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NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector

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Abstract

NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1) is a widely-distributed FAD-dependent flavoprotein that promotes obligatory 2-electron reductions of quinones, quinoneimines, nitroaromatics, and azo dyes, at rates that are comparable with NADH or NADPH. These reductions depress quinone levels and thereby minimize opportunities for generation of reactive oxygen intermediates by redox cycling, and for depletion of intracellular thiol pools. NQO1 is a highly-inducible enzyme that is regulated by the Keap1/Nrf2/ARE pathway. Evidence for the importance of the antioxidant functions of NQO1 in combating oxidative stress is provided by demonstrations that induction of NQO1 levels or their depletion (knockout, or knockdown) are associated with decreased and increased susceptibilities to oxidative stress, respectively. Furthermore, benzene genotoxicity is markedly enhanced when NQO1 activity is compromised. Not surprisingly, human polymorphisms that suppress NQO1 activities are associated with increased predisposition to disease. Recent studies have uncovered protective roles for NQO1 that apparently are unrelated to its enzymatic activities. NQO1 binds to and thereby stabilizes the important tumor suppressor p53 against proteasomal degradation. Indeed, NQO1 appears to regulate the degradative fate of other proteins. These findings suggest that NQO1 may exercise a selective “gatekeeping” role in regulating the proteasomal degradation of specific proteins, thereby broadening the cytoprotective role of NQO1 far beyond its highly effective antioxidant functions.

Keywords

antioxidant response element (ARE); benzene toxicity; estrogen quinone; Keap1; microtubule stability; Nrf2; oxidative stress; p53; proteasomal degradation

Introduction

The term antioxidant is familiar to more than 90% of the US population which spends billions of dollars annually on “antioxidant” dietary supplements, in the belief that they provide a health

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benefit [1,2]. Unfortunately there is almost no objective scientific evidence to support this belief.

From a chemical viewpoint, antioxidants may be defined as electron donors to molecular centers that are susceptible to the loss of electrons and therefore to the formation of free radicals and initiation of oxidations and oxidative chain reactions. The critical cellular importance of protective antioxidants became more fully appreciated with the discovery by McCord & Fridovich in 1969 [3] of superoxide dismutase (SOD) which was found to be a widely distributed - enzyme that converted superoxide ($O_2^{\cdot-}$) to H_2O_2 at diffusion-controlled rates. But the mechanisms that have evolved for protecting cells against oxidant stress, a term coined by H. Sies [4], are far more elaborate and complex, probably emphasizing the enormous dangers that oxidants impose to the integrity of living systems. Indeed, because of the presence of elaborate antioxidant defenses, Lane [5] refers to the aerobic cell as: "The Antioxidant Machine." Two properties of these overlapping networks of antioxidative protectors are of special interest. *First*, they do not operate at maximal capacities under basal conditions, but can be upregulated (induced) to much higher levels by the oxidative signals themselves or by a variety of chemical and phytochemical agents, thereby markedly enhancing the capacity of cells to withstand oxidative stress and other forms of toxicity. *Second*, the protective mechanisms not only counteract endogenous oxidative toxicities, but often also protect cells against attacks by electrophiles that are largely of extracellular origin.

A brief overview of the cellular defenses against oxidants and electrophiles will suffice here, since this subject has been discussed in detail elsewhere [6–8]. Aerobic cells express superoxide dismutase which converts superoxide to hydrogen peroxide and acts in concert with catalase and peroxidases that dispose of H_2O_2 . These might be designated as "housekeeping" enzymes that play major roles in combating oxidative stress. In addition to these ubiquitous enzymatic defenses, two types of small molecules provide antioxidant protection: (a) **Direct** antioxidants (e.g., glutathione, ascorbic acid, tocopherols, liponic acid, vitamins K, ubiquinol, and carotenes) which participate in redox reactions directly and scavenge oxidation products. Direct antioxidants are all redox active, are consumed or at least modified in the course of their antioxidative functions, and must be replenished or regenerated in order to regain their antioxidative capacities. (b) **Indirect** antioxidants comprise a wide range of chemical structures that are capable of inducing the cytoprotective (phase 2) response. These agents may or may not be able to participate in redox reactions. Their sole common property is their reactivity with thiol groups, and many have phenolic hydroxyl groups and/or Michael reaction acceptor functions (olefins or acetylenes conjugated to carbonyl or other electron-withdrawing groups). These molecules (inducers) transcriptionally upregulate a large number of genes that exert cytoprotective functions and are also concerned with the biosynthesis of glutathione. Indirect and direct antioxidants however, are not always distinguishable. There are also (c) **Bifunctional** antioxidants which contain chemical substructures that are oxidatively labile and others that are Michael reaction acceptors, and thus are both radical scavengers as well as inducers of cytoprotective enzymes. It is the ability to upregulate the expression of cytoprotective genes that makes such molecules particularly efficient antioxidants, because the "ultimate antioxidants," namely, the cytoprotective enzymes, act catalytically, are not consumed in the course of their antioxidant functions, have relatively long (usually several days) half-lives, and catalyze a wide variety of chemical reactions, such that their concerted actions protect cells and organisms and allow their adaptation to many types of stress. Conversely, deficiencies in these systems frequently result in adaptive failure and exacerbated toxicity.

There is now a wealth of experimental evidence that pharmacological induction of cytoprotective enzymes has multiple beneficial effects [9–12] by: (i) Increasing levels of enzymes that catalyze detoxification of electrophiles, e.g., glutathione *S*-transferases (GSTs),

epoxide hydrolase, NAD(P)H:(quinone acceptor) oxidoreductase 1 (NQO1; EC 1.6.99.2); (ii) Upregulating enzymes that catalyze direct inactivation of oxidants, e.g., catalase, superoxide dismutase, selenium-dependent glutathione peroxidase, and the glutathione peroxidase function of GSTs; (iii) Stimulating glutathione synthesis and regeneration through elevation of the activities of γ -glutamylcysteine ligase, glutathione reductase, and thioredoxin reductase; (iv) Generating additional direct antioxidants, e.g., bilirubin, and CO through induction of heme oxygenase and biliverdin reductase; (v) Preventing iron overload and potentially oxidative stress, e.g., by increasing levels of the iron-binding protein ferritin; (vi) Stimulating NADPH synthesis; (vii) Enhancing the export of toxic drugs via multidrug efflux transporters; (viii) Inhibiting cytokine-mediated inflammation, e.g., via leukotriene B₄ dehydrogenase; and (ix) Enhancing the recognition, repair, and removal of damaged proteins. We focus in this review on the extraordinarily versatile types of protection provided by NQO1, a single multifunctional enzyme, not only through its catalytic activity, but also through totally unanticipated and apparently unrelated properties of this protein.

NQO1, a marker cytoprotective enzyme

NQO1 exemplifies a protein with multiple protective roles that include and extend beyond its catalytic function [13]. It is a widely distributed FAD-dependent flavoprotein that catalyzes the reduction of quinones, quinoneimines, nitroaromatics, and azo dyes. NQO1 was discovered and named DT-diaphorase by Lars Ernster in the late 1950s [14], and shown to be identical to the dicoumarol-inhibited vitamin K reductase described by Märki and Martius [15]. The classical direct antioxidant role of NQO1 is inherent in its catalytic mechanism: the obligatory two-electron reduction [16] of a broad array of quinones to their corresponding hydroquinones by using either NADPH or NADH as the hydride donor [17,18]. In doing so, NQO1 diverts quinone electrophiles from participating in reactions that could lead to either sulfhydryl depletion, or to one-electron reductions that can generate semiquinones and various reactive oxygen intermediates as a result of redox cycling. In addition, the hydroquinone products of the NQO1 reaction can be further metabolized to glucuronide and sulfate conjugates, thereby facilitating their excretion. Notably, quinone reductases have been found in a wide range of eukaryotic organisms ranging from yeast to mammals [19]. Although in many systems much remains to be learned about the intricate details of the function and regulation of these enzymes, it is clear that in all cases quinone reductases are at the forefront of the cellular defense by providing multiple layers of protection (Figure 1).

Around the time of the discovery of NQO1, Williams-Ashman and Huggins [20] found that this enzyme (referred to by them as menadione reductase) was highly inducible in rat liver by azo dyes and polycyclic aromatic hydrocarbons. Furthermore, the potencies of these molecules as inducers of NQO1 correlated with their efficacy as protectors against the toxicity and carcinogenicity of polycyclic aromatic hydrocarbons, leading Huggins to suggest the use of quantifying the activity of NQO1 as a screening method for the identification of potential protective agents [21]. In the late 1980s, Hans Prochaska and his colleagues [22,23] developed a highly quantitative 96-well microtiter plate bioassay for NQO1 in murine hepatoma (Hepa1c1c7) cells (Figure 2). This assay is currently widely used for rapid screening for NQO1 inducer activity of pure compounds and complex mixtures, for activity-guided fractionation of such mixtures, and for the precise determination of inducer potencies [24]. Furthermore, many chemical entities that were found to induce NQO1 in this bioassay were subsequently shown *in vivo* to protect against the toxic and carcinogenic effects of a wide array of carcinogens in a number of target organs, and vice versa. The isothiocyanate sulforaphane represents a prominent example of this strategy [25,26].

Regulation of NQO1 by the Keap1/Nrf2/ARE pathway

The ability of a wide array of chemical inducers [27] and of caloric restriction [28] to upregulate NQO1 is mediated through the Keap1/Nrf2/ARE pathway (Figure 3). By controlling the expression of a battery of >100 cytoprotective genes, this pathway is essential for the adaptation of mammalian cells and organisms to various electrophilic and oxidative stressors [10–12, 29–31]. As the name of the pathway suggests, three cellular components are of central importance for the mechanisms by which the transcription of this gene battery is regulated: (i) antioxidant response elements (ARE), DNA sequences that are present in the upstream regulatory regions of these genes and have the consensus: TGAG/CNNNGC [32]. In the case of the murine *nqo1* gene, the exact sequence was revised by John Hayes and his colleagues [33] who showed that certain nucleotides previously thought to be redundant in the ARE function have essential roles, whereas others that were previously considered essential, were dispensable; (ii) Nrf2 (nuclear factor-erythroid 2-related factor 2), a basic leucine zipper transcription factor of the “cap-‘n collar” family that binds as a heterodimer with a small Maf protein, to the ARE, thereby signaling enhanced transcription [34,35]; and (iii) Keap1 (Kelch-like ECH-associated protein 1), the protein sensor for inducers, a Kelch family multidomain repressor protein that binds Nrf2 [36] and promotes its ubiquitination and proteasomal degradation [37,38] by functioning as an adaptor for Cul3-based E3 ligase [39–41]. Several different models have been proposed for the mechanism of regulation of the Keap1/Nrf2/ARE pathway [reviewed in 31]. The most widely accepted model postulates that inducers, all of which react with sulfhydryl groups [11], modify highly reactive cysteine residues of the sensor Keap1 [42] which then loses its ability to target Nrf2 for degradation. Consequently, Nrf2 is stabilized and accumulates in the nucleus where it binds to AREs and triggers the expression of cytoprotective genes. Interestingly, even though the Keap1/Nrf2/ARE pathway seems to have evolved relatively recently, there are many parallels between animals and plants regarding inducible gene expression [43], and quinone reductase represents a prominent example of a cytoprotective enzyme that is induced in both mammals and plants in response to electrophiles. Furthermore, in rodent and human cells and tissues, NQO1 is one of the most consistently and robustly inducible genes amongst the members of the family of cytoprotective proteins. This early finding [44] has been repeatedly confirmed by global gene expression profiling in a number of systems that employed both pharmacological inducers of the Keap1/Nrf2/ARE pathway, and Keap1 knockdown or knockout genetic approaches. Although the protective effects of Nrf2 activators against cancer and other chronic diseases that have been observed in numerous animal models are undoubtedly due to the concerted action of many cytoprotective proteins whose gene expression is controlled by this transcription factor, the role of NQO1 is prominent, and it has been called a “quintessential” cytoprotective enzyme [45].

The multiple antioxidant activities of NQO1

An unequivocal demonstration that NQO1 has a direct antioxidant activity came from the collaborative work between the laboratories of Helmut Sies and Paul Talalay [46,47]. When supernatant fractions of mouse liver homogenates were incubated with menadione (2-methyl-1,4-naphthoquinone) in the presence of NADPH, an oxygen-dependent low-level emission of red light was observed (Figure 4). This chemiluminescence arises from the formation of superoxide and singlet oxygen during the quinone redox cycling accompanying the one-electron reduction of menadione catalyzed by NADPH-cytochrome P-450 reductase. When liver homogenates from mice that were fed with BHA, a treatment that resulted in a 13-fold higher NQO1 activity in comparison to control mice, were used in a similar experiment, the chemiluminescence was much reduced. Conversely, adding the potent inhibitor dicumarol to control fractions increased the chemiluminescence signal, strongly suggesting that NQO1 was catalyzing the two-electron reduction of menadione thus, diverting it from oxidative one-electron redox cycling. This conclusion was unequivocally established by titration of purified

crystalline NQO1 into postmitochondrial mouse liver supernatant fractions, which resulted in progressive suppression of the menadione-dependent chemiluminescence. Importantly, the degree of suppression was identical between fractions prepared from BHA-treated animals and those from control animals that had identical added levels of exogenous NQO1.

Notably, in addition to its catalytic role in reduction of quinones, NQO1 has been reported to scavenge superoxide directly, albeit less efficiently than superoxide dismutase (SOD) [48]. This property could provide an additional layer of protection and could be especially important in tissues with low SOD expression. Thus, in cardiovascular cells, where expression of NQO1 is high and that of SOD is relatively low, induction of NQO1 was found to correlate with increased superoxide scavenging, whereas its inhibition led to a decrease in superoxide scavenging [49].

The protective role of NQO1 against the toxicity of benzene is widely recognized and has been recently reviewed [50]. Benzene is a procarcinogen and is converted by CYP2E1 in the liver to a number of metabolites including benzene oxide, catechol, 1,4-hydroquinone, and 1,2,4-benzenetriol. Upon transport to the bone marrow, these hydroquinones serve as substrates for myeloperoxidase giving rise to genotoxic benzoquinones. Thus, the combination of low NQO1 expression and high myeloperoxidase activity constitutes a major susceptibility factor for benzene hematotoxicity that has been reported in humans who were exposed to very low atmospheric levels of benzene, below 1 ppm [51].

Genetic variations in NQO1

Two types of polymorphisms in the gene encoding for NQO1 have been described in humans [reviewed in 13,52–54]. The more prominent one, both in terms of frequency and phenotypic consequences, is NQO1*2, a single nucleotide polymorphism, a C to T change at position 609 of the NQO1 cDNA. This nucleotide substitution results in a proline to serine substitution at position 187 of the amino acid sequence of the protein. The mutant NQO1*2 protein is very unstable, and is rapidly ubiquitinated and degraded by the proteasome [55]. Thus, both NQO1 protein and activity are virtually undetectable in humans carrying the NQO1*2/*2 genotype [52,56]. Individuals who have this null phenotype for NQO1 are more susceptible to the toxic and neoplastic effect of benzene [57]. NQO1 deficiency increases the risk of developing leukemia following occupational exposure to benzene [58], even though bone marrow cells normally do not have detectable levels of NQO1. This apparent paradox was explained by Moran et al. [59] who demonstrated a dose-dependent induction of NQO1 in a human promyeloblastic cell line upon exposure to hydroquinone, and proposed that it is the failure to induce functional NQO1 that may contribute to the increased risk of benzene poisoning in individuals homozygous for the NQO1 C609T substitution. In addition, NQO1 is present in stroma, especially in endothelial cells lining large blood vessels and sinusoids, as well as in adipocytes in human bone marrow [55] and thus could still play a protective role. Studies with NQO1-knockout mice have also found greater sensitivity to benzene-induced hematotoxicity in comparison with wild-type animals [60]. Mice are much more susceptible to benzene-induced hematotoxicity than are rats, and this increased susceptibility correlates with the much lower expression of NQO1 in murine bone marrow [61]. Conversely, induction of NQO1 protects cultured bone marrow stromal cells in culture against benzene-mediated toxicity [62].

Protection against estrogen quinones

It is now being increasingly recognized that NQO1 is essential for protection against the deleterious effects not only of exogenous, but also of endogenous quinone metabolites such as estrogen quinones (Figure 5). Although its physiological significance is still a matter of debate [63], there is now strong biochemical evidence that NQO1 participates in the reduction of

estradiol-3,4-quinone [64]. Furthermore, induction of NQO1 protects against estrogen-induced formation of depurinating N⁷-guanine and N³-adenine adducts [65,66], oxidative DNA damage, and mammary carcinogenesis [67]. Conversely, downregulation of NQO1 increases the levels of estrogen quinone metabolites and enhances the transformation potential of 17 β -estradiol [67]. In addition, lower NQO1 activity accompanied by higher CYP1B1 activity in breast tissue was documented when 5 cases of human breast cancer were compared to 4 healthy controls [68].

Curiously, hepatic NQO1 activity is higher in female than in male mice (our unpublished observations), most likely reflecting the occurrence of induction of its gene expression by the quinone metabolites of estrogens, as well as the protective role of the enzyme against the genotoxicity of such metabolites. Indeed, we have shown that estradiol-3,4-quinone induces ARE-dependent gene expression by ~6-fold in MCF-7 human breast cancer cells stably transfected with a luciferase reporter under the control of the antioxidant response element (ARE) [69]. In contrast, exposure to 2-hydroxyestradiol, 4-hydroxyestradiol, or 4-hydroxyestrone has only a modest effect on luciferase expression; however, induction is potentiated by ~4–5-fold under conditions that favor formation of the estrogen quinones, i.e., in the presence of Cu⁺⁺ and O₂, strongly suggesting that the quinone metabolites are the ultimate inducers. Recently, Singh et al. [70] generated stably transfected human breast epithelial cell lines that express either wild-type or various mutant isoforms of NQO1. Compared to cells transfected with the wild-type gene, cells transfected with mutant NQO1 expressed ~2-fold lower levels of the protein and, upon exposure to estradiol-3,4-quinone, showed lower ability to reduce the quinone and contained 2-fold lower levels of free catechols, ~3-fold lower levels of methylated catechols, and 2.5-fold greater amount of depurinating DNA adducts. Taken together, these findings suggest that NQO1 polymorphisms may increase the likelihood of mutagenesis in estrogen-exposed breast epithelial cells. Indeed, a recent Finnish study demonstrated one such polymorphic variant, P187S, to be a strong prognostic and predictive factor in breast cancer survival and metastasis [71]. Curiously, Lu et al. [72] reported that in the human breast epithelial cell line MCF-10F, exposure to resveratrol induced NQO1 through stabilization of transcription factor Nrf2, but in addition, led to subcellular redistribution of the (presumably newly synthesized) protein such that NQO1 was observed not only in the cytoplasm, but also in the nucleus of these cells. Alternatively, it is possible that even at basal expression levels, there exists a small pool of nuclear NQO1 that is below the limit of detection. It is thus tempting to speculate that NQO1 in the nucleus may be important in guarding against estrogen quinone-induced depurinating DNA adducts at the site of their formation.

Neuroprotective aspects of NQO1

Another class of potential endogenous NQO1 substrates are the quinone derivatives of dopamine, L-DOPA, norepinephrine, epinephrine, and 3,4-dihydroxyphenylacetic acid, as well as their respective cyclization metabolites aminochrome, dopachrome, noradrenochrome, adrenochrome, and furanoquinone. Zafar et al. [73] demonstrated that such cyclized quinones, and/or reactive oxygen intermediates generated during their redox cycling, inhibited the proteasome and that NQO1 guarded against this inhibition. In contrast, superoxide dismutase and catalase were not protective. In addition, overexpression of NQO1 protected SK-N-MC human neuroblastoma cells against the cytotoxicity of dopamine, whereas superoxide dismutase and catalase were ineffective [74]. Prior treatment of PC12 neuronal cells with dopamine increased NQO1 and glutathione levels and protected against cell death caused by toxic concentrations of dopamine or 6-hydroxydopamine [75]. Similarly, induction of NQO1 and GSH by dimethyl fumarate, ³H-1,2-dithiole-3-thione or *tert*-butylhydroquinone (tBHQ) protected against neurocytotoxicity caused by dopamine, 6-hydroxydopamine, 4-hydroxy-2-nonenal, or H₂O₂ [76–80]. In PC12 cells that were treated with H₂O₂, pharmacological

induction of NQO1 attenuated the formation of protein-bound quinones and protected against oxidative damage [81]. Similarly, in SH-SY5Y cells, the H_2O_2 – dependent formation of reactive oxygen intermediates was reduced by treatment with the neuroprotective agent ladostigil, with the concomitant induction of NQO1 and other antioxidant enzymes [82,83]. In a dopaminergic cell line derived from a brain tumor of transgenic mice carrying the SV40 T antigen under the transcriptional control of the rat tyrosine hydroxylase gene (CATH.a), methamphetamine treatment was reported to lead to the formation of protein-bound quinones that was accompanied by NQO1 induction [84]. Prior treatment with BHA was protective both against the methamphetamine-dependent formation of protein-bound quinones and subsequent cell death. Notably, pharmacological induction of NQO1 is usually accompanied by coordinate elevation of the gene expression of many other cytoprotective proteins, and thus the protective effects of small molecule inducers are most likely due to the combined cytoprotective effects of the induced proteins. Nevertheless, the direct ability to catalyze the detoxification of quinone metabolites places NQO1 at the forefront of cellular defense against their potential neurotoxic effects.

It has been suggested that the ability of endogenous metabolites of dopamine to induce proteasomal inhibition and apoptosis may contribute to the selective loss of dopaminergic neurons in Parkinson's disease [85]. NQO1 expression has been reported to be elevated in substantia nigra pars compacta in Parkinson's disease patients [86] and in neurofibrillary tangles and hippocampal neurons of Alzheimer's disease patients [87,88]. These high levels of NQO1 are most likely a consequence of enhanced gene expression triggered by dopamine quinone metabolites and may represent an adaptive response against their potential accumulation. We have recently shown that ARE-dependent gene expression is markedly enhanced upon exposure of cells to dopamine or L-DOPA in the presence of O_2 and Cu^{++} , conditions that favor the oxidation of the catechol moiety [69].

Since dopaminergic neurons are selectively damaged in certain neurodegenerative conditions, such as Parkinson's disease, and dopamine and its metabolites have been implicated in disease pathogenesis, it could be hypothesized that NQO1 may have a protective role against such conditions. Although the epidemiological data regarding the relationship of polymorphisms in NQO1 and the incidence of Parkinson's disease and Alzheimer's disease remain controversial [89–92], a recent study reported that the wild-type genotype for NQO1 has a possible protective effect against the development of Alzheimer's disease, whereas the C609T allele might constitute a risk factor [93].

NQO1 has been also implicated in the maintenance of coenzyme Q (CoQ) and vitamin E in their reduced and active forms. Thus, pure rat NQO1 was able to reduce homologues of CoQ with both short (e.g., CoQ₁, CoQ₃) and long (e.g., CoQ₉, CoQ₁₀) isoprenoid side chain lengths when incorporated into phospholipid vesicles, and protected against 2,2'-azobis(2,4-dimethylvaleronitrile)-induced lipid peroxidation [94–96]. In addition, whereas reduced CoQ protected isolated hepatocytes against adriamycin-induced oxidative damage, this protection was abolished by addition of the NQO1 inhibitor dicumarol [97]. Pure recombinant human NQO1 catalyzed the reduction of α -tocopherylquinone (a product of the oxidation of α -tocopherol) to α -tocopherylhydroquinone, a potent cellular antioxidant [98], suggesting a role of NQO1 in the metabolism of α -tocopherol.

In comparison with most nicotinamide nucleotide-dependent oxidoreductases, NQO1 is unusual in its ability to use both NADPH and NADH equally efficiently. It has been suggested that NQO1 may contribute to the regulation of the redox balance by modulating reduced/oxidized pyridine nucleotide ratios [99]. Inhibition or absence of NQO1 may lead to loss of NAD^+ which could affect polyADP-ribose polymerase and the function of other NAD^+ -dependent proteins [100]. In agreement with this supposition, NQO1-knockout mice, compared

to their wild-type counterparts, have lower levels of nicotinamide nucleotides in liver, kidney, and skin [101,102]. Conversely, induction of NQO1 by sulforaphane also coordinately induced genes encoding for cellular NADPH regenerating enzymes such as glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malic enzyme [103].

Curiously, by using a chemical approach that involved the screening of >1500 purine derivatives, NQO1 was identified as a potential novel regulator of microtubule stability in extracts of *Xenopus* eggs [104]. Although the underlying mechanism remains unknown, the authors suggested the possibility that NQO1 might protect microtubules by catalyzing the reduction of certain endogenous quinones that could otherwise cause microtubule depolymerization.

NQO1, a gatekeeper of the 20S proteasome

Stabilization of the tumor suppressors p53, p73 α , and p33 is a relatively recently recognized role of NQO1 that could be especially important under conditions of stress when NQO1 expression is robustly elevated. In 2001, Asher et al. [100] reported that NQO1 inhibits the degradation of p53 in HCT-116 human colon cancer cells. Exposure to the potent NQO1 inhibitor dicumarol [3,3'-methylenebis(4-hydroxycoumarin)], as well as other inhibitors that compete with NAD(P)H, enhanced p53 proteasomal degradation, and this effect was reversed by proteasome inhibitors. In contrast, transfection with the wild-type, but not mutant enzyme resulted in p53 stabilization [105]. Stabilization of p53 by NQO1 was: (i) especially prominent under conditions of oxidative stress [100], or upon acute exposure to γ -irradiation or benzo [a]pyrene [106], (ii) occurred via a distinct mechanism that was independent of both Mdm-2 and ubiquitin [106,107], and (iii) was not observed in cells isolated from NQO1-knockout mice [106]. Interestingly, compared to wild-type p53, some of the "hot spot" p53 mutants that are frequently found in human cancers, i.e., p53 R175H and p53 R273H, exhibited higher affinity for binding to NQO1 and greater resistance to dicumarol-induced degradation, although they remained sensitive to Mdm-2-ubiquitin-mediated degradation [108].

The observation that the specific irreversible NQO1 suicide inhibitor ES936 (5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione) had no effect on p53 degradation [109] strongly indicated that the catalytic function of the enzyme is not required for stabilization of p53 and prompted the search for a physical interaction between the two proteins. Immunoprecipitation experiments in HCT-116 human colon cancer cells, primary human keratinocytes, and mammary epithelial cells, as well as studies in an *in vitro* transcription/translation system utilizing rabbit reticulocyte lysates confirmed such an association, and that it was not affected by ES936 [109]. In contrast, dicumarol, and other inhibitors that compete with NAD(P)H, disrupted the binding of NQO1 and p53 and caused ubiquitin-independent p53 degradation [108,110], whereas binding of NQO1 to p53 (and p73 α , see below) was enhanced by addition of NADH, but not by NAD or FAD [110]. The crystal structure of human NQO1 in a complex with dicumarol revealed that the binding of this inhibitor induced conformational changes that involved tyrosine 128 and phenylalanine 232 [111]. Interestingly, these conformational changes were much larger than those that occurred upon binding of ES936, leading the authors to suggest that the native conformation around tyrosine 128 and phenylalanine 232 could be important for p53 binding. Further work will be needed to test this hypothesis, but the findings point to the critical importance of NAD(P)H which, in addition to serving as the electron donor for the catalytic function of NQO1, is also required for the association of NQO1 with p53.

In addition to p53, NQO1 also regulates the ubiquitin-independent 20S proteasomal degradation of p73 α [110] and p33 [112]. Furthermore, in fractionated murine liver homogenates the majority of NQO1 was found to be associated with the 20S proteasome, and

the authors proposed that NQO1 could function as a gatekeeper for protein degradation through the 20S proteasome [113]. This proposition was further strengthened by the finding that the ubiquitin-independent 20S proteasomal degradation of ornithine decarboxylase (ODC) was also regulated by NQO1 [113]. Thus, under basal conditions, overexpression of NQO1 stabilizes, whereas knockdown of NQO1 destabilizes endogenous ODC. In contrast to the prominent stabilization of p53 under stress conditions, however, treatment with H₂O₂ compromised the ability of NQO1 to protect ODC from degradation. Consequently, ODC is degraded, a process that prevents further exacerbation of oxidative stress due to the otherwise persistent ODC activation. Very recently, it was shown that NQO1 protects the eukaryotic translation initiation factor (eIF) 4GI from proteasomal degradation and thus may participate in the regulation of translation [114].

In Summary

In the more than 50 years that have elapsed since the discovery of NQO1, the functional significance and cellular importance of this enzyme have been an evolving story with many unexpected twists and turns. The initial beliefs that NQO1 might participate in cellular energy capture by regulating the balance and relations between nicotinamide nucleotides, or that it was involved in blood coagulation because of its potent inhibition by dicumarol, are no longer widely accepted. Instead, it has now been firmly established that NQO1 provides major antioxidant functions by virtue of its obligatory two-electron reduction mechanism, which diverts quinones from participating in oxidative cycling and generation of reactive oxygen intermediates. But the finding that the gene coding for NQO1 is highly inducible, and that such induction protected animals and their cells against the toxic and neoplastic effects of carcinogens and against oxidative stress, provided a major new perspective on the functional importance of this enzyme. It led to the development of a very useful bioassay system to measure the potencies, to isolate, and to identify inducers for NQO1. This assay has reliably predicted the chemoprotective properties of numerous new agents *in vivo*. It is therefore surprising that NQO1 also exerts a selective “gatekeeping” role in regulating the proteasomal degradation of specific proteins, apparently independently of its catalytic mechanism. This function appears to be important in the stabilization of p53, a broadly-functioning tumor suppressor gene. The last finding provides another example of the propensity of nature to endow functionally important proteins with novel and quite unrelated roles.

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Figure 1. The multiple cytoprotective functions of NQO1

NQO1 catalyzes the obligatory 2-electron reduction of various exogenous and endogenous quinones, quinoneimines, nitroaromatic compounds and azo dyes. NQO1 has superoxide scavenging activity, which although much less efficient than superoxide dismutase (SOD), can be important in tissues or under conditions where expression of NQO1 is high and that of SOD is low. NQO1 stabilizes p53 and other tumor suppressor proteins which are otherwise degraded via the 20S proteasome. In *Xenopus* egg extracts, NQO1 stabilizes microtubules. It has been proposed that NQO1 can modulate the ratios of reduced/oxidized nicotinamide nucleotide pools.

Figure 2. The principle of the NQO1 bioassay

Murine Hepa1c1c7 cells are cultured in 96-well plates. Twenty-four hours later, cells are exposed to a range of concentrations of inducers for 48 hours. Then, cells are lysed and the activity of NQO1 is determined using menadione as a substrate and an NADPH-regenerating system. The menadiol reaction product reduces the tetrazolium yellow dye MTT to a purple formazan, the formation of which is quantitatively determined by its characteristic absorbance (in the range of 490 to 640 nm). The specific activity of NQO1 is plotted as a ratio of treated over control wells against the inducer concentration. CD value, the Concentration of an inducer that Doubles the enzyme activity. G6PDH, glucose-6-phosphate dehydrogenase.

Figure 3. The Keap1/Nrf2/ARE pathway

Under basal condition (dashed arrow), the dimeric multidomain protein Keap1 binds transcription factor Nrf2 via its Kelch domain and promotes the ubiquitination and proteasomal degradation of the transcription factor by functioning as an adaptor for Cul3-based E3 ligase. Inducers, all of which react with sulfhydryl groups, chemically modify specific highly reactive cysteine residues of Keap1 which then loses its ability to target Nrf2 for degradation. Consequently, Nrf2 is stabilized and becomes available for translocation (solid arrow) to the nucleus where it binds to AREs and triggers the expression of NQO1 and a battery of >100 other cytoprotective genes.

Figure 4. The antioxidant activity of NQO1

Mouse liver postmitochondrial supernatant fractions (S9) from untreated (containing 1.7 units of NQO1, uppermost trace) or BHA-treated (10.9 units NQO1, lowest trace) animals were incubated with menadione in the presence of NADPH-generating system, and the chemiluminescence due to formation of superoxide and singlet oxygen during the quinone redox cycling was recorded. The intermediate traces represent the chemiluminescence when liver fractions of untreated mice were used to which pure crystalline enzyme was added to produce 4.9, 5.5, or 10.9 units of NQO1. Note that the degree of suppression of the chemiluminescence was proportional to the amount of enzyme activity added, and was identical in fractions prepared from BHA-treated animals and those from control animals that had identical added levels of exogenous NQO1. [Reproduced with permission from H.J. Prochaska, P. Talalay, H. Sies. *J. Biol. Chem.* 262 (1987) 1931.]

Figure 5. The role of NQO1 in cellular protection against the toxicity of estrogen quinones NQO1 catalyzes the obligatory 2-electron reduction of estrogen quinones to catechol estrogens which can then be conjugated and excreted. In doing so, NQO1 diverts the electrophilic quinones from participating in potentially deleterious processes that could lead to: (i) formation of depurinating mutagenic DNA adducts, (ii) sulfhydryl depletion, or (iii) one-electron reduction generating semiquinones and various reactive oxygen intermediates arising from redox cycling. In addition, the estrogen quinones themselves induce the gene expression of NQO1 and the synthesis of glutathione via the Keap1/Nrf2/ARE pathway. COMT, catechol *O*-methyl transferase; GSH/GST, glutathione/glutathione *S*-transferase; P450 R, cytochrome P450 reductase; SOD, superoxide dismutase. Adapted (with modifications) from Reference 70.