REVIEW ARTICLE

Nrf2: control of sensitivity to carcinogens

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Abstract Induction of enzymes that enhance the detoxication of chemical carcinogens has been a broadly effective strategy for chemoprevention of experimental carcinogenesis in rodent models. Several inducing agents are now in clinical trials to evaluate utility for prevention of cancers associated with unavoidable high exposures to environmental carcinogens. The successes of these pre-clinical and clinical interventions lead to studies to define the molecular basis for protection by these agents, which now include phenolic antioxidants, dithiolethiones, isothiocyanates, and triterpenoids. In the mid-1990s, the NF-E2-related factor 2 (Nrf2) transcription factor was identified as a key regulator of the inducible expression of enzymes such as glutathione S-transferases and NAD(P)H: quinone oxidoreductase in catalyzing the detoxication of reactive electrophiles and oxidants that contribute to the formation of mutations and ultimately cancers. Nrf2 is now recognized to regulate a broad cytoprotective, transcriptional response leading to prevention of damage to DNA, proteins and lipids; recognition, repair and removal of macromolecular damage; and tissue renewal following toxic assaults. Highlighting the importance of this pathway as a determinant of susceptibility to carcinogenesis, multiple studies now demonstrate enhanced incidence, multiplicity, and/or tumor burden in Nrf2-disrupted mice compared to wild-type in models of inflammation and colon cancer, bladder cancer, lung disease and cancer, stomach cancer, mammary cancer, skin cancer, and hepatocarcinogenesis.

Keywords Keap1 · Nrf2 · Cancer · Chemoprevention

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Introduction

The NF-E2-related factor 2 (Nrf2) transcription factor-signaling pathway has been targeted for prevention of chemical carcinogenesis, both in animal models and in clinical trials well before its initial molecular characterization in the late 1990s. In the early 1970s, Wattenberg and colleagues established that phenolic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene were effective anticarcinogens in rodents, especially when administered prior to carcinogen challenge (Wattenberg 1972). Early mechanistic studies focused on the possibility that elevation of cellular glutathione levels or of glutathione-utilizing enzymes such as glutathione S-transferases (GSTs) by these antioxidants would lead to protection against chemical carcinogenesis. GSTs, now known to be regulated in part through Nrf2, were known at that time to detoxify the electrophilic intermediates of some carcinogens. In particular, Benson et al. (1978) showed that liver cytosols from butylated hydroxyanisole-fed mice exhibited much higher GST activities than controls and that cytosols prepared from the livers of these rodents eliminated the mutagenic activity in urine from mice treated with the carcinogen benzo[a]pyrene (Benson et al. 1978). Subsequently,

the concerted induction of multiple carcinogen detoxication enzymes was found to occur in many tissues of the mouse leading to the hypothesis that a broad-based approach to chemical protection against carcinogenesis, mutagenesis, and other forms of toxicity would be the modulation of enzymes involved in the metabolism and disposition of the reactive intermediates of toxicants, namely, electrophiles and free radicals (Bueding et al. 1982; Benson et al. 1978). Substantial experimental evidence has been developed to support the view that induction of such cytoprotective enzymes is a critical and sufficient mechanism to engender protection against carcinogenesis provoked by environmental (i.e., dietary and airborne carcinogens) and endogenous (i.e., inflammatory states) factors. Since the validation of enzyme induction as a successful means for prevention in scores of animal models of chemical carcinogenesis, there has been an explosion of knowledge on several fronts. Foremost has been the molecular dissection of the pathway by which the initial classes of enzyme inducers acted. These studies have been abetted by the utilization of genetically engineered mice in which components of the Keap1-Nrf2-signaling pathway have been disrupted, either globally or by tissue-specific means. A general theme that emerges is that mice in which Nrf2 has been genetically disrupted are more

sensitive to chemical carcinogenesis. Moreover, the chemopreventive activities of several classes of enzyme inducers, including phenolic antioxidants, dithiolethiones, isothiocyanates, and triterpenoids, are lost in these pathway-deficient mice. Additionally, comparisons of the pharmacodynamic actions of these inducers, especially at low concentrations, to those of genetic activation of the pathway highlight a strong convergence of qualitative if not quantitative action. Pleiotropic effects of these inducers, such as induction of apoptosis, are seen at higher concentrations. Emerging insights into the dark side of the Nrf2 pathway, namely, its propensity to be usurped through mutation and epigenetic marks by cancer cells, wherein a pro-survival, chemoresistant phenotype is facilitated, also point to a key role for the pathway as a multifaceted determinant of susceptibility to carcinogenesis.

Keap1-Nrf2 signaling

The Keap1-Nrf2-signaling axis provides a broad-based cytoprotective response toward disruption of cellular homeostasis by extrinsic and intrinsic stresses. The current model of Keap1-Nrf2 interactions, as depicted in Fig. 1, and as addressed in recent reviews (Dinkova-Kostova et al.
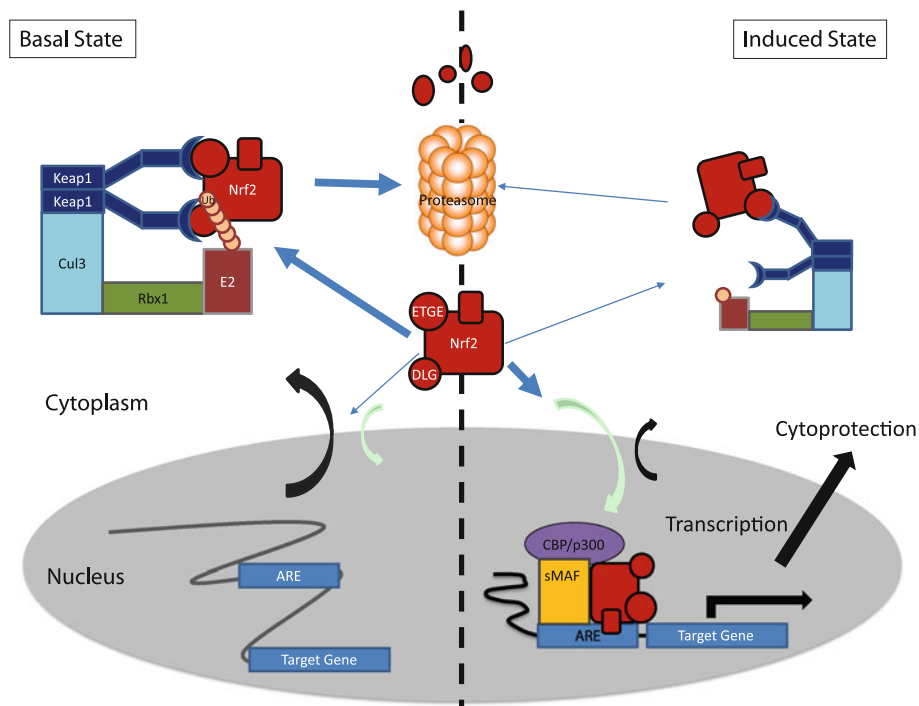

Fig. 1 General schematic for the Keap1-Nrf2 signaling axis. Under homeostatic conditions, Nrf2 is bound by Keap1 through the 'hinge' (ETGE) and 'latch' (DLG) domains of Nrf2. Following binding, Nrf2 is ubiquitinated by the Cul3/Rbx1/E2 ubiquitin ligase complex, marking it for proteasomal degradation. Induction of Nrf2 signaling by pharmacologic agents or endogenous cellular stress (see Table 1) leads to a disruption of the interaction between the 'latch' motif of Nrf2 and Keap1. This interaction is disrupted via tertiary or quaternary structural changes in the Keap1 dimer, likely by alteration of reactive cysteines within Keap1. This disruption leads to an inefficient ubiquitination of Nrf2, allowing for accumulated Nrf2 to translocate into the nucleus where Nrf2 interacts with small Maf and co-activator proteins, binds to AREs in promoters of target genes, and drives transcription of cytoprotective genes.

2005; Hayes and McMahon 2009), involves the Kelch domains of a Keap1 homodimer functionally interacting with two different sites within the Neh2 domain of Nrf2, the ETGE, or high affinity 'hinge' site, and the DLG, the lower affinity 'latch' site. Under normal cellular conditions, Tong et al. (2006) propose that Nrf2 first interacts with the Keap1 dimer through the ETGE hinge interaction, tethering Nrf2 to the Keap1 homodimer, and subsequently the Cul3-Rbx1 complex which, following the stable interaction of Nrf2 to Keap1 through the DLG latch motif, leads to the appropriate orientation of proteins to facilitate the ubiquitination and subsequent proteasomal targeting as well as destruction of Nrf2. Upon cellular stress or pharmacologic induction, the ability of Keap1 to maintain both points of contact, the hinge and the latch, is thought to be disrupted by the alteration of the tertiary or quaternary structure of the Keap1 homodimer, accomplished via alterations of the many reactive cysteines within Keap1 through oxidation or covalent modification. The disruption of this efficient turnover of Nrf2 allows for the accumulation of the protein and permits Nrf2 to translocate into the nucleus. Once within the nucleus, Nrf2 forms heterodimers with small Maf proteins, and drives the transcription of genes with a functional Antioxidant Response Element (ARE) within their promoters (Nioi et al. 2003; Malhotra et al. 2010). These genes include, but are not limited to, conjugation/detoxication proteins, antioxidative enzymes, anti-inflammation proteins, the proteasome, and cellular chaperones, creating a general cytoprotective response following pathway activation. Recently, the response of Nrf2 has been broadened in scope, with research documenting interactions between Nrf2 and Notch signaling (Wakabayashi et al. 2010a), p53/p21 (Chen et al. 2009), p62-based autophagy (Komatsu et al. 2010; Lau et al. 2010), aryl hydrocarbon receptor signaling (Shin et al. 2007; Miao et al. 2005), and other processes (Wakabayashi et al. 2010b). These interactions provide the means to elicit the broad-based cell survival responses that now typify the pathway.

Genetic mouse models

The genetic manipulations of the Keap1-Nrf2 pathway have produced a number of transgenic mice, with one of the most useful varieties being the knock out of Nrf2. This mouse has no gross phenotypes within the laboratory setting (Chan et al. 1996); however, it displays impaired homeostasis of reactive oxygen species (ROS), a lupus-like autoimmune syndrome (Ma et al. 2006) and is more sensitive to toxic insult. The sensitivity to toxic insult has been shown in a variety of circumstances, including demonstrations of acute toxicity as well as prolonged exposure to both toxic compounds and carcinogens including acetaminophen (Enomoto et al. 2001;

Chan et al. 2001), ovalbumin (Rangasamy et al. 2005), cigarette smoke (Rangasamy et al. 2004; Iizuka et al. 2005), pentachlorophenol (Umemura et al. 2006), 4-vinylcyclohexene diepoxide (Hu et al. 2006), benzo[a]pyrene (Ramos-Gomez et al. 2001), diesel exhaust (Aoki et al. 2001) and N-nitrosobutyl (4-hydroxybutyl) amine (Iida et al. 2004). In order to examine the situation where Nrf2 signaling is upregulated, a Keap1 knock-out mouse was created; however, this genetic construct is lethal at approximately 3 weeks post-birth, due to hyperkeratosis of the esophagus and forestomach. Prior to death, however, these mice demonstrate a high level of Nrf2 signaling and within the liver show an upregulation of detoxication enzymes including GSTs as well as Nqo1 (Wakabayashi et al. 2003). In order to circumvent this consequence of upregulated Nrf2 signaling, a hepatocyte-specific Keap1 knock-out mouse was created, utilizing flanking loxP sites and a Cre recombinase driven under the albumin promoter. This mouse, hereby referred to as a CKO mouse (conditional Keap1 knock-out), demonstrates no overt phenotypic effects of the upregulated Nrf2 signaling within the liver; however, the upregulation of signaling confers enhanced expression of cytoprotective enzymes and demonstrated protection against acetaminophen poisoning (Okawa et al. 2006). These mice do exhibit a shortened lifespan, indicating that sustained, constitutive activation of Nrf2 is disadvantageous to long-term survival (Taguchi et al. 2010). This transgenic mouse allows for a genetic upregulation of the Keap1-Nrf2-signaling axis, giving another angle of inquiry into the consequences of the alteration of Nrf2 signaling.

The general cytoprotective response of activation of the Nrf2 pathway has made it an attractive target for pharmacological intervention, with a major driving force behind the research being that of chemoprevention. Numerous classes of compounds are known to activate Nrf2 signaling through interaction with Keap1, with the more prominent molecules including dithiolethiones (e.g., olitpraz Egner et al. 1994), isothiocyanates (e.g., sulforaphane Dinkova-Kostova et al. 2007), and triterpenoids (Bensasson et al. 2010) (e.g., CDDO-Im Yates et al. 2007; Liby et al. 2005), with a detailed list in Table 1. These three examples demonstrate a dynamic range of activating potential with a 1,000-fold difference in Nrf2 induction as measured by the induction of ARE-driven genes (Kensler and Wakabayashi 2010). This difference in potency may reflect mechanism of interaction with sensor cysteines in Keap1 (Kobayashi et al. 2009; Dinkova-Kostova et al. 2010). Of these prototypic agents, oltipraz and sulforaphane have either undergone or are actively undergoing clinical trials as chemoprevention agents. Triterpenoids are just entering clinical development as therapeutic agents, but also have a huge potential for chemoprevention.

These drugs are often utilized in conjunction with Nrf2$^{-/-}$ mice in order to demonstrate that the effects

Table 1 Inducers of Keap1-Nrf2 signaling

Endogenous	Exogenous
15Δ-PGJ2	Diphenols, diamines
ER stress	Michael acceptors (e.g., CDDO-Im)
ROS	Isothiocyanates (e.g., sulforaphane)
NO	Thiocarbamates
Lipid aldehydes (e.g., 4-hydroxy nonenal)	Dithiolethiones (e.g., oltipraz)
Nitro-fatty acids	Hydroperoxides
Shear stress	Trivalent arsenicals
Nitrated cGMP	Heavy metals
Toxic bile acids	Vicinal dimercaptans
	Conjugated polyenes
	Phenolic antioxidants (green tea phenols)
	Flavonoids (curcumin, quercetin)

shown in a drug trial against carcinogens are due to upregulation of the Keap1-Nrf2-signaling axis. With that said, of the compounds utilized to induce Nrf2 signaling, many are relatively unspecific, with the exact mode of action being unknown. The use of pharmacologic induction in conjunction with Nrf2$^{-/-}$ mice provides solid evidence of the role of Nrf2 signaling within the experimental context being explored. Additionally, a comparison of gene expression patterns by microarray between the effects of pharmacologic dosing with CDDO-Im with that of genetic alteration via Keap1 disruption in the CKO mouse allow for the assertion that the primary mode of action of CDDO-Im at low doses is that of a specific activator of Nrf2 (Yates et al. 2009). Higher concentrations affect additional targets (Liby et al. 2007), which may in fact also contribute to their protective actions. Sulforaphane, for example, has been shown to cause apoptosis (Gamet-Payrastre et al. 2000; Pledgie-Tracy et al. 2007) and inhibition of proliferation, angiogenesis, and metastasis (Zhang and Tang 2007). Pharmacologic induction of the pathway, however, allows for pulsed induction rather than permanent induction of the Keap1-Nrf2-signaling axis, which may reduce any untoward effects of constant pathway activation.

The role of the Keap1-Nrf2-signaling axis on carcinogenesis

Inflammation and colon cancer

The role of Nrf2 in protection against numerous acute toxicities and carcinogenesis has been investigated, within the contexts of genetic ablation of Nrf2 as well as

pharmacologic interventions. For example, Nrf2 is an effective modifier of inflammatory-based promotion of carcinogenesis within the setting of colorectal cancer. Osburn et al. (2007) as well as Khor et al. (2006) have examined the effect of Nrf2 genotype on the susceptibility of mice to azoxymethane (AOM), and dextran sulfate sodium (DSS) induced colorectal cancer. Within this model of colon cancer, AOM is utilized as a potent initiator and is followed up by exposure to DSS as a promoter via induction of inflammation within the colon. Khor et al. (2006) found that following 1 week of AOM/DSS treatment of both wild-type and Nrf2$^{-/-}$ mice, Nrf2$^{-/-}$ mice demonstrate reduced length of colon as well as an increased severity of colitis. In an additional study, Khor et al. (2008) show that following 20 weeks of AOM/DSS treatment, Nrf2$^{-/-}$ mice show a 40% higher incidence of colonic tumors, with 80% of those tumors being adenocarcinomas versus adenomas. Osburn et al. (2007) also demonstrate that following dosage with the regimen of AOM/DSS, Nrf2$^{-/-}$ mice demonstrate significantly higher levels of inflammatory cytokine mRNAs, polymorphonuclear leukocyte invasion within the colon, and a higher level of protein oxidation when compared with wild-type mice. Within this experimental setting, Nrf2 was shown to attenuate inflammatory damage within the tissue by counteracting the generation of ROS and subsequent macromolecular damage, thereby removing the need to create a microniche of pro-growth within the tissue to compensate for any damage. The activity of Nrf2 thereby reduces ROS generation, a source of potential mutagens, and minimizes the generation of a tumor-promoting microniche within the colonic crypts.

Bladder cancer and interactions with p53

Iida et al. (2004) have shown that Nrf2 plays a role in the detoxification of N-nitrosobutyl(4-hydroxybutyl)amine (BBN), a carcinogen derived metabolically from an N-nitroso compound found in cigarette smoke which acts, when given orally to mice, as a urinary bladder carcinogen. Within the study, it was shown that bladder cancer incidence, following treatment with BBN, was highest within Nrf2$^{-/-}$ mice at 65% incidence, followed by wild-type at 36%, and the lowest incidence of cancer was present within wild-type mice dosed with oltipraz at 14%. Iida et al. then show that UDP-glucuronosyltransferase (UGT) is upregulated within the wild-type and oltipraz-treated mice when compared with Nrf2$^{-/-}$ mice, shunting the carcinogen through a known metabolic pathway leading to detoxication and excretion when Nrf2 is activated. This upregulation is also seen when examining cellular expression of GSTπ. Additionally, Iida et al. show through HPLC that within the urine of Nrf2$^{-/-}$ mice, N-nitrosobutyl(3-carboxypropyl)amine, a carcinogenic metabolite of BBN, is present in

significantly higher quantities when compared to wild-type mice in either the oltipraz treated or untreated groups. This reduction was not seen, however, in Nrf2$^{-/-}$ mice treated with oltipraz, demonstrating the efficacy of the Nrf2 pathway both alone and in combination with pharmacologic activation in reducing the carcinogenic potential of BBN. The carcinogenic potential of BBN in bladder cancer has also been shown to be modulated by Nrf2 through a synergistic effect with p53 (Iida et al. 2007). Iida et al. demonstrated that compound transgenic mice (Nrf2$^{-/-}$ genotype together with a mutation in a single p53 allele) dosed with BBN have a significantly higher incidence of bladder cancer than that of solely an Nrf2$^{-/-}$ mouse or a p53$^{+/-}$ mouse. The Nrf2$^{-/-}$::p53$^{+/-}$ mice also demonstrate a significantly higher rate of muscle-invasive cancer when compared with the Nrf2$^{-/-}$, p53$^{+/-}$ or wild-type mice. This demonstrates the cooperative nature of Nrf2 and p53 in defense against carcinogenic insults, with a two-pronged defensive mechanism. Nrf2 acts to detoxify compounds, and p53 initiates cell cycle checkpoints to either allow for DNA and cellular repair or begins the induction of cellular apoptosis in order to remove potentially harmful cells. Lines of direct interactions between these two pathways are beginning to emerge (Wakabayashi et al. 2010b; You et al. 2011).

Lung disease and cancer

Nrf2 has also been shown to be involved in the response of the lung to numerous toxicological insults including exposure to diesel exhaust and cigarette smoke (Iizuka et al. 2005; Sussan et al. 2009). Aoki et al. (2001) demonstrated that following exposure to diesel exhaust, Nrf2$^{-/-}$ mice have significantly higher levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-OHdG) DNA adducts as well as a higher extent of hyperplasia when compared with Nrf2$^{+/-}$, a genotype that is comparable with wild-type mice in expression of cytoprotective genes. Nrf2 has also been shown to modulate the effects of cigarette smoke exposure within the lungs. Lizuka et al. (2005) as well as Rangasamy et al. (2004) have shown that Nrf2$^{-/-}$ mice are significantly more susceptible to cigarette smoke-induced emphysema and lung damage when compared with wild-type mice. Iizuka et al. demonstrated this vulnerability following both an 8- and 16-week exposure, as shown by levels of inflammatory signals, macrophage, and neutrophil counts. This phenotype is accounted for by a reduction in the ability of Nrf2$^{-/-}$ mice to increase levels of antioxidant genes following exposure to smoke, as well as an impairment of macrophages to phagocytose neutrophils leading to an increase in elastase activity. Rangasamy et al. (2004) demonstrated an earlier onset, and increase in severity of cigarette smoke induced emphysema in Nrf2$^{-/-}$ mice versus their wild-type littermates. The effect of

pharmacological activation of the Nrf2 pathway on the effects of cigarette smoking has also been examined, where mice were dosed with CDDO-Im at 90 mg/kg of diet during a 6-month (5-day/week; 5-h/day) exposure to cigarette smoke. The CDDO-Im-treated mice showed a significant reduction in alveolar destruction, cigarette smoke induced apoptosis and oxidative stress, and DNA damage as shown by examination of 8-OHdG adducts (Sussan et al. 2009). This reduction in damage was attributed to an increase in antioxidant gene expression caused by the upregulation of Nrf2 signaling via pharmacologic induction.

Stomach cancer

Nrf2 has also been shown to be involved in the detoxication of benzo[a]pyrene, a potent pro-carcinogen formed by the incomplete combustion of carbon. The impact of Nrf2 on benzo[a]pyrene carcinogenicity has been examined both in Nrf2$^{-/-}$ mice as well as pharmacologically induced mice with different activators of the Nrf2 pathway, oltipraz (Ramos-Gomez et al. 2001, 2003) and sulforaphane (Fahey et al. 2002) via exposure to Nrf2-activating compound either prior to or concurrent with exposure to benzo[a]pyrene. The activation of the Nrf2 pathway leads to an increase in the activity of conjugating enzymes, allowing for increased conjugation and excretion of benzo[a]pyrene, thereby reducing the toxicological impact (Fig. 2). These induced changes allow for a reduction in tumor incidence within the forestomach that is not seen within the Nrf2$^{-/-}$ genotype, indicating that the pharmacological activation of Nrf2 is responsible for the diversion of benzo[a]pyrene through a less harmful metabolic pathway. Moreover, wild-type mice dosed with vehicle show a lower multiplicity of forestomach tumors than Nrf2$^{-/-}$ mice, further reinforcing the role of Nrf2 as a cytoprotective transcription factor.

Skin cancer

The effect of Nrf2 activation has also been examined within the context of the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) as an initiator of skin cancer. Xu et al. (2006) examined the consequences of both the pharmacological induction of Nrf2 by topical application of sulforaphane as well as the genetic disruption of Nrf2 on the carcinogenic potential of DMBA followed by promotion via the repetitive application of 12-O-teradecanoylphorbol-13-acetate (TPA). Within this experimental context, Nrf2$^{-/-}$ mice developed significantly more skin tumors than did wild-type mice, 95 to 45%, respectively. The induction of the Nrf2 pathway by treatment of the skin with sulforaphane prior to application of DMBA led to a threefold reduction in tumor burden in the wild-type mice, from 60 to 20%, but was ineffective in the
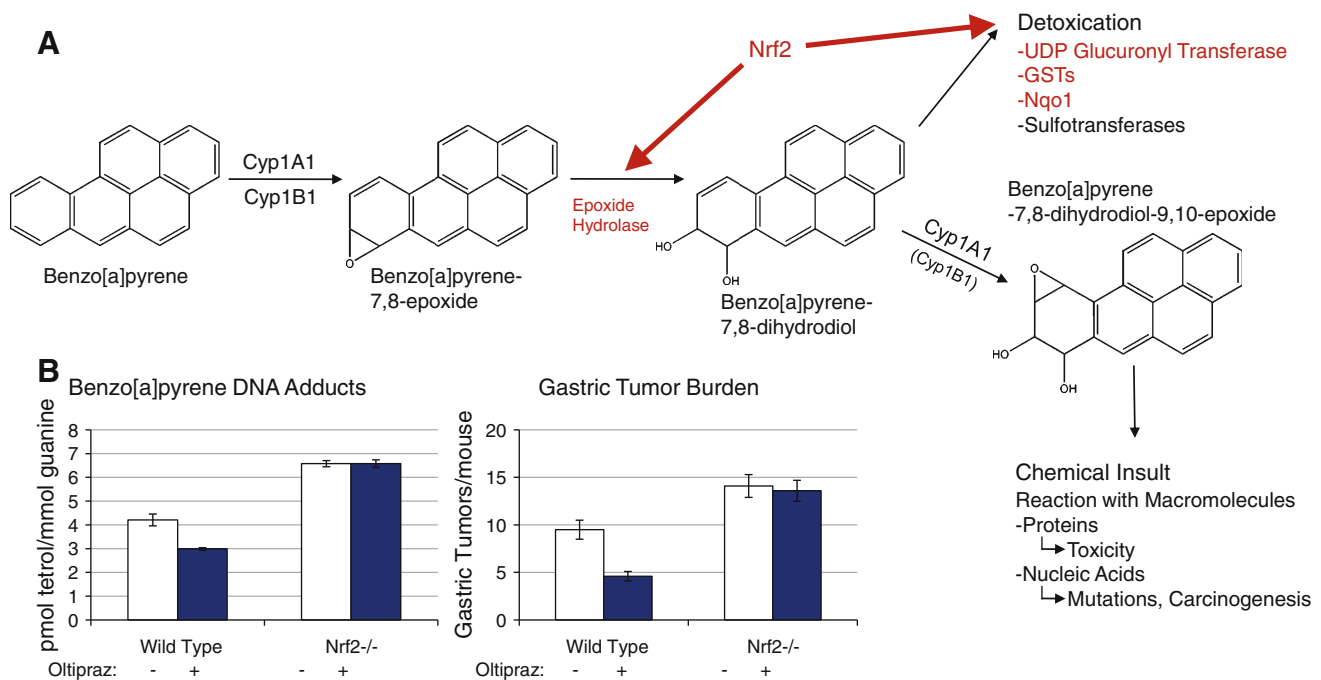
Fig. 2 The role of Nrf2 in the metabolism of B[a]P. **a** Nrf2 serves to upregulate various enzymes involved in the detoxication of B[a]P (Uppstad et al. 2010). **b** The effect of Nrf2 genotype on benzo[a]pyrene metabolism through DNA adducts or tumorigenesis (*left and right panel*, respectively). B[a]P adducts were measured as total tetrols released from DNA isolated from mouse forestomach 24-h post-exposure. Tumorigenesis was evaluated 30 weeks after the first of four weekly exposures to B[a]P. Oltipraz or vehicle was administered 48 h prior to B[a]P exposure (Ramos-Gomez et al. 2001, 2003)

Nrf2$^{-/-}$ mouse, demonstrating an Nrf2-dependent protection from tumorigenesis following DMBA/TPA treatment.

Mammary cancer

The role of Nrf2 has also been examined in mammary gland carcinogenesis. Becks et al. (2010) induced female mice with medroxyprogesterone acetate, followed by treatment with DMBA and examined the influence of both Nrf2 genotype, as well as the potential induction of Nrf2 via the citrus coumarin auraptene. The Nrf2$^{-/-}$ mice, when compared to the wild-type controls, had the same occurrence of tumors; however, the masses of the tumors in the Nrf2$^{-/-}$ mice were significantly larger than those within the wild-type. This suggests a potential role for Nrf2 in regulating the malignant progression of these mammary tumors. Activation of antioxidant signaling by auraptene treatment, as shown by GST activity, had no effect on carcinogenesis within this study.

Endogenous ROS

Nrf2 has also been shown to play a role in the modulation of endogenous stresses, specifically in the case of an increase in intracellular ROS when Nrf2 expression is lowered or knocked out. Frohlich et al. (2008) demonstrated this phenomenon within constructs of prostate cancer cell lines, as well as in Nrf2$^{-/-}$ cells. With a reduction or elimination of Nrf2 expression, intracellular ROS is increased due to a decrease in the expression of antioxidative enzymes. This creates an increase in oxidative damage within the cell, which manifests in increased DNA damage as shown by comet assay. It is easy to concede that an increase in oxidative damage to DNA has the potential to create mutagenic lesions, which if located in the correct area of the genome, can lead to harmful accrued mutations and subsequently, initiation and promotion of cancer (Yu and Kensler 2005; Kwak et al. 2004).

Hepatocarcinogenesis

Nrf2 has also been shown to play a role in mediating hepatocarcinogenesis following exposure to multiple toxins in the mouse. Umemura et al. (2006) have shown that Nrf2$^{-/-}$ mice are more susceptible to pentachlorophenol (PCP) toxicity than the wild-type controls within the study. Following exposure to 1,200 ppm doses of PCP for 4 weeks, Nrf2$^{-/-}$ mice showed significantly higher levels of 8-OHdG within the liver, as well as higher levels of fatty acid oxidation. Additionally, Nrf2$^{-/-}$ mice demonstrate

significantly higher levels of cellular proliferation following exposure to PCP as measured through BrdU-staining. Umemura, and colleagues observed a reduction in both Nqo1 as well as UDP-glucuronosyltransferase expression in the Nrf2$^{-/-}$ mice, demonstrating a reduction in detoxication enzymes that contribute to the increase in cellular insult by PCP. Additionally, Kitamura et al. (2007) have shown that Nrf2$^{-/-}$ mice demonstrate an increased susceptibility to hepatocarcinogenesis following exposure to 2-amino-3-methylimidazo[4,5-*f*-quinoline (IQ). Kitamura and colleagues show that Nrf2$^{-/-}$ mice display significantly higher levels of hepatocellular adenomas and hepatocellular carcinomas following a 52-week exposure of IQ in the diet. A second experiment within the study demonstrates a significantly higher incidence of preneoplastic and neoplastic lesions within the forestomach of Nrf2$^{-/-}$ mice following a 4-week exposure to IQ. This study also demonstrated a depression in UGT and GST activity within the liver of Nrf2$^{-/-}$ mice when compared to control following exposure to IQ. This result, in conjunction with a demonstration of increased fatty acid oxidation, a higher level of cellular proliferation, and higher levels of DNA adduct formation within the livers of Nrf2$^{-/-}$ mice following IQ treatment, show that Nrf2 deficiency leaves these mice in a compromised position of hepatocellular integrity. Within all of these examples, Nrf2$^{-/-}$ mice show an increased sensitivity to cellular stressors and carcinogens, demonstrating the importance of functional Nrf2 in combating toxicological insults through alterations of the cellular defense mechanism, and an increase in detoxification enzymes leads to a reduction in exposure of toxin, leading to lowered risks of mutations and subsequent disease.

The mycotoxin aflatoxin B$_1$, produced by the fungus Aspergillus, has been shown to be a potent hepatic carcinogen in humans (Ross et al. 1992; Sun et al. 1999), rats (Wogan et al. 1974), and infant mice (Vesselinovitch et al. 1972) among many species. This carcinogen is present within staple foods around the world, especially corn and peanuts. This widespread contamination makes aflatoxin an important target for chemopreventive efforts, as while the contamination in foods can be minimized, it is nearly impossible to eradicate. Aflatoxin, once ingested, is metabolically activated to the AFB$_1$-8-9-*exo* epoxide by Cyp450, which then rapidly binds to DNA, forming an N^7-guanine adduct, which in turn is rapidly depurinated from the DNA-yielding abasic sites which hold the potential to lead to mutations within the cell. Nrf2 has been shown to modulate this metabolic pathway. Yates et al. (2006) have shown that CDDO-Im confers protection against aflatoxin-induced tumorigenesis in the rat. Graded dosing with CDDO-Im resulted in reductions in the hepatic burden of GSTπ-positive foci, a presumptive marker of precancerous

lesions, within the liver from 39% up to a complete elimination when compared to control. In order to implicate the Keap1-Nrf2-signaling axis into the mechanism of cancer control in the rat, analysis of numerous Nrf2 target genes was examined; expression of Nrf2 targets Nqo1, Gstα, aflatoxin aldehyde reductase (AFAR) and Hmox1 expression were all elevated. Due to the lack of an Nrf2$^{-/-}$ rat model, the pharmacologic effects of CDDO-Im were investigated in both wild-type and Nrf2$^{-/-}$ mice by microarray, which implicated Nrf2 in the CDDO-Im-based induction of many of these cytoprotective enzymes. It is likely that Nrf2 activation by CDDO-Im is at least partially responsible for the reduction in aflatoxin-based carcinogenesis within the rat; however, the field of aflatoxin research would be well served with an Nrf2$^{-/-}$ rat in order to further investigate the role of Nrf2 in the resistance of aflatoxin toxicity and carcinogenesis.

In an effort to examine the effects of the upregulation of AFAR on the processing of AFB$_1$, one of the consequences of CDDO-Im intervention, a transgenic rat overexpressing AFAR was created. When this rat was challenged with AFB$_1$, higher levels of the AFB$_1$ dialcohol metabolites were excreted, with a concurrent reduction in AFB$_1$-lysine adducts which are formed from the same precursor aflatoxin-dialdehyde metabolite; however, this alteration in metabolism did not significantly impact either the acute toxic or tumorigenic effects of AFB$_1$ dosage as measured by bile duct proliferation or GSTπ positive foci, respectively (Roebuck et al. 2009).

A complication in the examination of the role of the Keap1-Nrf2-signaling axis in aflatoxin-induced carcinogenesis within the mouse is, with the exception of the infant mouse (Vesselinovitch et al. 1972), the wild-type mouse is refractory to the effects of aflatoxin due to high levels of expression of the mouse isoform of glutathione S-transferase A3 (Gsta3), an Nrf2-regulated gene that functions to detoxify the mutagenic aflatoxin-epoxide metabolites (Jowsey et al. 2003). Llic et al. (2010) have developed a Gsta3 knock-out mouse, which has been shown to be sensitive to the acute cytotoxic and genotoxic effects of AFB$_1$; however, as of yet, this mouse model has not been examined in its ability to develop AFB$_1$-induced tumors.

Dark side of Nrf2

Recently, Nrf2 has been shown to be upregulated in numerous types of cancer, indicating a potential role in the promotion of malignancy. A variety of tumor types, including primary head and neck cancers (Stacy et al. 2006) lung tumors (Shibata et al. 2008b; Ohta et al. 2008; Singh et al. 2006), skin (Kim et al. 2010), gallbladder

(Shibata et al. 2008a), breast (Nioi and Nguyen 2007), prostate (Zhang et al. 2010), and endometrial cancers (Jiang et al. 2010) have been shown to have upregulated Nrf2 signaling. Mutations in Keap1 and Nrf2, principally in sequences affecting the interaction domains between these two proteins, are observed in these settings.

It is hypothesized that this upregulation in Nrf2 signaling, and subsequent increase in cellular defensive enzymes, gives tumors an advantage, not only over a challenging tumor microenvironment, but leads to drug resistance, as many standard chemotherapeutics agents are cytotoxic. With the increased levels of protective enzymes as well as drug exporters, many of the cytotoxic effects of chemotherapeutics can be abrogated by the tumor cells by upregulation of Nrf2 signaling, thereby yielding a tumor more suited to aggressive growth and expansion and more capable of escaping anticancer therapy.

Nrf2 may also play into the phenotype of more aggressive growth directly through interactions with Notch1, a cell fate decision molecule, of which Nrf2 has been shown to directly contribute to its expression (Wakabayashi et al. 2010a). An upregulation of Nrf2 thereby has the potential to increase the expression levels of Notch1 within cells, leading to an increase in proliferation rate and a more malignant phenotype. Additionally, Nrf2 has been observed to interact with the p53 pathway via contribution to the basal expression of Mdm2, a direct inhibitor of p53 (You et al. 2011). An increase in the expression levels of Nrf2 could thereby increase the inhibition of p53, as well as confounding any ROS-based apoptotic signals through detoxication, enhancing cellular survival in the face of the challenging tumor microenvironment (Wakabayashi et al. 2010b). Any upregulation in Nrf2 signaling, therefore, has the potential to lead to a more aggressive growth phenotype of tumor in the face of both inherent (e.g., ROS) and external (chemotherapeutics) challenges.

Nrf2 has been shown to facilitate the drug-resistant tumor phenotype in numerous manners, with one instance being that of Nrf2 regulating the expression of the multidrug-resistant protein-3 (MRP3) in both human bronchial epithelial and non-small-cell lung carcinoma (Mahaffey et al. 2009). MRP3 is a member of the multidrug resistance protein family, which, when combined with an upregulation of detoxication enzymes, many of which are direct targets of Nrf2 signaling including GSTs, can lead to the increased hydrophilicity, and excretion of a variety of cytotoxic agents utilized in chemotherapy including chlorambucil, cisplatin, etoposide, and doxorubicin (Wang et al. 2008; Meijerman et al. 2008). The ability of Nrf2 to influence both of these actions, the increase in detoxication enzymes as well as the expression level of MRPs, demonstrates a powerful combination of alterations allowing for an increase in tumor cell survival in the face of cytotoxic agents.

The ability of Nrf2 to modulate the effects of chemotherapeutic drugs has also been shown within the context of pancreatic cancer cell lines by Hong et al. (2010). Within this study, upregulation of Nrf2 protein levels is shown in pancreatic cancer cell lines when compared with normal pancreatic cell lines indicating the phenotype of increased Nrf2 signaling promoting a pro-cell survival phenotype. This is reinforced by the examination of the protein levels of numerous direct downstream targets of Nrf2 including HMOX1, NQO1, and GCLC. Additionally, increasing the levels of Nrf2 through transfection in pancreatic cell lines conferred resistance to the cytotoxic agents cisplatin and camptothecin, while the reduction in Nrf2 signaling through the transfection of a dominant negative construct of Nrf2 leads to a diminution of cell survival rates in the face of such cytotoxic agents.

Upregulation of Nrf2 signaling within the tumor environment has also been observed within lung cancer. Shibata et al. (2008b) observed a number of mutations within the Keap1-Nrf2-signaling axis serving to upregulate Nrf2 signaling, both within lung tumors, and lung cancer cell lines. Shibata et al. also discovered reduced levels of expression of Keap1 within both primary lung cancer tissues and in lung cancer cell lines, indicated that Keap1 activity may be abrogated not only by introduced mutations, but by epigenetic or other methods of control of protein expression levels, in order to ensure the phenotype of increased Nrf2 signaling, and the benefits therein. Also, within the vein of lung cancer research, Singh et al. (2010) have shown that a gain of Nrf2 function confers radioresistance within non-small-cell lung cancer cells. Singh and colleagues hypothesize that this gain in radioresistance is acquired through the increased levels of antioxidative and cytoprotective enzymes that exist within the battery of Nrf2 target genes. This idea is supported by the observation that the disruption of Nrf2 signaling supplemented with the application of exogenous antioxidants prior to exposure to radiation leads to an abrogation of radiation-induced cell death. The utilization of Keap1$^{-/-}$ cells, mimicking the mutations found naturally within tumors, also confers radioresistance to cells, demonstrating an additional consequence of the upregulation of Nrf2 within tumors.

Table 2 Multitiered protection through Nrf2

Damage prevention: Prevent macromolecular damage by electrophiles and ROS (elevated GSH, GSTs, NQO1 etc.); enhanced drug/toxin export

Damage control: Induction of macromolecular damage recognition, repair and removal systems (proteasome, autophagy, DNA repair)

Cell renewal: Activation of tissue repair/regeneration pathways (e.g., Notch1)

Disruption of Keap1 either through genetic mutation or deletion has also been found to occur in biliary tract cancer, with Shibata et al. (2008a) finding somatic mutations within 11.6% of tumors analyzed. These mutations, when introduced into gallbladder cancer cell lines, were found to abrogate the ability of Keap1 to catalyze the ubiquitination and subsequent degradation of Nrf2, allowing for elevated activity of Nrf2 signaling. The upregulation of Nrf2-based cytoprotective signaling leads to a resistance to 5-fluorouracil (5-FU), via neutralization of 5-FU-generated mitochondrial ROS and subsequent apoptotic signaling. The opposite of this effect is true in cell lines with a reduction in Nrf2 signaling demonstrating a survival advantage conferred by an increased Nrf2 signaling.

Nrf2, a multitiered response signaling protein

Nrf2 may protect against carcinogenesis by means other than the detoxication of xenobiotics and cellular toxicants. Nrf2 has been shown to interact with numerous other cellular pathways, including the pro-growth cell differentiation factor Notch1 (Wakabayashi et al. 2010a). In the recent examination of this interaction, Wakabayashi and colleagues demonstrate that Nrf2 is at least partially responsible for the expression of Notch1 in adult mice, as demonstrated though the rescue of the delay in lever regeneration following partial hepatectomy in Nrf2$^{-/-}$ mice by expression of a liver-specific Notch1 intracellular domain (NICD) construct. This interaction demonstrates that Nrf2 not only plays a role in the immediate damage control response to cellular toxic via enzyme induction, but that Nrf2 plays a role in a damaged tissue recovery process. This specific example is not isolated, as Nrf2 has recently been described to interact with a host of cellular signaling networks and processes including p62-mediated autophagy (Komatsu et al. 2010; Lau et al. 2010), NF-κB signaling (Liu et al. 2008), and p21/p53-based signaling (Chen et al. 2009; Wakabayashi et al. 2010b). These interactions show that Nrf2 responds to toxicological insults in a multitiered manner (Table 2). The primary response involves damage prevention through detoxication, conjugation, and excretion of compounds to eliminate reactive intermediates of oxidants and electrophiles. A secondary response entails damage control through increases in DNA repair (Yates et al. 2009), proteasomal activity (Kwak et al. 2003) as well as autophagy (Komatsu et al. 2010; Lau et al. 2010) in order to facilitate recognition, repair and removal of damaged macromolecules from the cell. A tertiary response facilitates cell renewal through interactions with factors such as Notch1 in order to allow for the regrowth of damaged tissues (Wakabayashi et al. 2010a).

Nrf2 functions as a double-edged sword in cellular defense signaling. Under a disruption of cellular homeostasis within a normal, functioning cell, Nrf2 catalyzes the general cytoprotective response to any number of insults that may affect a cell. In the case of a malfunctioning Keap1-Nrf2-signaling axis, however, unfettered Nrf2 signaling allows for protection of cells against numerous chemotherapeutic and cytotoxic agents. The Keap1-Nrf2-signaling axis, thereby, allows two different potential outcomes to pharmacologic interventions. First, the transient upregulation of Nrf2 signaling within the context of chemoprevention against exposure to harmful agents, in order to heighten their detoxification and excretion, and second, the interruption of Nrf2 signaling allowing for the sensitization of chemoresistant tumors to chemotherapeutic and cytotoxic agents. In this setting, inhibitors of the Nrf2 pathway may exhibit therapeutic utility (Ren et al. 2011). Greater understanding of the impacts of diminished and amplified Nrf2 signaling in normal and malignant tissues will be required to safely and effectively utilize small molecule activators and inhibitors as modifiers of multiple stages of carcinogenesis.

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