Dynamics and regulation of the oxidative stress response upon chemical exposure

Bischoff, L.J.M.

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General introduction and aim of the thesis

There is an increasing number of chemicals that enters the society, including drugs, environmental chemicals and cosmetics, combined also referred as the chemical exposome. Likewise there is an increased hazard for chemically-induced health effects. Chemicals can interfere with biological systems and induce compound specific responses, either related to the pharmacological on- or off-target effects. In particular compounds with (in)direct electrophilic reactivity are of direct harm to cells. Such compounds will interfere with normal cellular physiological processes and activate adaptive cellular stress responses that try to repair the cellular injury. Understanding the fundamental relationship between activation of these cellular stress responses and ultimate onset of cytotoxicity can be used for constructing mechanism-based biomarkers.

CLASSIFICATION AND USE OF BIOMARKERS

To establish whether exposure to a certain chemical or drug did or did not occur, or what the unwanted consequences are from exposure to a chemical or drug, appropriate biomarkers are needed. The broad definition of a biomarker, as stated by the World Health Organization (WHO), is: a biomarker is almost any measurement reflecting an interaction between a biological system and an environmental agent, which may be chemical, physical or biological (WHO, 1993). A more specific definition of a biomarker given by the WHO is: a biomarker is any substance, structure or process that can be measured in the body or its products and influence or predict the incidence or outcome of disease (WHO, 2001). The role of a biomarker can be interpreted as a "fingerprint" left behind in the body after exposure (with the analogy of the body as a "crime scene"). Many different classification systems of biomarkers are described in literature based on the information they provide or their intended use. Although classification of biomarkers in certain categories might be useful, one has to keep in mind that biomarkers might fit in different categories, depending on the knowledge we have regarding their link to the chemical exposure or disease mechanisms, as well as their intended use in a particular situation.

Manno et al. (2010), describes a classification system where biomarkers are divided in three different groups depending on their toxicological significance: biomarkers of susceptibility, biomarkers of exposure and biomarkers of effect (Manno et al. 2010). Another method of classification is described by Baker et al. (2005), who classifies biomarkers concerning their applications, for example: disease biomarkers and toxicity biomarkers (Baker 2005). Furthermore, with advancement in technology and knowledge of biological pathways and disease mechanisms, came the use of

biomarker panels, constructed of multiple biomarkers e.g. multiple genes or proteins representing a specific stress pathway, like for example inflammation. Based on this, Robinson et al. (2013), introduced the concept of "actionable" biomarkers, biomarkers that can be used to guide clinical management of disease and could even be used to diagnose diseases in their early, asymptomatic state (Robinson et al. 2013) (Figure 1). Two types of "actionable" biomarkers described by Robinson et al. (2013), are mechanistic biomarkers, which play a role in the mechanism of the disease, and descriptive biomarkers, which are not directly involved in the mechanism of disease, but are rather products of the disease or the damage induced by the disease. Antoine et al. (2013), describe a mechanistic biomarker for early and sensitive detection of acetaminophen-induced acute liver injury. They described a plasma derived biomarker panel consisting of miR-122, a microRNA highly specific for the liver, high mobility group box 1 (HMGB1) a marker of necrosis, and caspase-cleaved keratin-18 (K18) a marker of necrosis and apoptosis (Antoine et al. 2013). This biomarker panel proved to be more sensitive than the measurement of alanine transaminase (ALT) a well-established biomarker for assessing the health status of the liver. This indicates that mechanistic biomarkers existing of several proteins and microRNAs related to certain stress response pathways, can provide information concerning the molecular mechanisms of action of a chemical upon exposure. In the field of pharmacology, there is great need for mechanistic biomarkers, as these markers might have the ability to predict the response of a drug and thereby provide information which can be used to develop personal-based medicine approaches (Amadoz et al. 2015). However, it might be clear that for the construction of these (mechanistic) biomarker panels a greater understanding is needed regarding the different players (proteins, genes, microRNAs) of the different stress response pathways, as well as their interactions and changes over time (dynamics of the stress response pathway). Therefore, guided by the advancement in omics techniques, much research is targeted on unraveling the mechanisms of stress response pathways such as the DNA-damage response, unfolded protein response, and oxidative stress response. Moreover, microRNAs are promising small non-coding RNAs that could serve as biomarkers for small injury and can also modulate cellular biology including toxic responses. Since oxidative stress and microRNAs are central in this thesis, below these topics will be specifically addressed in some detail.

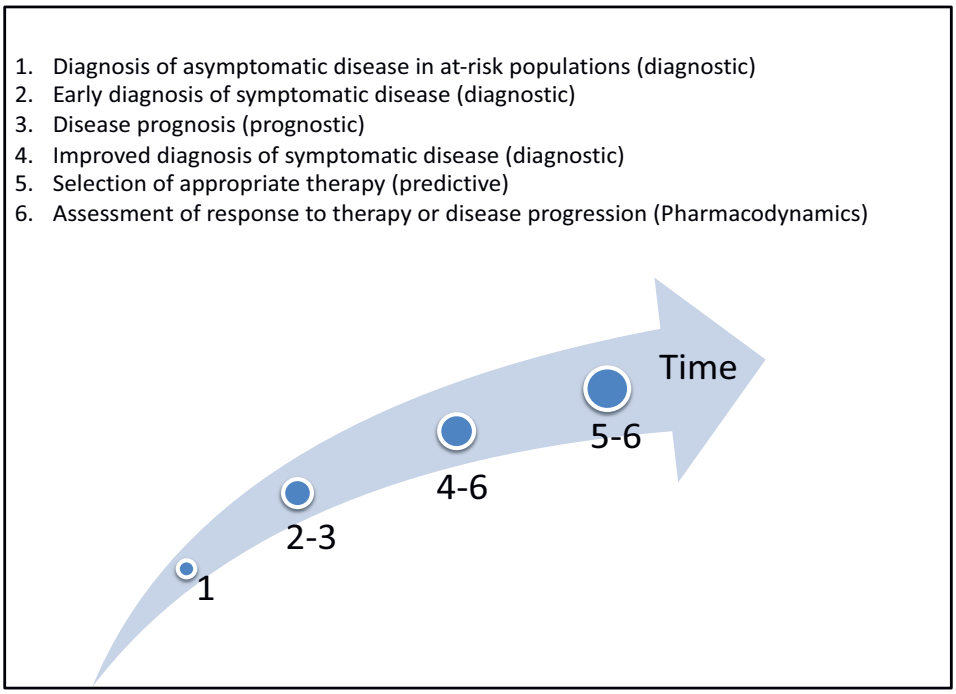

Figure 1. Use of actionable biomarkers over time.
(Modified from Robinson et al. 2013).

THE OXIDATIVE STRESS RESPONSE PATHWAY

In a cell there is a continuous production of reactive oxygen (ROS) and nitrogen species (RNS) (Finkel and Holbrook 2000). ROS and RNS are generated as a result of internal metabolism, for example aerobic respiration in mitochondria, and exposure to exogenous toxicants (Filomeni et al. 2015; Ma 2013; Turrens 2003). A controlled production of ROS and RNS has been described in literature to contribute to the regulation of various physiological processes in the cell like proliferation, autophagy and inflammation (Finkel 2011). However, uncontrolled production of ROS and RNS, called oxidative stress, can result in inflammatory responses and eventually lead to pathological conditions like cancer and neurodegenerative disorders (Prasad et al. 2017).

To overcome oxidative stress, a cell has several mechanisms to protect itself against oxidative stress. One of the most important mechanisms against oxidative stress is the Nrf2 pathway (Figure 2), named after its transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2). Not surprisingly, the Nrf2 pathway plays a role

in many diseases including cancer and neurodegenerative diseases like Alzheimer's disease (Bryan et al. 2013; Deshmukh et al. 2017). It is reported that in many different tumor cells Nrf2 is overexpressed, consequently making these cells less vulnerable for chemotherapy (Kensler and Wakabayashi 2010; Ren et al. 2011; Tang et al. 2011; Wang et al. 2008).

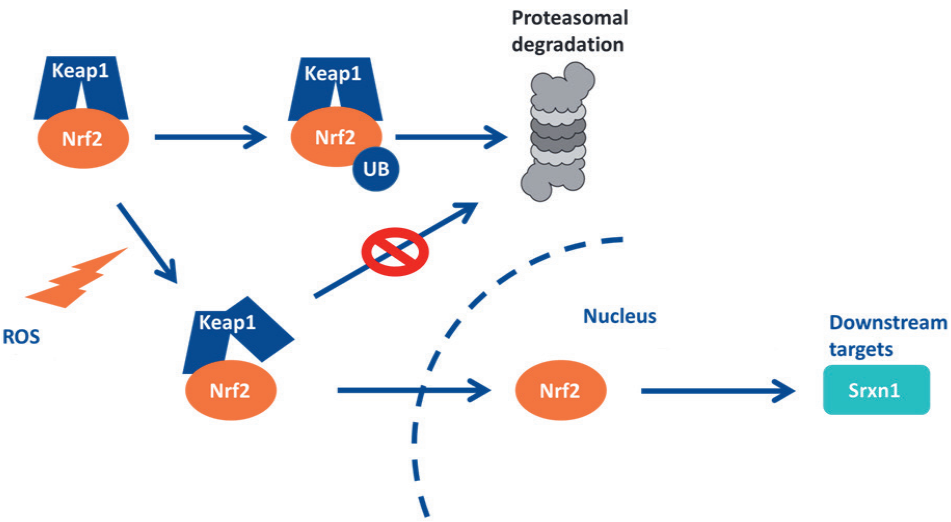

Figure 2. The Nrf2 pathway: the "cyclic sequential attachment and regeneration" model.

Nuclear factor E2-related factor 2 (Nrf2) is a transcription factor that upregulates expression of a battery of genes to combat oxidative and electrophilic stress. Modification of Kelch-like ECH-associated protein 1 (Keap1) by reactive oxygen species stabilizes Nrf2 by escaping from degradation. Nrf2 then is able to move freely to the nucleus, were it activates many different antioxidants like Srxn1.

Canonical activation of the Nrf2 pathway

In basal conditions, Nrf2 is bound in the cytoplasm to two Kelch-like ECH-associated protein 1 proteins (Keap1) (Keum and Choi 2014; Zipper and Mulcahy 2002). Nrf2 consist of seven functional domains (Neh1 – Neh7). Of these domains Neh2 contains seven lysine residues, which plays a role in the ubiquitination of Nrf2 (Itoh et al. 1999; Zhang et al. 2004), which facilitates the destruction of Nrf2 via the ubiquitin-26S proteasomal pathway (Kobayashi et al. 2004). Furthermore, the Neh2 domain contains two binding sites which interact with Keap1. These are the ETGE and DLG motives (McMahon et al. 2006).

Keap1 is an adaptor for Cullin-3 (Cul3)-based E3 ubiquitin ligase, which facilitates poly-ubiquitin conjugation to Nrf2, and therefore degradation by the proteasome

(Kobayashi et al. 2004). Keap1 consists of three functional domains. A broad complex/tramtrack/bric-a-brac (BTB) domain, which binds to Cul3 and is required for the dimerization of Keap1 (Zipper and Mulcahy 2002). An intervening region (IVR), and a kelch/double glycine repeat (DGR) domain, which interacts with the Neh2 domain of Nrf2 (Canning et al. 2015). Furthermore, human Keap1 contains 27 cysteine residues (Zhang and Hannink 2003). These cysteine residues can interact with ROS and electrophilic compounds, leading to Nrf2 pathway activation. Interestingly, chemicals show different affinity for the different cysteine groups (Takaya et al. 2012).

In literature, different models can be found describing how, upon activation, Nrf2 enters the nucleus to bind to the antioxidant response element (ARE) and starts the transcription of different antioxidants. Early models described total dissociation of Nrf2 from Keap1, but latest understanding suggest models like the 'two-site recognition hinge-and-latch' and abended the idea of total dissociation of Nrf2 from Keap1. The two-site recognition hinge-and-latch model, is named after the Nrf2 motives ETGE (hinge) and DLG (latch) (Tong et al. 2006b). Keap1 has a higher affinity for the hinge than for the latch (Tong et al. 2006a). Subsequently, Keap1 binds first to the ETGE domain and after the connection is established to the DLG domain.

Covalent binding of ROS or reactive metabolites to one of the cysteine groups of Keap1 is thought to induce a conformational change in the IVR domain of Keap1, decreasing the binding from Keap1 with Cul3 and dissociation of the DLG domain (Cleasby et al. 2014). The ETGE domain, which has a tighter interaction than the DLG domain, does not dissociate from Keap1 (Suzuki and Yamamoto 2015; Tong et al. 2007). Moreover, because of the dissociation of the DLG domain, Nrf2 is not targeted for degradation. As a consequence, *de novo* synthesized Nrf2 is able to translocate to the nucleus. In the nucleus Nrf2 will bind to the ARE, together with members of the masculoaponeurotic fibrosarcoma (Maf) proteins (MafF, MafG and MafK), which facilitates binding to the ARE. Binding to the ARE results in the transcription of different cytoprotective genes involved in e.g. glutathione metabolism, phase 2 drug-metabolizing enzymes and antioxidant response proteins as, for example, sulfiredoxin1 (Srxn1), hemeoxygenase 1 (Hmox1), and NAD(P)H-quinone oxidoreductase 1 (Nqo1) (Hayes et al. 2010; Zhang and Gordon 2004).

In parallel with the above described inhibition of ubiquitination of Nrf2, binding of an electrophilic compound can trigger the ubiquitination of Keap1 by the Cul3-Rbx1 complex decreasing the levels of Keap1, resulting in the movement of *de novo* synthesized Nrf2 into the nucleus (Hong et al. 2005). Unlike degradation of ubiquitinated Nrf2, Keap1 degradation is independent of the proteasome pathway (Zhang et al. 2005).

Different mechanisms of Nrf2 pathway termination are described in literature. Sun et al. (2007), suggest a mechanism whereby Nrf2 is transported back to the cytoplasm by Keap1, which has a nuclear export sequence (Sun et al. 2007). Furthermore, transcription regulator protein Bach1 can bind to the ARE and is therefore able to compete with Nrf2 (Tkachev et al. 2011). Kaspar and Jaiswal (2010), describes that Nrf2 regulates its own degradation through increasing Cul3-Rbx1 expression upon binding to the ARE and thereby inducing promoter activity of Cul3-Rbx1 genes (Kaspar and Jaiswal 2010).

Interactions with other adaptive pathways: non-canonical Nrf2 pathway activation

Numerous studies describe the interaction of the Nrf2 pathway with other adaptive stress response pathways like, for example, the DNA-damage response, the unfolded protein response and the NF-κB-signaling pathway.

Interaction with the DNA damage response

Faraonio et al. (2006), showed that p53, a key player in the DNA damage response, negatively regulates Nrf2-mediated gene transcription (Faraonio et al. 2006). However, the KRR motif in p21, a downstream target of p53, is able to bind to the DLG and ETGE motifs within Nrf2, blocking the binding of Nrf2 with Keap1. Consequently, ubiquitination cannot take place, which in turn leads to activation of the Nrf2 pathway (Chen et al. 2009).

Interaction with the unfolded protein response

Oxidative stress can result in endoplasmic reticulum stress (ERS) (Digaleh et al. 2013). ERS might in turn lead to activation of the unfolded protein response (UPR). The UPR roughly exists of three major branches, which consists of three transmembrane sensors: transcription factor 6 (ATF6), inositol-requiring enzyme-1α (IRE1) and protein kinase-like ER kinase (PERK) (Hetz 2012). PERK-dependent phosphorylation promotes the dissociation of Nrf2 from keap1, and therefore activation of the Nrf2 pathway (Cullinan et al. 2003; Zhu et al. 2015).

Interaction with the NF-κB-signaling pathway

Wardyn et al. (2015), described the crosstalk between NF-κB and Nrf2, with increased activity of NF-κB in the absence of Nrf2 (Wardyn et al. 2015). Furthermore, ROS can oxidize cysteine residues in the DNA binding domain of NF-κB (Hirota et al. 1999). Moreover, IκB kinase β (IKKβ) is a substrate analogue of Keap1 (Kim et al. 2010). Jiang et al. (2013), found that, like Nrf2, IKKβ has a ETGE motif (Jiang et al. 2013). This makes it possible for IKKβ to bind to Keap1, and therefore to compete with Nrf2. Consequently, Keap1 is responsible for IKKβ ubiquitination and therefore

degradation, and therefore downregulation of NF-κB (Lee et al. 2009). Furthermore, AP-1 factors as c-Fos and Jun-D are also known to bind to the ARE. Binding of these factors blocks the binding side of the ARE for Nrf2 resulting in a decrease of its downstream targets (Li and Jaiswal 1992; Venugopal and Jaiswal 1996; Wilkinson et al. 1998). Recently it became clear that besides genes and proteins another class of signaling molecules play an important role in the regulation of stress response pathways: microRNAs.

MICRORNAS

MicroRNAs (miRNAs or miRs) are small (~22-nt) non-coding RNAs (Starega-Roslan et al. 2010). MicroRNAs regulate gene expression at the post-transcriptional level and are involved in many biological processes. MicroRNA target sites are typically located on the 3'untranslated region of their target mRNAs. These target sites only needs to be partially complementary to the microRNA (Lam et al. 2015), which leads to target mRNA translational repression or degradation (Djuranovic et al. 2012; Filipowicz et al. 2008). A single microRNA can have about 100 target sites (Brennecke et al. 2005), and mRNAs can be targeted by more than one single microRNA (Peter 2010; Wu et al. 2010).

MicroRNAs are involved in many physiological processes including the immune response, metabolism, and development (Hou et al. 2011). Furthermore, microRNAs are involved in toxicological responses (Mendell and Olson 2012) including activation/inhibition of stress response pathways (Bartoszewska et al. 2013). Therefore microRNAs also play a role in diseases like, for example, (various types of) cancer (Meng et al. 2016) and other pathologies like acute myocardial infarction (Devaux et al. 2012). Moreover, some microRNAs exists which are highly "tissue specific", meaning they are abundantly present in a certain tissue type, as for example miR-122 is tissue specific for the liver. Measurement of these microRNAs might provide information regarding the organs which are damaged upon chemical exposure, because of their high concentration in the bloodstream after tissue damage occurred (Laterza et al. 2009). Altogether, these features make microRNAs interesting candidates for biomarkers for exposure and disease.

Understanding the fundamental relationship between activation of cellular stress responses and ultimate onset of cytotoxicity is of critical importance. As described above, knowledge of stress pathway behavior on protein, gene, and microRNA level can be applied in the construction of a mechanistic biomarker fingerprint.

THESIS OUTLINE

The focus of the described research in this thesis is on the oxidative stress response (Nrf2 pathway). The aim of the research presented in this thesis is to obtain more information concerning microRNAs which are involved in the Nrf2 pathway to determine and evaluate the application of microRNAs for the construction of novel mechanistic biomarkers. Furthermore, we aimed to obtain a better understanding with respect to the dynamics of the Nrf2 pathway to repeated xenobiotic exposure. In **Chapter 2**, microRNAs are introduced and their utility as biomarkers of chemical exposure and disease (effect) is reviewed in respect to the current knowledge of this upcoming field. As shown in **Chapter 2**, exposure to chemicals can lead to overexpression of certain microRNAs. In **Chapter 3**, to investigate the effect of overexpression of microRNAs on the Nrf2 pathway response in general and in combination with chemical exposure, a microRNA mimic screen was performed. In this screen overexpression of microRNAs was induced by using synthetic microRNA mimics. Since repeated exposure may drive adaptation programs and may lead to different responses between single and repeated exposures, in **Chapter 4** the effect of a second exposure on the dynamics of the Nrf2 pathway activation was conducted. In **Chapter 5** results of a study are shown where a panel of structurally different phenolic compounds were used to demonstrate the proof-of-concept that Nrf2 pathway reporters can successfully be applied as biomarkers to characterize the specific pro-oxidant responses of chemicals. Finally, in **Chapter 6** the findings of the studies described in this thesis are discussed and an overview is provided concerning future perspectives and implications of the included studies.

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