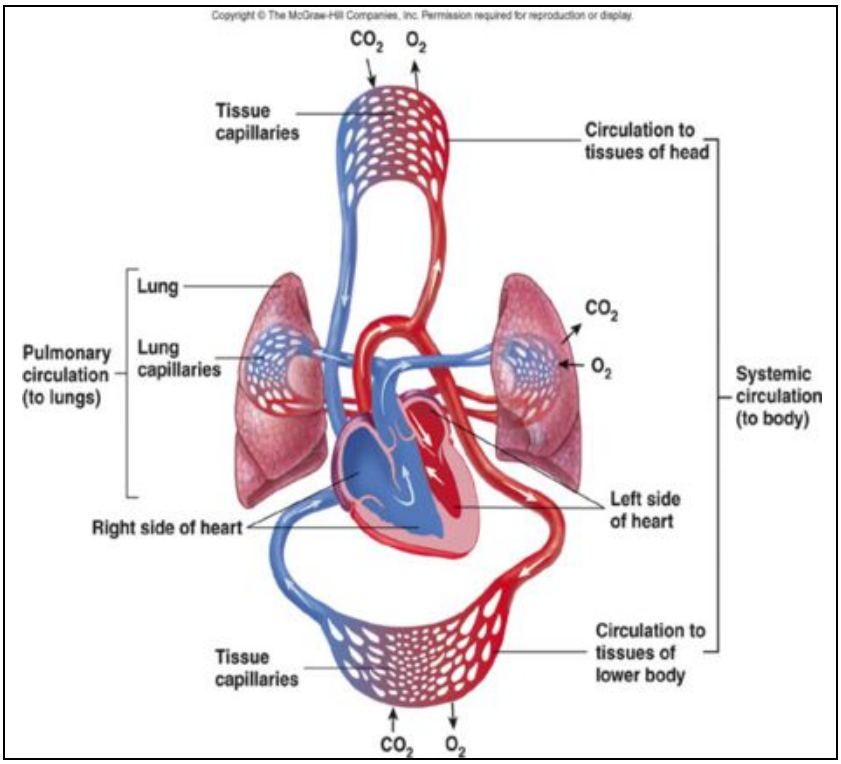

Fig 2.1 The cardiovascular system. A closed loop of oxygen rich blood traveling from the left side of the heart to all organs and parts of the body via arteries to the right side of the heart as oxygen deprived blood via enlarging veins. Source: studyblue.com

CVDs are caused when this cardiovascular system is malfunctioning, so when disorders of the heart and blood vessels occur. CVDs include for example, coronary heart disease (heart attacks), cerebrovascular disease (stroke), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. There is an array of CVDs one can suffer from, however the majority of the people die from heart attacks and strokes [4]. CVDs are caused by intrinsic and extrinsic risk factors such as age, gender, high blood pressure, high serum cholesterol levels, tobacco smoking, excessive alcohol consumption, family history, obesity, lack of physical activity, psychosocial factors, diabetes mellitus, air pollution etc. Unfortunately, some of these risk factors such as age, gender or family history are unchallengeable. Though, many important cardiovascular risk factors are modifiable by lifestyle change or drug treatment. So most CVDs can be prevented by addressing risk factors such as tobacco use, unhealthy diet and obesity, physical inactivity, harmful use of alcohol, high blood pressure, diabetes and raised lipids. Important to note is that tobacco consumption and smoking is even the largest risk factor for cardiovascular diseases worldwide [5]. Although CVD usually affects older people, the onset often begins early in life. Thus, this makes it even more important to put effort into prevention even from childhood.

I.2.1.3 Atherosclerosis

The main cause of most of those types of CVD is atherosclerosis, a disease of the vessel wall of the medium- and large-sized arteries. It is characterized by initial lesions of the intima, the inner endothelial wall, followed by secondary changes in the surrounding layers, the media and adventitia. These lesions, also called atherosclerotic plaques, can eventually obstruct the vascular lumen. This obstruction can lead to acute or chronically reduced arterial perfusion leading to severe complications. Acute symptoms occur not because the plaques rises acutely but because an atherosclerotic plaque, remaining silent for decades, suddenly can become unstable (also called vulnerable), shear and trigger thrombus formation. This thrombus can obstruct the vessel and completely cut-off the blood flow resulting in death of the tissue that is supplied by that vessel. A thrombus can also dislodge and become an embolism. The latter will flow into the more narrow capillary vessels and cause blockage in a distant part of the body. Even when the plaques are not unstable they can also obstruct the vessel walls as they keep on growing in time. Moreover, if the enlargement of the atheroma exceeds the dimension of lumen, then an aneurysm is created [6]. The buildup of an atheromatous plaque is a slow process, developed over a period of several years through a complex series of cellular events occurring within the arterial wall and in response to a variety of local vascular circulating factors. These atherosclerotic plaques are the result of a chronic inflammatory process, triggered by infiltration of inflammatory cells, lipid accumulation, proliferation of vascular smooth muscle cells (VSMC) and matrix and calcium accumulation [6].

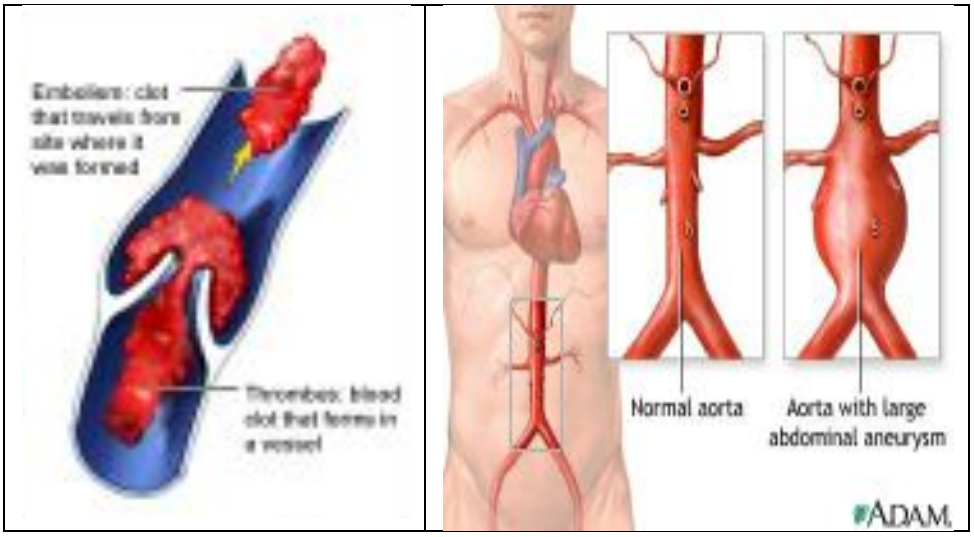

Fig 2.2 Formation of a thrombus, embolism or aneurism. A thrombus will decrease or even completely cut-off the blood flow through the vessel . An embolism is a dislodged thrombus traveling from the site where it was formed causing blockage in more narrow capillary vessels in a distant part of the body. An aneurysm, a localized, blood-filled balloon-like bulge, increasing in size over time can lead to rupture of the artery. Source: www.cvvcenters.com

Formation of an atherosclerotic plaque

The inner endothelial wall or intima, is a continuous layer of endothelial cells, seated on a basement membrane. In general, the intima is a thin layer but can be thickened at certain loci. Some of the thickenings are physiological adaptations to changes in flow and wall tension, but these intimal thickenings could also be the precursors for the atherosclerotic plaques as they are seen in atherosclerosis prone places [7, 8]. One of the earliest events in atherosclerosis is dysfunction of the endothelium. The endothelial cells exert an arsenal of functions, including maintaining balance in vasodilation and vasoconstriction, inhibition or stimulation of proliferation and migration of VSMC and thrombogenesis or fibrinolysis [9-11]. A quantifiable feature of endothelial dysfunction is for example malfunction of the vasodilatation-vasoconstriction balance. Dysfunctional endothelium has an inability to dilate fully in response to stimuli. This is often due to a reduced activity of the endothelial nitric oxide synthase (eNOS) as NO is one of those important endothelium-produced relaxing factors.

Initiation of plaque formation

An important event in the initiation of the formation of an atherosclerotic plaque is the recruitment of leucocytes to the endothelial wall. This recruitment is followed by migration of the leucocytes into the subendothelial layer. This phenomenon is called the leucocyte adhesion cascade. In response to various stimuli, endothelial cells express adhesion molecules, chemokines and other pro-inflammatory proteins such as VCAM-1, intercellular adhesion molecule-1 (ICAM-1), E-selectin, MCP-1 etc. This will facilitate recruitment, binding and migration of the leucocytes to the endothelial wall. There is a whole range of stimuli that can activate the endothelium such as LDL, hypercholesterolemia, high glucose levels, increased amount of pro-inflammatory factors such as TNF or IL-6 etc. The continuous presence of even small amounts of these various stimuli can already be sufficient to trigger inflammation. It launches initial damage to the endothelium, resulting in an advanced inflammatory response [12, 13].

Progression

When the monocytes transmigrate into the intima they differentiate into macrophages [13]. This differentiation is induced by macrophage colony-stimulating factor (M-CSF) that is produced initially by the endothelium and at a later stage by monocytes and VSMCs. Meanwhile, LDL particles from the blood plasma invade the endothelium and become oxidized. Those LDL particles enable transport of multiple different fat molecules, including cholesterol, within the water-based bloodstream. They are often informally called “bad cholesterol” particles. OxLDL can promote atherogenesis via multiple mechanisms. It can serve as a ligand for scavenger receptors such as macrophage scavenger receptor class A (SR-A) and cluster of differentiation 36 (CD36) resulting in the uptake of oxLDL into intimal macrophages [14]. This phagocytosis helps to protect the body from those high amounts of oxLDL. However, extreme uptake of this oxLDL turns macrophages into foam cells, lipid loaded macrophages. These foam cells start to accumulate and contribute to the formation

of a fatty streak, the first visible lesion in the development of atherosclerosis. Foam cells eventually die, and thereby propagate further the inflammatory process. Another effect of oxLDL is that it activates macrophages, endothelial cells and VSMCs to produce pro-inflammatory mediators, thereby aggravating the inflammatory state within the lesion [15, 16]. This early lesion is characterized by subendothelial accumulation of foam cells, lipids and immune cells [17]. Later on, this early lesion turns into a more advanced atherosclerotic lesion defined by deposition of fibrous tissue. VSMCs are migrating from the media into the intima in response to cytokines that are secreted by damaged endothelial cells. Once in the tunica intima, they proliferate and synthesize extracellular matrix [18]. Thus, the atherosclerotic plaque develops from a lipid rich atherosclerotic lesion into a fibrotic plaque [17]. As discussed earlier, this plaque can stay stable for a life time but can also lead to complications as it keeps on growing. Hereby, it can obstruct the lumen just by his size or it can form an aneurysm. If the plaque becomes unstable, it can from a thrombus, which leads to an infarction or ischemia of the area that is downstream of the lesion site or jammed embolism.

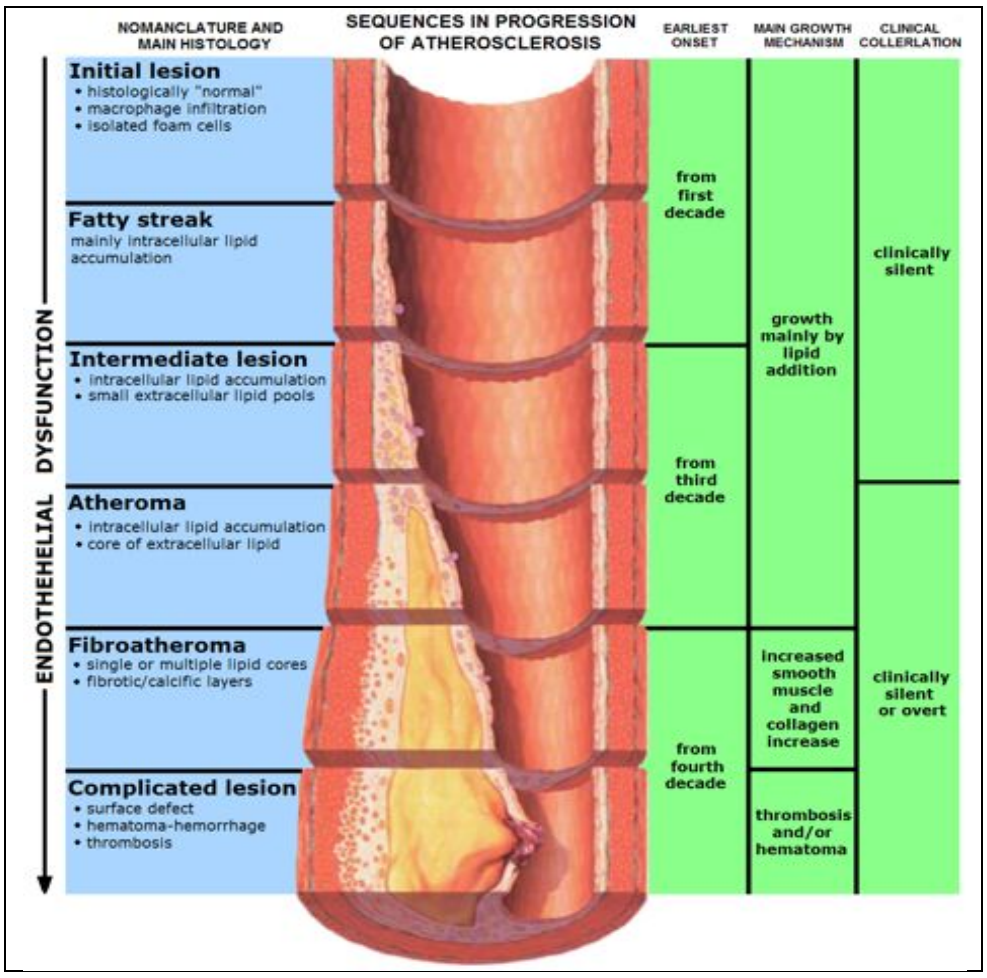

Fig 2.3 Evolution of an atherosclerotic plaque. Source: www.wikipedia.org

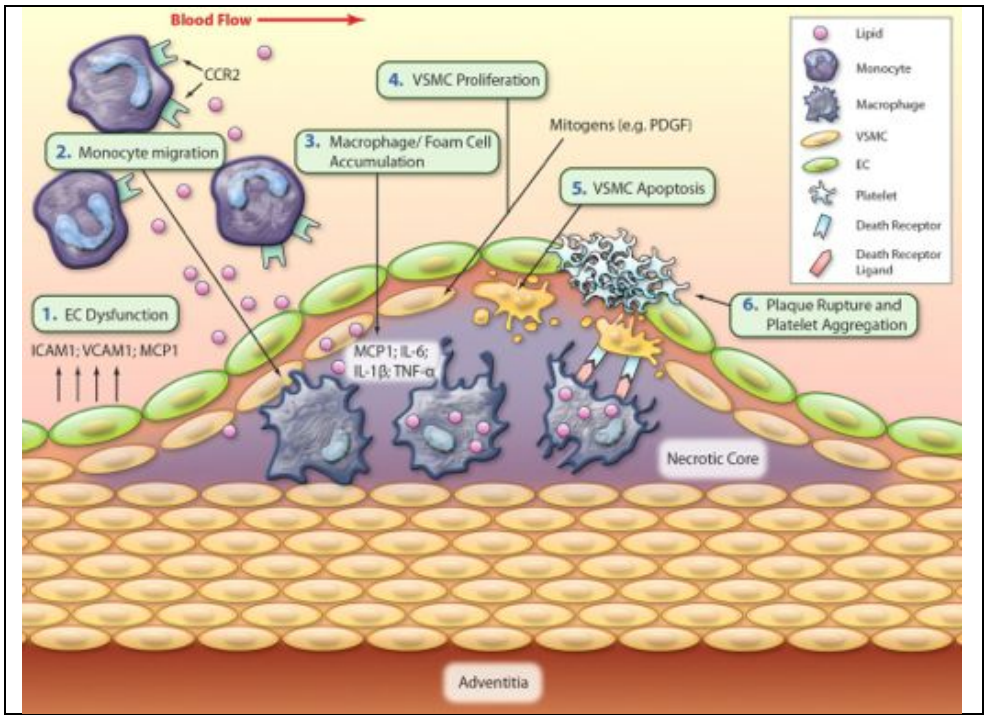

Fig 2.4 Formation of an atherosclerotic plaque. Dysfunctional endothelial cells produce more pro-inflammatory proteins, including adhesion molecules, chemokines and chemo-attractant proteins. This will facilitate recruitment, binding and migration of leucocytes to the endothelial wall, followed by transmigration into the subendothelial layer. Monocytes differentiate into macrophages and engulf oxLDL until they turn into foam cells. VSMCs migrate from the media into the intima and start to proliferate, synthesize extracellular matrix and go eventually into apoptosis [19].

I.2.1.4 Oxidative stress in cardiovascular disease

Although oxidative stress and free radicals are frequently mixed terms, they have a different meaning. Oxidative stress in general is a broader term than free radical biology, as few oxidants are actually free radicals. The term oxidative stress often refers to the imbalance of a redox couple such as GSH/GSSG (glutathione in reduced (*GSH*) and oxidized (*GSSG*) states) or NADPH/NADP+ (nicotinamide adenine dinucleotide phosphate and its reduced form NADPH), where no overproduction of free radicals are involved. From all free radicals, superoxide is the most known radical. However, not all free radical linked processes in the body are involving superoxide, for example lipid peroxidation. Therefore, oxidative stress is no synonym for free radical damage and free radicals are not similar to reactive oxygen species as many ROS are no free radicals [20]. ROS are generated during reduction of oxygen and are categorized into two groups: 1) free radicals with short half-lives (e.g. superoxide, nitric oxide) and 2) non radicals with a longer half-live (e.g. hydrogen peroxide, peroxynitrite) [21]. Over the last two decades, it has become increasingly clear that besides inflammation also ROS are involved in CVDs. Subsequently, there has been a growing interest in clinical implications in CVD of these oxidants. Various animal models of oxidative stress support that ROS have a causal role in atherosclerosis and other cardiovascular diseases. Thus, oxidative modifications present in atherosclerotic plaques are causal to lesion formation and not only consequential, as it was suggested by some researchers [22]. Oxidative stress is one of the

most potent inductors of endothelial dysfunction and is involved in all stages of atherosclerotic plaque evolution. There is even a clear association between the amount of oxidative challenge and reversible vascular dysfunction that can be observed before permanent alterations of the vessel wall occur [23]. ROS contribute to CVD in several aspects. ROS can have direct adverse effects on vascular function and integrity. But besides this, they can also oxidize LDL entrapped in the arterial wall [15].

Vascular enzymes, including xanthine oxidase (XO), NADPH oxidases (NOX) and uncoupled eNOS are involved in production of ROS. They are the main molecular sources of ROS in the vessel wall, leading to the development of oxidative stress and associated endothelial dysfunction. XO catalyze the oxidation of hypoxanthine to xanthine and xanthine to uric acid. It makes use of O_2 as an electron acceptor producing NADH and $O_2^{\circ-}$ and H_20_2. Pro-inflammatory stimuli upregulate transcription of XO and patients with CVD have significantly elevated XO levels. High amounts of $O_2^{\circ-}$ react with NO forming $ONOO^-$, leading to reduced NO, a crucial modulator of vascular disease, as it has vasorelaxation, endothelial regeneration, inhibition of leukocyte chemotaxis and platelet adhesion properties. Peroxinitrite excerts toxic effects at high concentrations. Upon activation of NOX O_2 is reduced to $O_2^{\circ-}$ by the transfer of one electron from the switch of NADH to NADPH. All NOX isoforms are transmembrane proteins transporting electrons across the membrane to reduce O_2 to $O_2^{\circ-}$. The activity of NOX are stimulated by angiotensin II (Ang II), vasoactive hormones, growth factors and mechanical stimuli. Uncoupled eNOS occurs when the electron flow within eNOS is disturbed leading to generation of $O_2^{\circ-}$ instead of NO. This uncoupled state is found in patients with hypertension, hypercholesterolemia-associated endothelial dysfunction and chronic smokers [21].

I.2.2 Nrf2 and HO-1 in atherosclerosis

I.2.2.1 Nrf2

Since the causal role of free radicals and ROS in the pathogenesis of CVD, various clinical trials were performed studying the beneficial role of supplementing antioxidant vitamins. Surprisingly, these treatment of scavenging oxidants seemed rather harmful than helpful [24-27]. Nonetheless, one antioxidant, probucol, had positive effects as an antioxidant drug as it gave a scavenging free radical-independent reduction in atherosclerotic lesions and notably via upregulation of HO-1 [28]. Thus, restoring the oxidative balance by targeting the endogenous antioxidant defense mechanism is probably a better therapeutic strategy than administration of antioxidant enzymes, synthetic mimetics of antioxidant enzymes or natural or synthetic antioxidants. Therefore, an interesting treatment for CVD could be targeting the Nrf2 pathway. Nrf2 is ubiquitously expressed in the cardiovascular system and is a critical regulator of vascular homeostasis via suppression of oxidative stress [29]. In addition, several Nrf2 downstream genes have been demonstrated to be implicated in protection

against CVD [29]. Thus, it is clear that Nrf2 plays a crucial role within the cardiovascular system. However, its contribution within CVD pathology is complex.

Experimental evidence indicate that the Nrf2 pathway is playing an important role in regulation of endothelial function. Since dysfunction of the endothelium is a critical factor for initiation of formation of an atherosclerotic plaque, it is plausible that Nrf2 is involved in protection against development of atherosclerosis. For example, Nrf2 activation is linked with the cardioprotective effect of high shear stress. It is known that atherosclerosis only develops in certain predisposed areas. Those regions that are prone to atherosclerotic plaque formation experience low shear stress linked with an oscillatory blood flow. This environment will activate procoagulant and pro-inflammatory transcription factors NF-κB and AP-1. In contrast, high shear stress linked with laminar blood flow, contributes to healthy endothelial cells, expressing anticoagulant and anti-inflammatory gene expression, a condition where Nrf2 and kruppel-like factor 2 (KLF2) are two main transcription factors [30]. In addition, in human aortic endothelial cells laminar flow can inhibit oxidative stress-induced cytotoxicity or TNF-induced expression of MCP-1, VCAM-1 and monocyte adhesion. This effect was shown to be driven by the Nrf2 pathway. Interestingly, oscillatory flow was not able to activate the Nrf2 pathway and those associated cardioprotective effects [31-35]. In addition, activation of KLF2 induced by laminar flow in endothelial cells, even enhanced the antioxidant activity of Nrf2 [36]. Thus, Nrf2 is activated by high shear stress, a potent anti-atherogenic force, *in vitro* as well as *in vivo*. This is a nice example of the idea that stress is not necessarily negative, because high shear stress is beneficial in comparison to low shear stress. Supporting the atheroprotective role of Nrf2, Nrf2 is also suggested to be involved in protection against hyperglycemia. High sugar levels or hyperglycemia is a condition in which an excessive amount of glucose circulates in the blood plasma. Chronic hyperglycemia can lead to complications such as cardiovascular damage. One of the most common causes of chronic hyperglycemia is diabetes mellitus, a disease that doubles the risk of cardiovascular disease. Nrf2 activation was seen to mediate insulin protection against hyperglycemia-induced endothelial cell apoptosis. This mediation was suggested to be via the Nrf2-dependent GCLC [37]. GCLC is the first rate limiting enzyme of glutathione synthesis. Glutathione (GSH) is the principal antioxidant in our cells. GSH is able to diminish damage to oxygen-deprived tissue during and after an attack. Moreover, it also protects against lipid peroxidation. Overall, GSH has been shown to prevent and even reverse atherosclerosis. Moreover, biochemical dysfunction of endothelial cells induced by hyperglycemia could be reversed by Nrf2 activation [38]. Thus, this data suggest that Nrf2 can contribute to the protection against endothelial dysfunction.

Furthermore, after initiation of an atherosclerotic plaque, a prominent role for Nrf2 in atherosclerotic plaque progression has been suggested. So are VSMCs that are involved in advanced atherosclerotic plaques influenced by the Nrf2 pathway. Similar as to in endothelial cells, Nrf2 is able to induce antioxidant genes and to diminish apoptosis. Moreover, an additional atheroprotective effect of Nrf2 in VSMCs that is suggested is its

ability to inhibit cell proliferation [39, 40]. Nrf2 gene transfer induces expression of antioxidant enzymes and suppresses VSMC growth *in vitro*. Interestingly, the inhibitory effect on proliferation was partially reversed by the HO-1 inhibitor Sn(IV) protoporphyrin. Therefore, HO-1 is at least partially responsible for the inhibitory effect of Nrf2 on VSMC proliferation. Moreover, Nrf2 gene transfer reduced oxidative stress in rabbit aorta *in vivo*. However, the antiproliferative effects of Nrf2 *in vivo* were counterbalanced by diminished apoptosis in neointimal VSMCs, resulting in no change in neointimal hyperplasia [39]. On the other hand, Ashino and colleagues suggest that Nrf2 has indeed protective effects on neointimal hyperplasia. *In vivo*, Nrf2-deficient mice showed enhanced neointimal hyperplasia in a wire injury model. They suggest that the Nrf2 system is important for platelet-derived growth factor (PDGF)-stimulated VSMC migration and that Nrf2 activation may contribute to a reduced neointimal hyperplasia after vascular injury [41]. It is quite obvious that Nrf2 facilitates VSMC survival as it induces expression of several prosurvival proteins. Thus, it is feasible that Nrf2 contributes to accumulation of VSMCs. However, whether this counteracts the inhibitory effect of Nrf2 on VSMC proliferation or not needs further investigation [42-44]. Moreover, in general, apoptosis of VSMC introduce a more advanced inflammatory respons, contributing to formation of an antherosclerotic plaque.

Despite its controversial effect on neointimal hyperplasia, there are several other mouse models supporting evidence for cardioprotective effects of Nrf2. Many Nrf2-regulated genes, have been shown to be protective in mouse models of atherosclerosis. Overexpression of catalase and Cu/Zn superoxide dismutase inhibited progression of atherosclerosis in apolipoprotein E (ApoE) mice [45]. Moreover, HO-1 determines the molecular regulation for the transition from stable to vulnerable plaque. HO-1 induction reversed plaque progression from a vulnerable plaque to a more stable phenotype in patients with carotid artery disease and in a murine model of vulnerable plaque development [46]. Furthermore, bone marrow transplantation experiments performed in lethally irradiated LDL-Receptor (LDLR) null female mice, reconstituted with bone marrow from HO-1(-/-) versus HO-1(+/+) mice, revealed that HO-1(-/-) reconstituted animals exhibited atherosclerotic lesions with a greater macrophage content. This data is in line with the vulnerability as unstable plaques are rich in macrophages and foam cells where stable ones are richer in extracellular matrix and VSMCs [47]. Similarly, peroxiredoxin1 (Prdx1), another antioxidant enzyme, can prevent excessive endothelial activation and early atherosclerosis. Prdx1(-/-)/apoE(-/-) mice developed larger, more macrophage-rich aortic sinus lesions than Prdx1(+/+)/apoE(-/-) mice, despite similar amounts and size distributions of cholesterol in their plasma lipoproteins [48]. Prdx2 as well has shown to have atheroprotective effects in both vascular and immune cells. Prdx2 deficiency exacerbates atherosclerosis in ApoE-deficient mice. Prdx2 has already been reported to regulate pro-inflammatory responses, vascular remodeling, and global oxidative stress [49]. In addition, increased expression of glutathione reductase in macrophages decreases atherosclerotic lesion formation in LDLR-deficient mice [50]. Also, a synthetic derivative (ADTM) of danshensu out of the root of Chinese sage was found to have cardioprotective effects through activation of Nrf2 *in vitro* and *in vivo*. In the presence of

ADTM, oxidative stress-induced cell injury in cardiomyoblast was reduced. Moreover, ADTM treatment significantly alleviated myocardial infarction in rat myocardial ischemia model [51].

In contrast to these reported protective actions of Nrf2-related genes in several cell and animal models, there are some data promoting the idea that Nrf2 has potentially pro-atherogenic functions. Loss of Nrf2 has been shown to protect against atherogenesis in apoE-deficient mice by some researchers [52-55]. It has been reported a few times by independent research groups that Nrf2-deficient mice when crossed with apoE-null hypercholesterolaemic mice are protected against atherosclerosis. However, the proposed mechanism varies from one report to another. One mechanism that is proposed is the positive regulation of CD36 via Nrf2 leading to a net pro-atherogenic effect of Nrf2. Sussan and colleagues noticed that ApoE(-/-)Nrf2(-/-) mice exhibited significantly smaller plaque area and this was associated with a significant decrease in uptake of modified LDL by isolated macrophages from those mice. Furthermore, atherosclerotic plaques and isolated macrophages from ApoE(-/-)Nrf2(-/-) mice exhibited decreased expression of the scavenger receptor CD36 [54]. Also Barajas and colleagues showed that antioxidant effects of Nrf2 were overshadowed by its effects on plasma lipoproteins and cholesterol transport [53]. Another mechanism suggested, is the activation of Nrf2 by cholesterol crystals leading to the induction of pro-inflammatory IL-1 in macrophages [52]. These data together suggest a pro-atherogenic effect of Nrf2, specifically in macrophages. Similarly, mice transplanted with Nrf2−/− bone marrow and fed a high-fat diet for 6 weeks exhibited significantly larger atherosclerotic lesions than WT bone marrow transplanted mice. Moreover, expression of pro-inflammatory mediators such as MCP-1 and IL-6 are increased in Nrf2−/− vs. WT macrophages. Therefore, all together this data suggests that Nrf2 in macrophages enhances foam cell formation and promotes a pro-inflammatory phenotype [15]. Though, these pro-atherogenic effects of Nrf2 are noted specifically in Nrf2-deficient mice when crossed with apoE-null hypercholesterolemic mice. This combined systemic and local vascular effects on lesion macrophages as the mechanism by which Nrf2 deficienty could afford atheroprotection was investigated in more detail by Ruotsalainen and colleagues. They investigated bone marrow-specific loss of Nrf2 on early atherosclerosis in LDLR-deficient mice. They noted that mice transplanted with Nrf2-deficient bone marrow and fed a high-fat diet for 6 weeks exhibited significantly larger atherosclerotic lesions. Moreover, uptake of LDL was increased in Nrf2 knockout macrophages with an increased expression of this uptake responsible receptors and pro-inflammatory proteins [15]. This experiment clearly shows opposite result as to those in Nrf2-deficient mice crossed with apoE-null hypercholesterolemic mice suggesting that these pro-atherogenic effects of Nrf2 are specifically seen in this type of mouse model and that pro-atherogenic effects are not attributable to Nrf2 in general.

Nontheless, based on the results obtained from these Nrf2 -/- / ApoE -/- mice more investigation is needed and care should be taken with application of systemic treatment of CVD based on Nrf2 inducers.

Moreover, Nrf2 activity appears to decline with aging leading eventually to deregulation of stress responses. There are several reports suggesting that old animals have impaired Nrf2 activity [56, 57]. As we know, age is a known and important risk factor for atherosclerosis. The risk of strokes for example, doubles every decade after the age of 55 [58]. So reduced Nrf2 levels might contribute to the impact of aging on atherosclerosis. However, the explanation why age has such a great contribution is complex and multifactorial and has direct and indirect links to other risk factors. One of the explanations are serum cholesterol levels, as these increase with age. Aging is also associated with changes in structural and mechanical properties of the vascular wall, leading to loss of elasticity and reduced arterial compliance. Also, formation of an atherosclerotic plaque is a matter of time, which can be clinically silent for decades before problems arise at a later stage [20]. Thus, a decline in Nrf2 levels is probably only partly explaining the effect of aging on atherosclerosis.

To conclude, it is clear that Nrf2 is playing a crucial role within the cardiovascular system. Thereby, it could be an excellent target to combat CVDs. However, its contribution within the pathology is complex, thus the exact contribution of Nrf2 within atherosclerosis and different CVDs still needs further in depth investigation. The question whether Nrf2 inducers or Nrf2 inhibitors should be applied still can be asked. Though, most publications are granting atheroprotective characteristics to Nrf2. Most of those articles are linked with its direct effect with oxidative stress. On the other hand, there are some researchers that published pro-atherogenic effects of Nrf2. However, a possible explanation for some of these inconsistencies and different degrees how atherogenesis is affected in all these studies may have to do with differences in experimental designs and inherent characteristics of the animal models that were employed. Of importance, pro-atherogenic effects of Nrf2 are specifically seen in one type of mouse model, Nrf2-deficient mice crossed with apoE-null hypercholesterolemic mice. Moreover, whether treatment should be systemic, treating all cells involved in atherosclerotic plaque formation or if it would be better to treat some cells in specific can also be questioned. Also, one should question whether treatment should be before initiation and deregulation of endothelial cells or if targeting Nrf2 would still be as effective during an advanced atherosclerotic plaque. Therefore, would prophylactic treatment of individuals with increased risk factors be beneficial? It is however clear that changes in Nrf2 and its target genes have major implications in the development of CVDs. Thus, with some prudence, one can state that Nrf2 is a good target for treatment of CVDs.

I.2.2.2 HO-1

In the previous chapter, a few studies were already mentioned where atheroprotective effects were specifically assigned to the upregulation of HO-1. For example, treatment with probucol as an antioxidant specifically upregulated HO-1 with concomitant protection from

atherosclerosis [28]. In another study, HO-1 was demonstrated to induce reversed plaque progression from a vulnerable plaque to a more stable phenotype [46]. Furthermore is has been demonstrated that HO-1 is at least in part responsible for the anti-proliferative effect of Nrf2 in VSMCs [39]. Since possible cardioprotective effects of Nrf2 are at least in part mediated via its capacity to combat oxidative stress, it can be expected that HO-1, an important phase II detoxifying/ antioxidant enzyme, has a major contribution in this process. Nonetheless, HO-1 is not only involved in adaptive cellular response against toxicity of oxidative stress. It is also recognized to exhibit important immunomodulatory, anti-apoptotic, antiproliferative, pro-angiogenic and anti-inflammatory functions increasing the probability of a protective role in the formation of atherosclerosis.

There are different types of malignancies linked with deregulation of HO-1. In 1999 the first human case of HO-1 deficiency was reported [59]. It was a boy of 26 months old having recurrent fever and generalized rash. Moreover, growth retardation was apparent and after examination several abnormal conditions in different organs were stated. When he got the age of six years, abnormalities aggravated and a general presence of severe, persistent endothelial damage was noted. When cells were investigated there was a complete absence of HO-1 production. This was due to deletions and a complete loss of an exon present in the HMOX1 gene. Later on, equal characteristics were seen in HO-1-deficient mice [60]. Patients with HO-1 deficiency in general have severe abnormalities like growth retardation, anemia, persistent endothelial damage and vulnerability to stressful injury. This emphasizes the importance of the protein at several levels [59]. A large size (GT)n repeat in the HMOX1 gene promoter reduces the inducibility of HO-1 by ROS in cigarette smoke and is resulting in chronic pulmonary emphysema [61]. The same mutation is seen in type 2 diabetics causing, besides the hyperglycemia, an increased susceptibility to develop coronary artery diseases due to higher oxidative stress [62]. In general, a lower number of GT repeats within the GT-microsatellite polymorphism is associated with a higher inducibility of the HO-1 protein expression in response to stress stimuli [61-66]. Individuals with low repeat numbers seem also to be more protected against CVD indicating an important protective role of HO-1 for CVD [67].

The protective effect of HO-1 has been investigated and shown in various other *in vitro* and *in vivo* models of atherosclerosis [47, 68-76]. Interestingly, a link between HO-1 and inflammation has been observed. This link has initially been shown in an animal model where specific upregulation of HO-1 diminished complement-dependent inflammation [77]. HO-1 knockout models in general develop a chronic inflammatory status where they are highly vulnerable to sepsis [60, 78, 79]. In parallel, targeted overexpression of HO-1 has been shown to have beneficial effects in various experimental animal models of inflammation [80, 81]. Treatment of sickle mice with HO-1 generated metabolites, CO or biliverdin, inhibited NF-κB, VCAM-1 and ICAM-1 expression and stasis [82]. A plethora of publications can be found describing the regulation of chemoattractants and adhesion molecules by HO-1. Moreover, reduction of those pro-inflammatory proteins by HO-1 correlated often with

reduced monocyte recruitment to the endothelial wall in *in vitro* and *in vivo* models [83-91]. Although HO-1 is expressed in all cell types and tissues of the body the salutary effects of HO-1 appears to be dependent on cell type-specific functions. HO-1 appears to have versatile functions in different cardiovascular-related cell types. As mentioned, in endothelial cells it is capable of reducing expression of chemo-attractants and adhesion molecules leading to reduced monocyte binding. In rodent macrophages, HO-1 is leading to the attenuation of different pro-inflammatory proteins like COX-2, iNOS, TNF and IL-6 [92, 93]. Interestingly, HO-1 expression can also inhibit maturation of dendritic cells [94] and may modulate antigen presentation and participate in macrophage differentiation/polarization [76].

The mechanisms that mediate the anti-atherogenic effects of HO-1 are still under intensive investigation. Yet, it has been assumed that a great contribution is due to degradation of pro-inflammatory free heme and production of protective metabolites. As mentioned before, HO-1 is the stress inducible form of the enzyme that catalyses degradation of heme into iron, CO, and biliverdin that subsequently is converted into bilirubin.

Although, heme is absolutely essential and required in aerobic life, playing a physiological role as part of hemoglobin, myoglobin and cytochromes in oxygen- and mitochondrial electron- transport, non-protein bound free heme can be cytotoxic [95]. Thus, it is of great importance that enzymatic synthesis and degradation of heme is tightly regulated. The vasculature is one of the sites in the body that has the greatest risk of being exposed to free heme as erythrocytes contain high amounts of heme and can undergo unexpected lysis. Furthermore, extracellular hemoglobin releases heme when it gets oxidized. When heme is released, it can intercalate in the membrane and cause severe damage. Several researchers have shown detrimental effects of heme in framework of the pathogenesis of atherosclerosis [95-100]. For instance, hemoglobin-derived heme can act as a catalyst of LDL oxidation [101]. In cultured endothelial cells, heme was able to induce several adhesion molecules and pro-inflammatory cytokines [102, 103]. In contrast, HO-1 generated metabolites of heme induce protective biological responses related to atherosclerosis. Although bilirubin and biliverdin were originally considered to be only waste products of heme degradation, later on they were demonstrated to acts as antioxidants. At high concentrations, these metabolites have toxic effects in neonates [104]. However, at physiologic concentrations bilirubin strongly suppresses oxidation of chemically-induced peroxyl radicals [105]. Later on, several other publications described cardioprotective effects of bilirubin and biliverdin and many epidemiological studies demonstrated that higher serum levels of bilirubin are inversely related with the incidence of CVDs [106-111]. Bilirubin has been implicated in protection against endothelial activation and dysfunction in human aortic endothelial cells [112, 113]. Moreover, it was suggested that biliverdin and bilirubin are able to have an inhibitory effect on LDL oxidation [114]. It has also been suggested that bilirubin reduces leukocyte transendothelial migration via interaction with adhesion molecules [91, 115]. The antioxidant actions of bilirubin have been demonstrated *in vivo* in a variety of

diseases, including multiple sclerosis and other immune disorders, ischemia-reperfusion injuries and coronary heart diseases [116-118]. Another metabolite, CO is considered as a toxic gas but it has also been recognized to have major physiological functions as a signaling molecule [119-121]. CO derived by HO-1 activation can arrest cellular respiration [122]. Furthermore, CO has been implicated in regulation of apoptosis, vasodilatation and inflammation. Similar to NO, CO can upregulate cyclic guanosine monophosphate (cGMP) leading to vasodilatation and inhibition of VSMC proliferation [123, 124]. However, cardioprotective actions of CO can also be explained by cGMP-independent mechanisms. CO is able to directly activate Ca^{2+}-dependent potassium channels, leading to vascular relaxation via Ca^{2+} desensitization. Another action of CO is activation of the p38 MAPK pathway. Via this pathway, CO can lead to an inhibitory effect on LPS-induced pro-inflammatory cytokine production in macrophages and exerts an antiproliferative effect on VSMCs [93]. HO-1-mediated heme degradation also releases a molecule of iron from the core of heme porphyrin rings. Free iron itself has not been described to be cardioprotective but rather acts as an oxidant. However, it stimulates ferritin synthesis that in turn has antioxidant effects by reducing the bioavailability of free radicals, such as heme and Fe2+ itself [125].

Thus, HO-1 reaction products are capable of regulating several responses leading to a protective effect against atherosclerosis. This is via modification of the activation, differentiation and maturation of several cell types. Nonetheless, contribution of HO-1 in the development of atherosclerosis is probably even more complex. For example, a cross talk between HO-1 and the prostaglandin I2 (PGI2) has been suggested. The latter can inhibit platelet activation, VSMC contraction and proliferation, leukocyte-endothelial cell adhesion and induces thrombomodulin (an inhibitor of blood coagulation) [126-128]. Some researchers provide evidence that COX-2-dependent PGI2 can upregulate HO-1 which inhibits TNF induction. They suggest that PGI2 atheroprotection is at least in part via HO-1 [129, 130]. Moreover, the atheroprotective effects of estrogens are explained in a similar manner [131].

Therefore first of all, a major contribution to atheroprotective effects that are assigned to HO-1 are mediated via its function in adaptive cellular response against toxicity of oxidative stress. Secondly, HO-1 possesses important immunomodulatory capacities via anti-inflammatory, anti-apoptotic, antiproliferative, and pro-angiogenic effects as explained above. To conclude, upregulation of HO-1 has an overall cardioprotective effect due to several beneficial effects. As ROS generation, tissue oxidative stress and inflammation have been implicated in all stages of atherosclerosis, it appears that HO-1 expression is protective against development of both early and advanced atherosclerotic plaques. Therefore, targeted modulation of HO-1 for potential cardioprotective therapeutic intervention would be of great interest. However, one has to be careful with general modulation of HO-1 as it may lead also to some possible side effects, in particular for tumor growth since HO-1 promotes angiogenesis and exerts anti-apoptotic effects. Also, importance of tissue and

cellular localization as well as precise mechanisms responsible for the atheroprotective effects of HO-1 within those specific targeted cells needs further in depth investigation.

I.2.3 NF-κB in atherosclerosis

The molecular mechanism behind different CVDs have not been fully elucidated. Though, one of the major transcription factors that has been linked to both cardiovascular health and disease is NF-κB. NF-κB controls multiple processes, including immunity, inflammation, cell survival, differentiation and proliferation. NF-κB regulates cellular responses to stress, hypoxia, stretch and ischemia. It is therefore not surprising that NF-κB has been shown to influence numerous CVDs, including atherosclerosis, myocardial ischemia/reperfusion injury, ischemic preconditioning, vein graft disease, cardiac hypertrophy and heart failure [132-136]. It is generally believed that activated NF-κB is contributing to pathologies. However, NF-κB can sometimes also be cardioprotective depending on cellular and physiological context. The NF-κB target genes can differ greatly according to stimulus and cell type. In this way, there are some conditions where activation of NF-κB can contribute to protection against CVDs [12, 137, 138]. In most cell types involved in different stages of atherosclerotic lesion development the NF-κB pathway is activated. This suggest that NF-κB is involved in initiation and progression of the atherosclerotic plaque formation [87, 132, 139-142]. The link between NF-κB regulated inflammation and atherosclerosis is quite evident. There are many pro-inflammatory proteins under regulation of NF-κB that are important in all steps of an atherosclerotic plaque formation. If those pro-inflammatory proteins are inhibited by pharmacological inhibition or via genetic knockdown reduction in formation of an atherosclerotic plaque, macrophage infiltration and disease progression is noticeable. This phenomenon is investigated in many experimental models of atherosclerosis [143-151]. NF-κB is already involved in initial steps in formation of the atherosclerotic plaque. NF-κB has a contribution in the activation of endothelial cells to facilitate leucocyte adhesion via expression of adhesion molecules and chemokines. Those genes that recruit inflammatory cells to the vessel wall include ICAM-1, VCAM-1, P-selectin, E-selectin, chemokines (such as MCP-1), cytokines (such as TNF, IL-1 IL-6, IL-8) and growth factors [139, 140, 143]. Circulating activated platelets strongly contribute to NF-κB activation in endothelial cells. Progression in atherosclerotic plaque formation in mice is robustly increased in the presence of those circulating activated platelets [152, 153]. Interaction between endothelial cells and these platelets result in an NF-κB-dependent increase of ICAM-1, MCP-1 and E-selectin [153-157]. Endothelium specific inhibition of p65 activation in apoE knockout mice lead to cardioprotective effects. Overexpression of a dominant-negative IκBα or inhibition of IKKγ led to suppression of adhesion molecules on endothelial cells, chemokines and cytokines produced by endothelial cells. This led to a reduced monocyte recruitment to the atherosclerotic plaque [141]. These results suggest that NF-κB activation in endothelial cells plays an essential role in vascular inflammation and formation of an atherosclerotic plaque.

This contribution is via regulation of an array of pro-inflammatory molecules. However, NF-κB is also responsible for regulation of anti-apoptotic genes enhancing the viability of endothelial cells. If NF-κB is therapeutically inhibited, then expression of anti-apoptotic genes becomes reduced leading to sensitization of endothelial cells to apoptosis. This could finally have adverse effects in formation of the atherosclerotic plaque.

Nuclear NF-κB was not only detected in endothelial cells in early lesions, it was also demonstrated in VSMCs, macrophages and T-lymphocytes in advanced lesions [158, 159] [160]. NF-κB was found inactive in VSMCs of healthy tissue but p65 and p50 were found in the nucleus in VSMCs from human atherosclerotic plaques [161]. Monaco and colleagues suggested that mainly activation of the canonical pathway of NF-κB selectively regulates pro-inflammatory and prothrombotic responses in human atherosclerosis. Via development of a short-term culture system of cells isolated from human atherosclerotic tissue they investigated signaling pathways in human atherosclerosis. Active p65, p50, and c-Rel were detected but not p52 or RelB. This NF-κB activation can be blocked by overexpression of IκBα or via a dominant-negative IKKβ. However, it could not be blocked by a dominant-negative IKKα [140]. On the other hand, inhibition of NF-κB in macrophages by use of IKKβ deletion in LDLR-deficient mice, increased atherosclerosis, suggesting that activation of NF-κB via the canonical pathway in macrophages can be beneficial [138]. It is possible that NF-κB exerts its protective effect in macrophages via IL-10-dependent suppression of inflammation. The genetic deletion of IKKβ in macrophages led to a reduced expression of this anti-inflammatory cytokine. Also genetic deletion of p105/p50 in the same cells led to a decrease in atherosclerosis. Those cells had a reduced uptake of oxLDL but a prolonged production of TNF after LPS induction [162]. Thus, the consequences of NF-κB activation in atherosclerotic plaques are likely to be complex. Depending on cellular and physiological context the outcome can be pro- or anti-atherogenic [163].

During formation of the atherosclerotic plaque a rupture can occur which might be correlated with increased NF-κB activity. Indeed, in unstable coronary plaques increased NF-κB activity could be detected [164]. Moreover, patients with unstable angina, who have a high risk of plaque rupture, have high levels of activated NF-κB in their white blood cells [165-167]. Furthermore, plaque rupture is linked with increased activity of matrix metalloproteinases (MMPs) in macrophages. Excessive production of MMP by NF-κB can lead to the destruction of extracellular matrix what plays an important role in plaque rupture [168, 169].

I.2.3.1 Triggers

During initiation phase, NF-κB is activated in endothelial cells in response to an array of pro-atherogenic molecules, including TNF, bacterial and viral infections, cytokines, oxLDL, ROS and advanced glycation endproducts (AGE). Interesting to note, activation of NF-κB by ROS and oxLDL can also be indirectly through uncoupling of eNOS. Hence, there is a reduced production of NO, which is a negative regulator of NF-κB [170-172]. Besides pro-

inflammatory cytokines, NF-κB can also be activated via pro-atherogenic mechanical conditions. For example, NF-κB activity is elevated in endothelial cells at regions that are exposed to disturbed blood flow [173, 174]. Cultured human aortic endothelial cells that are exposed to low shear stress show significantly higher activated NF-κB levels compared to cells exposed to high shear stress [175]. In addition, NF-κB activation in endothelial cells is also linked with hypertension. The latter is a major risk factor of atherosclerosis that can increase Ang II, leading to increased ROS and activated NF-κB [176-179]. Thus, although NF-κB and Nrf2 have similar stimuli, there outcome concerning CVDs is completely different.

It is clear that in CVDs involvement of NF-κB is extensive. This led to the establishment that this pathway would be an excellent therapeutic target. Several clinical therapies to treat cardiovascular diseases have been shown to have their effects on NF-κB [180, 181]. However, the function of NF-κB and its influence on disease varies according to the cell type in which NF-κB is activated. As we mentioned earlier, in endothelial cells NF-κB exerts pro-atherogenic functions, while it can be anti-atherogenic in macrophages. Moreover, complete blockage of the pathway can also be detrimental as NF-κB is also responsible for other functions such as immune responses and homeostasis. Thus, targeting NF-κB in humans to treat CVDs should be in a very controlled way and with cell specific targeted therapies. A treatment that inhibit detrimental effects of NF-κB, but that will still preserve the beneficial ones.

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I.3 *Withania somnifera*, Withanolides and Withaferin A

I.3.1 Ancient traditional medicine

In ancient times, human beings relied on local fauna and flora for their wellbeing. Not only did they use fauna and flora as food supply, they also used it for treatment of diseases. They experimented with various leaves, berries or roots from plants, minerals and even animal parts. This led to an arsenal of crude drugs used by the local healer or shaman. A high percentage of those drugs often only had a ceremonial or placebo effect or was even in the worst case rather detrimental and dangerous instead of beneficial. Some of the drugs however, contained pharmaco-active compounds exerting therapeutically beneficial effects. Nowadays, one can still find these different traditional medicines completely sophisticated and deep-rooted within several cultures and the daily lives of its people. In the Western countries also alternative herbal medicine*, based on ancient traditional medicine, is receiving growing attention. Scientific studies of plants used in those traditional medicines provide us already with some clues which plants or active constituents are worth studying in more molecular detail. Intensive research of some plants gave insight in the activity of their individual compounds as plants often contain an array of active and inactive compounds. Those studies led to a number of drugs used in Western medicine that are derived from a biological source. One example is morphine, a potent drug used to relieve severe pain. It is an opiate found in papaver poppies. Thus, for some commercial drugs used in our Western medicine we can find a traditional medicinal equivalent. Out of those crude traditional plant extracts, also some commercially available alternative drugs have been developed. However, some features explain why the commercial drugs in our modern medicine are considered more efficacious than the crude traditional plant extract and the commercial alternative drugs. First of all, modern medicinal drugs contain only the active compound, whilst the traditional and alternative drugs contain often an extraction of the complete plant, including other active, non-active or even detrimental compounds. Thereby, modern medicinal drugs will give higher specificity than its traditional or alternative equivalents. Secondly, the content of crude traditional drug is susceptible to variations. The amount of active compound may vary greatly depending on the extraction. Variabilities of the plant itself can lead to differences in content and percentages of the different compounds. Location of growth, moment of extraction, extraction solvent or growth conditions of the plant can make real differences [1-6]. This will lead to changes in efficacy. A nice example of differences in constitution of commercially available alternative drugs is given by the work of Sangwan and colleagues. They quantitatively analyzed several mono- and poly-herbal

products commercially available on the Indian market for a number of chemical constituents. Interestingly, a more than 70-fold variation of the active compound Withaferin A was found in the *Withania somnifera* products. This study emphasizes the need for stringent standardization of herbal products [7]. In contrast to this, the active compound for commercial drugs used in the modern medicine is mostly produced synthetically in bulk in a highly controlled manner leading to exactly the same product quality over and over again. Modern Western medicine can develop drugs with an exact dose of the active compound, leading to a controlled therapy using exact doses within the therapeutic range, taking side effects of the compound into account. Traditional medicine however is by far not inferior to the modern medicine, as it is the origin of our modern medicine. Traditional medicine offers science an array of study subjects which might finally lead to an enlargement of the modern western medicine. In other words, crude drugs can lead to precise fine-tuned drugs to combat modern diseases in a focused purposeful way.

*To my opinion, there is a general misconception about alternative herbal medicine in our modern western world. If Belgians speak about homeopathy, they often refer to a mixture of homeopathy and herbal therapy. It is a logic result as there are no clear regulations concerning alternative therapies. Therapists often juggle with fancy terms, are treating with a mix of homeopathy and herbal medicine at once and are not defining it in a clear and transparent way to their patients. Hereby, people are sometimes thought to be passionate supporters of homeopathy when they actually believe in the good of herbal medicine. However, there are distinct differences between both. Homeopathy is a (questionable) therapy based on the memory of a water molecule and doesn´t have anything to do with herbs. Thus to be clear: alternative herbal medicine is herbal therapy based on the ancient traditional medicines.

I.3.2 *Withania somnifera*

Sangwan and colleagues investigated herbal products from the plant *Withania somnifera* (WS), also known by its Sanskrit name “Ashwaganda”, “Indian ginseng” or “winter cherry”, which is commercially available on the Indian market. The plant *WS* occurs in Africa, Mediterranean sea and South Asia. As a result from this wide range of places, there are morphological and chemical changes in composition of local species. As we mentioned in the introduction of this chapter, this can affect the relative content of different bioactive phytochemicals and so also the activities of the plant [1-6]. *WS*, is a plant of the nightshade family from the genus *Withania*. Many members of the nightshade family are used by men as food or spices. Potato, tomato, eggplant, chili pepper and tobacco or some of the known important agricultural members of this family. Nonetheless, some species of the family are toxic for human beings, ranging from mildly irritating to fatal in small quantities. *WS* is a member of GRAS (Generally Regarded As Safe) plants. Acute toxicity of WS is modest [8-10].

WS is used as a therapy in Ayurveda, a traditional medicinal system of Indian origin. Ayurveda is one of the oldest and still practiced methods of herbal medicine. Roughly translated into "Knowledge of life", it is based on the use of natural herbs and herb products for therapeutic measures to boost physical, mental, social and spiritual harmony and improve quality of life. *WS* is applied in different forms, such as infusions, ointments, powders, syrups, etc. [6, 11]. The roots of *WS* are used to treat a wide range of disorders like chronic fatigue, gastrointestinal diseases, infertility, dehydration, arthritis, menstrual disorders, rheumatism etc. Overall it is used to promote general wellness. The berries and leaves of *WS* are locally applied to tumors, ulcers, burns and wounds.

Today, various health beneficial effects of *Ashwagandha* are scientifically evaluated in diet intervention studies. Today, scientific evidence has been presented in case of reduced inflammation and immunomodulation, reduced anxiety, periodontitis and arthritis pain, anti-cancer activity and cardioprotection [12-18] .

Family: Solanaceae (nightshade family)
Genus: Withania
Species: Somnifera
Scientific Name: Withania Somnifera (Dunal)

Fig 3.1 Withania somnifera, a plant from the nightshade family from the genus *Withania*. Roots, berries and leaves are used as therapy in Ayurveda, a traditional medicinal system of Indian origin. Source: www.wikipedia.org (plant), stsn.scnatweb.ch (berries), psychoactiveherbs.com (roots).

I.3.3 Withaferin A

The main bio-active chemical constituents of *WS* are the withanolides, a class of chemicals containing a steroidal alkaloid and lactone structure. Steroids contain a specific arrangement of four cycloalkane ring structures. From these four structures there are three cyclohexane rings and one cyclopentane ring. The lactone part is a cyclic ester. Although withanolides are

present in almost all parts of the plant, roots and leaves are mainly used in Ayurveda. Those roots and leaves are quantitatively rich in withanolides. From the various withanolides Withaferin A (WA), a highly oxygenated withanolide, is by far the most studied bioactive compound. It was surprisingly the first member of the withanolides discovered and it is one of the main biologically active withanolides. In case of WA the cyclic ester is built up with five carbon atoms and a single oxygen atom.

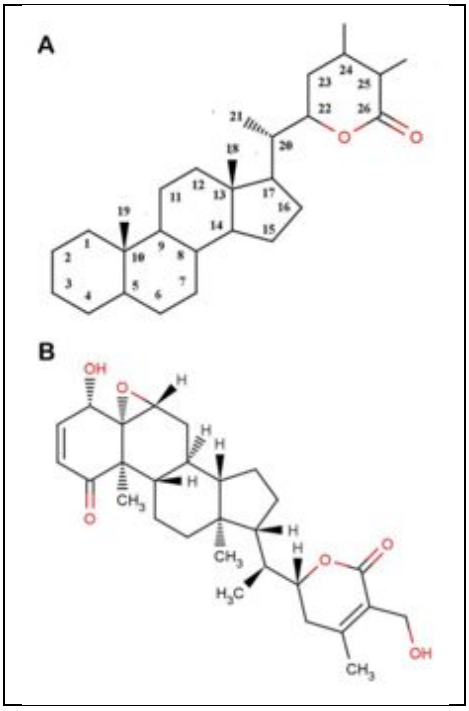

Fig 3.2 (A) Main structure of withanolides, a class of chemicals containing a steroidal alkaloid and lactone structure. (B) Structure of WA, a highly oxygenated withanolides, where the cyclic ester is built up with five carbon atoms and a single oxygen atom [19] .

I.3.3.1 Molecular mechanisms

WA was demonstrated to exert a broad spectrum of health promoting effects, including anti-inflammatory, anticarcinogenic, anti-angiogenic, antibacterial, anticonvulsive, immunosuppressive and pro-apoptotic properties [20-30]. Despite this wide variety of bioactivities of this compound, the molecular mechanisms remain largely unknown. In the context of modern pharmacology there is a strong need to gain better insight in these underlying mechanisms. Several explanations though have been proposed for the unknown molecular mechanisms of WA. So is acylation (0=C-R) or alkylation (C-R) of critical structures or enzymatic active sites by covalent attachment proposed [31]. Chemical structure analysis of WA pinpointed 3 positions that could be involved in a thio-alkylation reaction with nucleophilic sites like sulfhydryl groups of cysteine residues in target proteins. These positions are the epoxide structure at C5 and the unsaturated ketone at C3 and C24, being all 3 highly susceptible for nucleophilic attack by the target protein leading to a covalent binding with WA by a Michael addition alkylation reaction. Via UV spectrophotometry the capacity of WA in adduct formation with cysteine residues was confirmed. When WA was co-incubated with L-cysteine, a hypochromatic shift could be observed [32]. NMR spectral

analysis identified C3 as the main nucleophilic target site for ethyl mercaptan, thiophenol and L-cysteine ethyl ester *in vitro*. In contrast, structure-function analysis of several withanolides indicated that the epoxide structure at C5-C6 mainly contributes to the biological activities of WA. In line with this analysis, reaction of WA with 2-mercaptoethanol specifically affects the C5-C6 epoxy structure leading to loss of the anticancer activity of WA [33]. In parallel, co-treatment of WA with the strong reducing agent Dithiothreitol (DTT) or N-acetyl cysteine (NAC) resulted in abrogation of the biological anti-inflammatory, pro-apoptotic or heat shock response inducing effects of WA. This strengthens the hypothesis of the covalent interactions with WA leading to loss of activity of the target proteins [32, 34, 35]. In contrast to the description of WA reactivity with small thiol-containing compounds, hitherto only few cellular target proteins have been identified so far, including vimentin and annexin II [36, 37]. For some proteins with whom WA can directly interact, also modulation of their activity and related signal transduction pathways was demonstrated. Several transcription factors, structural proteins, proteases and kinases are suggested as target proteins of WA [24].

In combination with pentafluorophenyl-biotin WA can be modified at position C27 creating a biotinylated form of WA. This can be used to investigate potential molecular target proteins of WA via affinity purification [38, 39]. According to a structure-function analysis this modification is not playing a critical role in the activities of WA.

I.3.3.2 Targets

Transcription factor: NF-κB

One important target pathway of WA is the NF-κB pathway. As mentioned in a previous chapter, NF-κB is involved in several cellular processes such as inflammation, differentiation, cell survival, growth and angiogenesis. Tight regulation of the transcription factor occurs via its inhibitor protein IκBα. The latter is regulated by the IKK complex composed of IKKα, IKKβ and IKKγ. *In silico* studies suggested that inhibition of the NF-κB pathway by WA is a result of the inactivation of the IKK complex by binding of WA to IKKγ and IKKβ [40]. Our lab demonstrated in 2007 that WA interferes with TNF-induced NF-κB activation in *in vitro* studies at the level of IKKβ or upstream of it, leading to hyperphosphorylation of IKKβ. The absence of an active IKK complex, prevented phosphorylation and degradation of IκBα leading to inhibition of NF-κB release. Also, reducing agents reversed WA-mediated IKKβ hyperphosphorylation and suppression of TNF-induced NF-κB activation. These data are in line with the hypothesis that WA is binding covalently to its target proteins [34]. Moreover, a mechanism for suppression of the IKKβ kinase activity by WA was proposed. Cysteines in IKKβ are suggested to be the target of WA. Cysteine 179 has been described as a target of several natural compounds to directly suppress the IKKβ kinase activity as it is a cysteine residue located in the activation loop of the kinase domain. Another possibility suggested are the cysteines 622 and 716 affecting the formation of the IKKα en IKKβ complex [41-45]. IKKγ rather than IKKβ was suggested by others as the target protein of WA in the NF-κB

pathway. A strong binding between WA and IKKγ was predicted *in silico* via molecular docking analysis and molecular dynamics simulations studies [40]. It was predicted that the binding between the two was via an Van der Waals interaction resulting in a steric and thermodynamic hindrance. Due to this hindrance IKKγ was prevented to enter the IKK complex. Moreover, docking of WA into an active IKKγ/IKKβ complex also suggests the disruption of the active complex. Thus, WA could be able to prevent the formation of an IKK complex and to disrupt a well formed active IKK complex. The observation that WA is also able to block constitutively active NF-κB is noticed in several *in vitro* studies [26, 46, 47]. The latter is an interesting observation as constitutive activation of NF-κB is observed in cancers and other chronic inflammatory diseases.

Thus, WA is clearly able to inhibit the NF-κB pathway. However, the exact target of WA at the level of the IKK complex leading to its inhibitory effect on the NF-κB pathway needs further in depth study.

Proteasome

A crucial player in the regulation of the NF-κB transcription factor is the proteasome. Ubiquitination and proteasomal degradation are important for tight regulation of NF-κB. It is suggested that also the proteasome could be one of the targets of WA. This implicates that WA is potentially able to regulate NF-κB at more than one level. The 26S proteasome is build up with a cylindrical 20S core part and two 19S cap particles that are docking at both ends of the 20S core unit. 3 β-subunits in the 20S are mediating 3 different proteolytic activities: β1 mediating a caspase like activity, β2 a trypsin like activity and β5 a chemotrypsin like activity. The proteasome has an important role in several essential biological functions, including mitosis, differentiation, signal transduction, apoptosis and inflammation. Deregulation of the system is related to various human diseases. Thus, the proteasome is a therapeutic target used in treatment of various diseases. Bortezomib, is an example of a proteasome inhibitor used as a treatment for cancer such as multiple myeloma. It would be promising if WA could also inhibit the proteasome giving it possible therapeutic applications. However, this research demonstrates a lot of inconsistencies with some researchers claiming inhibitory effects for WA whilst others claim the opposite.

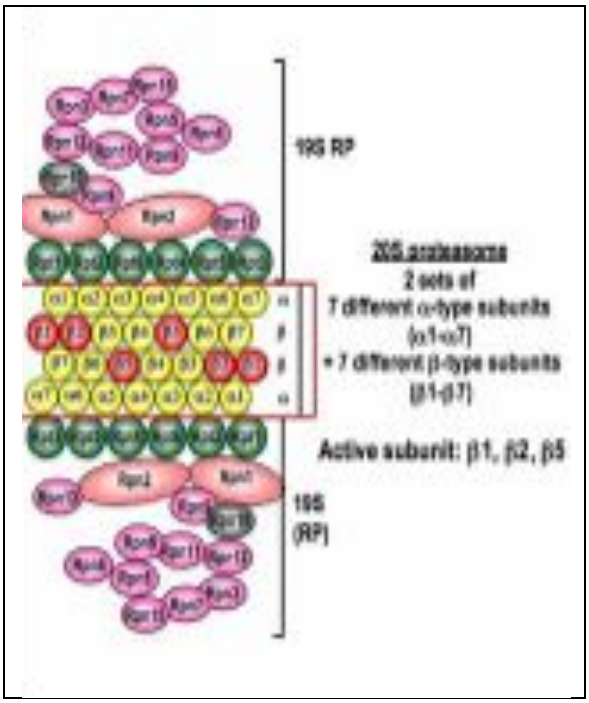

Fig 3.3 The 26S proteasome is build up with a cylindrical 20S core part and two 19S cap particles that are docking at both ends of the 20S core unit. 3 β-subunits in the 20S are mediating 3 different proteolytic activities: β1 mediating a caspase like activity, β2 a trypsin like activity and β5 a chemotrypsin like activity. Source: www.f.u-tokyo.ac

When investigating the effect of WA on angiogenesis in human umbilical vein endothelial cell (HUVEC) cells, a link between WA and the proteasome was suggested. WA was able to inhibit TNF-induced NF-κB DNA binding activity. Interestingly, WA did not interfere with upstream phosphorylation events, but did act possibly at the level of protein ubiquitination and degradation. WA increased global levels of ubiquitinated protein species in TNF-treated HUVECs, suggesting that the ubiquitin–proteasome pathway (UPP) is one of the major targets of WA [25, 38]. Binding of WA with the proteasome is suggested to be mediated via a nucleophilic attack by the hydroxyl group of N-terminal threonine of the proteasomal chymotrypsin subunit β5. Moreover, treatment of WA under reducing conditions could reverse the binding, strengthening the hypothesis that WA can covalently link with its target proteins [26, 48]. At high doses, WA was able to inhibit the chymotrypsin like activity in a cell free assay system, in human prostate cancer cells and xenografts. Inhibition of the proteasome activity by WA resulted in accumulation of ubiquitinated proteins, including three proteasome target proteins (IκBα, Bax, p27) accompanied by apoptosis induction. Treatment of human prostate PC-3 xenografts with WA for 24 days resulted in 70% inhibition of tumor growth in nude mice, associated with 56% inhibition of the tumor tissue proteasomal chymotrypsin like activity [48]. Another *in vitro* and *in vivo* study on the suppression of mesothelioma growth by WA also suggested inhibition of proteasome activity which may account at least partially for the known anticancer effect of WA [23]. Moreover, it is suggested that WA is inducing endoplasmic reticulum stress together with proteasome inhibition [21].

Several publications also presented conflicting data, related to WA-dependent proteasomal degradation of client proteins. For example, Noh *et al.* showed that CHOP down-regulates cFLIP(L) expression by promoting ubiquitin/proteasome-mediated cFLIP(L) degradation. Interestingly, WA appears to be a CHOP inducing agent. The WA-induced down-regulated cFLIP(L) expression appeared to be mediated by suppression of cFLIP(L) transcription and increased cFLIP(L) protein instability and degradation [49]. Moreover, down regulation of ERα protein levels by WA in MCF-7 breast cancer cells is caused by proteasome-dependent ERα degradation. Cotreatment with a protein synthesis inhibitor cycloheximide or proteasome inhibitor MG132 revealed that depletion of ERα by WA is posttranslational, due to proteasome-dependent ERα degradation [50] Altogether, in contrast to earlier papers, various papers suggest increased proteasomal degradation of WA client proteins [21, 23, 26, 39].

In conclusion, discrepancies in WA-dependent regulation of proteasomal functions require further study, taking in account different cell lines, concentrations or exposure times applied for WA.

Structural proteins

WA is also able to target cytoskeleton organizing and structural proteins. Vimentin, the intermediate filament protein is probably the most intensively studied target protein of WA. It is a protein that can make up the cytoskeleton together with microtubules and actin microfilaments. Vimentin is responsible for maintaining cell shape, integrity of the cytoplasm and stabilizing cytoskeletal interactions. Moreover, vimentin can also function as an organizer of proteins involved in attachment, migration and cell signaling. Interesting to note, is that WA is able to bind vimentin through a Michael addition at cysteine 328 [36]. Also other intermediate filament proteins, such as the glial fibrillary acidic protein (GFAP) and desmin are demonstrated to be covalently linked with WA in a similar manner [51]. Several biological effects of WA could be partially explained by the binding of WA to vimentin. There was a decrease of inhibition of NF-κB and anti-angiogenic and pro-apoptotic properties of WA in vimentin knockout mice or knockdown cells compared to wild type cells [36, 51, 52]. In addition to the direct binding and consequent cleavage of vimentin, WA is also able to induce phosphorylation of vimentin. This phosphorylation leads to reorientation and disassembly of vimentin and is suggested to lower invasive activity of cancer cells. Also annexin II and F-actin, targeted by WA, are contributing to this phenomenon [53]. Vimentin is defined as a novel anticancer therapeutic target giving additional strength to the possible therapeutic applications of WA as an anticancer drug. Though, more research is needed especially concerning the complex interactions with other intermediate filament proteins and the exact contribution of WA to it.

Kinases

In addition to the previously discussed IKK kinase, several studies have investigated the effect of WA on additional kinases, such as MAP kinases p38, JNK, ERK and Akt [21, 23, 34,

39, 50, 54-68]. Remarkably, many conflicting data have been published. A possible explanation of these inconsistencies are the various cell types and differences in WA exposure time and concentrations applied. Also, most studies are based on the phosphorylation states of the kinases via Western blotting or flow cytometry. In most cases, direct effect on kinases or direct interaction of WA with kinases were not analyzed, except in case of PKC, for which direct inhibition by WA was demonstrated *in vitro* [69]. As such, in many conditions we cannot conclude whether these observations are direct or indirect effects of WA. Further identification of direct kinase targets of WA will increase the pharmacological interest for therapeutic applications.

Heat shock proteins

A possible explanation for indirect effects of WA on several kinase pathways maybe explained via the CDC37-Hsp90 complex. This cochaperone-chaperone complex is shown to play an important role in modulating kinase function [25, 70-74]. It is suggested that WA is able to dissociate the CDC37-hsp90 complex leading to downregulation of hsp90 target proteins, favoring the idea that WA promotes rather than inhibits proteasome function. In line with these results, the effect of WA could be reversed by the proteasomal inhibitor, MG132. The effect of WA on the CDC37-Hsp90 complex was mediated via a direct binding with hsp90 [39]. In contrast, *in silico* analysis rather suggested binding of WA to CDC37 [19, 75].

Other transcription factors

The transcription factor NF-κB is one of the well-established targets of WA. Besides NF-κB, WA actions have been described on activity of heat shock factor 1 (HSF-1), liver X receptor alfa (LXRα), members of the signal transducer and activator of transcription family (STAT1 and STAT3), Notch1, AP-1 and the forkhead box transcription factor FOXO3A. For most of them it is not clear yet if the transcription factor itself is the target of WA, rather proteins and enzymes involved in the signaling cascade or combinations thereof. WA was indicated as one of the most potent inducers of a heat shock response as it induces the transcription factor heat shock factor 1. Santagata and colleagues evaluated over 80,000 natural and synthetic compounds as well as partially purified natural product extracts using a reporter cell line optimized for high-throughput screening. Interestingly, the most potent compounds identified shared the same chemical motif, an α,β-unsaturated carbonyl functionality, with a strong potential for thiol-reactivity [32]. It can be considered surprising that WA combines anticarcinogenic properties with stimulation of a heat shock response as HSF-1 activation is typically a marker of cancer which enables survival under stressful hyperproliferative conditions. However, products that further tax these stress responses can overwhelm the stress capacity that cancer cells can deal with and may as such result in cancer cell death. Another paradox related to HSF-1 activation is WA-dependent regulation of the heat shock proteins like hsp90. Paradoxically, WA combines positive effects on HSF-1 activity with a negative effect on hsp90 activity. This apparent controversy might be a consequence of the applied concentration of WA as well as the cellular context. Alternatively, the induction of

the heat shock response by WA maybe an indirect consequence of hsp90 inhibition, since there is a reciprocal regulation of hsp90 and HSF-1 [76].

The liver X receptors, containing LXRα and LXRβ, form a subfamily of the nuclear receptor superfamily. These two isoforms of LXR form obligate heterodimers with retinoid X receptors (RXRs) and regulate expression of target genes containing LXR response elements. Those genes are involved in cholesterol, fatty acid and glucose metabolism, transport and homeostasis. WA is suggested to act as an agonistic LXRα ligand. Molecular docking studies of the ligand binding domain of LXRα revealed a possible interaction with WA [77]; [our unpublished results]. This direct binding of WA to LXRα is possibly the reason for the WA-induced activation of LXRα. LXRs influence inflammation and immune responses partially via transrepression of NF-κB. Thus, this suggest another regulatory manner how WA can further regulate NF-κB activity [77, 78]. Important to note, the ligand binding domains of nuclear receptor family members are structurally highly conserved across the family members. Thus, the specificity of WA for LXRα has to be further investigated.

Moreover, WA could inhibit constitutive as well as induced STAT3 and STAT1. Interesting for future therapeutic applications is that WA could trigger apoptosis and largely inhibit cell migration/invasion of breast cancer cells via STAT3 even after IL-6 induction [79]. Also in human renal carcinoma Caki cells, the JAK/STAT3 signaling induced by WA could lead to apoptosis [22]. In non-cancerous cells, inhibition of LPS-induced STAT1 and STAT3 phosphorylation by WA could lead to a downregulation of the LPS-induced COX-2 expression and prostaglandin E2 (PGE2) production reducing the inflammatory state [55].

WA is also suggested to inhibit Notch-1, another therapeutic target against carcinogenesis. Notch signaling plays a crucial role in the development of colon cancer [57, 63]. On the other hand, WA was shown to cause activation of Notch2 and Notch4 in human breast cancer cells. Although Notch signaling is often hyperactive in human breast cancers, it was observed that activation of Notch2 and Notch4 by WA is inhibiting breast cancer cell migration [80].

An investigation in our lab led to the observation that WA could repress IL-6 gene transcription in metastatic breast cancer cells upon dual inhibition of NF-κB and AP-1 [47]. Via electrophoretic mobility shift assay (EMSA), inhibition of DNA binding of NF-κB and AP-1 by WA were noticed [35]. Similar results were obtained with a crude extract of *WS* that could inhibit NF-κB and AP-1 in human peripheral blood and synovial fluid mononuclear cells [81]. However, how WA exerts its effect on AP-1 activity needs further investigation.

In our lab WA and other natural compounds were tested in myelogenous leukemia cells for their pro-apoptotic and anti-cancer effects. As Nrf2 has a role in the expression of the drug efflux protein, P-glycoprotein that can lead to multidrug resistance in cancer, this transcription factor was taken along in the investigation. Via EMSA an inhibition of DNA binding of Nrf2 was noticed after an induction of 6 µM of WA [35]. The profile of WA showed high similarity to the profile of the traditional Chinese medicinal formula Si-Wu-Tang (SWT)

in the connectivity map database. Via pathway analysis the expression of genes of Nrf2, includin HMOX1, GCLC, GCLM, SLC7A11 and NQO1 were identified to be the most significantly affected by SWT [82]. In 2012 Vaishnavi *et al.* published differential activities of WA and WN using bioinformatics and experimental evidence. Molecular docking studies conducted on the three-dimensional structure of Nrf2 showed that both WA and WN could interact with amino acids Ala 69, Phe 71 and Gln 75 in the active site region of Nrf2. WA could bind more efficiently, in terms of binding affinity, with Nrf2 than WN [83]. This was validated in human normal and cancer cells where similar effects where noticed. WA could bind strongly to the selected targets, acting as a strong cytotoxic agent. Further studies are required to understand how WA and WN can differentially regulated the Nrf2 pathway. In conclusion, we can state that WA is a multi-targeting molecule, which modulates activity of several transcription factors.

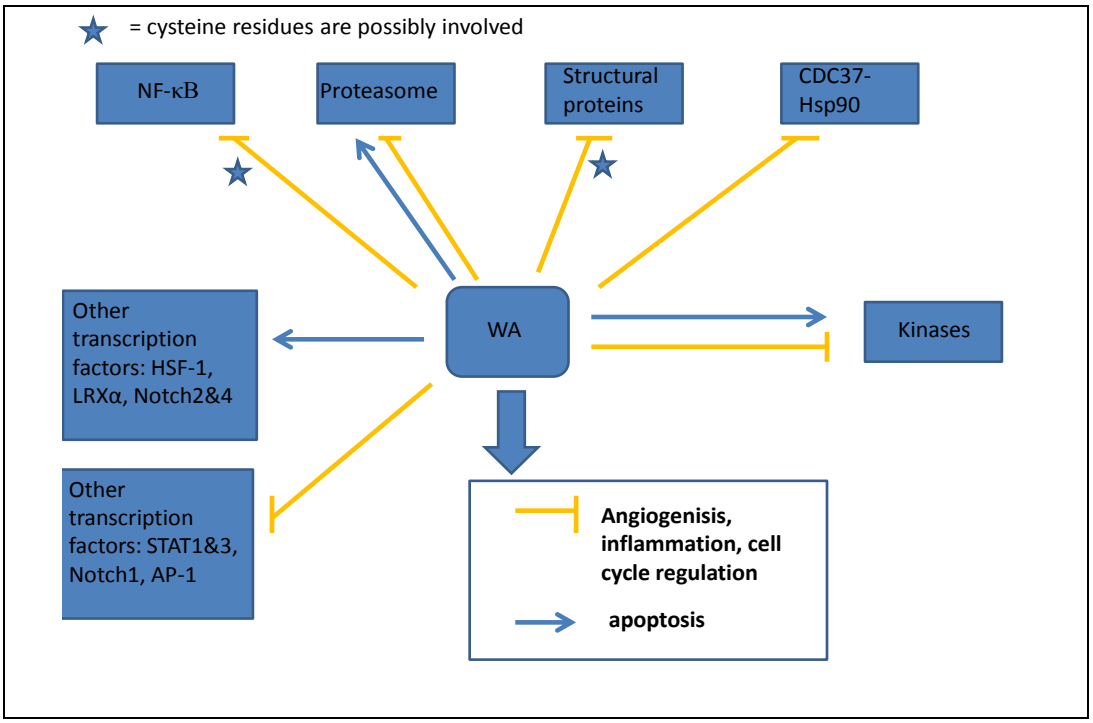

Fig 3.4 Overview of the most important WA targets and effects. WA is influencing the NF-κB pathway possibly via binding to IKKγ or IKKβ. An inhibitory and activatory effect on the proteasome is described for WA. Inhibition is suggested to be via nucleophilic attack of the hydroxyl group of N-terminal treonine of β5. WA inhibit several structural proteins, including vimentin, annexin II, GFAP, F-actin and desmin via direct binding or phosphorylation. An inhibitory effect on CDC37-Hsp90 by WA is suggested via binding to Hsp90 or CDC37. Several kinases, including p38, JNK, ERK, Akt and PKC are suggested to be directly or indirectly influenced by WA in an inhibitory or activatory fashion. Several transcription factors, including HSF-1, LXRα and Notch2&4 are suggested to be activated by WA and several transcription factors, including STAT1&3, Notch1 and AP-1 are suggested to be inhibited by WA.

I.3.3.3 ROS formation

WA was suggested to generate ROS leading to apoptosis in *leishmanial* cells [69], in human myeloid leukemia HL-60 cells [84], human kidney carcinoma caki cells [85], human breast cancer cells such as MDA-MB-231 and MCF-7 cells [86] and in glioblastomas [61]. These publications suggest that WA-induced apoptosis in cancer cells can be mediated at least partially by induction of ROS. This hypothesis was further confirmed by the observation that NAC rescued the apoptotic events [84]. Moreover, WA-induced ER stress was suggested to be mediated by ROS [87]. In contrast, in the gouty arthritis model an anti-oxidative effect of WA was observed [88]. Similarly, in differentiated PC12 cells a protective effect of *WS* root extract against hydrogen peroxide cytotoxicity was noted, leading to neuroprotection. WA was suggested to be the main derivative responsible for these neuroprotective properties of *WS* [89]. Interesting to note is that other researchers focused on the differences between cancerous and healthy cells, which revealed important difference concerning the WA-mediated ROS production in both cell types. Apoptosis-related events, through the upregulation of ROS were detected in human renal Caki cells, but not in human normal mesangial cells [85]. Moreover, WA treatment caused ROS production in MDA-MB-231 and MCF-7 cells, but not in a normal human mammary epithelial cell line (HMEC) [86]. Thus, it appears that WA is able to induce ROS, but that this event is presumably limited to cancerous cells. Although some publications describe WA-induced formation of ROS also in non-cancerous cells, they often make use of relative high WA concentrations. For example, when investigating cell death in erythrocytes Jilani and colleagues noticed ROS formation after induction for more than 48 hours with 10 µM WA [90].

I.3.3.4 Structurally similar withanolide: withanone

Besides WA one can find Withanone (WN) in *Withania somnifera*, a structurally similar withanolide. Despite the high structural similarity, these withanolides have diverse pharmacological properties. Often the effects of the alcoholic extract of Ashwagandha leaves are investigated where WN is defined as the major component. Widodo and colleagues identified anti-cancer activity in the leaf extract (i-Extract) of Ashwagandha and demonstrated WN as a cancer inhibitory factor. They fractionated the i-Extract to its components by silica gel column chromatography and subjected them to cell based activity analyses. At least seven components could cause cancer cell killing. They suggested that the main effect of i-extract is caused by the presence of WN [91]. This selective killing of cancer cells by Ashwagandha leaf extract and WN required the involvement of ROS signaling [92]. Surprisingly, the same research group also published that WN was able to suppress ROS levels, DNA and mitochondrial damage. Moreover, it could even lead to induction of cell defense signaling pathways, including activation of Nrf2 and increased proteasomal degradation [93]. By this, WN can protect normal human cells against toxicity of methoxyacetic acid, a major industrial metabolite [93]. These exciting observations suggest that WN could induce ROS in cancer cells and reduce ROS in normal cells. A potential protective effect of i-Extract, and WN via reduction of oxidative stress was confirmed by

others [94]. Abrogation of mortalin-p53 complex by WN resulting in nuclear translocation and functional reactivation of p53 in human cancer cells was shown by *in silico* prediction, molecular dynamic simulations and experimental evidence [95]. Moreover, WN was suggested to target the TPX2-Aurora A complex, a complex playing a critical role during mitosis and cytokinesis, which is upregulated in several cancer types [96].

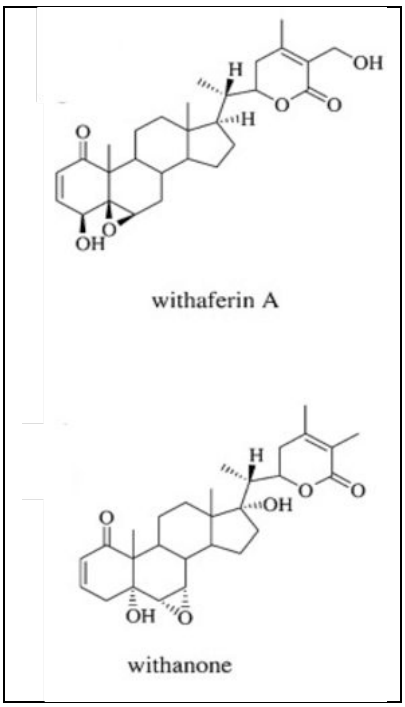

Fig 3.5 Comparison of molecular structure of WA and WN [97].

These articles investigating i-extract and WN suggest a protective and anticarcinogenic effect of both the extract and its main compound WN, as observed for WA. However, WA and WN have distinct anticancer properties. For example, WA could induce activation of Notch2 and Notch4 in human breast cancer cells but no effect was seen with WN [80]. Worth mentioning is, that in this article they compare similar concentrations of WA and WN (e.g. 0-4 µM). Often studies comparing bioactivities of WA with WN, apply different concentrations of WN and WA (for example konar shah 2011 6 : withaferin A (0.4 µM) and withanone (i-Factor, 10 µM). To compare potency and specificity of WN and WA activities in cancer cells, it would be interesting to apply IC50 concentrations for both compounds. Via bioinformatics and experimental evidences Vaishnavi and colleagues have focused on the differences between the two closely related withanolides. They employed bioinformatics tools to predict interactions with four cancer-related proteins, i.e., mortalin, p53, p21 and Nrf2. A clear difference in binding properties was predicted and experimentally observed, where WA revealed the strongest interactions with the proteins of interest. When validating these finding in human normal and cancer cells similar effects where noticed. WA could bind strongly to the selected targets, acting as a strong cytotoxic agent both for normal and cancer cells. Whereas WN only had a weak binding to the targets, it showed milder cytotoxicity towards cancer cells and was safe for normal cells [83]. Notably, despite its described selectivity, in these experiments WA is capable to induce cytotoxicity in normal

cells and not only in cancer cells. The explanation can probably be found in the concentration as they use concentrations up to 10 µM.

I.3.3.5 Therapeutic applications

Despite the broad spectrum of health promoting effects of WA investigated in *in vitro* models, characterization of molecular targets just start to be unraveled. Nevertheless, all targets that are here described are already partially filling in the puzzle on how WA exerts his bioactivities. However, to introduce WA as a therapeutic drug increased insight is needed in the targets and exact molecular mechanisms of action. Nevertheless, some *in vivo* studies in rodent animal models give promising results for WA as a treatment for different types of diseases, including for instance different types of cancers such as medullary thyroid cancer [59], breast cancer [98-100], soft tissue sarcoma [52], glioma [32], cervical cancer [101], pancreatic cancer [39], mesothelioma [23], ovarian cancer [102], lung cancer [103, 104] and melanoma [62]. In *in vivo* models, WA exerts a selective killing towards cancer cells. The therapeutic doses of WA induced only minimal toxicity to normal tissue [98]. Moreover, the same selectivity of pro-apoptotic effect of a similar dose of WA for cancer cells was seen in *in vitro* studies. Normal lymphocytes and monocytes did not respond to WA, but in cancerous cells WA could induce pro-apoptotic signaling [56, 78]. This is a very notable characteristic since most of the cancer therapies are confronted with “collateral damage” as many healthy cells suffer from cancer therapy as well. Further investigation is required as it is not known how WA exerts this selectivity in killing. Moreover, as mentioned previously, This selectivity is largely dependent on the dose administered, as toxicity to normal cells was also observed when WA was administered at high doses [83]. Pharmacokinetic studies of WA in mice revealed plasma peak concentrations of 2 µM, with a half-life of less than 1.5 hours [98]. For some therapies this short half-life could be an obstacle if continuous exposure maybe needed. This problem can probably be circumvented by the use of polycaprolactone implants embedded with WA [103, 104]. In 2012 Gupta and colleagues observed that there was a significant growth inhibition seen in lung tumor xenografts when such implant with WA was used, where intraperitoneal administration of the same WA concentration was not effective. This device allows stable systemic release of WA which holds promise for future therapeutic applications. Moreover, recently a stable derivative of WA has been introduced, 3-azido Withaferin A (3-azidoWA) [105]. To what extent 3-azidoWA effects are similar as WA needs to be further studied. Also, concentration of both compounds needs to be taken into account, as the cellular effects (potency, specificity) of WA strongly depend on the concentration range applied.

The therapeutic effect of WA has also been studied in a cellular model of Cystic Fibrosis (CF), where the inhibition of NF-κB had a main contribution to the protective effects of WA. CF is one of the most common autosomal genetic disorders in humans. It is caused by mutations within a single gene, coding for the cystic fibrosis transmembrane conductance regulator (CFTR) protein. Patients with CF are suffering from chronic lung infections with associated inflammation from opportunistic microbes such as *Pseudomonas aeruginosa*, leading

eventually to death. Thus, NF-κB as an important player of chronic inflammation, is a potential anti-inflammatory drug target in CF. New agents that can be used to control chronic inflammation in CF are needed in the absence of a cure for the disease, surely since the current anti-inflammatory agents in CF have severe limitations due to adverse side effects. The researchers demonstrated that WA blocks activation of NF-κB induced by *Pseudomonas aeruginosa* [106]. The therapeutic value of WA is also demonstrated in animal models of gouty arthritis and chronic proliferative arthritis. Gouty arthritis is an intense acute inflammatory reaction as a result from formation of sodium urate crystals in the joint cavity. The effect of WA on monosodium urate crystal-induced inflammation was investigated in mice, which is used as an experimental model for gouty arthritis. WA could reverse monosodium urate crystal-induced characteristics, such as increased paw volume, lysosomal enzyme levels, lipid peroxidation, and TNF together with decreased antioxidant status [88]. *WS* and WA were tested in several other inflammatory models, including systemic lupus erythematosus, amyotrophic lateral sclerosis and retinal gliosis [13, 14, 20, 34, 107-111].

In general, the effect of WA in these different animal models is not unexpected. Since WA has a strong NF-κB inhibiting capacity it is expected that WA exerts benefits in chronic or acute diseases related to inflammation with an important contribution of NF-κB. Nonetheless, more research is needed since WA has pleitotropic activities beyond its anti-inflammatory effects via inhibition of NF-κB.

To conclude, natural medicinal products have an important contribution in drug discovery for treatment of various human diseases. Several alternative herbal crude drugs have led to production of drugs used in modern medicine. This is yet only the tip of the iceberg and many research still has to be done. Several pharmaco-active compounds are still to be discovered and from most known natural compounds the mechanisms of action remains largely unclear. The exact knowledge of the molecular mechanisms of those phytochemicals is a necessity for their therapeutic use. At the time of writing pubmed gave 196 hits for the term Withaferin A. Our knowledge of this natural compound has increased in a fast rate over the last years. Nevertheless, there is still a long way to go if we want to unravel the molecular network responsible for its medicinal effects. We hope that this work will have a contribution to this research and that in time WA could be used as a treatment of a wide range of diseases.

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Part II. Aims of research

CVDs are nowadays the most deadly diseases in the world. More people die annually from CVDs than from any other cause. Therefore, research concerning prevention or treatment of CVD is a major public health concern. The main cause of CVD is atherosclerosis. An early step in the formation of an atherosclerotic plaque is monocyte adhesion to a deregulated endothelial wall. Adhesion molecules on the cell membrane of these endothelial and monocytic cells are playing an important role in this binding.

One of the important stress factors involved in the onset of atherosclerosis is oxidative stress. Under physiological conditions oxidative stress is strongly controlled by adaptive cellular responses involving upregulation of antioxidant enzymes, including HO-1. Based on its importance against the toxicity of oxidative stress, HO-1 has several cardioprotective effects. Moreover, HO-1 is recognized to exhibit important immunomodulatory, anti-apoptotic, antiproliferative, pro-angiogenic and anti-inflammatory functions increasing its protective role in the formation of atherosclerosis. Numerous HO-1 inducers activate Nrf2, the main transcription factor regulating expression of HO-1. Under homeostatic conditions, Nrf2 is associated with a KEAP1 homodimer in a KEAP1/Cul3/Rbx1-E3 complex in the cytoplasm. This association is leading to constant ubiquitination and degradation of Nrf2. KEAP1 possesses a dual function: I) it functions as a sensor and II) it regulates Nrf2 and switches the Nrf2-mediated response on or off. Several of the Nrf2 inducers target crucial cysteine residues in KEAP1 leading to activation of Nrf2 and upregulation of HO-1.

WA is one of the main withanolides derived from *WS*. The roots, leaves and berries of *WS* are used to treat a wide range of disorders in Ayurvedic medicine. Those roots and leaves are quantitatively rich in withanolides and WA is one of the main biologically active withanolides. WA is demonstrated to exert a broad spectrum of health promoting effects, including anti-inflammatory, anticarcinogenic, anti-angiogenic, antibacterial, anticonvulsive, immunosuppressive and pro-apoptotic properties. Despite this wide variety of bioactivities

of this compound the molecular mechanisms remain largely unknown. Moreover, its potential beneficial effects to CVDs are not intensively investigated.

In this project, we aimed to search for specific cardioprotective players regulated by WA thereby focusing on endothelial cells, macrophages and monocytes. Therefore, we first defined optimal concentrations of this withanolide which does not exert cytotoxic effects in *in vitro* analysis. Using these optimized conditions, transcriptional regulation of gene expression by WA was evaluated in primary HUVEC cells via mRNA microarray. Differential gene expression indicated important pathways that might be affected by WA and that can be related to CVD. Gene expression analysis of some prominent players in CVD in relation to inflammation was studied by qPCR analysis to investigate the regulatory effect of WA. For one of these genes, more specifically HO-1, a more in depth analysis of its expression at mRNA and protein level was performed in endothelial cells. Furthermore, the contribution of the regulation of HO-1 by WA in monocyte adhesion to endothelial cells was investigated. Finally, we contributed to the elucidation of the transcription factor involved in WA-induced expression of HO-1 and the underlying molecular mechanism of WA activity within this signal transduction pathway.

Part III. Materials and methods

Chemicals WA was purchased from Chromadex (Irvine, US), WN and EGCG from Sigma-Aldrich (St. Louis, MO) and MG-132 from Calbiochem (Darmstadt, DE). In combination with pentafluorophenyl-biotin WA was modified at the position of C27 creating a biotinylated form of WA. This was performed by Peter Van Der Veken (Department pharmaceutical science, Antwerp University, BE). All compounds were solubilised in DMSO. LPS was purchased from Sigma-Aldrich (St. Louis, MO) and recombinant human TNF was obtained from the department of Molecular biology of Ghent University (DMBR, Ghent, BE). 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was obtained from Sigma-Aldrich (St. Louis, MO). Anti-Nrf2 and anti-flag antibody were purchased from Abcam (Cambridge, UK), anti-HO-1 from Gentaur (kampenhout, BE), anti-KEAP1 from Bio Connect (TE huissen, NL), anti-HA clone 16B12 from Covance Research Products (Berkeley, CA) and anti-tubulin was from Sigma–Aldrich (St. Louis, MO). siRNA HO-1 plus SMART pool (L-012453-00-0005), siRNA Nrf2 plus SMART pool (L-003755-00-0005) and non-targeting pool (D-001810-10-05) were obtained from Dharmacon (Lafayette, CO). The WT and cysteine mutant flag-tagged KEAP1 expression plasmids; P3x-FLAG-mKEAP1-WT, P3x-FLAG-mKEAP1-C257S, P3x-FLAG-mKEAP1-CYS273S, P3x-FLAG-mKEAP1-C288S, P3x-FLAG-mKEAP1-C151S and P3x-FLAG-mKEAP1-C489S were a kind gift of Emilia Kansanen (Department of Biotechnology and Molecular Medicine, A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, FIN-70211 Kuopio, FI). The plasmid HA-Nrf2-FL was given by Donna Zhang (Department of Pharmacology and Toxicology, University of Arizona, Tucson, 85721, USA). Neutravidin agarose beads were purchased from Thermo Scientific (Rockford, U.S.A) and red anti-flag affinity beads from Sigma–Aldrich (St. Louis, MO).

Cell culture The human EA.hy926 cell line was a kind gift of Annelies Janssens (Laboratory of Ion Channel Research, Department of Cell and Molecular Medicine, Leuven University, BE). This hybridoma of HUVEC with the human epithelial cell line A549 has shown to retain

several native endothelial cell properties [1]. Cells were cultured in dulbecco´s modified eagle´s medium, supplemented with 10% foetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and HAT selection supplement. Cells were passaged using 0.05% (w/v) of trypsin in 0.4% (w/v) EDTA (all products from Invitrogen, Carlsbad, CA). Human HUVEC cells were cultured in EBM-PRF medium supplemented with EGM-2 single quot kit suppl&growth factor and cultured with the reagent pack subculture reagents (all, including cells, obtained from Lonza). HUVEC cells were used for experiments at passage 3. Mouse Raw264.7, human U937 and THP-1 cells were maintained in RPMI-1640 with Glutamax-I, supplemented with 10% foetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin. Raw264.7 cells were passaged using cell dissociation buffer (all products from Invitrogen, Carlsbad, CA). Hek293T were cultured in dulbecco´s modified eagle´s medium, supplemented with 10% foetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and HAT selection supplement. Cells were passaged using 0.05% (w/v) of trypsin in 0.4% (w/v) EDTA (all products from Invitrogen, Carlsbad, CA). All cells were grown in humidified atmosphere of 5% CO2/95% air at 37C in mycoplasma- and endotoxin-free conditions.

MTT Cells were seeded in 96-well plates at a density of 10,000 cells/well in 100 µl medium and allowed to attach overnight. Various concentrations of WA, WA-BIOT and WN were added to the cells. As a positive control for cell death sterile water and a high concentration of WA were used. After 24 hours incubation, 10 µl MTT reagent was added to each well and incubated for an additional 4 hours at conditions which allows the cells to grow further. Then 40 µl of SDS/HCL solution was added to the wells to solubilize the MTT crystals. The plates were incubated overnight at room temperature. Absorbance was read at 595 nm using a plate reader (victor 3 wallac 1420, perkinelmer life and analytical sciences).

Toxilight bioassay kit U937 cells were seeded in 96-well plates at a density of 30,000 cells/well in 100 µl medium. Immediately, cells were induced with various concentrations of EGCG and WA for 24 hours. After this incubation time, the plate was spinned down for 10 seconds at 900 rcf to pull down the cells. 20 µl of cell supernatant was transferred to a luminescence compatible 96 well plate. 100 µl of reconstituted AK detection reagent was added and incubated for 5 minutes. Absorbance was read at 595 nm using a plate reader (victor 3 wallac 1420, perkinelmer life and analytical sciences).

RNA isolation and qPCR Cells were seeded in 6-well plates at a density of 150,000 cells/well in 2 ml medium and grown overnight. The next day the cells were stimulated for 6 or 8 hours with various concentrations of WA or with 1 µM WA at several time points. WN and DMSO were tested, both at a concentration of 1 µM for 6 or 8 hours. After incubation total RNA was extracted with the acid-guanidinium-thiocyanate-phenol chloroform method using Trizol Reagent (Invitrogen, Carlsbad, CA). 0.5 µg of total mRNA was used to perform Reverse transcription using MMLV (Promega, Madison, WI). Real-time polymerase chain reaction (qPCR) was performed on the light cycler 480 II system (Roche, Vilvoorde, BE) using the SYBR green Master Mix (Biorad, Hercules, CA) and the following primers: mouse primers: mEL fw

GGGGTCGCTGCGAGATGAGC, mEL rv GGGGCAGCCAGTCAACCACC, mMCP-1 fw CCAGCACCAGCACCAGCCAA, mMCP-1 rv GGGGCGTTAACTGCATCTGGC, miNOS fw GGCAGCCTGTGAGACCTTTG, miNOS rv GCATTGGAAGTGAAGCGTTTC, mHO-1 fw AAGACCGCCTTCCTGCTCAAC, mHO-1 rv CGAAGTGACGCCATCTGTGAGG, mIL-6 fw ATGGATGCTACCAAACTGGAT, mIL-6 rv TGAAGGACTCTGGCTTTGTCT, mGAPDH fw GAACATCATCCCTGCCTCTACTGG, mGAPDH rv TCCACCACCCTGTTGCTGTA and human primers: hHO-1 fw CCAGCGGGCCAGCAACAAAGTGC , hHO-1 rv AAGCCTTCAGTGCCCACGGTAAGG, hGAPDH fw GCTCTGCTCCTCCTGTTC, hGAPDH rv ACGACCAAATCCGTTGACTC, hIL-6 fw GACAGCCACTCACCTCTTCA, hIL-6 rv AGTGCCTCTTTGCTGCTTTC, hICAM-1 fw GTCCCCTCAAAAGTCATCC, hICAM-1 rv AACCCCATTCAGCGTCACC. Efficiency and relative mRNA amounts were calculated for every run by the use of a standard (dilution serie of a cDNA mix). Relative mRNA values were normalized to a GAPDH reference gene. Primers used for qPCR validation of the mRNA array data were produced by Qiagen. KLF2 PPH02566A-200; HSPB8 PPH11188B-200; DNAJB9 PPH05919A-200; HERPUD1 PPH12606A-200; GCLM PPH02099A-200; SQSTM1 PPH02107A-200; ENC1 PPH00878A-200; ZFP36 PPH17294C-200; LDLR PPH00503E-200; CD55 PPH05711A-200; EDN1 PPH00653A-200; THBS1 PPH00799F-200. Relative mRNA values were normalized to a cyclophilin reference gene.

Western Immunoblotting Cells were seeded in the same way as the cells prior to RNA isolation and qPCR. At the end of the incubation time, whole cell lysates were prepared in SDS sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 0.5% DTT). Proteins were separated by loading equal amounts of the lysates on a 10% SDS-page, and transferred onto a nitrocellulose membrane. Non-specific binding sites on the membrane were blocked with licor/TBST for 1 hour. Afterwards membranes were probed with anti HO-1, ICAM-1, Nrf2, KEAP1, HA, flag or tubulin specific primary antibodies and visualized with fluorophore-coupled secondary antibodies (Rockland, Gilbertsville, PA). Immunofluorecent detection was performed by the use of the Odyssey Imaging System (Licor, Lincoln, NA). Tubuline was used as a loading control protein. Quantification of the bands was performed using the odyssey software.

siRNA silencing Cells were seeded in 6-well plates at a density of 150,000 cells/well in 2 ml medium and grown overnight. The next day the cells were transfected with 25 µM siRNA Nrf2 or HO-1 siGENOME plus SMART pool via the dharmafect transfection reagent number 1 following the protocol as described by thermo scientific. ON-TARGET plus non-targeting pool was used as a control to assess for aspecific effects. 24 hours after transfection cells were induced with WA for the indicated time periods. Subsequently, cells were lysed in SDS sample buffer or RNA was isolated, as described previously.

Nuclear en cytoplasmic extraction EA.hy926 cells were seeded in 10 cm plates at a density of 1,200000 cells/well in 10 ml medium and grown overnight. The next day the cells were stimulated with 1 µM WA at several time points before they were lysed in B1 buffer (10 mM

HEPES pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 5% glycerol, 0.5 mM EDTA pH 7.5, 0.1 mM EGTA pH 7.5, 0.5 mM DTT, protease inhibitors and 0.65%, v/v, NP40). Nuclei were pulled down by centrifugation at 800 rcf for 15 minutes and nuclear pellets were lysed in B2 buffer by shaking for 15 minutes at 4°C (20 mM HEPES pH 7.5, 1% NP40, 1 mM $MgCl_2$, 400 mM NaCl, 10 mM KCl, 20% glycerol, 0.5 mM EDTA pH 7.5, 0.1 mM EGTA pH 7.5, 0.5 mM DTT and protease inhibitors). Concentration of protein samples were measured with the Pierce BCA protein assay kit (thermo scientific, rockford, U.S.A) and subsequently analyzed by Western Blotting. Tubulin, as a cytoplasmatic protein and PARP, as a nuclear protein were used as a loading control and to verify the fractionation.

Binding affinity study endogenous KEAP1 levels HUVEC cells were seeded in 10 cm plates at a density of 1,000000 cells/well in 10 ml medium and grown overnight. Plates to define bindingaffinity between WA and KEAP1 were incubated for 2 hours of 1 µM WA-BIOT. Pre-incubation for 1 hour with 1 µM WA and co-incubation with 1 µM DTT was also tested. After incubation cells were washed with PBSA and lysed in buffer A (5 mM tris ph7.5, 1% triton-X100, 5 mM EDTA and 50 mM NaF, aprotinin, pefabloc and leupeptin), incubated on ice for 20 minutes and supernatant was collected after centrifugation for 15 minutes at full speed. Input was prepared with 2x SDS sample buffer and pull down samples were treated with neutravidin beads overnight at 4 degrees. Before dissolving the pull down samples in 2x SDS sample buffer the beads were washed several times in lysis buffer. Subsequently, samples were analyzed by Western blotting.

Bindingaffinity study exogenous KEAP1 levels Plates to define the importance of certain cysteine residues in the binding of WA and KEAP1 were prior to induction transfected with WT or cysteine mutant plasmids. Cells were transfected with 12.5 µl Polyethylenimin (PEI) and 5ug plasmid DNA (2.5 µg KEAP1 expression plasmid and 2.5 µg Empty vector). 24 hours later, cells were incubated with 1 µM WA-BIOT for 2 hours. After incubation cells were washed with PBSA and lysed in buffer A (5 mM tris ph7.5, 1% triton-X100, 5 mM EDTA and 50 mM NaF, aprotinin, pefabloc and leupeptin), incubated on ice for 20 minutes and supernatant was collected after centrifugation for 15 minutes at full speed. Input was prepared with 2x SDS sample buffer and pull down samples were incubated on ice for 15 minutes with 0.5% SDS and afterwards treated with neutravidin beads overnight at 4 degrees. Before dissolving the pull down samples in 2x SDS sample buffer the beads were washed several times in lysis buffer with 0.5% SDS. Subsequently samples were analyzed by Western blotting.

Coimmunoprecipitation for Nrf2 or Cul3 Cells were seeded in 10cm plates at a density of 1,000000 cells/well in 10ml medium and grown overnight. Cells were transfected with HA-Nrf2-pcDNA3/HA-Cul-3-pcDNA3 and P3x-FLAG-mKEAP1-WT or P3x-FLAG-mKEAP1-C151S plasmid using PEI and a total plasmid DNA concentration of 5ug (2.5 µg Nrf2 or Cul3 expression plasmid and 2.5 µg KEAP1 expression plasmid). After 18 hours cells were pre-incubated for 6 hours with 2.5 µM MG132 and incubated afterwards for another 2 hours

with 1 µM WA. After incubation cells were washed with PBSA and lysed in buffer A (50 mM tris ph7.5, 150 mM NaCl, 0.5% triton-X100, protease inhibitor cocktail, 1 mM DTT and 50 mM NaF), incubated on ice for 20 minutes and supernatant was collected after centrifugation for 15 minutes at full speed. Input was prepared with 2x SDS sample buffer and pull down samples were treated with flag beads overnight at 4 degrees. Before dissolving the pull down samples in 2x SDS sample buffer the beads were washed several times in lysis buffer. Subsequently samples were analyzed by Western blotting.

Monocyte adhesion binding HUVEC cells were seeded in 12-well plates at a density of 45,000 cells/well in 1 ml medium and grown overnight. The next day the cells were induced for 4 hours with TNF (2000IU/ml) with or without 2 hours pre-induction of WA (1 µM). THP-1 cells were dyed with BCECF-AM for 30 minutes (resuspended THP-1 cells in HEPES buffer solution at a concentration of 40 000 000 cells/ml with 1 mM BCECF-AM/DMSO solution) and washed afterwards with hepes buffered saline (HBS) for several times. HUVEC medium was refreshed before addition of 1 000 000 THP-1 per well for 30 minutes. Afterwards HUVEC cells were washed for 3 times with HBS. Cells were lysed with 1% SDS buffer. As negative control, fluorescence of HUVEC cells in absence of THP-1 cells was evaluated. Fluorescence was quantified by the use of a spectrophotometer (excitation 480nm emission 520nm) .

mRNA microarray processing and data analysis mRNA microarray processing and hierarchical clustering was performed by the Department of Biomedical Sciences, in Human Molecular Genetics Group of Guy Van Camp. Following trizol extraction from HUVEC cells left untreated (non-treated controls) or exposed for 6 h to 1 µM WA from three independent experiments, RNA was quality controlled on a Bio-Rad experion (Bio-Rad, Hercules, CA, USA). Samples with 28S/18S rRNA ratio > 1.8 and A260/A280 ratio between 1.9-2.1 were used for further analysis. 500ng of total RNA was amplified using the Illumina TotalPrep RNA Amplification kit (Life Technologies, Carlsbad, CA, USA). RNA was reverse transcribed using T7 oligo(dT) primers after which biotinylated cRNA was synthesized through *in vitro* transcription reaction. 750ng of amplified cRNA was hybridized to a corresponding array of a HumanHT12 beadchip (Illumina, San Diego, CA, USA). The beadchip was incubated for 18 hours at 58°C in a hybridization oven whilst continuous rocking. After several consecutive washing steps, bead intensities were read on Illumina Hiscan. Raw data intensities were read in R using the “beadarray” package. Intensities were normalized and differential gene expression between samples was estimated using “limma”. Resulting p-values were corrected for multiple hypothesis testing using the Benjamini Hochberg procedure. Next to estimating gene expressions, euclidean distances between samples were calculated and used as a distance metric in a hierarchical cluster analysis. Transcriptomic profiling was done by importation of the raw data set and computational analysis of this set by IPA. Each identifier was mapped to its corresponding gene object in the Ingenuity knowledge base. A fold change cutoff of 2 as well as false discovery rate of 0,05% were set to identify genes whose expression was significantly differentially regulated. These genes, called focus genes,

were overlaid onto a global molecular network developed from information contained in the Ingenuity knowledge base. Networks of these focus genes were then algorithmically generated based on their connectivity. The Bio Functional Analysis identified the biological functions and/or diseases that were most significant to the data set. Genes from the dataset that met the abovementioned cut-off and were associated with biological functions and/or diseases in the Ingenuity knowledge base were considered for the analysis. Fischer‘s exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone.

*Molecular modeling of human KEAP1 with WA and W*N This modeling was performed by Adjunct Professor Maija Lahtela-Kakkonen (School of Pharmacy (Pharmaceutical Chemistry) University of Eastern Finland, P. O. Box 1627, 70211 Kuopio, Finland). Homology models for human KEAP1 were built using the crystal structures of BTB domain (PDB code 2NN2 [2], 1BUO [3], 1CS3 [4]) and the crystal structure of KLHL3/Cul3 complex (PDB code 4HX1 [5]]. The sequence of human KEAP1 was obtained from sequenced expression plasmids. The sequences of templates were aligned with human KEAP1 using ClustalW2 [6] based on sequence similarity, (30%, 33%, 33%) and (37%) respectively for the alignment. ClustalW2 is a general purpose multiple sequence alignment program for proteins. There were no templates for the residues 1-35 and 604-621 and these C- and N-terminals were not included in the model. Several homology models for human KEAP1 were generated using Discovery Studio 3.0 [7]. The homology models were evaluated with Profile-3D available in Discovery Studio 3.0. Few models having more than 92% residues in allowed region in Ramachandran map were selected for docking studies. Before docking studies homology models were polished with Protein Preparation Wizards available in the Schrödinger suite version 9.2.112 [8]. The models were subjected to a restrained minimization using OPLS2005 force field. The structures of small molecules: WA and WN were generated with MOE (version 2011.10)[9]. The geometric optimization was carried out using MMFF94 forcefield, and the chiralities specified in the input structures were retained. The structures were having MMFF94 charges. The docking studies were performed with MOE (version 2011.10). The grid center was defined by residue C151 as it has been notified in mutagenesis study to be important for the binding of these compounds. In docking studies compounds were treated flexible.

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9. *Molecular Operating Enviroment (MOE)*, 2011, Chemical Computing Group Inc: Montreal.

Part IV. Results

IV.1 Characterization of cellular toxicity of WA in CVD-related *in vitro* models

IV.1.1 Introduction

Since the majority of publications involving WA are studying the cell death inducing capacity of WA in several cell types and at different concentrations, the compound became mostly famous by its anti-carcinogenic and pro-apoptotic properties. However, in this study we want to investigate the potential protective effects of WA in the context of cardiovascular diseases, hence avoiding cell death-induced side effects of WA. Therefore, it is indispensable to evaluate first cell viability of the cell types of interest after WA treatment. As sensitivity to cell death is largely dependent on the cell type, cell viability was evaluated in all cell types used in this project exposed to WA being endothelial cell types (HUVEC, EA.hy926) and monocytes/macrophages (U937, Raw264.7), to define a safe concentration range of WA which is not harmful to the cells investigated. To define indisputably a concentration of WA that does not cause any cell death, cells for these cell viability assays were exposed for longer incubation times with WA than the exposure times applied in further induction experiments described in this thesis.

An indirect parameter for cell death is decreased cellular metabolic activity. The intrinsic respiratory activity is a characteristic of viable cells and the activity of mitochondrial membrane enzymes, involved in this intrinsic respiratory activity, can be a readout for viability. Tetrazolium salts, such as yellowish MTT, can be cleaved into a blue colored

product formazan by the succinate dehydrogenase complex II enzyme of the inner mitochondrial membrane. The absorbance of the colored solution is afterwards quantified by the use of a luminometer. This method allows to quantify viable cells of which amounts are affected by effects on cell cycle progression as well as induction of cell death also defined as cytotoxicity. The applied method does not distinguish between different types of cellular cell death mechanisms.

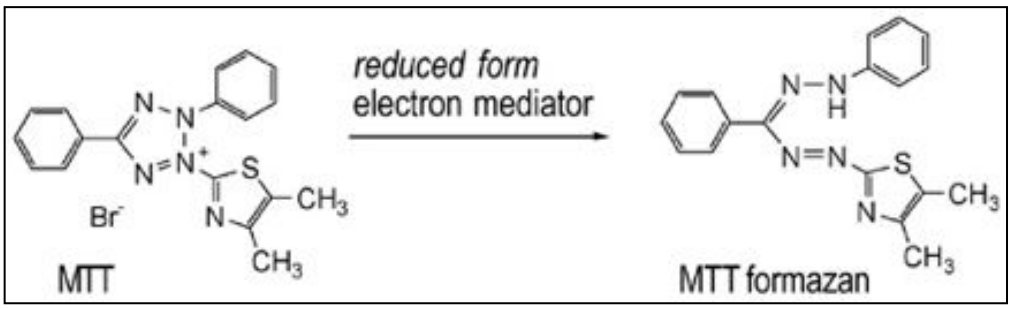


Fig 1.1 Yellow MTT conversion to blue/purple formazan. Source: www.itsbio.co.kr

It is known that some compounds containing intrinsic reductive potential, can reduce MTT into formazan by themselves and mask thereby cell death inducing effects [1, 2]. This often leads to conflicting results between cell numbers (crystal violet staining) and cell viability (MTT). More particularly, upon increasing compound concentration, cell number may decrease as a consequence of toxicity, whereas levels of formazan formation increase as a consequence of intrinsic reducing properties of the compound. Since the well known antioxidant epigallocatechin gallate (EGCG) interferes with reduction of MTT salts, cell death by EGCG was alternatively evaluated by a ToxiLight bioassay (Lonza) in our experiments. The latter is a bioluminescent, non-destructive cytolysis assay kit designed to measure release of the enzyme, adenylate kinase (AK), from damaged cells. AK is released in the medium when cells are dying where it can phosphorylate ADP to form ATP. Finally, converted ATP levels, which are a measure for cell death, are quantified via a bioluminescent firefly luciferase reaction, which requires ATP as a cofactor for its enzymatic activity and light production.

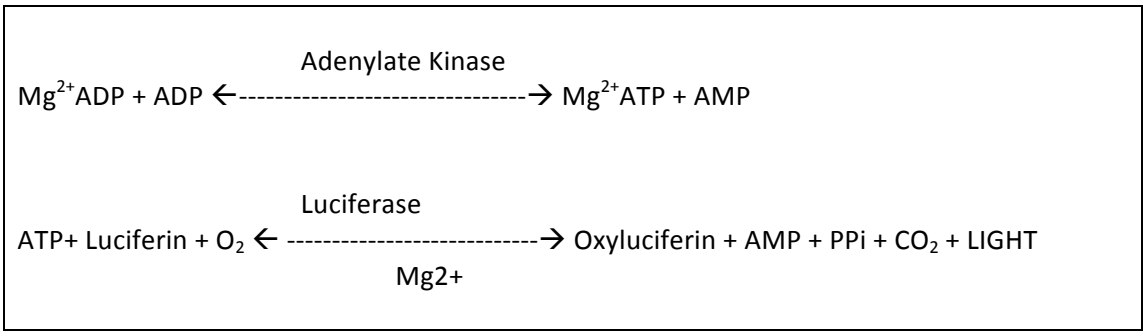


Fig 1.2 Principle of the Toxilight bioassay. This reaction involves two steps. The first involves addition of ADP as a substrate for AK. In the presence of the enzyme AK, ADP is converted to ATP. Secondly, the bioluminescent method utilizes an enzyme luciferase, which catalyses formation of light from ATP and luciferin. By combining these two reactions, the emitted light intensity is linearly related to AK concentrations and is measured by the use of a luminometer.

IV.1.2 Results

In this chapter, toxicity effects of WA were studied in primary endothelial HUVEC cells, EA.hy926 hybridoma cells (originating from a fusion of HUVEC cells with alveolar basal epithelial A549 cells), monocyte cell type U937 and macrophage cell line Raw264.7.

As shown in Fig 1.3, the endothelial cell line EA.hy926 remained viable at concentrations up to 3 µM of WA and no adverse effects on cell growth were visible. In contrast, 10 µM of WA gave reduced cell viability concomitantly with rounding up of the cells. WN had no effect on cell viability up to 10 µM. Since we also will use a biotinylated version of WA (WA-BIOT) during the course of this project, we also included this WA-variant in our toxicity studies. From Fig 1.3, it can be noted that 1 µM of a biotinylated form of WA gave similar results compared to its non biotinylated equivalent.

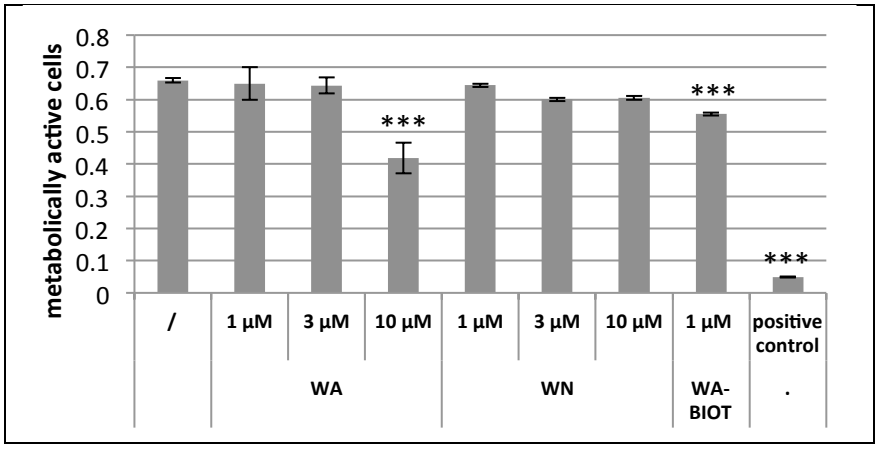


Fig 1.3 MTT assay in EA.hy926 following 24 hours incubation with various concentrations of WA, WN or WA-BIOT. Data depicted here are representative for 3 repeated experiments. Error bars are standard deviations of triplicate biological repeats. Control cells incubated with sterile water were used as a positive control. Metabolically active cells in the Y-axis represent the absorbance quantified by the use of a spectrophotometer. Statistics: ANOVA test, Dunnett's Multiple Comparison Test, One-way analysis of variance (*p<0.05, **p<0.01, ***p<0.001).

In Fig 1.4. it appears that primary endothelial HUVEC cells exposed to 10 µM WA reveal stronger cytotoxicity than observed in EA.hy926 cells. Remarkably, also WN induced cytotoxicity in HUVEC cells. WN is giving a reduced cell viability already at a concentration of 3 µM. WA-BIOT gave a slight reduction in viability compared to the same concentration of its non biotinylated equivalent (Fig 1.4).

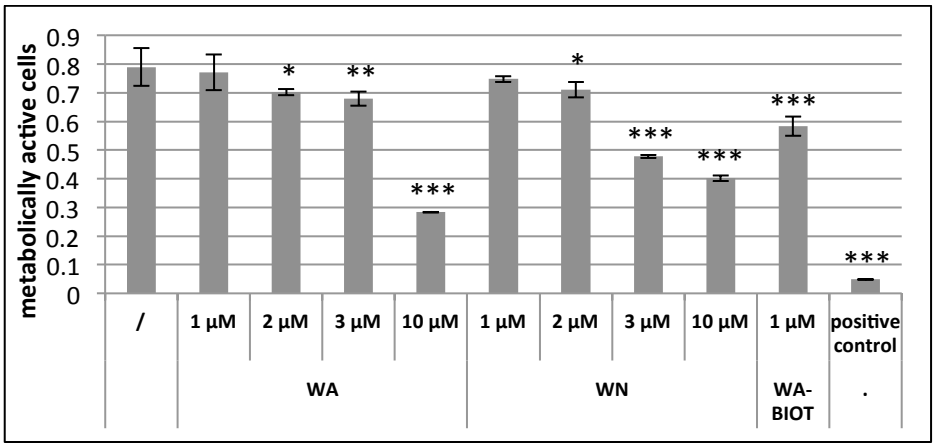


Fig 1.4 MTT assay in HUVEC cells following 24 hours incubation with various concentrations of WA, WN or WA-BIOT. Data depicted here are representative for 3 repeated experiments. Error bars are standard deviations of triplicate biological repeats. Control cells incubated with sterile water were used as a positive control. Metabolically active cells in the Y-axis represent the absorbance quantified by the use of a spectrophotometer. Statistics: ANOVA test, Dunnett's Multiple Comparison Test, One-way analysis of variance (*p<0.05, **p<0.01, ***p<0.001).

In macrophage Raw264.7 cells, 3 µM of WA slightly reduced cell viability. WA-BIOT was not tested on this cell type as no experiments with the biotinylated form of WA were foreseen (Fig 1.5 (A)). In U937 monocyte cells, WA and EGCG were tested by the use of ToxiLight bioassay kit from Lonza. No cell death was detected with 1 and 2 µM of WA or 100 µM EGCG (Fig 1.5 (B)).

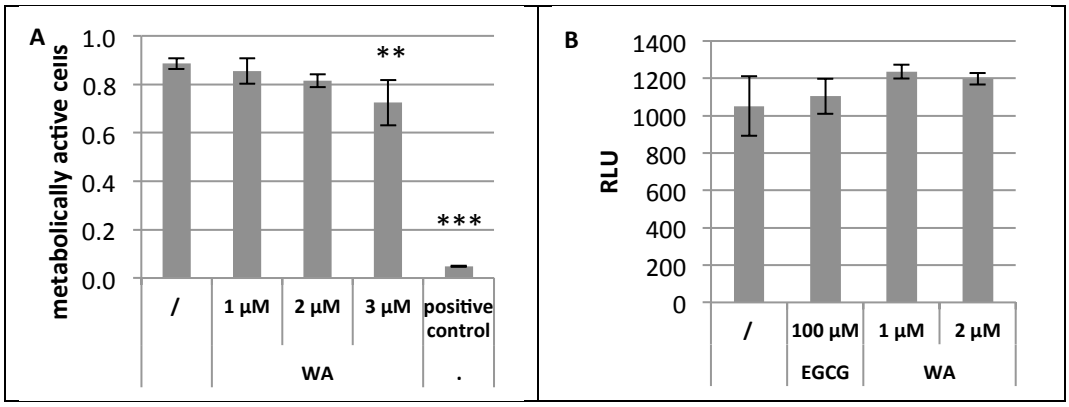


Fig 1.5 (A) MTT assay in Raw264.7 following 24 hours incubation with various concentrations of WA. Data depicted here are representative for 3 repeated experiments. Error bars are standard deviations of triplicate biological repeats. Control cells incubated with sterile water were used as a positive control. Metabolically active cells in the Y-axis represent the absorbance quantified by the use of a spectrophotometer. Statistics: ANOVA 1-way test, Dunnett's Multiple Comparison Test ($p<0.05$). (B) ToxiLight bioassay in U937 following 24 hours incubation with various concentrations of WA and 100 µM EGCG. Error bars are standard deviations of triplicate biological repeats. Emitted light as a result of a luciferase reaction was quantified by the use of a luminometer. (RLU: relative light unit) Statistics: ANOVA test, Dunnett's Multiple Comparison Test, One-way analysis of variance (*p<0.05, **p<0.01, ***p<0.001).

IV.1.3 Discussion and conclusion

In summary, we found that none of the cells tested revealed any cytotoxicity by WA or WN at concentrations below or equal to 2 µM. At a WA concentration of 3 µM, EA.hy926 and HUVEC cells remained unaffected. Validation of our assays is performed by including a high toxic concentration of WA, to rule out possible compound interference with the MTT assay. A concentration-dependent decrease of formazan, representing reduced respiratory activity, demonstrated no compound interference with the assay system. Sterile water was used as an internal positive control for cytotoxicity leading to osmotic shock, showing efficacy of the MTT assay to measure cell death/survival. Appropriate results obtained with the control setups, indicate that performed cytotoxicity assays are reliable. Based on our cytoxicity assays with WA, showing lack of cytotoxicity at a concentration of 2 µM (or below) and taking in account pharmacokinetic studies of WA in mice, reporting peak concentrations of 2 µM WA in plasma [3], a working concentration of maximum 2 µM (or lower) WA can be recommended for following experiments throughout the thesis. This is a nontoxic physiological concentration, which can also be achieved *in vivo* in therapeutic applications.

Recently, Vaishnavi and colleagues have shown via bioinformatics and experimental evidence the differences of two closely related withanolides, WA and WN. A clear difference in binding properties to different target proteins was noticeable. WA could bind with higher affinity to selected targets, thereby acting as a strong cytotoxic agent both for normal and cancer cells. In contrast, WN displayed only weak binding affinity to the targets correlated with milder cytotoxicity towards cancer cells and no cytotoxicity in normal cells [4]. Our data from the MTT assay in EA.hy926 cells correspond to the observations of Vaishnavi and colleagues. WA induced cell death when administered at high doses whereas WN lacks the capacity to induce cell death at time point used and concentration administered in this assay. However, a clearly different pattern was observed in the primary endothelial HUVEC cell culture. WN was even more potent than WA in inducing cell death in HUVEC cells. Overall, HUVEC cells are more sensitive than EA.hy926. Possibly, different cell death pathways may account in differences of sensitivity to WA of both cell types as well as higher sensitivity to WN in case of HUVEC cells. Similarly, previous studies indicated that oxidized LDL-induced apoptosis in HUVEC cells but not in EA.hy926 cells. In contrast, EA.hy926 cells were associated with necrosis. Moreover, a lower antioxidant capacity of EA.hy926 cells suggests even a higher sensitivity to oxidized LDL cytotoxicity [5]. Since we see a higher sensitivity in HUVEC cells, it is likely that WA and WN induce cell death through other mechanisms as oxidized LDL.

1. Bruggisser, R., et al., *Interference of plant extracts, phytoestrogens and antioxidants with the MTT.* Planta Med, 2002. **68**(5): p. 445-8.
2. Wang, P., S.M. Henning, and D. Heber, *Limitations of MTT and MTS-based assays for measurement of antiproliferative.* PLoS One, 2010. **5**(4): p. e10202.
3. Thaiparambil, J.T., et al., *Withaferin A inhibits breast cancer invasion and metastasis at sub-cytotoxic.* Int J Cancer, 2011. **129**(11): p. 2744-55.
4. Vaishnavi, K., et al., *Differential activities of the two closely related withanolides, Withaferin A and Withanone: bioinformatics and experimental evidences*, in *PLoS One*. 2012: United States. p. e44419.
5. Claise, C., et al., *Oxidized-LDL induce apoptosis in HUVEC but not in the endothelial cell line EA.hy.* Atherosclerosis, 1999. **147**(1): p. 95-104.

IV.2 Transcriptome profiling and pathway analysis of WA-treated HUVEC cells

IV.2.1 Introduction

During recent years, evidence steadily increases that many therapeutic compounds do not target single proteins but rather protein networks or pathways. To characterize pathways affected by WA in HUVEC cells, Illumina mRNA array analysis was performed to define WA responsive gene clusters. HUVEC cells were treated with or without 1 µM WA for 6 hours in absence of pro-inflammatory stimuli and were tested in 3 biological triplicates. Furthermore, following mRNA array data processing, raw gene expression data were imported into ingenuity pathways analysis (IPA) to identify pathways enriched with WA responsive genes. For IPA analysis, the log ratio cut off was set on 1 (minimum 2-fold change) and a false discovery rate of 0,05 % was set to identify genes whose expression was significantly differentially regulated. Significance values were calculated via Fisher Exact test. Thereby, 53 significant up or down regulated genes remained following exposure to WA. Via this analysis we have an indication on what genes, networks, biological functions, diseases and canonical pathways are affected by WA treatment in HUVEC cells.

Illumina mRNA array analysis was performed by the Human Molecular Genetics Group of Guy Van Camp, Department of Biomedical Sciences, Antwerp University. Confirmation by qPCR was performed by Katarzyna Szarc vel Szic of Laboratory of Protein science, Proteomics and Epigenetic Signalling, Department of Biomedical Sciences, Antwerp University.

IV.2.2 Results

IV.2.2.1 Genes, pathways, networks, biological functions and diseases affected by WA treatment in HUVEC cells analyzed through IPA

As a first step, we aimed to determine overrepresented signaling of metabolic canonical pathways containing genes differentially expressed by WA. In Fig 2.1 a graph of most significantly involved pathways are shown. Via canonical pathway analysis by IPA software, the Nrf2-mediated oxidative stress response pathway was shown to be most significantly impacted by WA treatment. Consequently, genes differentially expressed from WA treatment group were most significantly enriched in the Nrf2-mediated oxidative stress response pathway. Additional top pathways of interest are the mineralocorticoid (aldosterone) and glucocorticoid receptor, which suggests potential hormone modulating activity of the steroidal backbone of WA.

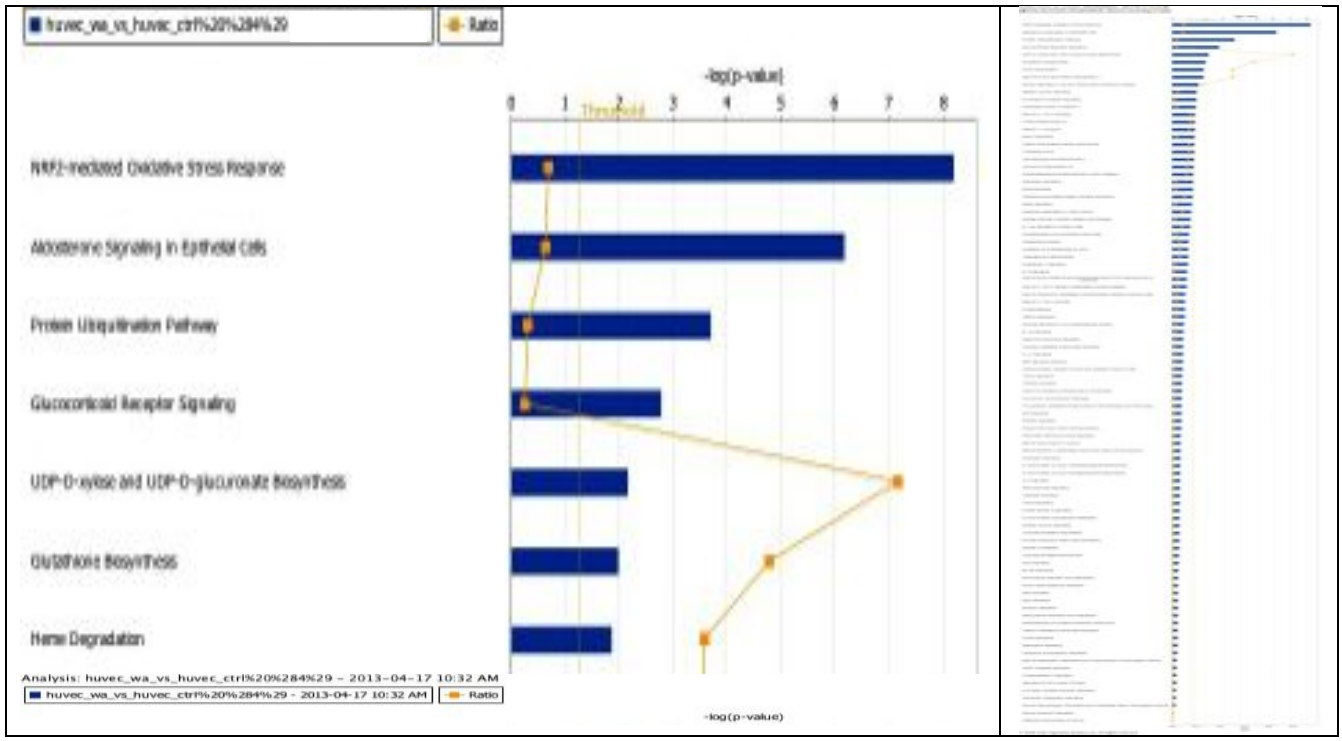


Fig 2.1 Top IPA pathways enriched with differentially expressed genes by WA. Only the top 7 of most significantly affected pathways are zoomed in.

In Fig 2.2 the top 7 enriched pathways are alternatively presented, including relative percentage of up- and downregulated genes. WA is able to simultaneously increase or decrease specific genes within the Nrf2 pathway.

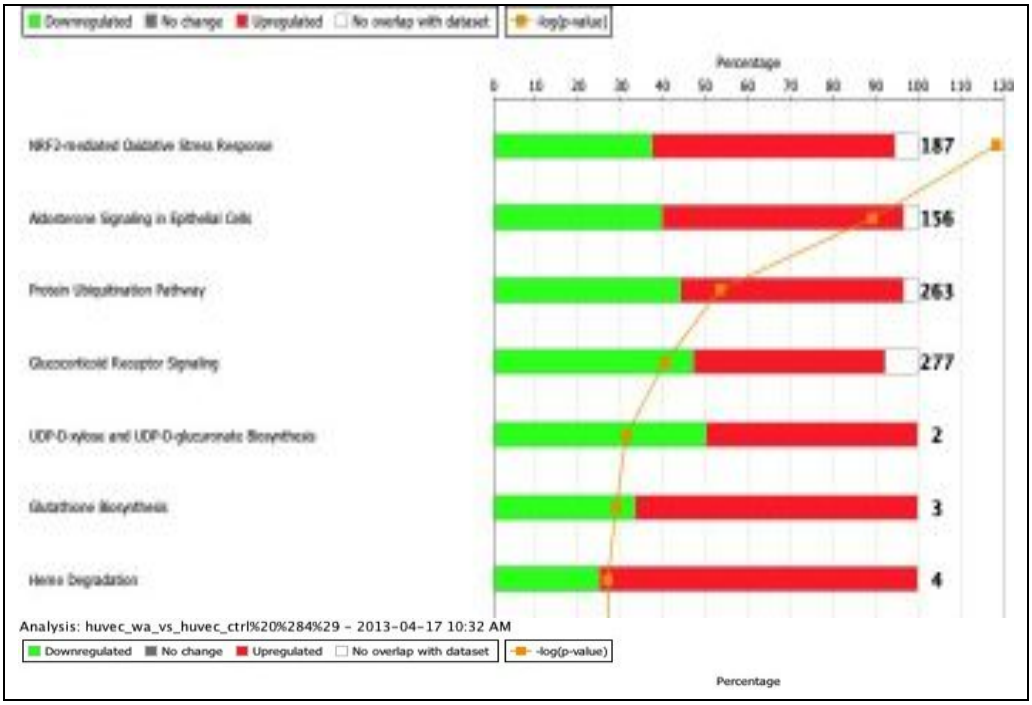


Fig 2.2 Top IPA pathways enriched with differentially expressed genes by WA, indicating relative fraction of up- or downregulated genes in each pathway.

A graphical presentation of the Nrf2-mediated oxidative stress pathway is summarized in Fig 2.3 and Fig 2.4. Transcription levels of the signaling proteins within the Nrf2 pathway in the cytoplasm remain unaffected upon treatment with WA. In the nucleus, several Nrf2 target genes or genes involved within the Nrf2 pathway are influenced by WA induction. In table 2.1, genes influenced by WA and involved within the Nrf2 pathway are shown. In total, 7 genes are regulated by WA, where 6 out of 7 are upregulated. The expression of those 6 upregulated genes are regulated by the Nrf2 transcription factor. The downregulated ENC1 gene coding for NRPB protein is not under transcriptional control of Nrf2, but is involved within the Nrf2 pathway. The most prominently upregulated gene is HMOX1, encoding HO-1 protein. It is the second strongest regulated gene in the top 10 list with about a 20-fold induction. An overview of the top 10 up and down-regulated genes are listed in table 2.2. Both HMOX1 and ENC1 can be found back in this top 10 gene list.

Gene	**protein**	**P-values**	**FC**	**Ranking**	**Up or down**
HMOX1	HO-1	0.000000435	20.94	2	Up
HSPB8	HSP22	0.00105	2.87	17	Up
DNAJB9	HSP40	0.00171	2.70	20	Up
HERPUD1	HERPUD1	0.010192	2.62	22	Up
GCLM	GCLM	5.26E-05	2.53	24	Up
SQSTM1	sequestosome1	0.000957	2.08	45	Up
ENC1	NRPB	0.001105	0.48	4	Down

Table 2.1 Genes, with their corresponding protein name, that are influenced by WA and whose expression is regulated by Nrf2 transcription factor or in case of ENC1 involved within the Nrf2 pathway. (FC: Fold change)

Upon analysis of top biological networks involved, the highest score was assigned to the network “Cell death and survival, cardiovascular disease and amino acid metabolism”. From 53 significant genes, 46 genes are involved in those network functions (table 2.3 (A)). These genes, called focus genes, were overlaid onto a global molecular network developed from manually curated information contained in the Ingenuity knowledge base. Networks of these focus genes were then algorithmically generated based on their connectivity. In Fig 2.5 a graphical connectivity map of this top network is shown. All genes are involved in cell death and survival and their interplay is specifically linked with cardiovascular diseases. Interestingly, some genes in this connectivity map, including HMOX1, HERPUD1 and HSPB8, are also regulated by Nrf2. Moreover, KLF2 a transcription factor having an interplay with Nrf2 is also upregulated.

After analyzing our data related to diseases and biological functions that are overrepresented, we noticed that more genes are linked with cardiovascular disease than to cancer. 7 genes are related to an inflammatory response and 11 genes are related to cardiovascular diseases (table 2.3 (B)). Genes that are related to cardiovascular diseases are further subdivided in different cardiovascular-related diseases in table 2.4, resulting in seven genes related to the category atherosclerosis. All genes can also be traced back in the

connectivity map of Fig 2.5, where EDN1 and THBS1 are the only two down regulated genes (table 2.5). From the atherosclerosis-related genes, only HMOX1 is Nrf2 regulated.

Log Ratio up-regulated

Molecules	Exp. Value	Exp. Chart
HSPA1A/HSPA1B*	↑4.475	
HMOX1	↑4.388	
DNAJB1	↑2.426	
ZFAND2A	↑2.334	
HSPA6*	↑1.941	
KLF2	↑1.941	
ZFP36	↑1.926	

Log Ratio down-regulated

Molecules	Exp. Value	Exp. Chart
EDN1	↓-2.382	
ENC1	↓-1.036	
SERTAD4	↓-1.033	
THBS1	↓-1.024	
FJX1	↓-1.020	
DLC1*	↓-1.000	
NCOR2*	↓-0.936	
CCL2	↓-0.916	

Table 2.2 List of top 10 up- and down-regulated genes induced by WA. (expression value= logFC)

A Top Networks

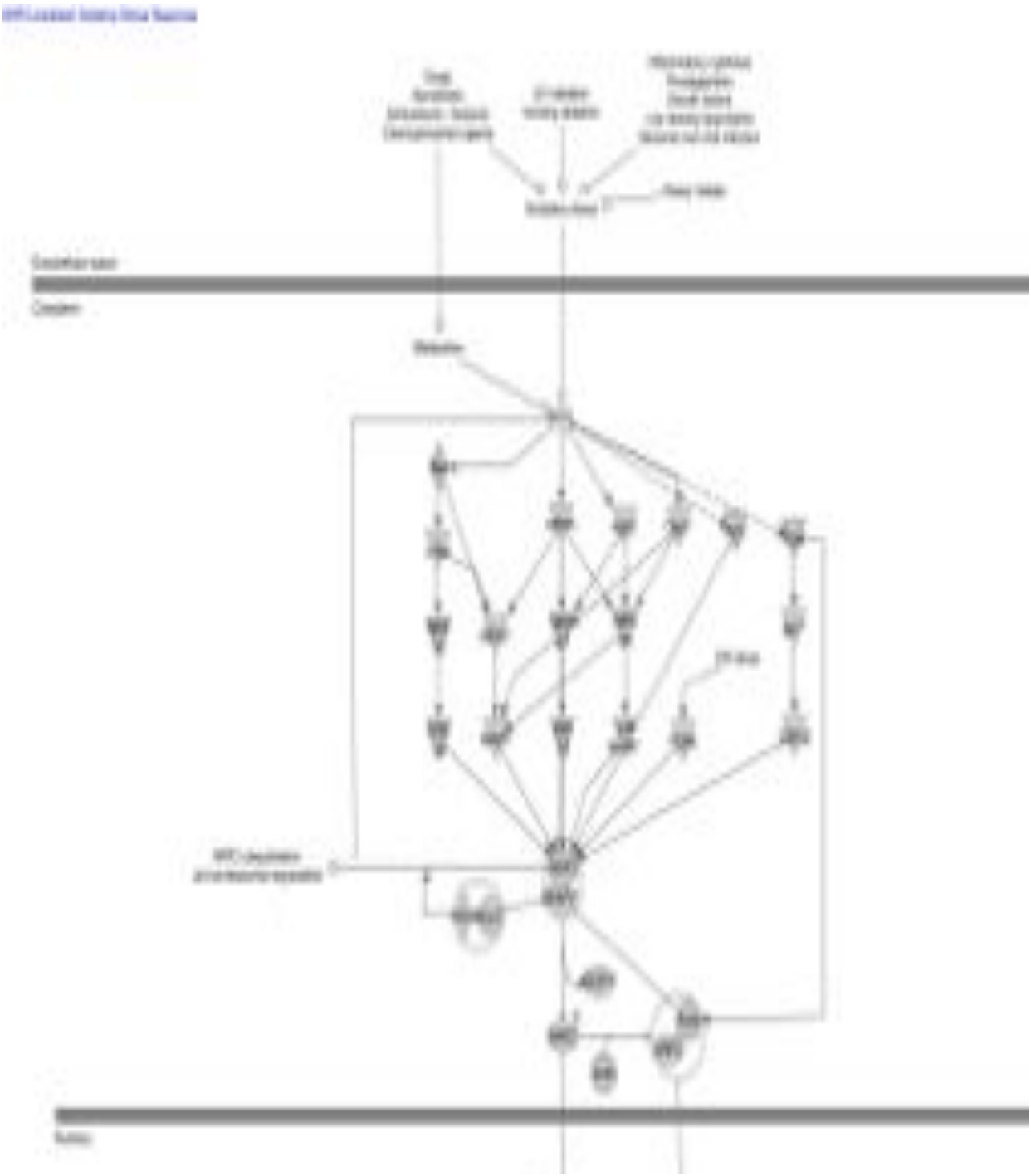
ID	Associated Network Functions	Score
1	Cell Death and Survival, Cardiovascular Disease, Amino Acid Metabolism	46
2	Cell Death and Survival, Cancer, Renal and Urological Disease	31
3	Cellular Development, Cellular Growth and Proliferation, Cell Death and Survival	17
4	Cancer, Cardiac Fibrosis, Cardiac Proliferation	2
5	Cellular Assembly and Organization, DNA Replication, Recombination, and Repair, Embryonic Development	2

B Top Bio Functions

Diseases and Disorders

Name	p-value	# Molecules
Inflammatory Response	1.22E-04 - 3.96E-02	7
Cardiovascular Disease	1.50E-04 - 3.63E-02	11
Connective Tissue Disorders	2.04E-04 - 2.66E-02	11
Immunological Disease	2.04E-04 - 3.96E-02	13
Inflammatory Disease	2.04E-04 - 2.33E-02	11

Table 2.3 List of top networks (A) and top bio functions (B) linked with significantly regulated genes.



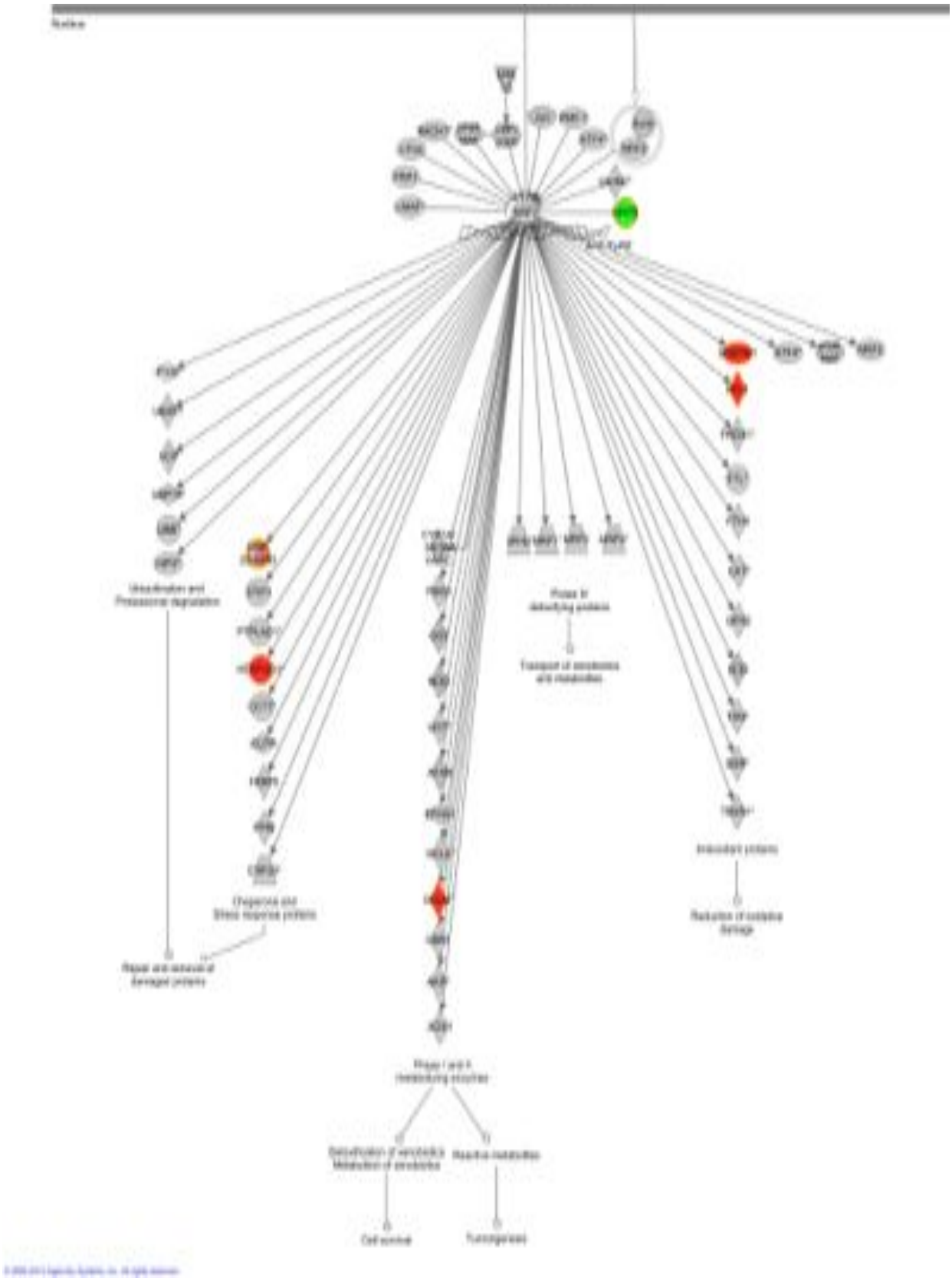


Fig 2.3 and Fig 2.4 The Nrf2-mediated oxidative stress response pathway in IPA database. The red color and green color indicate the up- and down-regulated genes after treatment of WA in this pathway, respectively.

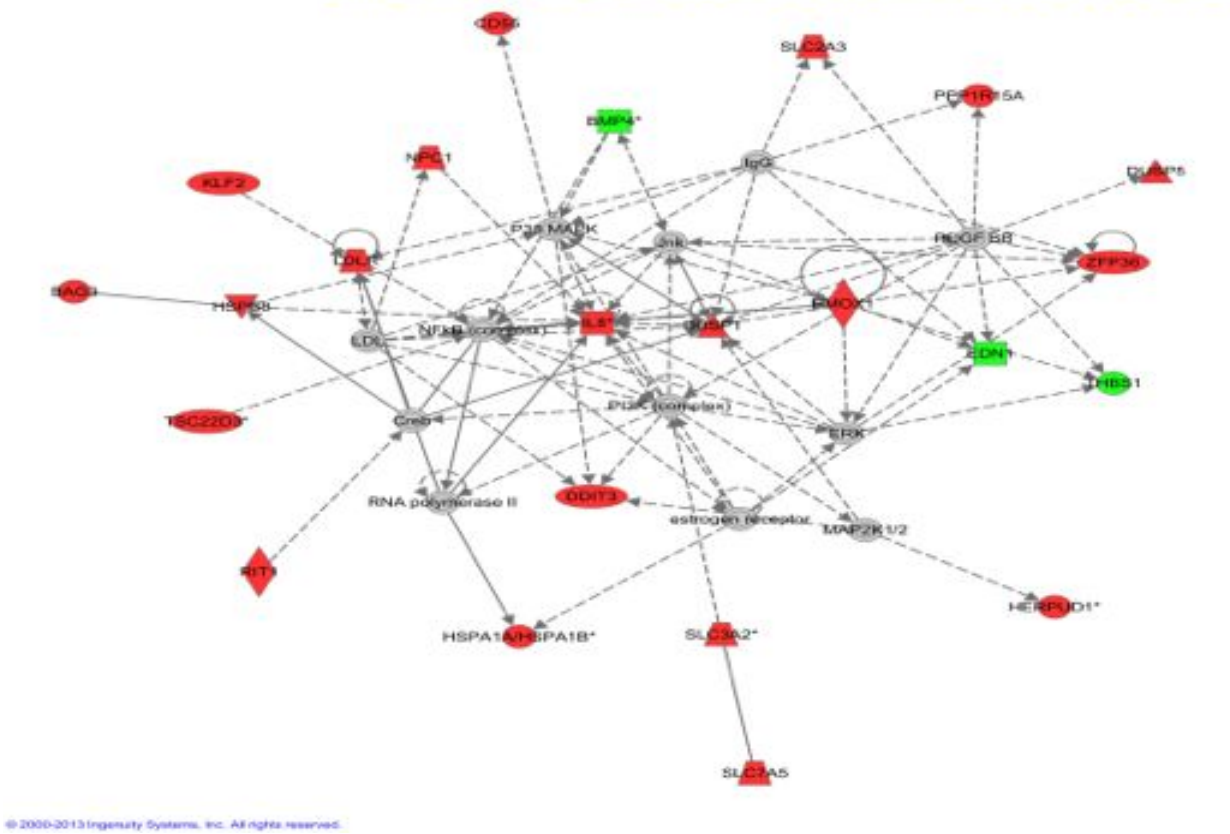


Fig 2.5 Connectivity map of top enriched network, i.e. cell death and survival, cardiovascular disease and amino acid metabolism. The red color and green color indicate the up- and down-regulated genes after treatment of WA in this network, respectively.

Category	Functions Annotation	p-Value	Molecules	# Molecules
Cardiovascular Disease	atherosclerosis	1.50E-04	CD55,EDN1,HMOX1,KLF2,LDLR,THBS1,ZFP36	7
Cardiovascular Disease	heart Disease	2.65E-04	BAG3,CD55,DDIT3,EDN1,GCLM,IL8,KLF2,LDLR,THBS1	9
Cardiovascular Disease	vascular disease	5.04E-04	CD55,EDN1,HMOX1,IL8,KLF2,LDLR,THBS1,ZFP36	8
Cardiovascular Disease	hypercholesterolemia suppressor	3.36E-03	LDLR	1
Cardiovascular Disease	stabilization of atherosclerotic lesion	3.36E-03	HMOX1	1
Cardiovascular Disease	coronary artery disease	1.10E-02	CD55,KLF2,LDLR,THBS1	4
Cardiovascular Disease	antineutrophil cytoplasmic antibody-associated vasculitis	1.67E-02	IL8	1
Cardiovascular Disease	primary pulmonary hypertension	2.66E-02	EDN1	1
Cardiovascular Disease	atherosclerotic lesion	3.63E-02	HMOX1	1
Cardiovascular Disease	hypertrophy of cardiac muscle	3.63E-02	EDN1	1

Table 2.4 List of WA regulated target genes with relevance for different cardiovascular-related diseases.

Gene	protein	P-values	FC	Ranking	Up or down
HMOX1	HO-1	0.000000435	20.94	2	Up
KLF2	KLF2	0.000488	3.58	10	Up
ZFP36	ZFP36	0.001044	3.50	11	Up
CD55	CD55	0.001119	2.26	30	Up
LDLR	LDLR	0.003855	2.14	41	Up
EDN1	EDN1 or ET-1	0.011359	0.19	1	Down
THBS1	THBS1	0.031145	0.49	6	Down

Table 2.5 List of atherosclerosis-related genes and corresponding proteins. (FC: fold change)

IV.2.2.2 Confirmation through qPCR analysis

Regulation of genes influenced by WA and involved within the Nrf2 pathway were reanalyzed by qPCR to confirm the mRNA data (Fig 2.6). In line with the mRNA array data, HMOX1, HSPB8, DNAJB9, HERPUD1, GCLM and SQSTM1 are upregulated and ENC1 is downregulated. All genes are regulated in a comparable FC as to those in the mRNA array. Furthermore, via this method HMOX1 also appears to be the strongest upregulated gene of all genes tested.

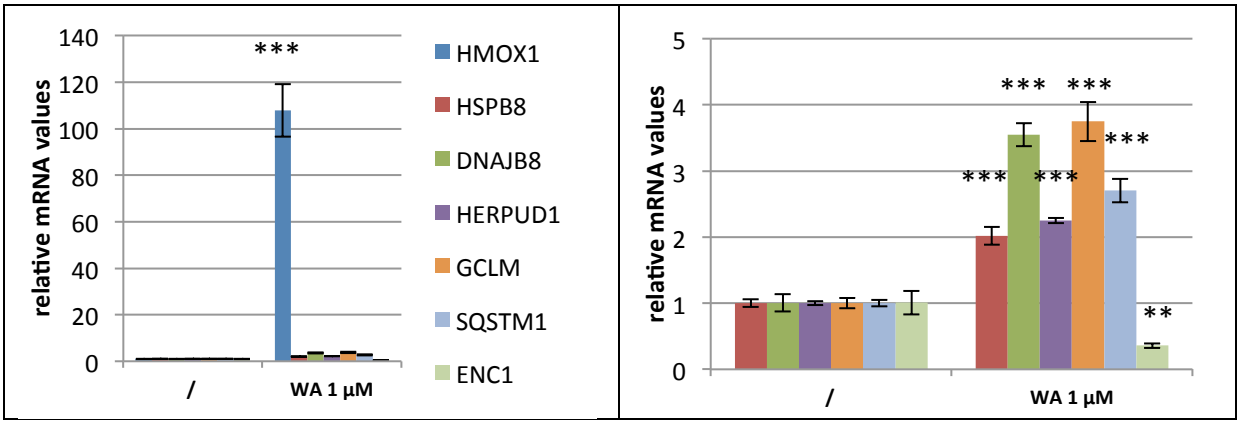


Fig 2.6 qPCR analysis of the genes HMOX1, HSPB8, DNAJB9, HERPUD1, GCLM, SQSTM1 and ENC1, genes influenced by WA and involved within the Nrf2 pathway. Values were normalized using cyclophilin reference gene. Statistics: T-test (*p<0.05, **p<0.01, ***p<0.001).

Confirmation through qPCR was also performed for all genes influenced by WA and related to the category atherosclerosis (Fig 2.7). In line with the mRNA array data, HMOX1, KLF2, CD55 and LDLR are upregulated and THBS1 downregulated. Other results were obtained for ZFP36 through qPCR in comparison to the mRNA array data, though not significant.

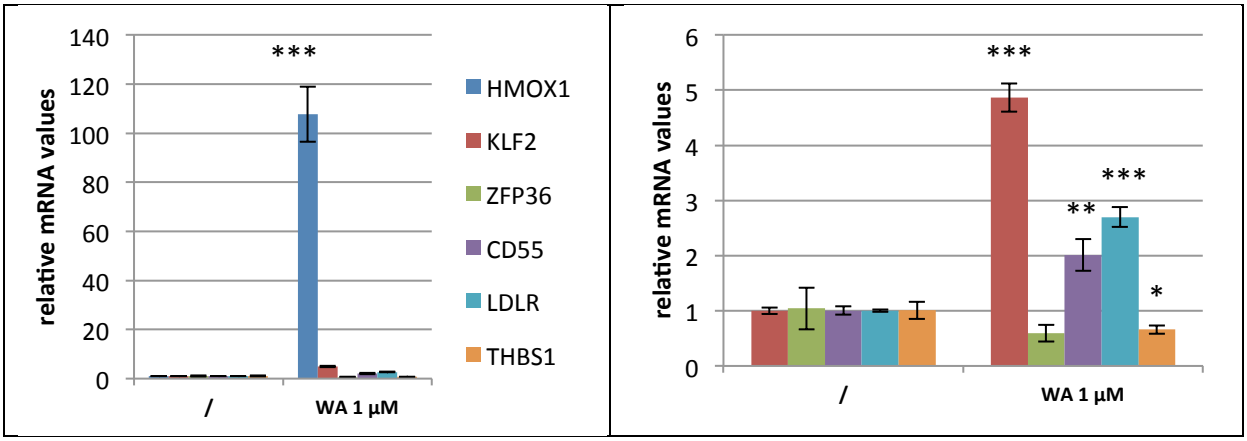


Fig 2.7 qPCR analysis of the genes HMOX1, KLF2, ZFP36, CD55, LDLR en THBS1, genes influenced by WA and related to the category atherosclerosis. Values were normalized using cyclophilin reference gene. Statistics: T-test (*p<0.05, **p<0.01, ***p<0.001).

IV.2.3 Discussion

To get insight into potential cardiovascular-related molecular targets and/or pathways affected by WA in endothelial HUVEC cells, we performed an mRNA array followed by an IPA analysis. The first observation was that genes differentially expressed from WA treatment were most significantly enriched in the Nrf2-mediated oxidative stress response pathway. Although strong enrichment of aldosterone (mineralocorticoid) and glucocorticoid pathways are also of high interest in relation to CVD and cardioprotection, these aspects fall beyond the scope of this thesis and are under investigation in a parallel PhD research project [1]. In total, there were 7 genes regulated by WA treatment and involved in the Nrf2-mediated oxidative stress response pathway. 6 out of 7 are upregulated and under control of the Nrf2 transcription factor. From those 7, HMOX1 encoding the antioxidant enzyme HO-1 is the second strongest WA regulated gene of all significant upregulated genes. From the cluster of heat shock proteins marked in red on Fig 2.4 hsp22 and hsp40 could be traced back in the list of significantly upregulated genes, on the 17th and 20th place in ranking, respectively. From the cluster of heat shock proteins, hsp90 was not present in the top list of WA-dependent upregulated genes. However, independently of transcriptional regulation, WA may regulate hsp90 chaperone function of client proteins via its direct binding with hsp90 [2]. Interestingly, the top WA regulated gene is hsp70 isoform HSPA1A/B (hsp72), which is a marker for oxidative- and cysteine-dependent proteotoxic stress, although its link with Nrf2 is not clearly established [3]. HERPUD1 or homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein, is a protein that plays a role in unfolded protein response (UPR), and destruction of misfolded proteins by the ER-associated protein degradation (ERAD) system. Its expression is induced by UPR. Also, it does have an ER stress response element in its promoter region and the encoded protein has an N-terminal

ubiquitin-like domain which may interact with the ERAD system. Glutamate cysteine ligase regulatory subunit (GCLM) forms together with glutamate cysteine ligase catalytic (GCLC) a heterodimer. This is the first rate limiting enzyme of glutathione synthesis. As glutathione is one of the most important antioxidants in the body, the upregulation of GCLM is an important response to oxidative stress. The last upregulated gene is SQSTM1, coding for the protein sequestosome1 or p62. The only downregulated gene is ENC1 or Ectoderm-neural cortex protein 1 encoding NRPB or ENC1 protein, also playing a role in the oxidative stress response. Its transcription is not under control of the Nrf2 transcription factor. ENC1 appears to downregulate Nrf2 on transcriptional level. Downregulation of ENC1 by WA, reducing its inhibitory effect on transcription of Nrf2, is in line with increased expression of Nrf2 regulated genes [4]. From all Nrf2 regulated genes, HMOX1 is by far the most prominently regulated gene with a fold change of about 20.

Notably, in the connectivity map of the top network “cell death and survival, cardiovascular disease and amino acid metabolism”, several Nrf2-mediated genes, including HMOX1, HERPUD1 and HSPB8 can be traced back. From those 3 genes only HMOX1 is a gene directly linked with cardiovascular diseases and in more particular atherosclerosis. All genes directly linked with atherosclerosis can be traced back in this connectivity map, marking their additional role in cell death and survival. Moreover, from those genes, the transcription factor KLF2 is not only an important transcription factor in atherosclerosis, it also has some interplay with Nrf2. Both are two main transcription factors related to high shear stress contributing to healthy endothelial cells [5]. In addition, activation of KLF2 induced by a laminar flow in endothelial cells enhances the antioxidant activity of Nrf2 [6]. Of this list, EDN1 or endothelin1 is the strongest WA downregulated gene. It is produced by vascular endothelial cells as a potent vasoconstrictor, explaining an atheroprotective role of its downregulation.

From the significantly regulated genes by WA, HMOX1 is the most prominent Nrf2 regulated gene related to cardiovascular diseases, more particularly in atherosclerosis.

IV.2.4 Conclusion

The Nrf2 pathway was identified as the major pathway which is most significantly affected by WA treatment in HUVEC cells. Furthermore, the Nrf2 target gene HMOX1 encoding HO-1 protein is the second strongly upregulated gene by WA and plays a critical role in atherosclerosis. In this perspective, future experiments in the following chapters aim to further elucidate WA´s relation to atherosclerosis and how WA is driving Nrf2-dependent HO-1 gene regulation.

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IV.3 Investigation of WA effects on atherosclerosis-related gene expression

IV.3.1 Introduction

Formation of an atherosclerotic plaque starts by an inflammatory process in response to high amounts of LDL that are susceptible to oxidation by free radicals and oxidants making them highly toxic to the cells. This damage of endothelial cells triggers an immune response which attracts monocytes and macrophages to the site of inflammation. This recruitment of white blood cells is coordinated by a wide range of chemo-attractants, chemokines, adhesion molecules and pro-inflammatory proteins.

As the formation of an atherosclerotic plaque comprises involvement of different cell types, it was of our interest to study the effects of WA in both endothelial cells and macrophages exposed to a pro-inflammatory stimulus. From a therapeutic point of view, modulation of lipoprotein metabolism, redox balance and an immune response to prevent attraction of white blood cells to the inflamed site could be a promising strategy to prevent the formation of an atherosclerotic plaque. In this respect, we have evaluated WA effects on various genes related to these events. As a gene involved in lipoprotein metabolism we selected the phospholipase, endothelial lipase (EL). It is regulating lipoprotein metabolism and mainly the one of high density lipoprotein (HDL) and high density lipoprotein cholesterol (HDL-C). Inhibition of EL in humans is leading to an increase of HDL-C and high plasma levels of HDL-C are reducing the risk of CVD [1]. HDL has several anti-oxidative, antiproliferative and anti-inflammatory properties [2]. It is expressed by endothelial cells, macrophages, VSMCs and hepatocytes [3]. A second important event that can be targeted is the redox balance. We selected the protein HO-1, encoded by the HMOX1 gene, as it is an important antioxidant enzyme discussed earlier on in the introduction. Another interesting strategy to prevent atherosclerosis is to target the immune response and the attraction-adhesion of white blood cells to endothelial cells. The cytokine IL-6 and iNOS have a strong contribution to the inflammatory process and MCP-1 and ICAM-1 contribute specifically to the attraction and adhesion of immune cells, respectively. IL-6 is produced by most nucleated cells but especially by macrophages and lymphocytes. It can modulate cell adhesion and promote coagulation of blood platelets [4, 5]. Increased plasma concentrations of this cytokine are an indicator of plaque instability [6]. Chronically increased IL-6 levels are suggested to be associated with a higher CVD risk and a link is seen between IL-6 levels with established risk factors like smoking or dyslipidemia [7]. iNOS is expressed mainly on macrophages and is

responsible for release of high amounts of NO. Low NO concentrations produced by endothelial eNOS can lead to vasodilatation but excessive production due to elevation of iNOS exert cytotoxic effects on vascular cells [8]. In Raw264.7 macrophages, cholesterol lowering statins inhibit expression of LPS-induced iNOS and upregulates HO-1 [9, 10]. MCP-1 is a chemo-attractant that is secreted by endothelial cells, VSMCs and macrophages [11]. The protein can be induced by TNF and it is strongly linked with atherosclerosis. MCP-1 serum levels are elevated in coronary artery disease patients and mRNA levels are significantly increased in macrophage-rich atherosclerotic plaques [12, 13]. ICAM-1 is facilitating leukocyte endothelial binding and transmigration and is expressed on vascular endothelium, macrophages and monocytes. As an endothelial adhesion molecule it can bind to leukocyte integrins, enabling a strong adhesion of leukocytes to the endothelium [14]. It is continuously expressed in low levels in membranes of endothelial cells and leukocytes, but in inflammatory environment expression levels increase tremendously. Thus, influencing all these particular genes, would contribute greatly to the formation of an atherosclerotic plaque.

To mimic the inflammatory process we used TNF in endothelial cells and LPS in macrophage experiments. Cytokines in general and TNF in particular are playing an essential role in CVD [15]. There is a wide range of clinical studies emphasizing this importance of TNF [16-18]. Increased levels of the cytokine are linked with different types of cardiovascular-related pathologies [19]. The direct link of CVD to TNF is more straightforward than to LPS. However, there are some indications that LPS can contribute to CVD. Activation of toll like receptors (TLRs) contribute to the development and progression of atherosclerosis, cardiac dysfunction and heart failure [20]. As LPS is a major pathogen-associated molecular pattern, binding to TLR4, it plays an important role in the pathogenesis of CVD. It is known that bacterial infections, such as periodontitis, can contribute to the onset of systemic diseases, including CVD. Systemic challenge with gram-negative bacteria or LPS induces major vascular responses, including an inflammatory cell infiltrate in the vessel walls, vascular smooth muscle proliferation, intravascular coagulation etc. LPS upregulates expression of endothelial cell adhesion molecules and pro-inflammatory proteins which results in platelet aggregation, monocyte adhesion, formation of lipid-laden foam cells, and deposits of cholesterol [21].

In this chapter we want to investigate more specifically the effect of WA on atherosclerosis-related gene expression. Next, we will evaluate time- and WA concentration-dependent regulation of HMOX1 mRNA and HO-1 protein levels.

IV.3.2 Results

IV.3.2.1 Regulation of EL, HMOX1, MCP-1, IL-6 and iNOS in the murine macrophage cell line Raw264.7 by WA

Here, we aimed to assess whether WA induction modulates EL, HMOX1, MCP-1, IL-6 and iNOS expression in macrophages in inflammatory-induced conditions. qPCR analysis was performed on RNA of Raw264.7 after induction of WA and LPS. As depicted in Fig 3.1, in line with literature, LPS induction resulted in a strong increase of the pro-inflammatory mediators, EL, MCP-1 and IL-6. Surprisingly, LPS only weakly stimulated iNOS about two-fold. Pre-treatment with WA, completely abolished the LPS driven upregulation of all pro-inflammatory genes. Moreover, WA was also able to reduce basal gene expression of MCP-1, IL-6 and iNOS. HMOX1 mRNA levels were not influenced by the presence of LPS in macrophages and WA was increasing the levels to a similar extent in both LPS-induced and non-induced samples.

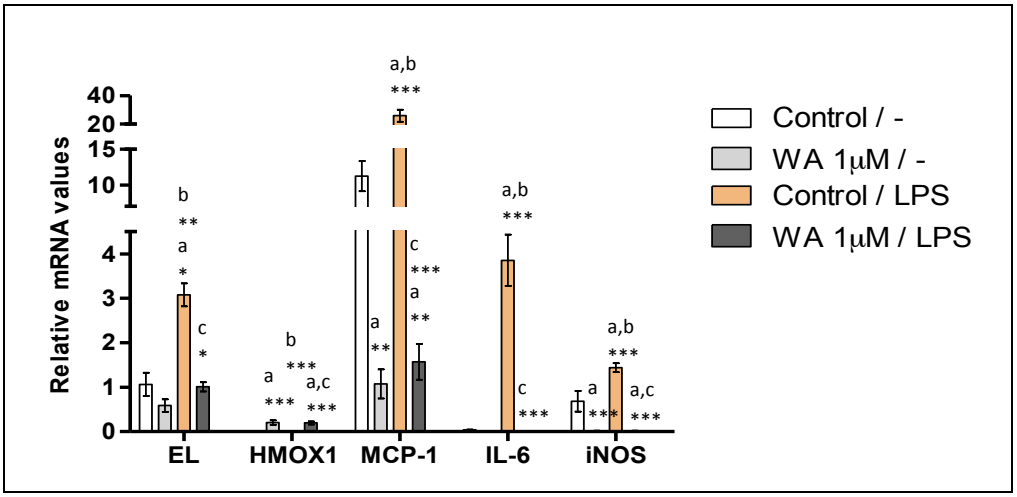

Fig 3.1 WA reverses the mRNA expression of pro-inflammatory-induced atherosclerosis-related gene expression in macrophages. Raw264.7 cells were induced for 4 h with LPS (1 μg/ml) alone or together with a pretreatment of 1 μM WA for 4 h. RNA was extracted using trizol. After reverse transcription, cDNA was amplified and mRNA transcription levels of EL, HMOX1, MCP-1, IL-6 and iNOS were analyzed by SYBR Green qPCR. Values were normalized using GAPDH reference gene. Data depicted here are representative for 3 repeated experiments. Error bars are standard deviations of triplicate technical repeats. Statistics: ANOVA test, Tukey's Multiple Comparison Test, One-way analysis of variance. a: Control vs 1 μM WA ;LPS and LPS+1 μM WA, b: 1 μM WA vs LPS and LPS+1 μM WA, c: LPS vs LPS+1 μM WA (*p<0.05, **p<0.01, ***p<0.001).

IV.3.2.2 Regulation of HMOX1, ICAM-1 and IL-6 in the human endothelial cell line EA.hy926 by WA

To explore whether WA similarly elicits anti-atherogenic responses in endothelial cells we investigated its influence on HMOX1, ICAM-1 and IL-6 gene expression in endothelial cells in inflammatory conditions. Again, qPCR mRNA analysis was performed on RNA of EA.hy926 after induction of WA and TNF. As presented in Fig 3.2, TNF induced an upregulation of the

pro-inflammatory proteins IL-6 and ICAM-1, in line with literature. Furthermore, IL-6 and ICAM1 transcription were strongly inhibited by WA. Finally, WA was able to stimulate HMOX1 mRNA transcription in the presence or absence of TNF. No additional or opposite effect by TNF in regulation of HMOX1 was noted.

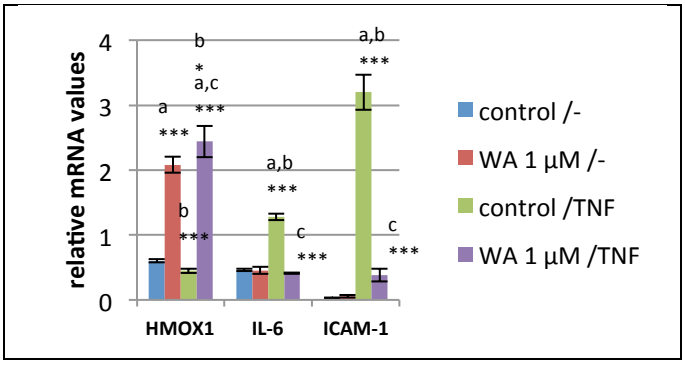

Fig 3.2 WA reverses the mRNA expression of pro-inflammatory-induced atherosclerosis-related gene expression in endothelial cells. EA.hy926 cells were induced for 4 h with TNF (2000IU/ml) alone or together with a pretreatment of 1 µM WA for 4 h. RNA was extracted using trizol. After reverse transcription, cDNA was amplified and mRNA transcription levels of HMOX1, IL-6 and ICAM-1 were analyzed by SYBR Green qPCR. Values were normalized using GAPDH reference gene. Data depicted here are representative for 3 repeated experiments. Error bars are standard deviations of triplicate technical repeats. Statistics: ANOVA test, Tukey's Multiple Comparison Test, One-way analysis of variance. a: Control vs 1 µM WA ;TNF and TNF+1 µM WA, b: 1 µM WA vs TNF and TNF+1 µM WA, c: TNF vs TNF+1 µM WA (*p<0.05, **p<0.01, ***p<0.001).

IV.3.2.3 WA triggers HO-1 expression in a time- and concentration-dependent manner

Following initial experiments in Raw264.7 and EA.hy926 exposed for 8 hours to 1 µM WA, we performed time kinetic and concentration gradient experiments to determine the time-frame and WA concentration of maximal gene induction of HMOX1 mRNA (and HO-1 protein levels) in endothelial EA.hy926 and HUVEC cells. Treatment of HUVEC and EA.hy926 cells with WA lead to a concentration- and time-dependent increase in HMOX1 mRNA levels (Fig 3.3). Induction of HMOX1 mRNA by WA was associated with an increased HO-1 protein expression in a similar concentration- and time-dependent manner (Fig 3.4). After 6 hours of WA administration, HO-1 protein levels reached a plateau phase prolonged till 24 hours (Fig 3.5). WN, a structural-related withanolide of WA, was also tested at a concentration of 1 µM for 6 hours. Remarkably, WN failed to activate HO-1, as HO-1 protein and mRNA levels detected were similar as to the non-induced sample, indicating that the effects seen on HO-1 expression are highly specific to WA. DMSO, the vehicle in which WA and WN was dissolved, did not change HO-1 protein or mRNA levels. These results indicate that WA induces HO-1 protein expression in endothelial cells in a concentration- and time-dependent manner in a highly specific fashion.

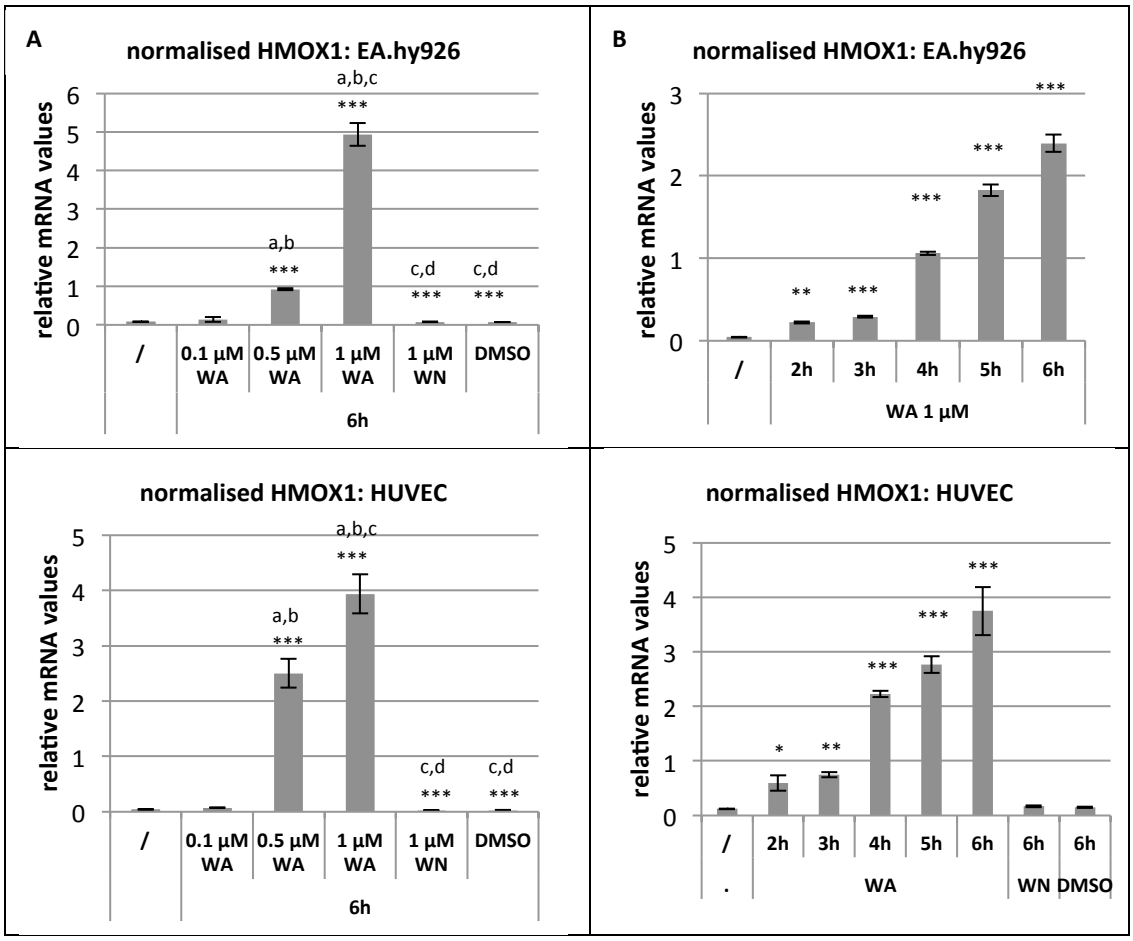

Fig 3.3 WA increases HMOX1 mRNA levels in a time- and concentration-dependent manner. (A) EA.hy926 and HUVEC cells were treated for 6 h with various WA concentrations or 1 µM WN. DMSO (1 µM, 6 h) treatment functions as a negative control. Statistics: ANOVA test, Tukey's Multiple Comparison Test, One-way analysis of variance. a: control vs 0.1 µM WA; 0.5 µM WA; 1 µM WA; 1 µM WN and DMSO. b: 0.1 µM WA vs 0.5 µM WA; 1 µM WA; 1 µM WN and DMSO. c: 0.5 µM WA vs 1 µM WA; 1 µM WN and DMSO. d: 1 µM WA vs 1 µM WN and DMSO. e: 1 µM WN vs DMSO (*p<0.05, **p<0.01, ***p<0.001). (B) EA.hy926 and HUVEC cells were treated with 1 µM WA for different time points as indicated. DMSO (1 µM, 6 h) treatment functions as a negative control. Statistics: ANOVA test, Dunnett's Multiple Comparison Test, One-way analysis of variance (*p<0.05, **p<0.01, ***p<0.001). RNA was extracted using trizol. After reverse transcription, cDNA was amplified and mRNA transcription levels of HMOX1 were analyzed by SYBR Green qPCR. Values were normalized using GAPDH reference gene. Data depicted here are representative for 3 repeated experiments for each experimental set-up. Error bars are standard deviations of triplicate technical repeats.

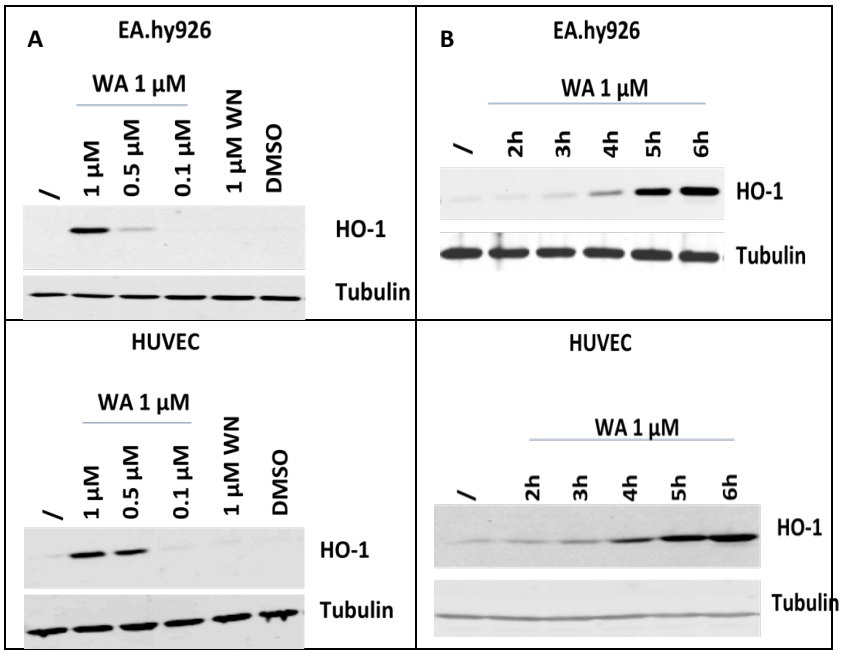

Fig 3.4 WA increases HO-1 protein levels in a time- and concentration-dependent manner. (A) EA.hy926 and HUVEC cells were treated for 6 h with various WA concentrations or 1 µM WN. DMSO (1 µM, 6h) treatment functions as a negative control. (B) EA.hy926 and HUVEC cells were treated with 1 µM WA for different time points as indicated. HO-1 protein levels were evaluated by Western blotting. Expression of tubulin was used as a loading control. Data depicted here are representative for 3 repeated experiments for each experimental set-up.

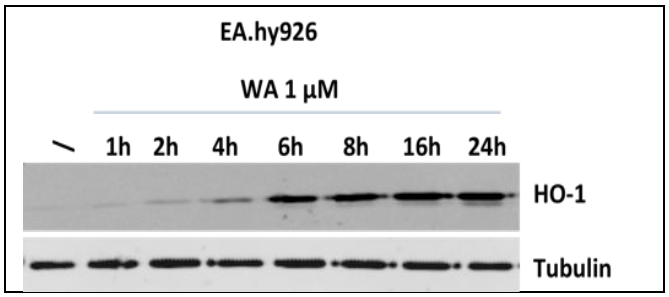

Fig 3.5 WA-induced HO-1 protein levels reach a plateau phase after 6 h prolonged till 24 h. EA.hy926 cells were treated with 1 µM WA for different time points as indicated. HO-1 protein levels were evaluated by Western blotting. Expression of tubulin was used as a loading control. Data depicted here are representative for 3 repeated experiments.

IV.3.2.4 WA is a more potent HO-1 stimulator than the reference phytochemical compound EGCG, derived from green tea

Besides WA, there are numerous natural compounds, including EGCG, sulforaphane, curcumin etc. capable of inducing HO-1 [22-34]. To estimate the relative potency of WA to induce HMOX1/HO-1 expression, as compared to well established phytochemicals, we compared the efficacy of WA in upregulating HMOX1 gene expression to EGCG, the major constituent of green tea and a known regulator of HO-1. In U937 cells, a dose range of EGCG from 10 µM to 100 µM was compared to 1 µM WA for 6 hours induction (Fig 3.6). The maximal mRNA transcription levels of HMOX1 induced by 100 µM EGCG reached only 5% of the value obtained with 1 µM WA. These results indicate that WA is a more potent

stimulator of HMOX1 than the established phytochemical EGCG at the time point investigated. Moreover, no significant difference in HMOX1 regulation between TNF-induced and non-TNF-induced cells was noted.

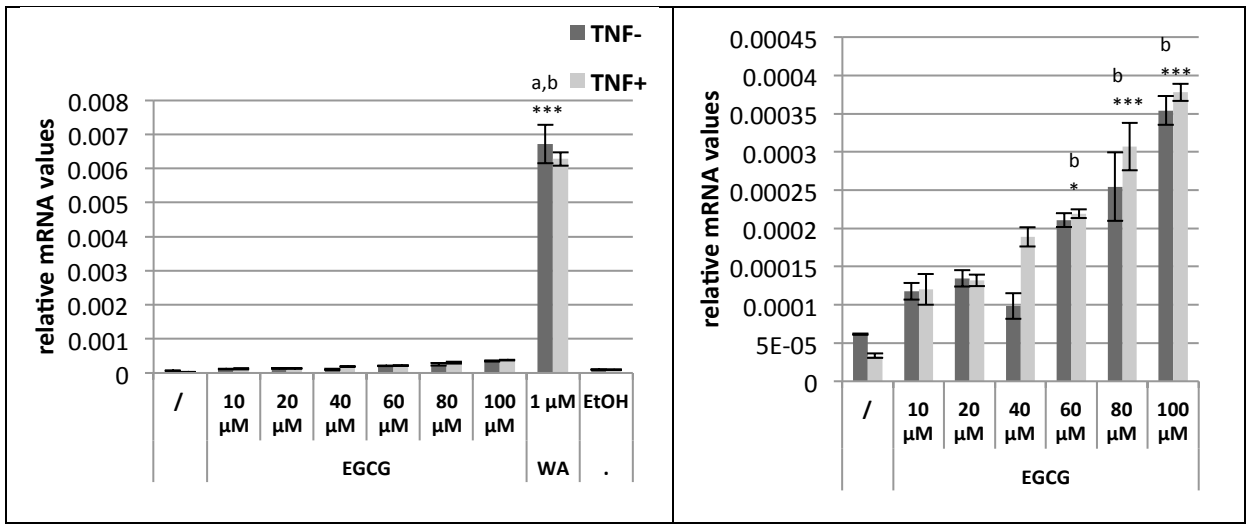

Fig 3.6 WA is a more potent stimulator of HMOX1 gene expression than the reference phytochemical EGCG. U937 cells were treated for 6 h with various EGCG concentrations and with 1 µM WA. ETOH treatment functions as a negative control. RNA was extracted using trizol. After reverse transcription, cDNA was amplified and mRNA transcription levels of HMOX1 were analyzed by SYBR Green qPCR. Values were normalized using GAPDH reference gene. Statistics: Two-way ANOVA, Dunnett's Multiple Comparison Test. a: TNF untreated samples, control vs 10-100 µM EGCG; 1 µM WA and EtOH. b: TNF-treated samples, control vs 10-100 µM EGCG; 1 µM WA and EtOH (*p<0.05, **p<0.01, ***p<0.001). Data depicted here are representative for 3 repeated experiments. Error bars are standard deviations of triplicate technical repeats.

IV.3.3 Discussion

We have first investigated the effect of WA on several atherosclerosis specific genes, selected on literature based evidence. We examined the expression pattern of those genes in macrophages and endothelial cell types, playing a crucial role in the formation of atherosclerotic plaques in the presence or absence of pro-inflammatory stimuli (TNF, LPS) following WA pretreatment. WA clearly reversed the mRNA expression of inflammation responsive proteins EL, MCP-1, IL-6, iNOS and ICAM-1, confirming previous data obtained by various labs (including our group) in other cell lines for IL-6 [35], ICAM-1 [36] and iNOS [37]. All those pro-inflammatory genes are regulated in common by the transcription factor NF-κB. As WA was already reported to inhibit NF-κB, the anti-inflammatory effects of WA on EL, MCP-1, IL-6, iNOS and ICAM-1 can be logically attributed through inhibition of the NF-kB signaling pathway. Potential cardioprotective activities of WA in atherosclerosis may in part be explained via suppressing NF-κB-dependent inflammation processes, for example at the interface of monocyt-endothelial cell attraction-adhesion. Next, we further focused on WA-dependent regulation of another atherosclerosis-related HMOX1 gene encoding the HO-1

protein. Although the HMOX1 gene promoter contains a potential binding motif for NF-κB [38], LPS or TNF failed to stimulate HMOX1 mRNA transcription in contrast to other NF-κB responsive genes which are stimulated, suggesting that the classical NF-κB pathway is a weak activator of the HMOX1 gene in the cell models tested [39]. As such, the WA-induced HO-1 upregulation in immune and endothelial cells suggests the involvement of additional cardioprotective pathways, alternative to NF-κB. Bargagna-Mohan and colleagues showed that WA could induce HO-1 in HUVEC and HCEC (human corneal epithelial cells) following combination treatment with VEGF or TNF [40]. We found that single treatment of WA, but not WN is sufficient to increase HO-1 mRNA and protein levels in a concentration- and time-dependent manner. The lack of effect of WN on HO-1 expression indicates highly specific structural requirements of withanolides to trigger HMOX1/HO-1 gene induction.

We investigated HO-1 mRNA and protein regulation in 2 alternative types of endothelial cells (HUVEC and EA.hy926). Since experiments in HUVEC cells are more labor intensive and more prone to intervariabilities between different samples we investigated whether we could observe similar expression patterns in EA.hy926. HUVEC cells have limited dividing capacity and every experiment was performed on the same passage number to avoid experimental variation. EA.hy926 cells on the other hand have unlimited dividing capacity and similar results were obtained from experiments on different passage numbers. WA induction in this fusion cell line of HUVEC and A549 resulted in similar expression patterns of HO-1 on mRNA and protein levels as compared to HUVECs.

Upon estimation of relative efficacies of WA and EGCG in upregulating HMOX1, we found that exposure of U937 cells for 6 hours with 1 μM WA is far more potent that 10-100 μM of EGCG. The relative HMOX1 gene induction observed with EGCG is within the expected range, as previously described in literature [22, 29, 34].

It is plausible that regulation by WA of the several other atherosclerosis-related genes is more complex than the known inhibitory effect of WA on the activation of NF-κB. For example, anti-atherogenic effects of HO-1 have been demonstrated through the attenuation of the production of MCP-1. HO-1 induction by hemin resulted in a significant decrease of the lysophosphatidylcholine-induced MCP-1 and inhibition of HO-1 attenuated this effect [41]. Similar effects were seen with EGCG-induced HO-1 [29]. Also, HO-1 was found to inhibit NO production in LPS-stimulated macrophages presumably through inhibition of iNOS by degrading heme, the cofactor of iNOS. Since NO is inducing HO-1 this phenomenon can be seen as a negative feedback mechanism to protect from NO-dependent toxicity [42-45]. Finally, TNF-induced ICAM-1 was downregulated both in *in vitro* and *in vivo* studies by HO-1 [31, 32, 46-48].

IV.3.4 Conclusion

Taken together, we can conclude that WA is regulating atherosclerotic-related gene transcription in macrophage and endothelial cell lines probably through NF-κB-dependent and -independent pathways. Furthermore, we demonstrated WA induced upregulation of HO-1 mRNA and protein in a dose- and time-dependent manner in the endothelial cell line EA.hy926 and the primary cell culture HUVEC, likely through NF-κB-independent mechanisms.

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IV.4 Investigation of WA effects on monocyte cell adhesion to endothelial cells

IV.4.1 Introduction

Monocytes and macrophages play an important role in the development of atherosclerosis. Recruitment of monocytes to endothelial cell walls of the arteries is one of the earliest and critical events in the pathogenesis of atherosclerosis. In this process ICAM-1 plays a crucial role. ICAM-1 is continuously expressed at low levels in the membranes of endothelial cells and leukocytes, but under inflammatory conditions the expression level increases tremendously. Under these conditions ICAM-1 facilitates leukocyte endothelial binding and their transmigration.

To investigate the potential cardiovascular effects of WA, we want to investigate whether WA can reduce monocyte adhesion to endothelial cells. Previous gene expression experiments already demonstrated that WA has an inhibitory effect on ICAM-1 expression. Interestingly, for several natural compounds it has been suggested that they reduce ICAM-1 expression and monocyte adhesion specifically by upregulation of HO-1 expression [1-6]. Therefore, in this chapter we want to evaluate whether WA may inhibit monocyte cell adhesion to endothelial cells through downregulation of ICAM1 expression via upregulation of HO-1.

IV.4.2 Results

IV.4.2.1 WA reduces monocyte adhesion to endothelial cells

To investigate whether WA is inhibiting monocyte adhesion to endothelial cells, we optimized a cell adhesion assay with primary endothelial HUVEC and monocytic THP-1 cells. By the use of a fluorescein BCECF-AM we could label THP-1 cells making it possible to quantify the amount of adhering monocytes. Fluorescence can be measured following 30 minutes co-incubation of fluorescent labeled THP-1 monocyte cells with HUVEC cells and washing out not adhered THP-1 cells. Fluorescence intensities directly correlate with the number of adhered THP-1 monocytes. Even under conditions without inflammatory stimuli, some adherence of monocytes could be observed (Fig 4.1). After 4 hours of TNF induction, monocyte binding to the endothelial cells is drastically increased, as expected. Notably, pre-induction of endothelial cells with 1 µM WA for 2 hours strongly inhibits monocyte adhesion

to basal levels. We can conclude that TNF-induced binding of monocytes to endothelial cells is severely impaired by pretreatment with WA.

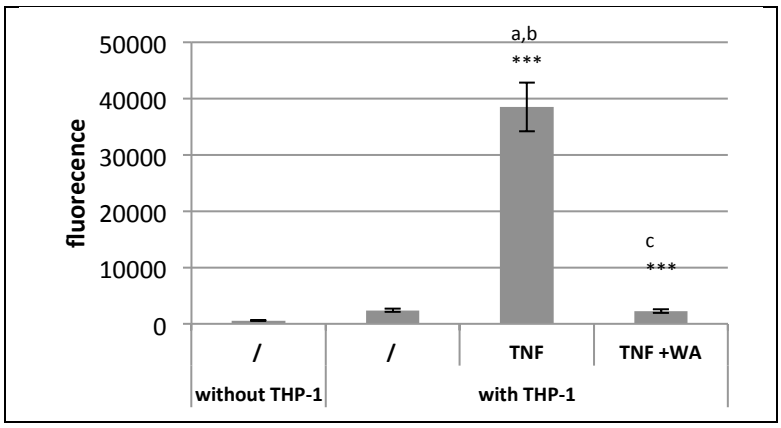

Fig 4.1 WA inhibits monocyte adhesion to endothelial cells. HUVEC cells were left untreated, exposed for 4 h with TNF (2000IU/ml) alone or pretreated for 2 h with 1 µM WA as indicated. After induction, HUVEC cells were exposed to BCECF-AM dyed THP-1 cells for 30 min. As negative control, fluorescence of HUVEC cells in absence of THP-1 cells was evaluated. Fluorescence was quantified by the use of a spectrophotometer. Statistics: ANOVA test, Tukey's Multiple Comparison Test, One-way analysis of variance. a: control without THP-1 vs THP-1-treated control, TNF and TNF +1 µM WA. b: THP-1-treated control vs TNF and TNF +1 µM WA. c: TNF vs TNF +1 µM WA (*p<0.05, **p<0.01, ***p<0.001).

IV.4.2.2 WA mediates reciprocal effects on ICAM-1 and HO-1 gene expression

Since treatment of endothelial cells with WA leads to a significant increase of HO-1 mRNA and protein levels (Fig 3.5) and downregulation of TNF-induced ICAM-1 mRNA levels (Fig 3.2), we wanted to further investigate whether the upregulation of HO-1 coincides with downregulation of ICAM-1. HUVEC cells were either treated with different concentrations of WA for 6 hours or exposed for different time frames with 1 µM WA. In addition, TNF treatment was used to mimic the inflammatory environment resulting in upregulation of ICAM-1 expression. Using qPCR analysis we observed that upregulation of HO-1 mRNA coincides with downregulation of TNF-induced ICAM-1 mRNA levels in a dose- and time-dependent manner (Fig 4.2). A similar pattern was observed at protein level as revealed by Western blot analysis (Fig 4.3). In contrast, in line with previous results, no effect of the WA-related withanolide WN could be observed. Similar data were also obtained in the EA.hy926 cell line (data not shown).

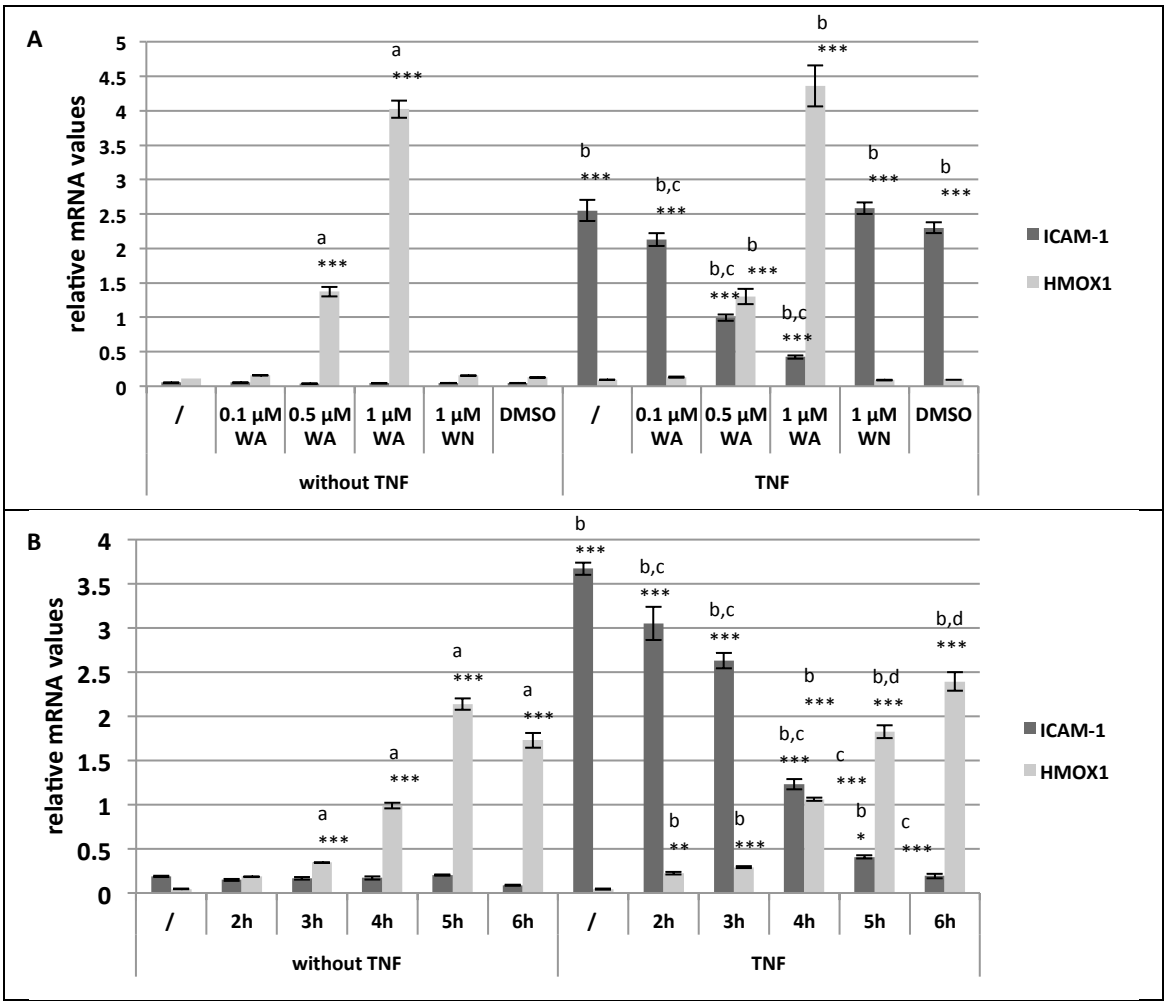

Fig 4.2 WA-induced HMOX1 expression coincides with ICAM-1 downregulation at mRNA level. (A) HUVEC cells were left untreated, exposed for 4 h with TNF (2000IU/ml) alone or pretreated for 2 h with various concentrations of WA or 1 µM WN as indicated. DMSO (1 µM, 6 h) treatment functions as a negative control. Statistics: ANOVA test, Tukey's Multiple Comparison Test, One-way analysis of variance. a: Control vs 0.1 µM WA; 0.5 µM WA; 1 µM WA; 1 µM WN and DMSO. b: Control vs TNF; 0.1 µM WA +TNF; 0.5 µM WA +TNF; 1 µM WA +TNF; 1 µM WN +TNF and DMSO +TNF. c: TNF vs 0.1 µM WA +TNF; 0.5 µM WA +TNF; 1 µM WA +TNF; 1 µM WN +TNF and DMSO +TNF (*p<0.05, **p<0.01, ***p<0.001). (B) HUVEC cells were treated with 1 µM WA for different time frames, with or without 4 h TNF (2000IU/ml) treatment. RNA was extracted using trizol. After reverse transcription, cDNA was amplified by SYBR green qPCR and values were normalized using GAPDH as reference gene. Statistics: ANOVA test, Tukey's Multiple Comparison Test, One-way analysis of variance. a: Control vs 2h WA; 3h WA; 4h WA; 5h WA and 6h WA. b: Control vs TNF; 2h WA +TNF; 3h WA +TNF; 4h WA +TNF; 5h WA +TNF and 6h WA +TNF. c: TNF vs 2h WA +TNF; 3h WA +TNF; 4h WA +TNF; 5h WA +TNF and 6h WA +TNF. d: 2h WA vs 2h WA +TNF; 3h WA vs 3h +TNF; 4h WA vs 4h WA +TNF; 5h WA vs 5h WA +TNF and 6h WA vs 6h WA +TNF (*p<0.05, **p<0.01, ***p<0.001).

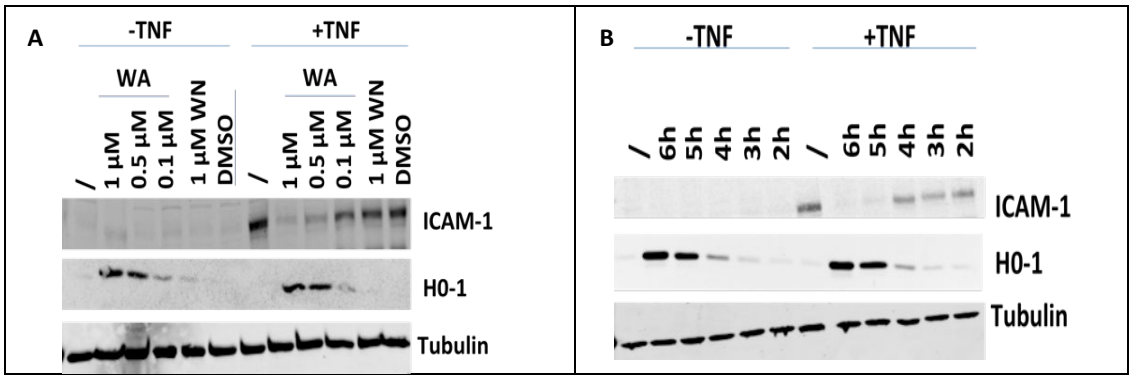

Fig 4.3 WA-induced HO-1 expression coincides with ICAM-1 downregulation at protein level. (A) HUVEC cells were left untreated, exposed for 4 h with TNF (2000IU/ml) alone or pretreated for 2 h with various concentrations of WA or 1 µM WN as indicated. DMSO (1 µM, 6 h) treatment functions as a negative control. (B) HUVEC cells were treated with 1 µM WA for different time frames as indicated with or without 4 h TNF (2000IU/ml) induction. HO-1 and ICAM-1 protein levels were evaluated by Western blotting. Expression of tubulin was used as a loading control.

IV.4.2.3 Effect of inhibition of HO-1 on WA-induced downregulation of ICAM-1 expression in endothelial cells

To assess whether WA-induced upregulation of HO-1 is implicated in TNF-induced ICAM-1 downregulation, a knockdown experiment of HO-1 was conducted. HUVEC cells were transiently transfected with non-targeting or HO-1 specific siRNA. HO-1 and ICAM-1 protein levels were analyzed by Western blotting. HO-1 siRNA clearly inhibited protein expression of HO-1. In contrast, transfection of non-targeting siRNA had no effect on HO-1 protein levels. The observation that WA can downregulate ICAM-1 expression only when it is administered before TNF treatment, indicates that WA interferes with the TNF signaling cascade at an early phase. Pre-induction of WA for 2 or 8 hours reduced ICAM-1 expression almost to baseline. When a pre-induction for 20 hours was applied, WA could reduce ICAM-1 expression to a lesser extent. However, since knockdown of HO-1 did not change inhibition of ICAM1 levels, this inhibitory effect by WA was likely via a HO-1-independent mechanism, most presumably by its inactivation of the IKK complex within the NF-κB signaling pathway [7] (see discussion) (Fig 4.4).

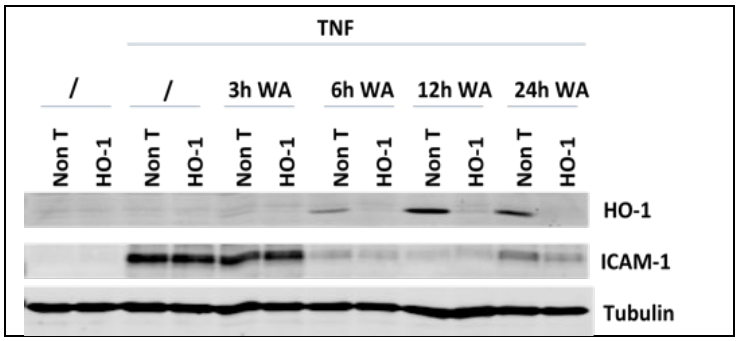

Fig 4.4 WA stimulation of HO-1 expression is not responsible for inhibition of ICAM-1. HUVEC cells were treated with 1 µM WA for different time frames as indicated together with 4 h TNF (2000IU/ml) stimulation. HO-1 and ICAM-1 protein levels were evaluated by Western blotting. Expression of tubulin was used as a loading control.

IV.4.3 Discussion

Several research groups appointed upregulation of HO-1 responsible for downregulation of TNF-induced ICAM-1 upregulation by natural compounds, resulting in decreased monocyte adhesion to endothelial cells [1-6]. Possibly, metabolites generated by HO-1, including carbon monoxide, bilverdin and bilirubin are involved as they exert NF-κB inhibiting activity. Since *in silico* studies suggested that inhibition of the NF-κB pathway by WA is a result of the inactivation of the IKK complex by binding of WA to IKKγ and IKKβ leading to a disruption of the IKK complex [7], it is convincing that a great contribution of WA-induced ICAM-1 inhibition is mediated by this mechanism. Nonetheless, we questioned whether WA-induced upregulation of HO-1 could have an additional contribution on WA-dependent suppression of ICAM-1 expression. Along the same line, some researchers described additional contribution of HO-1 on ICAM-1 regulation by natural compounds which also exert NF-κB inhibiting capacities. For instance, Yu and colleagues demonstrated inhibitory effects of andrographolide (AP) on ICAM-1 expression both via HO-1 upregulation and inhibition of NF-κB in EA.hy926 cells [2, 4]. Similar to AP also curcumin [5], genistein [1] and cinnamaldehyde (cin) [6] combine both activities to suppress ICAM-1 expression. Moreover, research on Cin suggested a biphasic inhibition on ICAM-1 expression [6]. More specifically, where short pretreatments result in reduced ICAM-1 upregulation via blocking IκBα degradation, long term effects on ICAM-1 repression were dependent on Nrf2-dependent stimulation of HO-1 expression. Until 3 hours of pre-induction with cin, IκB degradation was completely inhibited but at longer time points effects on IκB levels diminished coinciding with an upregulation of HO-1. Strikingly, after 12 hours of Cin induction, p65 levels were still completely inhibited and this effect was abolished when an HO-1 inhibitor was introduced. Keeping these observations in mind we have set up a knockdown experiment combined with different exposure times of WA treatment. Since specific knockdown of HO-1 did not affect WA-dependent reduction of ICAM-1 protein levels, we believe that WA reduces ICAM-1 induction via a HO-1-independent mechanism, presumably via its suggested inhibition of the NF-κB pathway via inactivation of the IKK complex.

IV.4.4 Conclusion

Altogether, we conclude that monocyte adhesion to endothelial cells is strongly reduced to background levels upon treatment with WA treatment. Whether this WA-induced inhibition of monocyte adhesion is exclusively via ICAM-1 needs to be further investigated. As functional silencing studies did not demonstrate a significant role for HO-1 in ICAM-1 repression, further silencing studies are required to proof a possible role for WA-dependent NF-kB inhibition in ICAM-1 gene repression.

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4. Yu, A.L., et al., *Induction of heme oxygenase 1 and inhibition of tumor necrosis factor.* J Agric Food Chem, 2010. **58**(13): p. 7641-8.
5. Olszanecki, R., A. Gebska, and R. Korbut, *The role of haem oxygenase-1 in the decrease of endothelial intercellular.* Basic Clin Pharmacol Toxicol, 2007. **101**(6): p. 411-5.
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IV.5 Molecular characterization of WA-dependent HO-1 gene induction via the Nrf2 signaling pathway

IV.5.1 Introduction

To clarify the upregulation of HO-1 by WA we focused on the main regulator of HO-1 transcription, the transcription factor Nrf2 and its activation mechanism. Recently, *in silico* studies suggested a potential binding of WA on Nrf2, providing additional evidence for regulation of the Nrf2 activation pathway by WA [1].

Under homeostatic conditions, Nrf2 is associated in the cytoplasm with a KEAP1 dimer leading to constitutive ubiquitination and degradation of the transcription factor. Thereby, KEAP1 acts as an adaptor for the recruitment of the Cul3 based E3 ubiquitin ligase complex. Several natural compounds have already been described to activate the Nrf2 pathway via specific binding to KEAP1. This interaction is mostly mediated via a Michael addition reaction whereby the natural compound becomes covalently linked to crucial cysteine residues within KEAP1. Chemical structure analysis of WA pinpointed 3 positions that could be involved in this type of alkylation reaction with nucleophilic sites like sulfhydryl groups of cysteine residues in target proteins. These positions are the epoxide structure at C5 and the unsaturated ketone at C3 and C24. KEAP1 contains several redox-sensitive cysteine residues that are crucial for its function and that upon modification lead to activation of Nrf2. Most of these KEAP1 cysteines and their flanking residues are highly conserved between human and mouse further reflecting their importance [2]. Bearing this in mind, we hypothesized that WA-induced HO-1 protein expression might be the result of covalent WA-KEAP1 binding, leading to increased stabilization and consequent activation of the Nrf2 transcription factor.

In silico molecular modeling of WA and WN at the center of C151 in KEAP1 was performed by Adjunct Professor Maija Lahtela-Kakkonen (School of Pharmacy (Pharmaceutical Chemistry) University of Eastern Finland, P. O. Box 1627, 70211 Kuopio, Finland).

IV.5.2 Results

IV.5.2.1 Role of Nrf2 in WA-induced upregulation of HO-1

To assess whether activation of the transcription factor Nrf2 is implicated in WA-induced HO-1 upregulation, a knockdown experiment of Nrf2 was conducted. EA.hy926 were transiently transfected with non-targeting and Nrf2 specific siRNA. Nrf2 en HO-1 protein

levels from untreated or WA-treated cells were analyzed by Western blotting. Transfection of EA.hy926 cells with Nrf2 specific siRNA clearly inhibited Nrf2 protein expression and concomitantly reduced WA-mediated induction of HO-1 protein expression levels. In contrast, transfection of non-targeting siRNA had no effect on Nrf2 protein levels nor on HO-1 induction by WA (Fig 5.1).

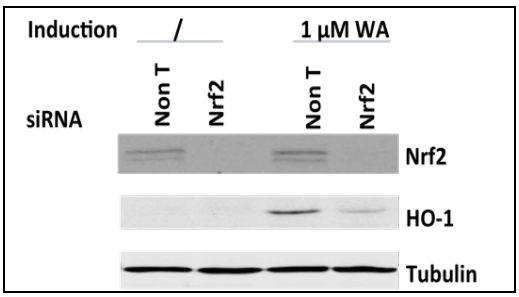

Fig 5.1 WA stimulation of HO-1 expression is Nrf2-dependent. EA.hy926 cells were transiently transfected with non-targeting or Nrf2 specific siRNA. 24 h after transfection, cells were treated with 1 µM WA for 6 h. Protein expression levels were evaluated by Western blotting. Expression levels of tubulin were used as a loading control.

As activation of Nrf2 requires several steps, including inhibition of its degradation, leading to its accumulation and nuclear translocation, we further investigated at which stage WA confers its effect within the Nrf2 pathway. First, we noted that WA treatment results in a time-dependent increase of total endogenous levels of Nrf2 (Fig 5.2 (A)). DMSO, used as a solvent control, did not influence Nrf2 expression levels. As expected MG132, a potent proteasome inhibitor, was able to increase the Nrf2 levels strongly. Secondly, levels of Nrf2 were evaluated in cytoplasmic and nuclear fractions of WA stimulated and unstimulated cells. Via Western blotting, a nuclear increase of the transcription factor was seen in a time-dependent manner (Fig 5.2 (B)). These results demonstrate that WA treatment of endothelial cells leads to increased nuclear accumulation of Nrf2 which plays a key role in the HO-1 upregulation by WA. Interestingly, via Western blotting two bands of Nrf2 are detected. Endogenous increase of the transcription factor is specially noted in the upper band, whereas in the cytoplasmatic fraction the lower band is most obvious. The existence of these different molecular weight bands might involve posttranslational modifications. However, so far their exact identity remains unclear.

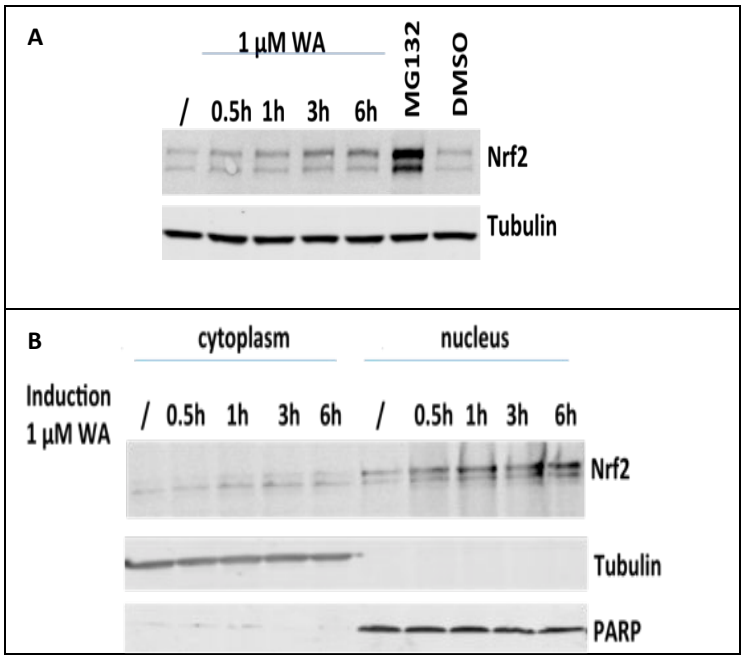

Fig 5.2. WA increases total endogenous Nrf2 levels leading to nuclear accumulation of the transcription factor. (A) EA.hy926 cells were treated with 1 µM WA for different time frames. DMSO (1 µM, 6 h) and MG132 (10 µM, 6 h) treatments function respectively as positive and negative control. Nrf2 protein levels were evaluated by Western blotting. Expression of tubulin was used as a loading control. (B) EA.hy926 cells were treated with 1 µM WA for different time frames. Nuclear and cytoplasmic fractions were extracted. Expression levels of Nrf2 were evaluated in both fractions by the use of Western blotting. To confirm correct fractionation of nuclear and cytoplasmic proteins, tubulin and PARP were used as a cytoplasmic and nuclear marker, respectively.

IV.5.2.2 Effect of WA on KEAP1 expression

To gain further insight in the effects of WA on the Nrf2 signal transduction pathway, we first checked whether WA could also influence expression levels of the Nrf2 inhibitor KEAP1. Therefore EA.hy926 cells were stimulated for different time points and with different concentrations of WA with or without MG132 (Fig 5.3). Remarkably induction of cells for 2 hours with 1 µM and 2 µM of WA gave a minor but noticeable shift to a slightly higher molecular weight level. This shift was more pronounced if cells were induced for 6 hours concomitantly with a reduced lower band intensity. The doublet observed on western blot using KEAP1 specific antibodies became less visible in a dose- and time-dependent manner. Notably, MG132 did only marginally influence the reduced KEAP1 levels. To conclude, WA induces a shift to a higher molecular weight level and a reduction of KEAP1 protein bands in a time- and dose-dependent manner. Moreover, WA-induced reduction of KEAP1 levels are only slightly influenced by proteasome inhibition.

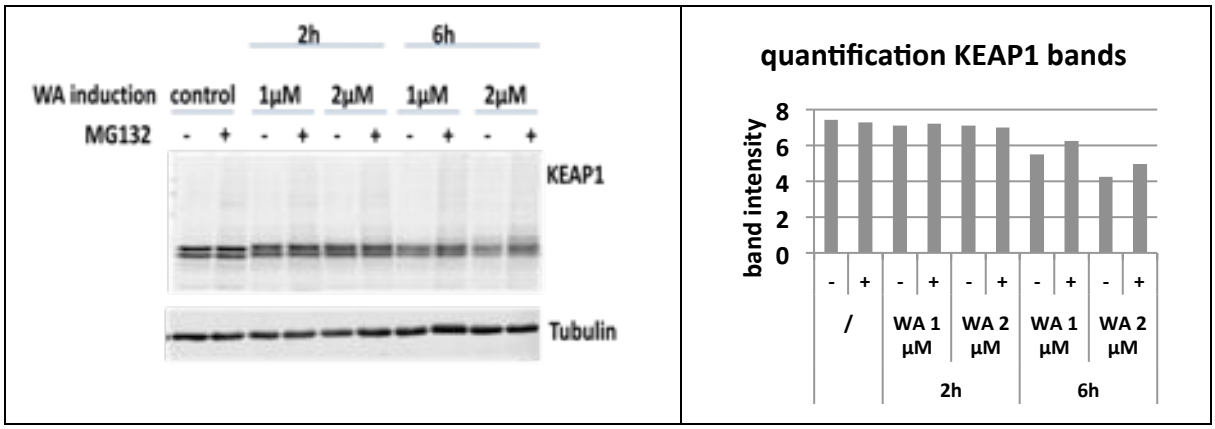

Fig 5.3 WA stimulation triggers a weak dose-and time-dependent shift in molecular weight of KEAP1 concomitantly with a weak reduction of total KEAP1 expression levels. EA.hy926 cells were treated with 1 µM and 2 µM WA for two different time frames as indicated. MG132 (10 µM, 6 h) was used to inhibit the proteasome to verify restoration of KEAP1 protein levels. KEAP1 protein levels were evaluated by Western blotting. Expression of tubulin was used as a loading control. Odyssee software was used for quantification of the KEAP1 protein bands.

IV.5.2.3 Binding of WA to KEAP1

To unravel the mechanism by which WA is able to increase endogenous Nrf2 levels, leading to nuclear accumulation of the transcription factor, we made use of a biotinylated form of WA (WA-BIOT) to identify potential binding partners. In combination with pentafluorophenyl-biotin WA was modified at position C27 creating a biotinylated form of WA (Fig 5.4). To confirm that the biological activity of WA was maintained in its biotinylated form, we investigated whether WA-BIOT was able to induce HO-1 to the same extent as WA. HO-1 expression levels induced by WA and WA-BIOT were analyzed by Western blot which demonstrated that both WA and WA-BIOT gave a comparable HO-1 induction at the concentration of 1 µM (Fig 5.5). Moreover, treatment with biotin alone had no effect on HO-1 protein levels. Thereby, we can conclude that WA-BIOT mimics WA-dependent stimulation of HO-1 expression.

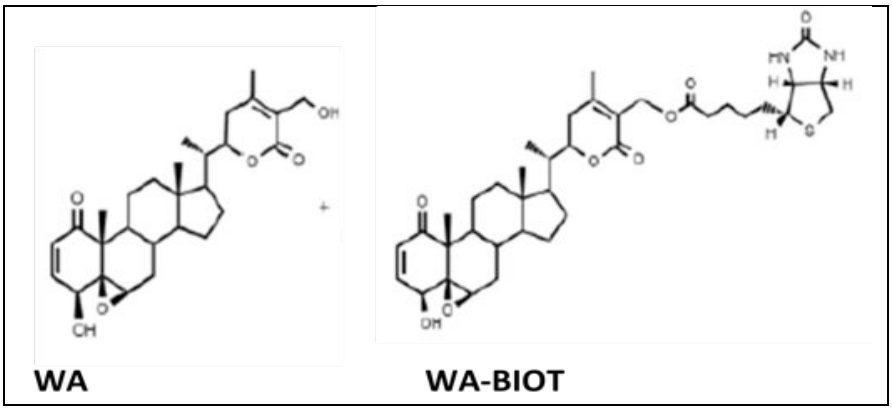

Fig 5.4 Structure of WA and biotinylated WA.

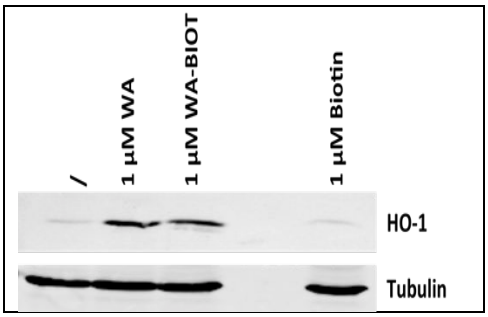

Fig 5.5 WA and WA-BIOT trigger comparable HO-1 protein induction. EA.hy926 cells were treated with 1 µM of WA, biotinylated WA or biotin for 6 h. HO-1 protein expression levels were evaluated by Western blotting. Detection of tubulin was used as an internal control.

Since KEAP1 is essential in the control of Nrf2 degradation, release, accumulation and translocation and based on the above described effects of WA on KEAP1 expression, we wanted to explore a potential direct interaction of KEAP1 with WA via cell based affinity purification using WA-BIOT and neutravidin beads. KEAP1 levels were analyzed in total cell lysates and following WA-BIOT pull down by Western blotting. As shown in Fig 5.6 (A), WA-BIOT is able to bind to KEAP1 after stimulation of EA.hy926 cells with 1 µM WA-BIOT for 2 hours. When these cells were pre-induced with 2 µM WA for 1 hour a reduced binding of WA-BIOT to KEAP1 was observed. The control setup in which cells were induced with 1 µM biotin for 2 hours did not result in any detectable KEAP1 protein following pull down. These data indicate strongly that WA-BIOT can interact with KEAP1 via its WA moiety and not via biotin. Interestingly, binding of WA-BIOT with KEAP1 is markedly interfered under reducing conditions, since no pull down of KEAP1 protein could be observed in the presence of DTT. Thus, reduction conditions hamper binding between WA-BIOT and KEAP1 indicating that this interaction might be based on a Michael addition reaction between the cysteine residues of KEAP1 and the active sites within WA. Remarkably, the KEAP1 bands in the input are a clear doublet in untreated, WA-BIOT- and WA-BIOT+DTT-treated samples. As described before these KEAP1 bands shift slightly to higher molecular weight level in WA-BIOT-treated samples which have been pretreated with WA. Remarkably , in the presence of WA-BIOT additional high molecular weight (HMW) bands of about 130kDa and 170 kDa appeared in total lysates as well as after pull down. The formation of this band is abrogated when DTT is present and is strongly reduced upon pretreatment with WA. The nature of these HMW bands of KEAP1 remains unclear.

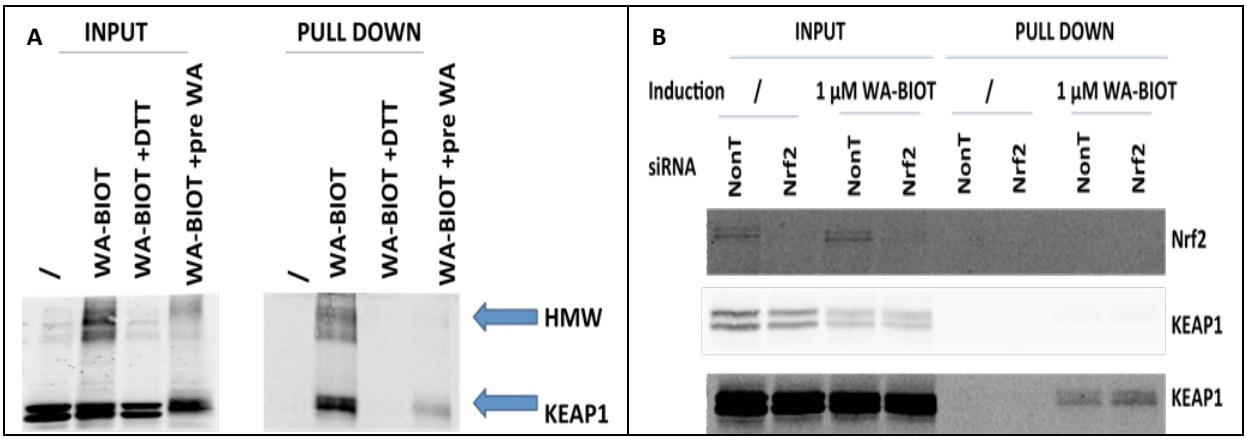

Fig 5.6 WA binds KEAP1 independently from Nrf2. (A) EA.hy926 cells were treated for 2 h with 1 µM WA-BIOT or 1 µM biotin (/). When indicated, 1 h pretreatment with WA (2 µM) or a simultaneous treatment with DTT (1 µM) was performed. After lysis of the cells, an affinity purification using neutravidin beads was performed. Western blot analysis on total lysate (input) and affinity purified proteins (pull down) were performed with the aim of detecting KEAP1 proteins. (HMW = high molecular weight bands). (B) EA.hy926 cells were transiently transfected with non-targeting siRNA or siRNA targeting specifically Nrf2. 24 h after transfection, cells were treated with 1 µM WA-BIOT or 1 µM biotin for 2 h. After lysis, WA-BIOT interacting proteins were purified by the use of neutravidin beads. Expression levels of Nrf2 and KEAP1 in both total cell lysates (input) and pull down lysates were evaluated by Western blotting.

Since *in silico* analysis predicted that Nrf2 could be a potential binding partner of WA, we further investigated whether WA-BIOT could pull down Nrf2 as well. In parallel, we investigated whether the observed binding of WA-BIOT with KEAP1 was not indirect via Nrf2. Therefore, EA.hy926 cells were transfected with non-targeting siRNA or Nrf2 specific siRNA before affinity purification using WA-BIOT. However, Western blot analysis of the pull down samples indicated no binding between WA-BIOT and Nrf2, disfavoring the *in silico* prediction. Furthermore, binding between WA-BIOT and KEAP1 is not alleviated in the presence of Nrf2 siRNA (Fig 5.6 (B)) favoring a direct interaction.

These data indicate that in endothelial cells WA can directly bind to KEAP1 independently of Nrf2. Moreover, cysteine residues within KEAP1 might play an important role in this interaction.

IV.5.2.4 Binding of WA to KEAP1 requires distinct cysteines

Since cysteine residues are predicted to play a role in the binding between WA and its target proteins and since some conserved cysteine residues in KEAP1 are critical for the Nrf2 activation by several Nrf2 inducers [3-6], we next investigated whether these specific cysteine residues within KEAP1 are also responsible for binding with WA. Using transient overexpression of KEAP1 mutated at different cysteine residues located in different domains of KEAP1, we investigated the contribution of these cysteines to WA-KEAP1 binding. EA.hy926 cells were transiently transfected with different triple FLAG-tagged KEAP1 expression plasmids and induced with 1 µM WA-BIOT for 2 hours before applying neutravidin based pull down. As demonstrated in Fig 5.7, no aspecific binding of biotin to

KEAP1 or non biotinylated WA to the neutravidin beads was observed. A strong affinity of WA-BIOT for wild type KEAP1 and its cysteine mutants C257S, C273S, C288S and C489S could be observed. In contrast, KEAP1 C151S mutant could not be pulled down by WA-BIOT. This suggests that cysteine 151 is of specific importance for the binding of WA-BIOT and KEAP1. While investigating the binding of WA-BIOT and endogenous KEAP1 we previously noticed some high molecular weight bands when cells were induced with WA-BIOT. In this experiment, also exogenous KEAP1 gave high molecular weight bands in both input and pull down. Notably, those bands are absent when the C151S mutant is expressed and when cells are induced with WA or biotin.

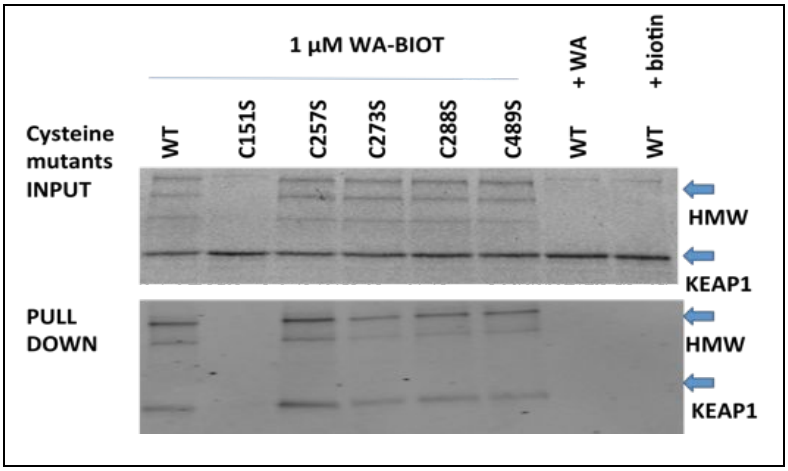

Fig 5.7 Cysteine 151 in KEAP1 is critically involved in KEAP1-WA binding. EA.hy926 cells were transiently transfected with triple FLAG-tagged expression plasmids coding for wild type KEAP1 (WT) or different cysteine mutants (C151S, C257S, C273S, C288S, C489S), where cysteine was mutated to serine. After 24 h, cells were treated with 1 µM WA-BIOT for 2 h. WT KEAP1 transfected samples were treated as indicated with 1 µM WA or 1 µM biotin as negative controls. Cells were lysed and WA-BIOT interacting proteins were purified by the use of neutravidin beads. Expression levels of overexpressed KEAP1 variants in both total cell lysates (input) and pull down lysates were evaluated by Western blotting using anti-FLAG antibody. (HMW=high molecular weight bands)

IV.5.2.5 Activation of Nrf2 by WA via KEAP1

To assess whether the binding of WA-BIOT to cysteine 151 of KEAP1 is functional and actually involved in the activation of Nrf2, we analyzed the effect of WA on Nrf2 protein expression levels upon overexpression of wild type or C151S mutated KEAP1. Since efficiency of transient transfection in EA.hy926 cells is rather low, these experiments were performed in HEK293T cells which can be transfected with high efficiency. HEK293T cells were transfected with an expression plasmid encoding HA-tagged Nrf2, either alone or under conditions of cotransfection of WT KEAP1 or C151S KEAP1 encoding expression plasmids. In the untreated samples, transfection of Nrf2 expression plasmid increased Nrf2 levels. As expected, co-expression of WT or C151S mutant of KEAP1 alleviates this increase. When cells co-expressing WT KEAP1 are induced with WA the inhibitory effect of KEAP1 on the Nrf2 levels is mostly abrogated, which could not be observed upon co-expression of the C151S mutant of KEAP1 (Fig 5.8). Thereby, we can conclude that WA is able to block the inhibitory

effect of wild type KEAP1 on Nrf2 stability. However, substitution of cysteine 151 to serine abrogates this regulating effect of WA, indicating that binding of WA to KEAP1 at cysteine 151 might indeed contribute to increased stability of Nrf2.

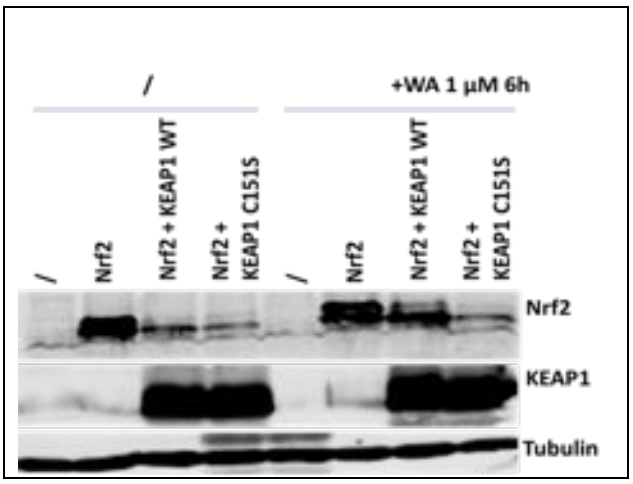

Fig 5.8 Cysteine 151 in KEAP1 might be important for the binding of WA resulting in increased stability of Nrf2. HEK293T cells were cotransfected with an expression plasmid for HA-Nrf2 and expression plasmids for triple FLAG-tagged wild type (WT) or cysteine 151 (C151S) mutant KEAP1. After 24 h, cells were treated with or without 1 µM WA for 6 h. Overexpressed Nrf2 and KEAP1 protein expression levels were evaluated by Western blotting by the use of anti-HA and anti-FLAG antibodies. Tubulin was used as an internal control.

IV.5.2.6 Effect of WA on Nrf2/KEAP1/Cul3 complex formation

Since stability of Nrf2 is regulated by complex formation with the KEAP1/Cul3/Rbx1-E3 ubiquitin ligase complex, we performed co-immunoprecipitation experiments to investigate the integrity of the Nrf2/KEAP1/Cul3 complex after WA induction. Thereby, we focused on the interaction between Cul3 or Nrf2 with KEAP1. Furthermore, we examined whether these regulatory effects of WA are abrogated when cysteine 151 of KEAP1 is mutated. Therefore, cells were transiently transfected with FLAG-tagged WT or C151S KEAP1 expression plasmids together with HA-tagged Cul3 or HA-tagged Nrf2 expression plasmid. Afterwards, the cells were induced with 1 µM WA for 2 hours. After pulling down KEAP1 proteins by the use of FLAG beads we could detect co-immunoprecipitation of Cul3 and Nrf2. As previously described, no difference is observed between the WT and the C151S mutant KEAP1 plasmid for these interactions. However, when cells were induced by WA, we did not observe reduced binding of Cul3 or Nrf2 to KEAP1 (fig 5.9).

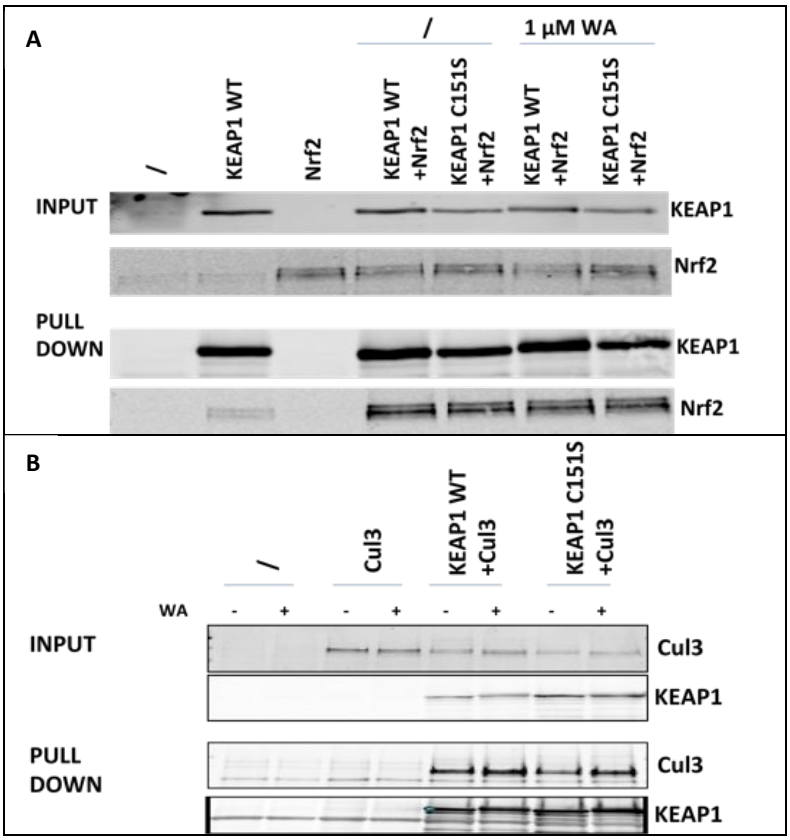

Fig 5.9 WA fails to reduce the binding of KEAP1 with Nrf2 or Cul3. (A) EA.hy926 cells were cotransfected with an expression plasmid for HA-Nrf2 and expression plasmids for triple FLAG-tagged wild type (WT) or cysteine 151 (C151S) mutant KEAP1. After 24 h, cells were treated with or without 1 µM WA for 2 h. Cells were lysed and FLAG-tagged KEAP1 interacting proteins were purified by the use of FLAG beads. Expression levels of overexpressed KEAP1 and Nrf2 in both total cell lysates (input) and pull down lysates were evaluated by Western blotting by the use of anti-Nrf2 and anti-FLAG antibodies. (B) EA.hy926 cells were cotransfected with an expression plasmid for HA-Cul3 and expression plasmids for triple FLAG-tagged wild type (WT) or cysteine 151 (C151S) mutant KEAP1. After 24 h, cells were treated with or without 1 µM WA for 2 h. Cells were lysed and FLAG-tagged KEAP1 interacting proteins were purified by the use of FLAG beads. Expression levels of overexpressed KEAP1 and Cul3 in both total cell lysates (input) and pull down lysates were evaluated by Western blotting by the use of anti-HA and anti-FLAG antibodies.

IV.5.3 Discussion

This study was undertaken to elucidate the underlying transcriptional mechanism of WA-induced HO-1 expression in endothelial cells. HO-1, whose expression is highly inducible, exhibits low basal expression levels in most cell types and becomes upregulated by a plethora of physiological and pathological stimuli, including oxidative stress signals, cytokines, bacterial and electrophilic compounds. In this process, several signal transduction pathways have been demonstrated to be involved but one of the main regulators is the transcription factor Nrf2.

In this chapter, we have demonstrated that downregulation of Nrf2 reverses HO-1 upregulation induced by WA (Fig 5.1). This indicates that Nrf2 is playing a major role in WA-induced HO-1 regulation. Nonetheless, a weak upregulation of HO-1 remains which can have different causes. Since Western blotting has only the sensitivity to detect proteins from a certain protein concentration level it is plausible to assume that knockdown of Nrf2 is not complete. So, there is still some residual Nrf2 which is not detectable via Western blotting but which is activated by WA. An alternative explanation could be that WA-induced HO-1 expression is not only regulated by Nrf2 but that additional regulatory mechanisms initiated by WA are involved in the HO-1 regulation. For example, Nrf2-independent HO-1 gene induction may rely on downregulation of Bach1. We cannot exclude that WA in addition to Nrf2 also influences Bach1 activity, thereby upregulating HO-1 in an Nrf2-independent manner. This regulation could be via cysteine residues in Bach1, since regulation of Bach1 via its cysteine residues as an alternative mechanism to induce multiple ARE-dependent genes is suggested by several other researchers [7-10]. Furthermore, it has been described that the promoter of HO-1 has several transcription factor responsive elements, including NF-κB [11] and AP-1 [12-17]. However, it is rather unlikely that WA will upregulate HO-1 via NF-κB or AP-1 as WA is known to inhibit NF-κB and AP-1 activation [18, 19]. Also, we could not observe TNF/LPS inducible HO-1 gene transcription, whereas other NF-κB targets genes could be stimulated.

Furthermore, although 6 hours exposure of endothelial cells with 1 µM WA only slightly increased Nrf2 levels, a strong increase of HO-1 levels is noticeable. A possible explanation is that HO-1 only needs minimal amounts of activated Nrf2 to get induced. It has been described that HO-1 contains the most ARE binding sites for Nrf2 of all Nrf2-dependent genes, strengthening this idea. This supports also the hypothesis that minimal amounts of Nrf2 are sufficient to upregulate HO-1 expression.

In line with observations of other researchers, clearly two bands of Nrf2 are visible after Western blot analysis [20-22]. The exact explanation for those two bands are still a matter of discussion. However, the explanation gaining most support is that the slower migrating form is a phosphorylated Nrf2 protein and the faster form an unphosphorylated Nrf2. Moreover, it has been suggested that unphosphorylated Nrf2 predominates in the cytoplasm, whereas the phosphorylated form is preferentially localized in the nucleus [21]. Our data are completely in line with this hypothesis (Fig 5.2 (B)). WA-induced increase of Nrf2 is specially noted in the upper band which is mainly observed in the nuclear fraction. These data indicate that WA might be able to regulate phosphorylation of Nrf2. Moreover, treatment with the proteasome inhibitor MG132 is able to increase both bands, as proteasomal degradation of both phosphorylated and unphosphorylated Nrf2 is inhibited.

Vaishnavi *et al.* suggested via molecular docking studies that both WA and its related withanolide WN interact with Nrf2 through its amino acids Ala 69, Phe 71 and Gln 75 in the active site region [1]. Interestingly, WA was suggested to bind to Nrf2 with higher affinity

than WN [1], which might explain the different regulation of HO-1 expression by WA and WN. However, in contrast to our observations in endothelial cells, the authors were unable to demonstrate an effect of WA on this Nrf2 activation pathway evaluated by analysis of Nrf2 expression levels and nuclear translocation in the human osteosarcoma cell line U2OS. Of special note, Nrf2 contains several conserved cysteine residues that are involved in the Nrf2 signaling [23]. Thus, besides Ala 69, Phe 71 and Gln 75 that are suggested by Vaishnavi and colleagues, it cannot be excluded that WA may react with cysteine residues of Nrf2. However, upon investigation of potential binding of WA to Nrf2 by pull down using biotinylated WA, we were unable to detect direct binding of WA-BIOT and Nrf2 (Fig 5.6 (B)).

Under homeostatic conditions, Nrf2 is kept inactive in the cytoplasm via its interaction with its inhibitor KEAP1. The latter functions as an adaptor between Nrf2 and the ubiquitin ligase Cul3 that constitutively ubiquitinates Nrf2 leading to its proteasomal degradation [24, 25]. By triggering with electrophilic reagents or oxidative stress, Nrf2 escapes from this control mechanism, becomes stabilized, accumulates and translocates to the nucleus. Based on the crucial role of KEAP1 in this pathway as well as the knowledge that several natural compounds can activate the Nrf2 pathway by chemically reacting with KEAP1, we evaluated this hypothesis for WA. Using the biotinylated form of WA, which exerts similar biological activities towards the regulation of HO-1 expression (Fig 5.5), we could identify a direct interaction between WA and KEAP1. This interaction is mediated by the WA moiety and probably involves an interaction to thiol groups since the interaction is hampered by pretreatment with WA or co-treatment with DTT, respectively. Remarkably, a slight increase in molecular weight of KEAP1 is apparent upon stimulation with WA-BIOT together with pre-induction with WA (Fig 5.6 (A)). Moreover, high molecular weight bands of KEAP1 in the range of 130 kDa and 170 kDa could be observed upon treatment with biotinylated WA. Similar observation have been previously obtained by using other small electrophilic compounds [5, 26-30]. Since the molecular weight of KEAP1 is around 65 kDa, these bands may be aggregates of 2 and 3 KEAP1 subunits. However so far, the composition of these bands remains elusive and requires further investigation. Furthermore, similar to Nrf2, endogenous KEAP1 is also visual as doublet bands on Western blot. This phenomenon is mentioned by several antibody suppliers and other researchers. It appears to be cell specific and in our hands can also be observed in endothelial cells (Fig 5.6 (A), Fig 5.6 (B), Fig 5.7) [31, 32].

Additionally, total protein levels of KEAP1 are slightly lowered upon WA treatment in a dose- and time-dependent manner (Fig 5.3). However, since Nrf2 and concomitant upregulation of HO-1 can already be observed at concentrations and time points earlier than degradation of KEAP1, we assume that this modification and downregulation is possibly not the initial cause of WA-induced HO-1 upregulation. Furthermore, WA-induced reduction of KEAP1 protein levels could only partially be inhibited by the proteasomal inhibitor MG132 . This suggests that also other degradation mechanisms are involved. Indeed, autophagy-dependent degradation has been demonstrated in case of KEAP1 [28, 33-35]. A physical and functional

relationship was elucidated for KEAP1 with p62 [36-41], suggesting autophagy as a possible mechanism of downregulation of KEAP1 as p62 can act as a scaffold protein, linking polyubiquitinated proteins to the autophagic machinery [42, 43].

In literature, several electrophilic compounds are described to activate the Nrf2 pathway via targeting distinct reactive KEAP1 cysteine residues [27, 44, 45]. Of particular interest, , we could demonstrate by WA-BIOT based affinity pull down assays that KEAP1 can interact with WA (Fig 5.6 (A)) via C151 whereas C257, C273 C288 and C489 were not affected (Fig 5.7).

Of particular interest, upon performing *in silico* molecular modeling of WA and WN at the center of C151 in KEAP1, highest docking scores were obtained with WA as compared to WN (Fig 5.10). Interestingly, based on the docking studies, WA seems to have several H-bonds with C151 whereas WN seems to form H-bonds towards other residues (Fig 5.11). Based on this molecular modeling, WA seems to have higher binding affinity to KEAP1 than WN. This is in line with our observation that WA but not WN is able to trigger HO-1 transcription via KEAP1/Nrf2 signaling.

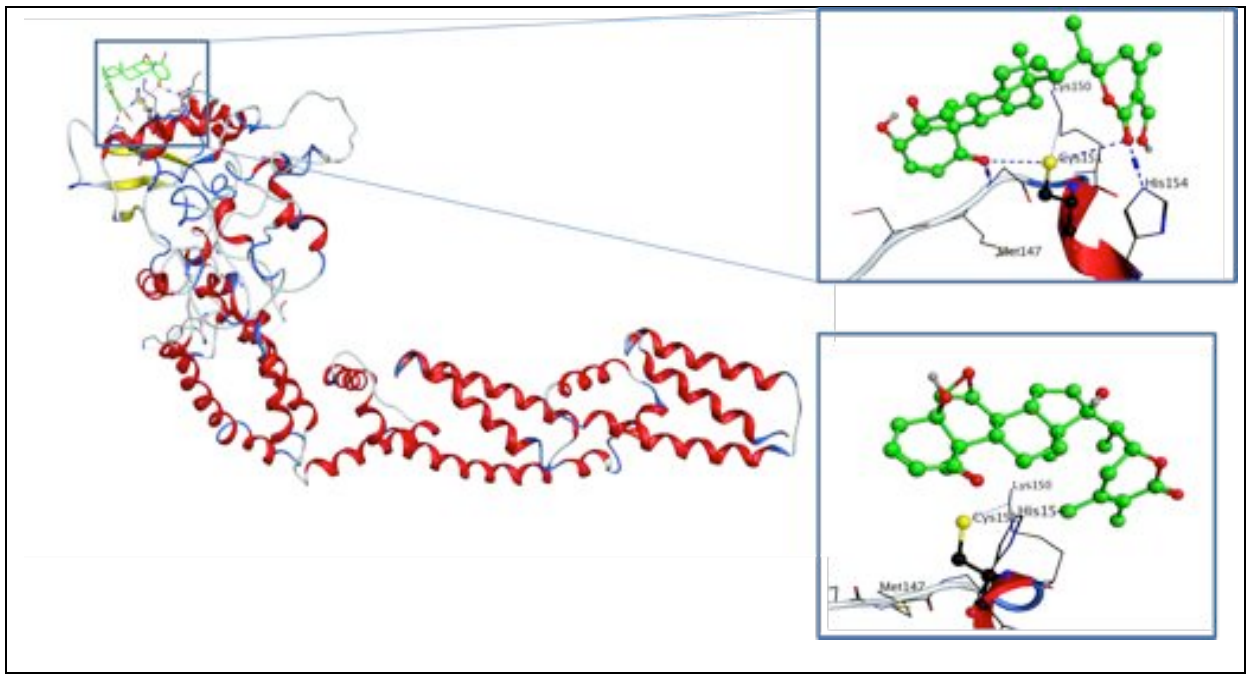

Fig 5.10 Homology model of human KEAP1 and the putative binding site of WA or WN.

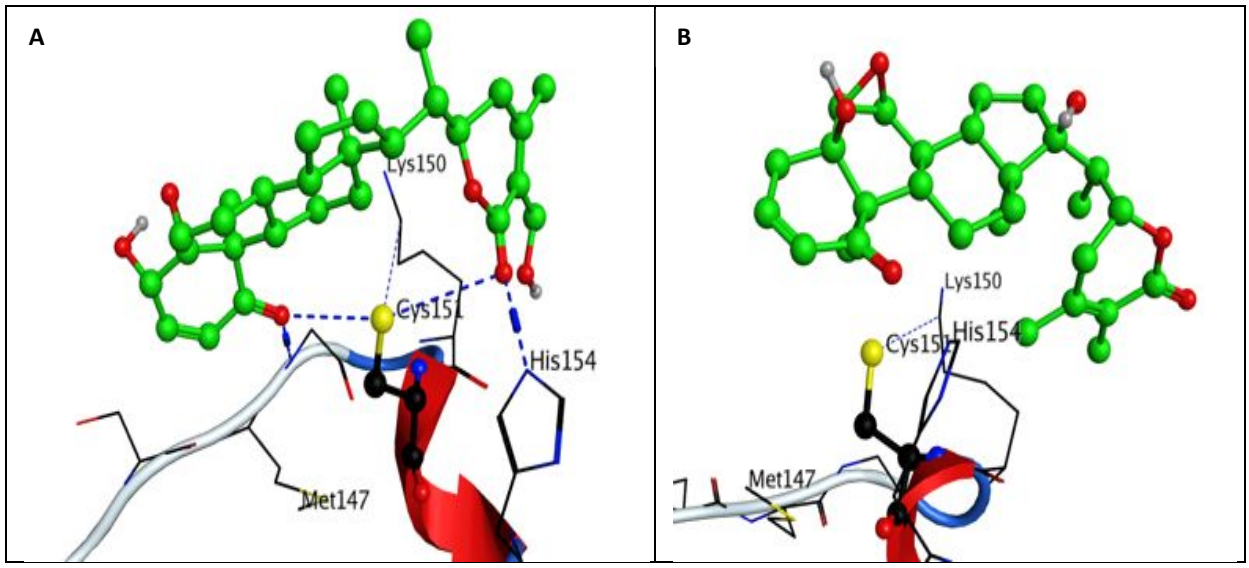

Fig 5.11 (A) The putative binding site of WA with the highest docking score shows several H-bonds with Cysteine 151 and additional H-bond with Histidine 154. (B) The putative binding site of WN with the highest docking score has no hydrogen bond with the homology model of human KEAP1.

This highly reactive C151 is located in the BTB domain, which is involved in the interaction between KEAP1 and the ubiquitin ligase Cul3. Despite quite low levels of primary sequence relatedness, this protein-protein interaction domain is structurally highly conserved during evolution in eukaryotes and can be found in more than 350 human proteins [46, 47]. Among these proteins, several have been described to interact with Cul3 and to act as an adaptor bringing more substrate proteins towards Cul3 to become ubiquitinated [48]. However, although C151 is conserved in KEAP1 through molecular evolution, it is a non-conserved amino acid within the BTB domain between paralogous BTB-Kelch proteins [46]. This lets us assume that the activity of WA is probably quite specific for KEAP1.

On the other hand, KEAP1 has recently been demonstrated to act as an adaptor protein for the recruitment of additional target proteins to Cul3, besides Nrf2. These include the NF-κB activating kinase, IKKβ, as well as the anti-apoptotic protein, Bcl-2, two proteins involved in key signal transduction pathways which are strongly affected by WA [49, 50]. However, the observation that IKKβ and Bcl-2 protein expression levels were unaltered or even reduced by WA treatment instead of the expected upregulation by WA-mediated inactivation of KEAP1, suggest that WA affects quite specifically the KEAP1-Nrf2 pathway [unpublished results][51, 52].

To assess whether the binding of WA to KEAP1 at C151 is functional and involved in the activation of Nrf2, we analyzed if binding of WA to KEAP1 can influence the stability of Nrf2 upon transient transfection in HEK293T cells (Fig 5.8). Whereas WT KEAP1 and C151S KEAP1 regulate Nrf2 degradation to the same extent, this Nrf2 inhibitory effect can be reversed by WA treatment only in KEAP1 wild type overexpressing cells. Mutation of C151 in KEAP1 reduced the WA-induced increase in Nrf2 levels, indicating a functional importance for the interaction of WA at C151 in KEAP1. In this context, we certainly want to mention that the

observed effects are strongly dependent on stimulation times and the ratio of Nrf2/KEAP1 expression levels. This importance is strongly underlined by the study of Takaya and colleagues, making use of a transgenic complementation rescue assay. They pointed out that overexpression experiments *in transfecto* can sometimes be misleading due to the arbitrary expression levels of KEAP1 mutant molecules and interference from endogenous KEAP1. [34]. To further unravel the mechanism of WA action, we investigated whether Nrf2 or Cul3 are released from the Nrf2/KEAP1/Cul3/Rbx1-E3 complex in the presence of WA. No difference in Nrf2 or Cul3 binding could be observed between WA-induced and non-induced samples (Fig 5.9). Moreover, there was no difference in Nrf2 or Cul3 binding between the WT and the C151S mutant KEAP1 plasmid in the WA-induced and non-induced samples. However, these experiments were so far performed using a single dose of WA and at one time point. Dose-and time-dependent analysis of these interaction patterns are certainly needed to provide conclusive data. So far, we could conclude that 1 µM WA could not release Nrf2 nor Cul3 after 2 hours of stimulation. In case of Nrf2, these data are in line with literature which already indicated that the cysteine 151 does not modify the binding of Nrf2 to KEAP1. To conclude, further research is required to unravel the molecular mechanism of action by which WA regulates Nrf2 activation in endothelial cells.

IV.5.4 Conclusion

In conclusion, we provide experimental evidence that Nrf2 plays a key role in the HO-1 upregulation by WA in endothelial cells. Moreover, WA is able to increase the endogenous Nrf2 levels leading to an increased nuclear translocation. This effect is mediated by interaction of WA to KEAP1, via cysteine 151 in KEAP1. So far, no effect of WA on the Nrf2-KEAP1-Cul3 complex could be observed. Thus, the exact molecular mechanism of action still requires further research.

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Part V. Discussion, summary and perspectives

V.1 Summary

CVDs are nowadays the most deadly diseases in the world. Therefore, research concerning prevention or treatment of CVD is a major public health concern. The main cause of CVD is atherosclerosis. One of the important stress factors involved in the onset of atherosclerosis is oxidative stress. An early step in the formation of an atherosclerotic plaque is the monocyte adhesion to a deregulated endothelial wall. Adhesion molecules on the cell membrane of these endothelial and monocytic cells are playing an important role in this binding.

A key transcription factor involved in managing environmental and endogenous oxidative and xenobiotic stresses is Nrf2. Under homeostatic conditions, Nrf2 is associated with a KEAP1 homodimer in a KEAP1/Cul3/Rbx1-E3 complex in the cytoplasm. This association leads to the constant ubiquitination and degradation of Nrf2. KEAP1 possesses a dual function: I) it functions as a sensor to stresses and II) it regulates Nrf2 ubiquitination. Thereby, KEAP1 switches the Nrf2-mediated transcriptional regulation on or off. Several Nrf2 inducers target crucial cysteine residues in KEAP1 leading to activation of Nrf2 and upregulation of ARE-dependent genes, including antioxidant enzymes. One of the important antioxidant enzymes is HO-1. Based on its importance against toxicity induced by oxidative stress, HO-1 has a cardioprotective effect. Furthermore, HO-1 is recognized to exhibit important immunomodulatory, anti-apoptotic, antiproliferative, pro-angiogenic and anti-inflammatory functions increasing its protective role in the formation of atherosclerosis.

WA is one of the main withanolides derived from *WS*. The roots, leaves and berries of *WS* are used to treat a wide range of disorders in Ayurvedic medicine. Those roots and leaves are quantitatively rich in withanolides and WA is one of the main biologically active withanolides. WA is demonstrated to exert a broad spectrum of health-promoting effects, including anti-inflammatory, anticarcinogenic, anti-angiogenic, antibacterial, anticonvulsive, immunosuppressive and pro-apoptotic properties. Despite the wide variety of bioactivities of this compound the molecular mechanisms remain largely unknown. Also, its potential beneficial effects for treatment of CVDs are not intensively investigated.

Since WA is mainly known for its cell death inducing effects, we first defined optimal concentrations of this withanolide which does not exert cytotoxic effects. In the endothelial cells (HUVEC and EA.hy926 cells) and monocytes/macrophages (Raw264.7 and U937 cells) no cytotoxic effects of WA were observed for 24 hours treatments with concentrations below 2 µM. Based on these data, in all subsequent experiments, the concentration of WA and related withanolide WN for 2 µM was never exceeded. Using these optimized conditions, transcriptional regulation of gene expression by WA was evaluated in primary HUVEC cells via mRNA microarray to define genes, pathways and diseases which might be regulated by WA treatment. The gene HMOX1, encoding the HO-1 protein, was the most significantly regulated and second most strongly upregulated gene by WA. Based on the complete set of differentially regulated genes, the transcription factor Nrf2 was indicated as the most significantly regulated pathway by WA. Moreover, CVD and atherosclerosis were in particular pointed out as the most prominent disease linked to dysfunctional regulation of WA-responsive target genes. We investigated the transcriptional effects of WA in two atherosclerosis-related cell types thereby focusing on genes related to atherosclerosis in the presence or absence of inflammation. We could conclude that WA exerted a global anti-inflammatory effect leading to potential anti-atherogenic effects. Further focusing on HO-1, we showed a WA-induced dose- and time-dependent upregulation of gene and protein levels of HO-1. Altogether, these transcription regulating effects of WA suggest that WA might exert a global anti-inflammatory and an anti-oxidative effect both potentially contributing to anti-atherogenic effects of WA. In an *in vitro* cellular system, WA treatment indeed reduced adhesion of monocytes to endothelial cells, a process in which ICAM-1 plays a major role. Although HO-1 upregulation has already been demonstrated to be involved in downregulation of ICAM-1 expression, we could not confirm this hypothesis in case of WA. Finally, we contributed to the elucidation of the transcription factor involved in WA-induced expression of HO-1 and the underlying molecular mechanism of WA activity within this signal transduction pathway. Knockdown experiments and investigation of endogenous levels and nuclear translocation pointed out the involvement of Nrf2 in the WA-induced upregulation of HO-1. With regard to the mechanism of Nrf2 activation, we identified KEAP1 as a binding partner of WA specifically via cysteine 151 in KEAP1, leading to stabilization of Nrf2.

V.2 Discussion and perspectives

Since CVD is defined as a chronic inflammatory disease it is apparent that WA could also have a protective contribution to CVD. The transcription factor NF-κB, which plays a primordial role in inflammation, is known to be inhibited by WA. NF-κB activation is linked with different stages of atherosclerosis. Inhibition of expression of adhesion molecules and monocyte adhesion to endothelial cells is an example on how inhibition of NF-κB can lead to potential protective effect in the formation of an atherosclerotic plaque. For example, Lee and colleagues demonstrated inhibition of upregulation of ICAM-1, VCAM-1 and E-selectin by WA in HUVEC cells which was suggested to be mediated by the NF-κB inhibiting capacity of WA. Moreover, they noted that WA reduced hyperpermeability of endothelial cells and transmigration of leukocytes *in vivo* [1]. Moreover, WA has been suggested to inhibit the development of myocardial fibrosis *in vitro* and *in vivo* via reduction of collagen type I. Excessive synthesis of collagen type I results in fibrosis of various organs, which is a major medical problem without an existing cure. This excessive synthesis of type I collagen in fibrosis is primarily due to stabilization of collagen mRNAs for instance by intermediate filaments composed of vimentin. Vimentin is one of the best described targets of WA, and thereby WA may reduce type I collagen production by disrupting vimentin filaments [2]. Nonetheless, the potential therapeutic applications of WA in CVD-related diseases are until now not extensively examined [3].

Though, other WA target proteins are plausible therapeutic targets for treatment of CVD. The UPS has been the subject of numerous studies to elucidate its role in cardiovascular physiology and pathophysiology. Malfunctioning of the proteasome has been described in many cardiovascular-related diseases. However, discussion remains whether to treath CVD with proteasome inhibitors or inducers. Also MAPKs have been the focus of extensive investigations concerning their contribution to CVD. However, there are still several contradictive results leading to a perception that they have both protective and detrimental effects in CVD [4]. Hsp90 inhibitors are suggested to attenuate inflammatory responses in atherosclerosis. So is Hsp90 expression associated with features of plaque instability in advanced human lesions [5]. On the other hand, Hsp90 is suggested to bind eNOS and soluble guanylate cyclase, leading to increased stability of both and so to increased endothelial integrity as both are involved in vascular relaxation [6]. Moreover, general overexpression of HSPs have shown to be protective against cardiac injury [7]. Studies on LXRα in the liver have recognized its crucial protective role in the initiation of a cross-talk between lipid metabolism and inflammation, important for development of atherosclerotic lesions. Interestingly, three critical mutations in the ligand binding domain of LXRα can be exploited for diagnosis of coronary heart diseases in human subjects and as a marker for exploring predisposition [8]. Similar mutations in notch1 and notch3 receptor have been associated with several types of cardiac diseases [9]. Animal studies implicate the transcription factors STAT1 and AP-1 as promising targets in treatment of atherosclerotic

disease [10, 11]. However, Meijer and colleagues published results from a clinical trial that do not characterize AP-1 as a therapeutic target for progressive human atherosclerotic disease [12]. Thus, although sufficient data are available of target proteins implicated in development of CVD which can be targeted by WA, the exact role within CVD and the mechanism of action has not completely been resolved. Therefore, further research is needed before we can conclude whether WA may exert cardioprotective effects via targeting those proteins. Moreover, novel chemoproteomic approaches which allow to map the WA interactome will undoubtedly discover more protein targets of interest.

In this project we obtained further evidence supporting the hypothesis of WA as a potential cardioprotective compound. An important observation is the inhibition of monocyte adhesion on endothelial cells by treatment with WA, an important early step in the development of atherosclerotic plaques. These results might be the consequence of a general downregulation of pro-inflammatory genes related to atherosclerosis. These downregulations, that we could detect in two atherosclerosis-related cell lines, are probably mainly due to the well described inhibition of NF-κB by WA. As the effect of WA on NF-κB is undoubtly a major contribution to WA´s potential protective contribution to CVD it seemed apparent to investigate this transcription factor. However, since many research groups reported already NF-κB inhibiting effects of WA in different cell lines, including HUVEC, upon stimulation by different pro-inflammatory conditions we chose not to investigate the effects of WA on NF-κB activation further. Therefore, besides these effects on expression of inflammatory genes, we performed a mRNA microarray in HUVEC cells, in order to unravel potentially new signal transduction pathways influenced by WA. This analysis revealed that different genes involved in CVD and Nrf2-dependent genes are differentially expressed in HUVEC cells by treatment with WA. Among these genes, HMOX1 was among the most interesting newly defined WA regulated gene. Of all Nrf2 regulated genes, HMOX1 is the most potently induced gene. We noticed that WA induced HMOX1 mRNA and HO-1 protein levels in a dose- and time-dependent manner and regulation of HO-1 is described as being cardioprotective. This antioxidant enzyme exhibits important immunomodulatory, anti-apoptotic, antiproliferative, pro-angiogenic and anti-inflammatory functions.

Based on the causal role of inflammation and oxidative stress within formation of an atherosclerotic plaque, an interplay between Nrf2 and NF-κB, two important pathways regulating both causal factors of atherosclerosis has also been evaluated in this PhD project. We questioned whether WA-induced upregulation of HO-1 could have an additional contribution on WA-dependent suppression of ICAM-1 expression. Along the same line, some researchers described additional contribution of HO-1 on ICAM-1 regulation by natural compounds which also exert NF-κB inhibiting capacities [13-17]. However, no additional effects were seen supporting the idea that the inhibitory effects on monocyte adhesion on endothelial cells by treatment with WA are probably mainly due to the well described inhibition of NF-κB by WA. From the obtained data we could conclude that WA positively influences the Nrf2 pathway and inhibits NF-κB activation in an independent manner. These

results suggest a regulatory effect of WA on oxidative stress and inflammatory conditions, making WA a potential compound for treatment for CVDs. Altogether, we expect that WA might excert anti-atherogenic effects. Though, as no proven anti-atherogenic effects of WA have yet been demonstrated in this work we therefore prefer to define it as potential anti-atherogenic effects of WA. Before addressing these anti-atherogenic effects to WA more research and *in vivo* experiments are needed.

These micro-array data identified the Nrf2 signaling pathway as a major affected signal transduction cascade of WA. A relationship between WA and Nrf2 has previously been suggested by different research groups. Comparison of gene expression profiling of a Chinese herb with WA suggested a regulation of Nrf2-dependent genes [18]. Our research group previously demonstrated via EMSA inhibition of DNA binding of Nrf2 in myelogenous leukemia cells after stimulation with 6 µM WA [19]. Vaishnavi *et al.* suggested an interaction of both WA and WN with amino acids Ala 69, Phe 71 and Gln 75 in the active site region of Nrf2. However, the authors were unable to demonstrate an effect of WA on this Nrf2 activation pathway evaluated by analysis of Nrf2 expression levels and nuclear translocation in the human osteosarcoma cell line U2OS [20]. The data described in this PhD project seems conflicting with these results in two aspects. No binding of WA to Nrf2 protein was noticed and we could observe effects of WA on the nuclear accumulation and activation of Nrf2.

Quite unexpectedly, only minimal changes of Nrf2 levels were induced by WA coinciding with strong HO-1 upregulation, suggesting that alternative mechanisms may also contribute to HO-1 upregulation. For example, Nrf2-independent HO-1 gene induction may rely on downregulation of Bach1. WA-induced Bach1 regulation could be via cysteine residues in Bach1, since regulation of Bach1 via its cysteine residues as an alternative mechanism is suggested by several other researchers [21-24]. In addition, possible WA effects on BRG1, influencing the recrutement of RNA polymerase II to the HO-1 promoter could affect HO-1 upregulation [25]. Also the high number of ARE-binding sites in the HMOX1 gene promoter and posttranslational modifications of Nrf2, including phosphorylation, might result in a synergistic effect of Nrf2 on HO-1 upregulation. From the mRNA array and qPCR confirmation, HMOX1 is by far the most strongly upregulated gene of all Nrf2-regulated genes influenced by WA in HUVEC cells. This suggest possible preference. However, future studies on the regulation of other Nrf2-related genes are required to gain better insight whether WA exerts specific gene preferences within the Nrf2 pathway or not.

We contributed to the investigation of the molecular effects of WA on the Nrf2 pathway. A direct binding of WA with KEAP1 was demonstrated leading to increased stabilization of Nrf2. Moreover, we defined cysteine 151 as an important cysteine within KEAP1 for the WA-KEAP1 binding. However, the exact mechanism of Nrf2 stabilization due to WA-KEAP1 binding remains unsolved. Co-immunoprecipitation experiments on overexpressed proteins did not reveal any effect of WA on the integrity of the Nrf2/KEAP1/Cul3/Rbx1-E3 complex. In contrast, disruption of the binding KEAP1-Cul3 interaction has already been demonstrated

for cysteine 151-targeting compounds. Also mutagenesis of cysteine 151 site to bigger amino acids was demonstrated to affect this interaction [26]. Indeed, cysteine 151 is located closely to the interface of KEAP1 and Cul3. However, to provide conclusive data, these interactions should be analyzed in more detail at the endogenous level and include time- and dose-responses of WA treatment to elucidate the exact mechanism of impairment of the structural integrity by WA on the complex. Furthermore, other cysteine 151-dependent mechanisms might also be involved since substitution of this residue with the small hydrophobic amino acid alanine could not disrupt the KEAP1-Cul3 interaction but it does inhibit degradation of Nrf2 [27]. Concerning the KEAP1-Nrf2 interaction, two models have been proposed: one model suggests a complete disruption between Nrf2 and the KEAP1/Cul3/Rbx1-E3 complex upon stimulation. Whereas a second model indicates that binding between Nrf2 and KEAP1 is only disrupted at the DLG binding sites, retaining a weaker interaction of Nrf2 with KEAP1. This second model certainly might explain our observation of lack of effect of WA on the integrity of the KEAP1-Nrf2 interaction.

Additional mechanisms might also be implicated in WA-mediated Nrf2 activation. Crosstalk between known targets of WA and Nrf2 have already been described. As mentioned before, an important target pathway of WA is the NF-κB pathway where *in silico* studies suggested that inhibition of the NF-κB pathway by WA is a result of the inactivation of the IKK complex by binding of WA to IKKγ and IKKβ [28]. An intensive interplay between NF-κB and Nrf2 has already been described often leading to opposite effects where upregulation of one transcription factor inhibits the other. The exact mechanism how this cross talk is regulated still requires further investigation, but direct effects and influence of co-activators (CBP) and co-inhibitors (HDAC3) might be involved in NF-κB´s regulating effect on Nrf2 [29-32]. Moreover, several kinases are involved in the Nrf2 activation pathway and regulation of their activity would certainly lead to fine tuning of the activity of this transcription factor. Several studies have investigated the effect of WA on different kinases, where the most examined kinases are the MAP kinases p38, JNK, ERK and Akt. Thereby, WA might further modulate Nrf2 activity via kinases/phosphatases [33-52]. Western blot analysis demonstrated the presence of a doublet band of Nrf2, of which the upper band might correspond to phosphorylated Nrf2. Since mainly the upper band of the Nrf2 doublet increases and predominates in the nucleus in the presence of WA, this might suggest an effect of WA on the phosphorylation status by modulation of kinase activity. However, this hypothesis certainly requires further investigation. Another WA target involved in the Nrf2 pathway is the proteasome. WA-mediated inhibition of the proteasome can also contribute to stabilization and accumulation of Nrf2. Nonetheless, whether WA is truly mediating inhibition is still a matter of debat. Alternatively, microarray data reveal regulation of the sequestosome1 (SQSTM1) or p62 by WA. This scaffold protein binds polyubiquitinated proteins and the autophagic machinery. Interestingly, an interaction between p62 and KEAP1 has been described, regulating KEAP1 expression via an autophagic degradation [53-58]. We noticed a molecular weight shift of KEAP1 and reduced expression in the presence of high concentrations of WA and/or when longer induction times were applied. This

reduction of KEAP1 level was also observed in the presence of the proteasome inhibitor MG132, indicating that non proteasomal degradation mechanisms are at least in part responsible for KEAP1 degradation. It is plausible that WA-induced upregulation of p62 would lead to an increased degradation of KEAP1. However, whether this phenomenon contributes to WA-induced HO-1 expression is still unclear since HO-1 upregulation occurs already at lower concentrations of WA and earlier time points than degradation of KEAP1 could be observed. Furthermore, it cannot be excluded that WA also regulates Bach1 activity via cysteine residues, thereby upregulating HO-1 in an Nrf2-independent manner. Or through BRG1, both mentioned earlier in this discussion. Since an Nrf2-related interactome and regulome study suggested at least 289 protein-protein, 7469 TF-DNA and 85 miRNA interactions [59], and the knowledge that proteins defined as WA targets are only a glance of potential binding partners of WA, it is likely that the effects of WA within the Nrf2 pathway are far more complex than illustrated here (Fig 1.1 and 1.2).

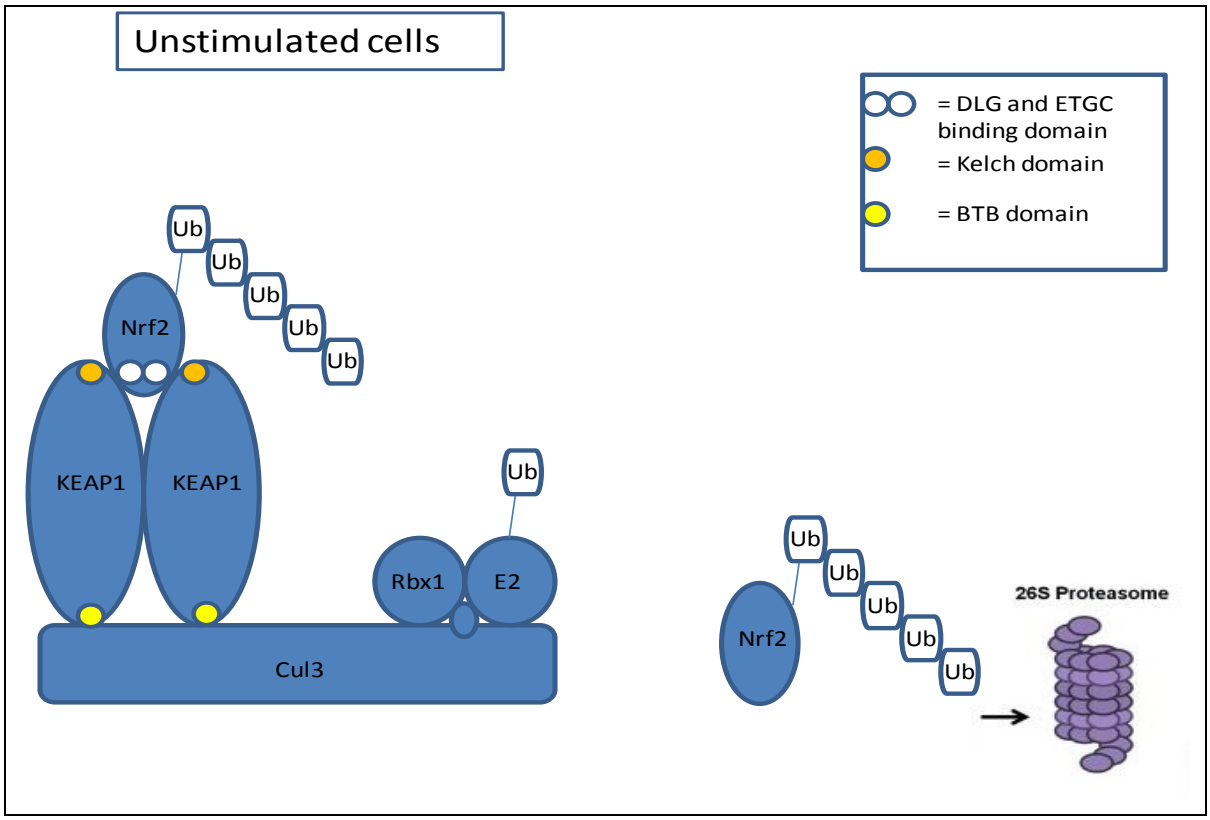

Fig 1.1 The KEAP1/Cul3/Rbx1-E3/Nrf2 complex leading to constant degradation of Nrf2 through proteasomal degradation in unstimulated cells.

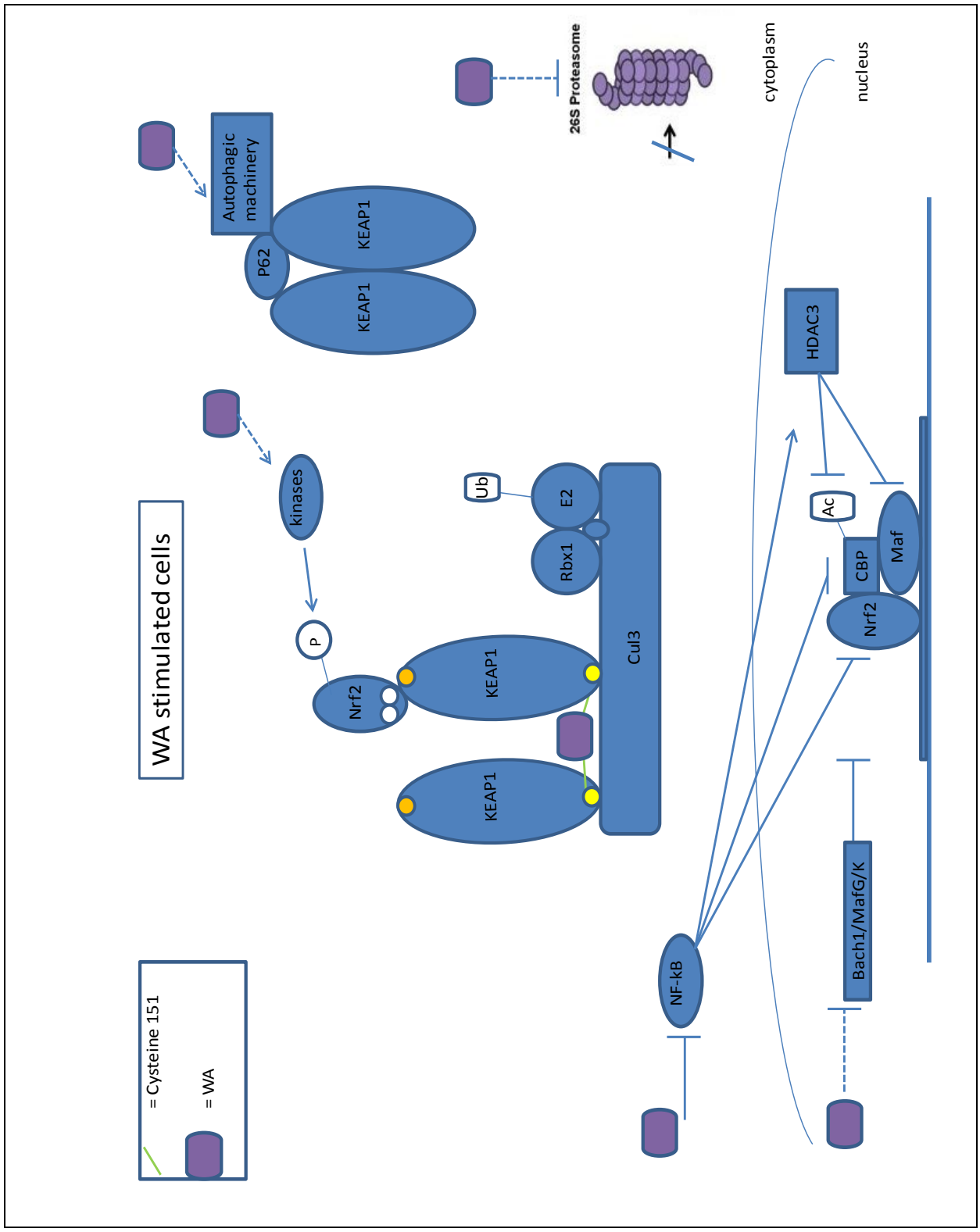

Fig 1.2 The KEAP1/Cul3/Rbx1-E3/Nrf2 complex in the presence of WA. Effects of WA on its targetproteins leading to possible changes in the Nrf2 pathway.

It is noted that WA is able to induce ROS. Most authors suggest a selective preference of WA for ROS induction and apoptosis in cancerous cells and not in normal cells [44, 60-63]. In contrast, some researchers mention also WA-induced ROS production in normal cells, however this occurs only at high concentrations and/or long time points of WA induction

[20, 64]. Various animal models and human *in vitro* cell studies have shown that oxidative stress due to excessive ROS production, is a major causative factor for the development and progression of CVD [65, 66]. We show that WA is able to bind KEAP1 via cysteine 151 leading to Nrf2 accumulation, possibly leading to HO-1 upregulation. However, we did not investigate whether there was additional ROS production by WA. It is not likely that the concentrations of WA used in this work would cause excessive ROS production in normal cells, including the endothelial cells used. Nonetheless, for further experiments, it would be interesting to repeat those experiments in the presence or absence of a ROS scavenger. If it appears that ROS is produced, it would probably only lead to mild oxidative stress and would even posses cardioprotective effects. As signal transmission of ROS takes place at free sulfhydryl groups of cysteine residues, including those present in KEAP1, leading to intra- and intermolecular disulifide bonds, altering the protein conformation it would lead to Nrf2 activation [67]. This WA-induced ROS, activating Nrf2, would counteract its own presence and put back the balance with the cellular antioxidant capacity. Along the same line, curcumin triggers an atheroprotective response through mild oxidative stress-induced HO-1 upregulation [68]. Only if ROS production is too strong this balance would be severely disturbed and ROS would lead to progression of atherosclerosis. Thus, administration in a highly controlled manner is obligatory if we want to introduce WA as a cardioprotective compound. Low concentrations of WA induction are likely to be sufficient to exert beneficial effects on the Nrf2 pathway. Cysteines in KEAP1 have an ultra-sensitive nature, so the Nrf2 pathway has a low concentration threshold. Compounds that undergo Michael addition have in general different binding affinities for their different target proteins. For example, low concentrations of triterpenoids, such as CDDO-Im, preferentially interact with targets such as KEAP1 leading to a cytoprotective effect whereas high concentrations interact with proteins with a lower binding affinity such as IKK to inhibit proliferation and induce apoptosis [69]. Moreover, endothelial cells do not become refractory to repeated activation of the Nrf2 pathway, so no increased concentration in time is needed [70]. Polycaprolactone implants embedded with WA was suggested to allow the administration of WA in a systemic, controlled and long term way. However, intermittent dosing for Nrf2 and HO-1 activation was preferred rather than long term administration, as chronic activation of Nrf2 and HO-1 is likely to be detrimental for health [70]. From our results we can conclude that the optimal time point for WA-induced upregulation of HO-1 is 6 hours. We did not investigate whether shorter time exposure of WA reveals a similar time-dependent expression profile of HO-1 after 6 hours. If expression profiles would be similar, the serum half-life of WA of 1.5 hours is not a therapeutic obstacle. If a minimum of 6 hours exposure of WA appears to be necessary for optimal HO-1 expression, we may experiment with the stable derivative of WA, 3-azido Withaferin A (3-azidoWA) [71].

Our data indicate that HO-1 is upregulated by WA in a dose- and time-dependent manner. Knockdown experiments and investigation of endogenous levels and nuclear translocation point out the involvement of Nrf2 in this WA-induced upregulation of HO-1. We indicate KEAP1 as a binding partner of WA specifically via cysteine 151 in KEAP1, leading to

stabilization of Nrf2. Together with WA´s global anti-inflammatory effect on genes related to atherosclerosis and WA-induced inhibition of monocyte adhesion to endothelial cells this data indicate that WA holds promise to elicit potential cardioprotective properties. Further *in vivo* experiments should clarify whether this molecular observations within the Nrf2 pathway result in effective short or long term cardioprotective actions.

Additional *in vivo* experiments should preferably be performed in atherosclerosis-related models. As several genetic and enviromental factors play a role in the pathophysiology, it makes it difficult to match CVD-related diseases with a single experimental model. Our preference would go to current mouse models for atherosclerosis, based on genetic modification of lipoprotein metabolism with additional dietary changes, including LDLR -/-, LDLR -/- / ApoE -/-, apoE or mouse models of diabetes-accelerated atherosclerosis to investigate the potential cardioprotective effects of WA. Future *in vivo* experiments are planned in collaboration with Prof. Marc Merx from Dusseldorf University Hospital to evaluate potential cardioprotective activities of WA in WT and Nrf2 KO mice, focusing on the effects of WA on the Nrf2 pathway.

In addition, analyses concerning stability, metabolite formation and toxicity must be performed. In a pilot experiment with WA in the CHIME (gastrointestinal simulator) in collaboration with Prof. T. Vandewiele (UGent), a progressive decrease in WA levels/activity was observed following passage to the CHIME. Moreover, upon oral administration only low plasma concentrations can be reached. In a recent published pharmacokinetic study improved WA formulations and analytical methods allowed to detect peak values up to 200 nM-2 µM WA in mouse plasma-serum following IP administration [72-74]. Consequently, preferably IP administration would be recommended for future *in vivo* experiments. So far, only pharmacokinetic studies have been focusing on WA concentrations in plasma after oral intake or IP injection and no data are available on WA derived metabolites. As clearance of WA in plasma is after 1 or 2 hours and since there is a progressive decrease in WA levels/activity following passage to the CHIME it is likely that WA is metabolized. This metabolisation could occur through micro-organisms in the gut or via the body, for example through sulfation or glucuronidation. Binding to plasma albumin is plausible, however experiments in serum free medium exerted comparable results. All together, this point out the importance of investigating metabolite formation.

To conlude with a quote of John Archibald Wheeler “We live on an island surrounded by a sea of ignorance. As our island of knowledge grows, so does the shore of our ignorance.” Many things still to be discovered concerning this research topic, but hopefully this Phd thesis added a grain of sand to this island.

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Part VI. Algemene discussie, samenvatting en toekomstperspectieven

VI.1 Samenvatting

Op dit moment sterven wereldwijd de meeste mensen aan cardiovasculaire ziekten (cardiovascular diseases = CVD). Hierdoor is onderzoek omtrent deze ziekten van uiterst belang. De meest voorkomende oorzaak van deze ziekten is atherosclerosis, dikwijls veroorzaakt door oxidatieve stress. Monocyten die adhereren aan de endotheliale vaatwandcellen vormen een van de vroegste fases bij de vorming van een atherosclerotische plaque. Hierbij vervullen adhesiemoleculen een sleutelrol.

Nrf2 fungeert als een belangrijke transcriptiefactor in de cel voor het bestrijden van endogene en exogene stress factoren. Deze transcriptiefactor is gebonden aan een KEAP1 homodimeer in een KEAP1/Cul3/Rbx1-E3-complex in het cytoplasma in cellen in rust. Hierdoor wordt Nrf2 constant geubiquitineerd en afgebroken. Het eiwit KEAP1 is verantwoordelijk voor deze constante afbraak van Nrf2 maar functioneert ook als sensor. Door deze combinatie van beide functies controleert KEAP1 de transcriptionele regulatie van Nrf2-gemedieerde genen als antwoord op verschillende stimuli. Verschillende van deze stimuli kunnen cysteines binnen KEAP1 beïnvloeden waardoor Nrf2 geactiveerd wordt en voor een opregulatie zorgt van verschillende ARE-afhankelijke genen zoals bijvoorbeeld antioxidant genen. Uit de groep van deze genen is HO-1 een belangrijk antioxidant enzym. Dit enzym heeft een sterk cardioprotectief effect omwille van zijn anti-oxidatieve,

immuunmodulerende, anti-apoptotische, antiproliferatieve, pro-angiogene en anti-inflammatoire functies.

WA is een van de belangrijkste medicinale withanolides die kunnen teruggevonden worden in de plant *WS*. De wortels, bladeren en besjes worden gebruikt in de ayurvedische geneeskunde voor het bestrijden van verschillende ziektes. WA bezit een heel arsenaal aan protectieve eigenschappen zoals anti-inflammatoire, anticarcinogene, anti-angiogene, anticonvulsieve, immuunsuppressieve en pro-apoptotische karakteristieken. Ondanks deze lange lijst van gezondheidsbeschermende eigenschappen is er nog weinig bekend over de moleculaire mechanismen die zorgen voor deze eigenschappen, alsook over potentiële anti-atherosclerotische eigenschappen van WA.

Aangezien WA gekend staat omwille van zijn celdood inducerende eigenschappen hebben we eerst gezocht naar een WA concentratie waarbij cellen geen cytotoxiciteit vertoonden. In endotheliale cellen, monocyten en macrofagen constateerden we dat WA aan concentraties tot 2 µM voor 24 uur geen celdood induceerde. Deze maximale concentratie hebben we dan ook in de volgende experimenten nooit overschreden. In HUVEC cellen hebben we de transcriptionele regulatie van de genexpressie in antwoord op WA onderzocht door middel van een mRNA microarray. Hieruit bleek dat HMOX1, een gen dat codeert voor de HO-1 proteïne en de transcriptiefactor Nrf2 het sterkst gereguleerd werden door WA. Uit deze array bleek ook dat verschillende genen die significant gereguleerd waren door WA eveneens een belangrijke rol spelen bij CVD en atherosclerosis. Naast deze globale genexpressie analyse onderzochten we ook meer specifiek de transcriptionele regulatie van atherosclerosis gerelateerde inflammatoire genen in immuuncellen en endotheelcellen betrokken bij plaquevorming. Verder hebben we fijnregulatie bestudeerd van HMOX1/HO-1 en konden besluiten dat WA-blootstelling HO1-expressie sterk kan induceren in een dosis en concentratie-afhankelijke manier. De anti-inflammatoire en anti-oxidatieve effecten van WA kunnen mogelijks een beschermende werking uitoefenen tegen CVD. Daarnaast konden we ook een verminderde binding van monocyten aan HUVEC endotheelcellen observeren. Daar ICAM-1 hier een sleutelrol in speelt en WA de expressie van ICAM-1 sterk inhibeert, is dit mogelijks de oorzaak. Verder hebben we onderzocht of WA-gemedieerde toename van HO-1 betrokken is bij afname van ICAM-1, maar dit bleek niet het geval te zijn. Als laatste onderzoeksdeel hebben we een tipje van de sluier proberen op te lichten omtrent het moleculaire mechanisme van WA binnen de Nrf2-signaaltransductieweg. Finaal konden we aantonen dat WA HO-1 kan opreguleren via activatie van Nrf2 na binding van WA aan KEAP1 via de cysteine 151.

VI.2 Discussie en toekomstperspectieven

De inflammatoire transcriptiefactor NF-κB speelt een cruciale rol in het ontstaan van CVD. Daar CVD als een chronisch inflammatoire ziekte wordt beschouwd, kunnen NF-κB-

inhibitoren zoals WA een gunstig (beschermend) effect uitoefenen in CVD. Nochtans zijn de potentiële therapeutische effecten van WA binnen CVD amper onderzocht [1]. Eén van de weinige onderzoeken hieromtrent toont aan dat inhibitie van ICAM-1, VCAM-1 en E-selectin door WA in HUVEC cellen te wijten is aan de inhiberende effecten van WA op NF-κB. Een ander onderzoek demonstreert dat WA de vorming van myocardiale fibrose kan inhiberen in *in vitro* en *in vivo* onderzoek via de vermindering van collageen type I. Aanmaak van collageen type I zou kunnen komen via stabilisatie van het mRNA door vimentine. Aangezien WA vorming van de vimentine-filamenten verhindert zorgt het zo voor verminderde aanmaak van collageen type I en fibrose van verschillende organen [2]. Van verschillende doelwitten van WA werden reeds verbanden met CVD aangetoond, waardoor het idee dat WA een beloftevolle cardioprotectieve molecule kan zijn wordt versterkt. Zo is de rol van het proteasoom en MAPKs in CVD meerdere malen beschreven, maar desondanks is het behandelen van CVD met proteasoom- of kinase- inhibitoren/inductoren nog steeds omstreden [3]. Ook over Hsp90 inhibitoren zijn er tegenstrijdige gegevens beschikbaar [4,5]. Naast inhibitie van hsp90, is bijvoorbeeld gebleken dat ook overexpressie van HSPs beschermend kan zijn bij hart aandoeningen [6]. WA werd ook beschreven als mogelijk ligand voor de hormoonreceptor LXR, die een cruciale rol speelt in vetmetabolisme en ontstekingsprocessen bij CVD [7]. Enkele publicaties tonen aan dat WA notch signalisatie reguleert en notch1 en notch3 receptoren zijn mogelijks betrokken bij CVD[8]. Ook STAT1 en AP-1 blijken beloftevolle doelwitten te zijn als behandeling voor CVD [9-11]. Ondanks al deze observaties moet er nog verder onderzoek gebeuren voor we kunnen aantonen dat WA een protectieve functie kan uitoefenen in CVD. Zonder enig twijfel zullen er ook nog een hele boel nieuwe proteïnen worden ontdekt die beïnvloed worden door WA.

Uit de mRNA-array genexpressie-analyse bleek dat de signaaltransductieweg van Nrf2 sterk gereguleerd werd door WA, in overeenstemming met enkele eerder gepubliceerde observaties [12]. Ook in ons labo werd eerder een inhibitie van DNA binding van Nrf2 aangetoond in leukemie cellen na een behandeling van 6 µM WA [13]. Een interactie van WA en WN met Nrf2 werd ook gesuggereerd via *in silico* predictie, maar de onderzoekers konden deze bevinding niet experimenteel aantonen [14]. Wij zagen daarentegen geen binding van WA met Nrf2 maar konden wel een activatie van Nrf2 waarnemen na blootstelling aan WA.

Ondanks de kleine hoeveelheden geactiveerd Nrf2 neemt de expressie van het targetgen HMOX1/HO-1 toch sterk toe. Ofschoon silencing van Nrf2 voor een sterke daling van HO-1 expressie zorgt en zijn betrokkenheid bewijst, kunnen we bijkomende regulatiemechanismen niet uitsluiten bvb. via regulatie van ARE binding via de transcriptiefactor Bach1, fijnregulatie door posttranslationele modificaties en of de relatief grote hoeveelheid ARE bindingsdomeinen in de promoter van HO-1.

Een directe binding van WA met KEAP1 via cysteine 151, zorgde voor een verhoogde stabilisatie van Nrf2. Uit verder onderzoek moet nog blijken hoe precies deze stabilisatie in zijn werk gaat. We konden namelijk geen veranderingen in de structuur van het

Nrf2/KEAP1/Cul3/Rbx1-E3 complex waarnemen na behandeling met WA. Nochtans is een verbreking van de verbinding aangetoond tussen KEAP1 en Cul3 voor Nrf2 stimuli die een voorkeur hebben voor cysteine 151, aangezien cysteine 151 dicht bij de binding ligt tussen beide proteïnes. Ook kon het veranderen van deze cysteine in aminozuren met een groter volume deze binding beïnvloeden [15,16]. Er zijn twee modellen beschreven voor de interactie tussen KEAP1 en Nrf2. Een mogelijkheid is dat deze binding volledig wordt verbroken en een andere dat er enkel een verbreking is ter hoogte van de DLG binding. Waarschijnlijk is deze laatste mogelijkheid een verklaring voor onze observaties.

Het is goed mogelijk dat er bijkomende mechanismen van belang zijn bij de activatie van Nrf2 door WA. Zo zijn er interacties van verschillende doelwit proteïnes van WA met Nrf2 beschreven. Zo blijkt dat Nrf2 en NF-κB, dat kan gëinhibeerd worden door WA [17] elkaar reciprook kunnen beïnvloeden. Het exacte mechanisme moet nog worden opgehelderd maar er zijn reeds indicaties dat NF-κB een inhiberend effect kan hebben op Nrf2 via het beïnvloeden van co-activatoren (CBP) en co-inhibitoren (HDAC3) [18-21]. Ook zijn er verschillende kinasen betrokken bij de regulatie van Nrf2 en is reeds aangetoond dat WA een invloed heeft op meerdere kinasen en fosfatasen die Nrf2/ NF-κB kunnen modificeren [22-41]. In Western analyse konden we een doublet van het Nrf2-eiwit observeren wat waarschijnlijk duidt op fosforylatie van Nrf2. Aangezien de bovenste band in dikte toenam en dit hoofdzakelijk te zien was in de nucleus, nemen we aan dat WA misschien Nrf2 kan fosforyleren via de modulatie van een kinase. Deze hypothese moet zeker nog verder onderzocht worden. Indien blijkt dat WA het proteasoom inhibeert is het mogelijk dat ook op deze manier WA kan bijdragen aan de stabilisatie van Nrf2. Uit de microarray analyse bleek dat WA sequestosome1 (SQSTM1) of p62 kan reguleren. Een interactie tussen p62 en KEAP1 is reeds beschreven en kan KEAP1-degradatie induceren via een autofagie-mechanisme [42-47]. In Western analyse, kon WA bij hoge concentraties of lange blootstelling, de proteïne band van KEAP1 doen opschuiven naar een hoger moleculair gewicht en de expressie ervan laten afnemen. Dit kon ook worden waargenomen in de aanwezigheid van de proteasoom-inhibitor MG132, mits in mindere mate. Dit doet vermoeden dat het proteasoom slechts partieel verantwoordelijk is voor de afbraak van KEAP1. Het is mogelijk dat de opregulatie van p62 door WA zorgt voor deze toegenomen afbraak van KEAP1. Aangezien dat WA geïnduceerde opregulatie van HO-1 reeds plaats vindt voor dat deze afbraak van KEAP1 waarneembaar is, suggereert dit dat het destabiliserend effect van WA op KEAP1 waarschijnlijk weinig bijdraagt aan de HO-1-effecten die wij waarnemen. Ook kunnen we niet uitsluiten dat WA een effect heeft op de cysteines van Bach1 en hierdoor deze zijn activiteit beïnvloedt [48-51]. Vermoedelijk is het effect van WA op de Nrf2 signaaltransductieweg nog complexer daar er minstens 289 proteïne-proteïne , 7469 TF-DNA en 85 miRNA interacties beschreven zijn in de Nrf2 signaaltransductieweg [52].

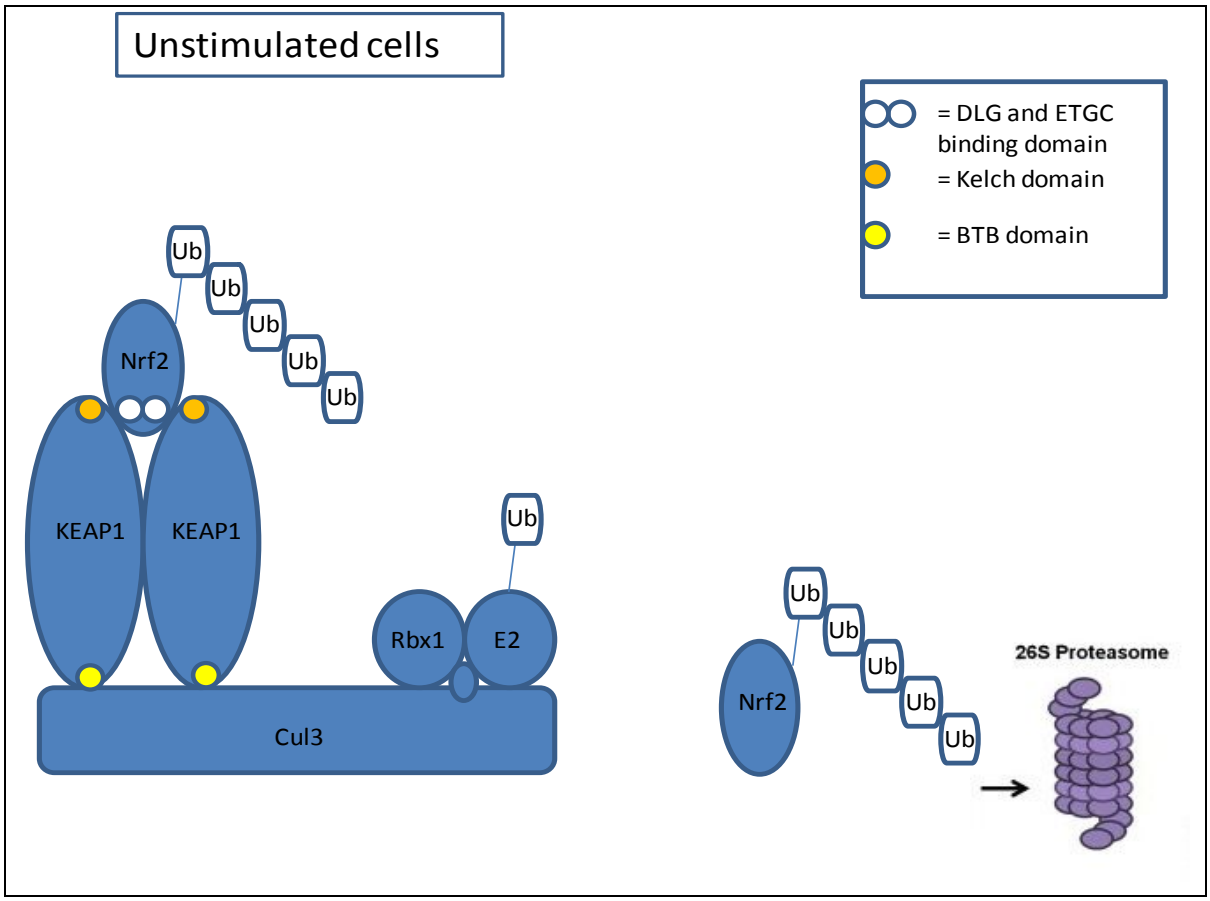

Fig 1.1 Weergave van het KEAP1/Cul3/Rbx1-E3/Nrf2-complex dat zorgt voor een constante afbraak van Nrf2 door middel van ubiquitinering in afwezigheid van een stimulus. Na stimulatie, wordt Nrf2 vrijgesteld en gestabiliseerd.

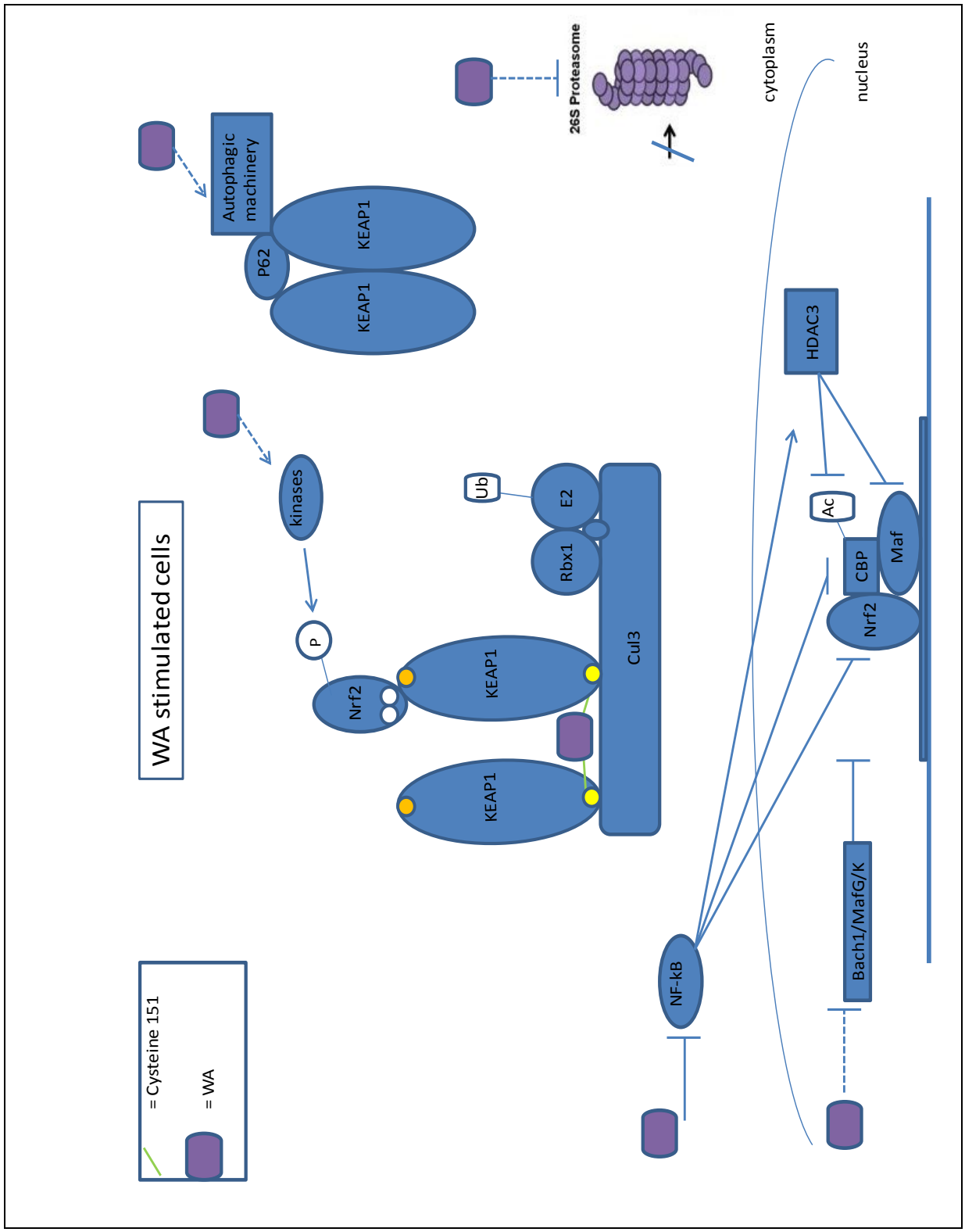

Fig 1.2 Samenvattend werkmodel van het KEAP1/Cul3/Rbx1-E3/Nrf2-complex in de aanwezigheid van WA. Mogelijke aangrijpingspunten van WA op de verschillende activatiestappen van de Nrf2-signaaltransductieweg zijn aangegeven in het schema.

Verschillende auteurs beschrijven dat WA ROS kan induceren wat leidt tot apoptosis in kankercellen en niet in gezonde cellen [33, 53-56]. Sommige onderzoekers beschrijven ook celdood via ROS geïnduceerd door WA in gezonde cellen maar zij maakten meestal gebruik van relatief hoge concentraties of lange inductie-tijden van WA [14, 57]. Daarnaast blijkt dat ROS ook een belangrijke oorzaak zijn voor CVD en dat ROS de Nrf2 signaaltransductieweg kunnen induceren [58, 59]. We hebben echter niet onderzocht of WA in onze experimenten ROS induceerde en of deze betrokken zijn bij de modulatie van KEAP1/Nrf2-signaaltransductiewegen. Het lijkt interessant om in de toekomst deze experimenten te herhalen in de aan- of afwezigheid van een ROS scavenger, ofschoon we bij de gebruikte lage WA concentraties geen en/of slechts lage ROS productie verwachten en lage concentraties ROS eerder een protectief effect vertonen [60]. De mogelijkheid van WA om hoge apoptose-gerelateerde ROS-concentraties te induceren zorgt wel dat de concentratie van WA sterk moet gecontroleerd worden indien het als therapeutisch middel wordt gebruikt. Meerdere factoren wijzen erop dat er slechts een lage dosis zal moeten worden toegediend. Zo blijkt dat cysteines in KEAP1 zeer gevoelig zijn voor variaties in concentraties van Nrf2 activatoren en dat de Nrf2-signaaltransductieweg even gevoelig blijft voor stimuli, zelfs na repetitieve blootstelling aan de stimuli [62]. Om deze concentratie sterk te kunnen controleren tijdens de behandeling kunnen we gebruik maken van polycaprolactone implantaten. Het enige nadeel hierbij is dat Nrf2 en HO-1 activatie niet chronisch mag gebeuren [62]. We hebben in ons onderzoek niet getest of de HO-1-hoeveelheden even sterk zouden zijn na 6 uur indien we de cellen slechts kortstondig blootstellen aan WA. Indien er een even sterke opregulatie zou zijn, zou de serum halfwaardetijd van WA in farmacokinetiekstudies (slechts 1.5 uur) geen obstakel vormen. Indien zou blijken dat een langere blootstelling van WA nodig is om een therapeutisch resultaat te bekomen dan kunnen we gebruik maken van een meer stabiele variant van WA, 3-azido Withaferin A (3-azidoWA) [63].

We kunnen besluiten dat WA zorgt voor een opregulatie van HO-1 in een concentratie- en tijdsafhankelijke wijze en dit via Nrf2. WA zorgt voor verhoogde stabiliteit van Nrf2 door een binding aan te gaan met KEAP1 en hierbij blijkt de cysteine 151 een cruciale rol in te spelen. Samen met de algemene anti-inflammatoire effecten van WA die we geobserveerd hebben kunnen we concluderen dat WA een molecule is met potentieel interessante therapeutische mogelijkheden voor de behandeling en/of preventie van CVD. Bijkomende *in vivo* experimenten zijn echter noodzakelijk om na te gaan of WA zijn beloftevolle verwachtingen kan inlossen in CVD, bij voorkeur in atherosclerose gerelateerde diermodellen. Voorbeelden hiervan zijn LDLR -/-, LDLR -/- / ApoE -/-, apoE of diabetes-geaccelereerde muis modellen. Het zijn modellen voor atherosclerose gebaseerd op genetische modificatie van het lipoprotein metabolisme met een bijkomend aangepaste voeding. Er is een samenwerking gepland met Prof. Marc Merx van de universiteit in Dusseldorf, waarbij de potentiële cardioprotectieve activiteiten van WA in WT en Nrf2 KO muizen zal worden onderzocht.

Het zou ook interessant zijn meer te weten te komen over de stabiliteit, de metabolisatie en de toxiciteit van WA. Een experiment met de CHIME (een simulator van het gastrointestinale tractus) bij Prof. T. Vandewiele (UGent) gaf aan dat er een daling is van WA hoeveelheid en activiteit. Dit, samen met het gegeven dat slechts lage plasma concentraties kunnen bereikt worden na orale inname en dat concentraties tot 2 µM kunnen bereikt worden na IP toediening leid tot het idee dat WA in toekomstige *in vivo* experimenten het best IP kunnen worden toegediend. Weinig is tot nu toe geweten over de metabolisatie van WA, maar er zijn verschillende indicaties waardoor we kunnen aannemen dat WA hoogstwaarschijnlijk wordt gemetaboliseerd.

Om af te sluiten met een quote van John Archibald Wheeler “We live on an island surrounded by a sea of ignorance. As our island of knowledge grows, so does the shore of our ignorance.” Zoals bij de meeste onderzoeken blijven we met nog meer vragen over dan waarmee we begonnen zijn, maar mogen we hopen dat deze thesis een kleine bijdrage heeft geleverd aan de groei van dit eiland!

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Part VII. Addenda

VII.1 Abbreviation list

ADTM	a synthetic derivative of danshensu out of the root of Chinese sage
AGE	advanced glycation endproducts
AK	adenylate kinase
Ang II	
AP	andrographolide
AP-1	activator protein 1
ApoE	Apolipoprotein E
ARE	antioxidant response element
ATF3	Activating transcription factor 3
Bach	breakpoint cluster region/abelson murine leukemia viral oncogene homolog
BCECF-AM	2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester
BG-12	dimethyl fumarate
BHA	Butylated hydroxyanisole
BRG1	Brahma-related gene 1
BSO	buthionine (S,R)-sulfoximine
BTB	Bric-a-brac tramtrack broad complex
bzip	basic leucine zipper
CARM1	co-activator arginine methyltransferase
CBP	cyclicAMP response element binding protein (CREB)-binding protein
CD-36	cluster of differentiation 36
CDDO-Im, CDDO-Me	imidazole and methyl ester derivative of 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO), CDDO-Me is also called bardoxolone methyl
CF	Cystic Fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	cyclic guanosine monophosphate
Cin	cinnamaldehyde
CK2	casein kinase 2
CNC	cap´n´collar
CO	carbon monoxide
COX-2	cyclo-oxygenase 2
CRE	cAMP response element
CREB	cyclicAMP response element binding protein
Crm1	chromosome region maintenance 1
CTR	C-terminal region
Cul3	cullin3
CVD	cardiovascular disease

DC	DGR and CTR together
DGR	double glycine repeat
DLG	low affinity motif
DNCB	dinitrochlorobenzene
DTT	Dithiothreitol
EDN1	endothelin1
EGCG	epigallocatechin gallate
EL	endothelial lipase
ENC1	Ectoderm-neural cortex protein 1
eNOS	endothelial nitric oxide synthase
ERAD	ER-associated protein degradation
ERK	extracellular signal-regulated kinase
ERα	estrogen receptor alfa
ETGE	high affinity motif
EMSA	electrophoretic mobility shift assay
FOXO3A	forkhead box transcription factor
GCLC	glutamate cysteine ligase catalytic subunit
GCLM	Glutamate cysteine ligase regulatory subunit
GFAP	glial fibrillary acidic protein
GRAS	Generally Regarded As Safe
GSH/GSSG	glutathione in reduced (GSH) and oxidized (GSSG) states
GSK3β	glycogen synthase kinase 3β
HDAC3	histon deacetylase 3
HDL	high density lipoprotein
HDL-C	high density lipoprotein cholesterol
HMEC	human mammary epithelial cell line
HMOX1	heme oxygenase 1 gene
HO-1	heme oxygenase 1 protein
HSF-1	heat shock factor 1
HUVEC	human umbilical vein endothelial cell
ICAM-1	intercellular adhesion molecule-1
IKK	IκB kinase
IL-10	interleukin 10
IL-1β	interleukin-1 beta
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
IPA	ingenuity pathways analysis
IVR	intervening region
IκB	inhibitor of kappa B

JNK	c-jun N-terminal kinase
KEAP1	Kelch-like ECH-associated protein 1
KLF2	kruppel-like factor 2
LDL-R	LDL-Receptor
LPS	Lipopolysaccharide
LXRα	liver X receptor alfa
maf	musculoaponeurotic fibrosarcoma oncogene homolog
MAPK	mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
M-CSF	macrophage colony-stimulating factor
MMP	matrix metalloproteinases
NAC	N-acetyl cysteine
NADPH/NADP+	nicotinamide adenine dinucleotide phosphate and its reduced form NADPH
NAPQI	N-acetyl-p-benzoquinineimine
Neh	Nrf2 erythroid-derived CNC homology protein homology domains
NFE2	nuclear factor erythroid derived 2
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NOX	NADPH oxidases
NQO1	NAD(P)H dehydrogenase quinone 1
Nrf2	nuclear factor erythroid-related factor-2
NTR	N-terminal region
oxLDL	oxidized low density lipoprotein
p53	protein 53 or tumor protein 53
p62 or SQSTM1	sequestosome 1
PDGF	platelet-derived growth factor
PEI	Polyethylenimin
PGI2, PGJ2, PGE2	prostaglandin I2, J2 and E2
PI3K	phosphatidylinositol 3-kinase-dependent pathway
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PPARγ	peroxisome proliferator-activated receptor gamma
Prdx1	peroxiredoxin1
PRMT1	protein arginine methyl-transferase
ProTα	prothymosin-α
RAC3	enhance the ability of receptor associated co-activator 3
RARα	Retinoic acid receptor alfa

Rbx1	RING box protein 1
ROS	reactive oxygen species
RXR	retinoid X receptors
SFN	sulforaphane
SIRT1	sirtuin 1
SOD	superoxide dismutase
SR-A	macrophage scavenger receptor class A
STAT	signal transducer and activator of transcription family
SWT	Si-Wu-Tang
tBHQ	tert-butylhydrochinon
TLR	toll like receptors
TNF	tumor necrosis factor
TRE	TPA response element
UPP	ubiquitin–proteasome pathway
UPR	unfolded protein response
UPS	protease complex of the ubiquitin and proteasome-dependent proteolytic sytem
VCAM-1	vascular cell adhesion molecule 1
VSMC	vascular smooth muscle cells
WA	Withaferin A
WA-BIOT	biotinylated version of Withaferin A
WN	Withanone
WS	*Withania somnifera*
WT	wild type
XO	xanthine oxidase

VII.2 Curriculum Vitae

Linde Sabbe

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Birth: 25/03/85 Bruges Belgium
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EDUCATION

- Master after Master in Molecular medical biotechnology, Ghent University. (2007-2008)
  Title thesis: Development of the P1 phagmid system for the delivery of genes in *Pseudomonas aeruginosa* enabling asRNA silencing as a therapy for biofilm.
  Promotor: Prof. Dr. M. Vaneechoutte

- Master in Biomedical Sciences, Ghent University (2003-2007)
  Title thesis: Proteomic research in mitochondrial disorders.
  Promotor: Prof. Dr. R. Van Coster

EXTRA TRAINING PROGAMMES

- Trees, maps and theorems, effective communication for rational minds by Jean-Luc Doumont, Ghent University. (2011)
- Advanced academic English: Conference skills- English proficiency for presentations at UCT Ghent, Ghent University. (2011)
- The conference poster at UCT Ghent, Ghent University. (2011)
- Advanced academic English writing skills in natural sciences at UCT Ghent, Ghent University. (2010)
- GROW PhD course Epigenetics, Maastricht University Medical centre, The Netherlands (2008)

WORK EXPERIENCE

- PhD student at Ghent University since 2008, Department Physiology, research group LEGEST, Ghent, Belgium.
  Title PhD: Modulation of Nrf2-KEAP1 signaling by the natural compound Withaferin A: potential therapeutic effect in cardiovascular diseases?

  Promotor: Prof. Dr. W. Vanden Berghe
  Supervisor: Dr. K. Heyninck

Funded by:
Oct. 2008 - Sep. 2012: Scholarship of the Agency for Innovation by Science and Technology, Flanders (IWT)
Oct. 2012 - Dec. 2012: Scholarship of Ghent Unversity.

SKILLS

- Laboratory skills: cell culture handling, inductions, transfections, RNA extraction, cDNA preparation, qPCR, CoIP, Western blotting.

- Computer skills: Basis knowledge of Word, Excel, Powerpoint

- Languages:

	Speaking	Listening	Reading	Writing
Dutch	mother tongue			
English	good	very good	very good	good
French	basic	good	good	basic

- Driver licence B

- Music degree (AMV, Algemene Muzikale Vorming and AMC, Algemene Muzikale Cultuur)

TEACHING AND GUIDANCE EXPERIENCE

- Annually throughout the PhD period teaching and guiding practical excercises of Bachelor- and Masterstudents in biochemistry.

- Supervision of Master thesis: Kevin Titeca, 2010, title: Evaluation of the effect of dietary polyphenols on atherogenic gene expression and epigenetic biomarkers in a monocyte/macrophage cell model.

- Supervision of Master project: Julie Goossens, 2011.

PUBLICATIONS

- Biochemical Pharmacology. 2012 Sep; Molecular insight in the multifunctional activities of Withaferin A. Wim Vanden Berghe, Linde Sabbe, Mary Kaileh, Guy Haegeman, Karen Heyninck.

- Mol Nutr Food Res. 2012 Aug; Dietary curcumin inhibits atherosclerosis by affecting the expression of genes involved in leukocyte adhesion and transendothelial migration. Coban D, Milenkovic D, Chanet A, Khallou-Laschet J, Sabbe L, Palagani A, Vanden Berghe W, Mazur A, Morand C.

- Bookchapter Phytochemicals : bioactivities and impact on health. p.159-198 (2011); Phytochemicals and cancer chemoprevention: epigenetic friends or foe? Katarzina Szarc vel Szic, Ajay Palagani, Behrouz Hassannia, Linde Sabbe , Karen Heyninck , Guy Haegeman, Wim Vanden Berghe

VARIA

- Student representative at the facultyboard of 2006-2007
- Degree Basic training first intervention at pbo fire brigate
- Personal interests: Traveling, doing sports (jazz dance, running, swimming, pilates…)