# Value of monitoring Nrf2 activity for the detection of chemical and oxidative stress

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## Abstract

**Beyond specific limits of exposure, chemical entities can provoke deleterious effects in mammalian cells via direct interaction with critical macromolecules or by stimulating the accumulation of reactive oxygen species (ROS). In particular, these chemical and oxidative stresses can underpin adverse reactions to therapeutic drugs, which pose an unnecessary burden in the clinic and pharmaceutical industry. Novel pre-clinical testing strategies are required to identify, at an earlier stage in the development pathway, chemicals and drugs that are likely to provoke toxicity in humans. Mammalian cells can adapt to chemical and oxidative stress via the action of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), which up-regulates the expression of numerous cell defence genes and has been shown to protect against a variety of chemical toxicities. Here, we provide a brief overview of the Nrf2 pathway and summarize novel experimental models that can be used to monitor changes in Nrf2 pathway activity and thus understand the functional consequences of such perturbations in the context of chemical and drug toxicity. We also provide an outlook on the potential value of monitoring Nrf2 activity for improving the pre-clinical identification of chemicals and drugs with toxic liability in humans.**

## Introduction

The process of evolution has enabled mammalian cells to withstand continuous exposure to the by-products of oxidative metabolism, environmental agents and dietary constituents that can, if left unchecked, cause acute and chronic injury. In recent years, following industrial and medical revolutions that have resulted in the development of many valuable chemical entities and therapeutic drugs, the hazards posed to mammalian cells have increased in nature and number. Many of these novel agents can provoke deleterious effects in cells via the induction of chemical stress (direct or indirect chemical interaction with one or more critical macromolecule) or oxidative stress (accumulation of reactive oxygen species (ROS) due to mitochondrial dysfunction, perturbation of cellular oxidase enzymes or the redox cycling of the chemical entity). Both chemical and oxidative stress can inhibit the physiological function of specific target proteins, cause damage to DNA and/or provoke lipid peroxidation and disruption of cell membrane integrity, ultimately leading to cellular dysfunction or death [1]. It is therefore imperative that the ability of a chemical entity to provoke chemical and/or oxidative stress can be determined prior to exposure of humans, in order to minimize the risk of toxicity and aid the design of safer alternatives. Mammalian cells can withstand moderate increases in local concentrations of electrophiles and ROS due to the concerted actions of numerous antioxidant proteins. The expression of most of the genes encoding these proteins is regulated by the ubiquitously-expressed transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) [2]. This article provides a brief overview of the Nrf2 pathway and considers how monitoring its activity could aid the identification of chemicals and drugs that carry the potential to provoke toxicity in humans.

**Key words:** antioxidant response element, hazard assessment, Kelch-like ECH-associated protein 1 (Keap1), nuclear factor erythroid 2-related factor 2 (Nrf2), stress response, toxicity.

**Abbreviations:** ADR, adverse drug reaction; AKR, aldo-keto reductase; AOP, adverse outcome pathway; ARE, antioxidant response element; GSK-3$\beta$, glycogen synthase kinase 3$\beta$; HMOX1, haeme oxygenase 1; Keap1, Kelch-like ECH-associated protein 1; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; NQO1, NAD(P)H dehydrogenase (quinone) 1; Nrf2, nuclear factor erythroid 2-related factor 2; PBMC, peripheral blood mononuclear cell; ROS, reactive oxygen species; SRXN1, sulfiredoxin 1; TXNRD1, thioredoxin reductase 1.

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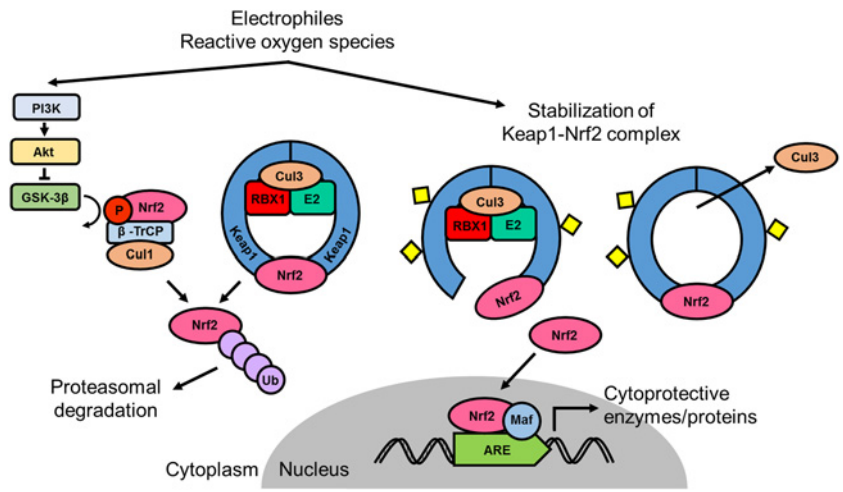
## Nrf2 and the antioxidant response

Nrf2 is a basic leucine zipper transcription factor of the cap'n'collar subfamily that, under basal conditions, is repressed via its cytoplasmic tethering and ubiquitination mediated by the redox-sensitive Kelch-like ECH-associated protein 1 (Keap1) [2]. Recent evidence also indicates a role for Keap1-independent mechanisms, including phosphorylation of Nrf2 mediated by glycogen synthase kinase 3$\beta$ (GSK-3$\beta$), in the regulation of Nrf2 activity [3] (Figure 1). Keap1 is endowed with many cysteine residues and is therefore sensitive to changes in local redox conditions [2]. As a result, increases in intracellular concentrations of electrophiles and ROS interfere with the ability of Keap1 to repress Nrf2, allowing the transcription factor to accumulate in the nucleus and up-regulate the expression of cell defence genes that contain the *cis*-acting antioxidant response element (ARE) in their promoter regions [2]. Conserved Nrf2 target genes include NAD(P)H dehydrogenase (quinone) 1 (NQO1),

**Figure 1** | **Overview of the Nrf2 pathway**

Under basal conditions, the cellular abundance and activity of Nrf2 is repressed through ubiquitination (Ub) via the cullin-dependant E3 ubiquitin ligase Cul3 bound to Keap1 homodimers or Cul1 bound to $\beta$-TrCP. The latter interaction is promoted via GSK-3$\beta$-mediated phosphorylation (P) of Nrf2. Chemical and oxidative stressors activate Nrf2 signalling via stimulation of the PI3K pathway or chemical/oxidative modifications of cysteine-containing pockets (diamonds) in Keap1. The latter process stabilizes the Keap1–Nrf2 complex, by inactivating Keap1 or stimulating the dissociation of Cul3, allowing newly-synthesized Nrf2 to accumulate in the cell and translocate to the nucleus, where it forms heterodimers with small Maf proteins and transactivates ARE-regulated genes to co-ordinate an adaptive response to chemical/oxidative stress.




thioredoxin reductase 1 (TXNRD1) and sulfiredoxin 1 (SRXN1), whereas members of the aldo-keto reductase (AKR) and GST families appear to be regulated by Nrf2 principally in human and mouse cells respectively (Table 1) [4]. As we and others have reviewed elsewhere [5–7], transgenic Nrf2 knockout mice exhibit lowered basal and inducible levels of ARE-regulated genes in multiple tissues and are highly susceptible to pathologies associated with exposure to chemical toxicants, such as acetaminophen hepatotoxicity [8,9], cisplatin nephrotoxicity [10] and bleomycin lung fibrosis [11]. Conversely, tissue-specific ablation of the Keap1 gene confers resistance to acute drug toxicity *in vivo* [12]. Based on these observations and evidence that Nrf2 pathway activity is perturbed in diseases (for example of the kidney [13–15]) with an oxidative stress component, there is an increasing interest in the therapeutic value of targeting Nrf2 with small molecules [16].

## Role of Nrf2 in the adaptive response to chemical stress

A number of toxic chemicals and drugs have been shown to induce Nrf2-mediated adaptive responses in cell and animal models. For example, the glutathione depletion and oxidative stress resulting from cytochrome P450-mediated bioactivation of acetaminophen to the chemically reactive and hepatotoxic metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI) has been associated with the rapid accumulation of Nrf2 and induction of target genes in mouse liver [17]. Importantly, dose-dependent activation of the Nrf2 pathway was evident in animals receiving non-toxic and toxic doses of acetaminophen [17], highlighting the ability of Nrf2 to report early chemical stress associated with drug-induced liver injury. Consistent with these findings, direct application of NAPQI to mouse hepatoma cells has been shown to cause chemical adduction of selected cysteine residues in Keap1 and the activation of Nrf2 signalling [18]. Accumulation of Nrf2 and/or induction of its target genes have been observed in response to other model hepatotoxins, including diclofenac [19] and carbon tetrachloride [20], nephrotoxins such as cisplatin [10,21] and cyclosporin A [22,23] and the neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [24] and 6-hydroxydopamine [25]. In addition, many electrophilic skin sensitizers appear to be highly capable of stimulating Nrf2 signalling; this is the rationale for use of the *in vitro* Keratinosens™ assay, in which a human HaCaT keratinocyte cell line equipped with an ARE-regulated luciferase reporter transgene is used to classify the skin sensitization hazard associated with new chemical entities [26]. Taken together, the above evidence demonstrates that Nrf2 can respond to diverse chemical toxins in multiple cell types, both *in vitro* and *in vivo*.

## Monitoring Nrf2 for the detection of chemical stress: *in vitro* models

Whereas established toxicological end-points, such as decreases in cellular adenosine triphosphate levels and leakage of

**Table 1** | **Examples of key Nrf2 target genes**
Summarized from [4].

| Category | Gene | Gene symbol |
|---|---|---|
| Antioxidant proteins | Glutamate cysteine ligase catalytic subunit | *GCLC* |
| | Sulfiredoxin 1 | *SRXN1* |
| | Thioredoxin reductase 1 | *TXNRD1* |
| Drug metabolizing enzymes and transporters | Aldo-keto reductases | *AKRs* |
| | Glutathione S-transferases | *GSTs* |
| | Multidrug resistance-associated proteins | *ABCCs* |
| | NAD(P)H:quinone oxidoreductase 1 | *NQO1* |
| | UDP-glucuronosyltransferases | *UGTs* |
| NADPH synthesis | Glucose-6-phosphate dehydrogenase | *G6PD* |
| | Malic enzyme 1 | *ME1* |
| Stress-response and metal-binding proteins | Ferritin | *FTL* |
| | Heat shock proteins | *HSPs* |
| | Haeme-oxygenase 1 | *HMOX1* |
| | Metallothionein | *MT1* |

lactate dehydrogenase into cell culture media, unquestionably demonstrate the ability of a compound to provoke cell death, their sensitivity is generally not sufficient to identify sub-lethal chemical insults that initiate adaptive stress responses but do not culminate in overt cytotoxicity. These stress responses encompass the Nrf2-driven antioxidant response, but also the DNA damage response (effected by the tumour suppressor p53), the unfolded protein response (effected by activating transcription factor 4/X-box binding protein 1, in response to endoplasmic reticulum stress) and the heat shock response (effected by heat shock factors), among others [27]. Such responses encapsulate some of the earliest biochemical signals that precede the initiation of toxic cascades and can thus provide sensitive and mechanistic insights into the deleterious effects of a chemical entity [28]. In this regard, stress responses represent important components of adverse outcome pathways (AOPs) that describe the molecular events leading from recognition of a chemical entity to a defined toxic outcome. In light of recommendations for the future direction of toxicity testing [29], there is much interest in the value of monitoring stress responses and other components of AOPs to minimize reliance on animal models and improve the mechanism-based identification of hazardous chemical entities [30].

To overcome some of the technical barriers of measuring Nrf2 directly (including a lack of sensitive antibodies for the detection of the low-abundance Nrf2 protein and relative stability of Nrf2 mRNA during activation of the pathway), researchers have developed novel strategies for monitoring the activity of the Nrf2 pathway. Such strategies include (a) the use of stable reporter cell lines in which the expression of luciferase is controlled by one or more ARE sequences [31,32], (b) automated, high-content imaging of cell lines expressing fluorescent-tagged Nrf2 or target gene products [28] and (c) transcriptomic analysis of dynamic changes in gene signatures that have been shown (for example, in ChIP data) to be representative of the battery of Nrf2-regulated genes [23,33,34]. However, whereas the cytoprotective genes regulated by Nrf2 are relatively well-conserved among mammals (Table 1), the majority of these genes are also regulated by other transcription factors, including those that govern the activity of discrete stress responses [34]. Therefore, the consolidation of these gene signatures is vital to unravelling the roles of different molecular pathways in the cellular response to chemicals that have diverse pharmacological and toxicological effects. These efforts will be enhanced by detailed assessments of the gene networks that are regulated by Nrf2 in human primary cells, of which few have been reported to date. As the transcriptomic phenotypes associated with activation of the Nrf2 stress response become clearer, it is envisaged that monitoring the activity of this pathway will contribute to the continuing drive toward *in vitro* and *in silico* approaches for predicting the human hazard posed by chemicals and new drugs.

## Monitoring Nrf2 for the detection of chemical stress: *in vivo* models

An important limitation of the above cell-based models is their lack of key physiological traits, which can limit their translational value. For example, in the case of drug-induced liver injury, the current lack of stable hepatocyte cell models that retain the capacity to metabolize many drugs, combined with the rapid dedifferentiation of primary hepatocytes following their isolation from liver tissue [35], limit the capacity of such systems to identify metabolism-dependent hepatotoxins. Whereas recent advances in stem cell technology are increasingly being used to address these shortcomings, state-of-the-art protocols generate cells

that can be best described as hepatocyte-like, given that their complement of cytochrome P450 enzymes and ability to metabolize drugs remains inferior to their primary counterparts [36]. At least until these barriers are overcome, animal models will continue to provide a unique opportunity to consider aspects of drug disposition in the toxicological effects of a given compound. Oikawa et al. [37] recently established the novel OKD48-Luc mouse model that expresses a transcriptionally inactive luciferase-tagged Nrf2, under the transcriptional control of endogenous Nrf2, mediated through multiple AREs. Whereas the transgene product is able to interact with Keap1 and is therefore repressed in the absence of an Nrf2-inducing stimulus, chemical or oxidative stressors cause the accumulation of luciferase-tagged Nrf2 via inhibition of Keap1-mediated turnover and transcriptional induction of the transgene by endogenous Nrf2 [37]. This dual-regulating mechanism produces a bioluminescent signal, detectable via whole animal and *ex vivo* organ imaging, upon activation of the Nrf2 response. By facilitating non-invasive, real-time measurement of Nrf2 activity *in vivo*, this model provides an excellent platform to examine the relationship between drug disposition, activation of Nrf2 stress response signalling and organ-specific drug toxicity.

## Relevance of Nrf2 for human hazard identification

A growing body of evidence indicates an association between dysfunction of the Nrf2 pathway and disease, particularly cancer [38] in humans. However, the invasive procedures required to obtain appropriate tissues means that the evidence for temporal modulation of Nrf2 signalling by pharmacological and toxicological agents in humans is currently far less substantial than in animals and cells. Of the limited studies performed to date, a 15-fold increase in the activity of NQO1 has been demonstrated in liver tissue obtained from acetaminophen overdose patients, compared to control liver [39], whereas daily oral administration of fumaric acid esters over 12 weeks has been associated with the increased expression of Nrf2 target genes in the skin of psoriasis patients [40]. In addition, a 5-fold increase in the mRNA level of NQO1 has been reported in peripheral blood mononuclear cells (PBMCs) obtained from patients with advanced solid tumours or lymphomas who received a daily dose of the triterpenoid Nrf2 inducer bardoxolone methyl for 3 weeks [41]. It is currently unclear if the levels of Nrf2 and/or its target genes in PBMCs are representative of the whole body status of the pathway or can describe stress responses taking place in specific target organs and therefore whether PBMCs can be useful surrogates for monitoring Nrf2 pathway activity in humans. In this regard, the lack of an accessible biomarker of Nrf2 activity currently hinders detailed assessment of the function and importance of this pathway in humans. Whereas it has been shown that haeme oxygenase 1 (HMOX1), which is partly regulated by Nrf2 at the transcriptional level and is known to be induced under conditions of oxidative stress [42], can be detected in the plasma and urine of animals and patients experiencing acute kidney injury [23], it remains to be determined if other Nrf2-regulated proteins and/or their metabolic by-products can (a) be detected in accessible biofluids and (b) can accurately inform on the response of Nrf2 to pharmacological and toxicological stimuli, particularly, in the latter case, before the onset of overt toxicity.

## Perspectives

Adverse drug reactions (ADRs), representing unintended, noxious responses to a therapeutic drug at doses normally used in humans, are a particularly burdensome manifestation of chemical and oxidative stress, accounting for up to 6.5 % of all U.K. hospital admissions [43] and the attrition of 30 % of new drug candidates [44]. To improve the pre-clinical detection of ADRs, particularly those of an off-target and idiosyncratic nature, a number of consortia (including the EU Innovative Medicines Initiative MIP-DILI project and the US Environmental Protection Agency ToxCast programme) are examining the value of novel test systems that could aid the identification of toxic drug candidates and minimize the human risks associated with new chemical entities. Monitoring the activity of stress response pathways, including the Nrf2 antioxidant response, will probably represent an important aspect of such test systems. Indeed, the Organisation of Economic Cooperation and Development (OECD) recently recommended the use of the Nrf2-based KeratinoSens™ assay for testing the skin sensitizing potential of chemicals [45], emphasizing that the value of monitoring Nrf2 activity for the detection of chemical stress is already being realized in certain settings. In terms of chemical and drug toxicity affecting other organs, it will be important to define the contexts in which activation of the Nrf2 pathway, particularly in cell and animal models, represents a signal with toxicological relevance to human health. Indeed, a consensus has yet to be reached on the threshold of modulation of Nrf2 activity that can be regarded as an early marker of cellular stress that pre-empts overt toxicity, rather than a benign adaptive response. Such criteria can only be formulated with the analysis of large data sets, comprising a broad range of training and test compounds (including suitable positive and negative controls), which consider both concentration and time-dependent chemical perturbations of stress response pathways, alongside other physiological readouts such as mitochondrial function and cell viability, in relevant models. It will also be critical to determine *in vitro–in vivo* correlations for such responses. Even with such a consensus in place, it appears unlikely that monitoring the response of the Nrf2 pathway in isolation will be sufficient to distinguish between chemical entities that are likely or not to provoke pre-clinical and/or clinical toxicity. This is underlined by the fact that relatively non-toxic, pharmacological inducers of Nrf2 have been identified and, in the cases of dimethyl fumarate [46] and bardoxolone methyl [41,47], are being developed as novel therapeutics in their

own right. Therefore, while its status as the master regulator of the antioxidant response will place Nrf2 at the forefront of modern hazard assessment for new chemical entities, the true value of monitoring the activity of this pathway will only be realized through a deeper understanding of its physiological, pharmacological and toxicological roles in relevant tissues and a greater comprehension of its interplay with other stress response pathways that determine the outcome of a cell's encounter with a given xenobiotic.

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