Potent Intracellular Oxidative Stress Exerted by the Carcinogen
4-Nitroquinoline-N-oxide

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Abstract

Oxidative stress exerted by superoxide-generating (redox-cycling) agents such as paraquat triggers the soxRS regulon of Escherichia coli. In this system, SoxR protein is the redox-sensitive activator of the soxS gene, the product of which then activates the −10 promoters of this regulon. We found that 4-nitroquinoline-N-oxide (4NQO) is a powerful inducer of soxS, >10-fold more potent than paraquat. The transcriptional induction of the soxS gene by 4NQO was tightly dependent on a functional soxR gene and on the presence of molecular oxygen, as found previously for several well characterized redox-cycling agents. Two 4NQO-related compounds were also shown to induce soxS:4-nitropyridine-N-oxide, with an efficiency only slightly less than 4NQO, and 4-hydroxyaminquinoline-N-oxide, at ~50-fold lower potency than 4NQO. E. coli strains that are hypersensitive to oxidative stress (owing to deficiency in either Superoxide dismutases or oxidative DNA repair enzymes) were hypersensitive to killing by 4NQO. Thus, considerable oxidative stress is induced in cells by 4NQO, which might contribute to the carcinogenic potency of this compound.

Introduction

4NQO (see Fig. 1) is a potent mutagen and carcinogen (1). In numerous studies, microgram quantities of 4NQO were sufficient to induce papillomas, squamous cell carcinomas, adenocarcinomas, fibrosarcomas, and lymphomas in mice, rats, hamsters, guinea pigs, and rabbits (1). Potent mutagenic activity of 4NQO has also been reported in Salmonella typhimurium (2). The carcinogenic action of 4NQO is thought to be initiated by the enzymatic reduction of its nitro group. The four-electron reduction product, 4HAQO (Fig. 1), may be the proximate carcinogenic metabolite of 4NQO and is implicated in the formation of DNA adducts (3, 4). These DNA damages are usually associated with the mutagenicity and carcinogenicity of 4NQO (1). Occasional reports have also suggested that 4HAQO in vitro can generate reactive oxygen species such as the superoxide radical (O2−) or hydrogen peroxide (H2O2). These secondary agents could then cause oxidative DNA damages or injure other cellular components to upset metabolic pathways and cause oxidative (redox) stress.

Chemical agents that increase the level of intracellular superoxide (redox-cycling compounds) switch on an oxidative stress response controlled by the soxRS locus (8–10). Induction of the soxRS system (which controls at least 10 promoters including sodA, nfo, zwf, micF, etc.) occurs in two stages: (a) existing SoxR protein is triggered by an intracellular redox signal to activate transcription of the soxS gene; and (b) the resulting elevated level of SoxS protein then activates the various soxRS regulon genes (11, 12). Using operon (transcriptional) fusions of the E. coli lac genes to the soxS promoter (11), we show here that 4NQO is a surprisingly potent inducer of the soxRS regulon system of E. coli. This 4NQO-induced oxidative stress might contribute to the tumor-promoting activity of 4NQO.

Materials and Methods

Bacteria, Phages, and Plasmids. The bacterial strain used to construct the lysogens carrying single copy soxS::lacZ was DJ901, which is a ΔsoxRS derivative of GC4468 [Δ(lac)U169 rpsL soxRS] (9). In the experiments measuring 4NQO sensitivity, the mutants AB1886 [as AB1157 but uvrB6] (13) and BW528 (RCP501) [AB1157 but Δnfo tar-1::kan] (14), and QC909 [as GC4468 but (sodA::Mu dpr)1325 (sodB::kan)Δ1–2] (15) were used, as well as the parental strains AB1157 and GC4468. The bacteriophage λRS45 [bla'::lacZ lacY'] (16), a gift from N. Kleckner, Harvard University, was used to insert the soxS::lacZ fusion into chromosomal DNA from MC4100 [Δ(lac)U169 rpsL soxRS'] carrying either pTN1520 [Φ(soxS' soxS::lacZ)] or pTN1530 [Φ(ΔsoxR soxS'::lacZ)] (11). The constructions were carried out as described by Simons et al. (16). Briefly, cells of MC4100/pTN1520 and MC4100/pTN1530 were infected with a λRS45 lysate. Recombination between ARS45 and the plasmids within homologous regions (lacZYa and bla) yields λ[Φ(soxS'::lacZ)] fusions and a still incomplete bla gene. Such recombinant phages were screened and identified by their Lac+ phenotype (blue plaques on LB agar supplemented with 40 µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Plaque-purified isolates, ATN1520 and ATN1530, were then integrated into the attB site of the chromosome of the ΔsoxRS strain DJ901. The resulting lysogens, TN521 and TN531, bearing Φ(soxS::soxS::lacZ) and Φ(ΔsoxR soxS::lacZ), respectively, were identified by their Lac+ Amp+ phenotype.

Induction Experiments. Overnight cultures of TN521 and TN531 in LB broth were diluted 100-fold into fresh LB broth and incubated at 37°C for ~45 min to reach OD600 ~0.1. Two ml aliquots of the cultures in 15-ml tubes were treated with various concentrations of agents at 37°C for 60 min, with vigorous shaking at ~200 rpm. For anaerobic experiments, the culture sample was transferred to AtmosBag (Aldrich) previously flushed and filled with argon gas (Ar), and bubbled with Ar at room temperature for 2–3 min. The indicated agent, 4NQO or PQ, was then added; the tubes were sealed with serum caps and bubbling tubes and then transferred to a 37°C bath where bubbling with Ar was continued. After a 60-min incubation, chloramphenicol was added to each sample to a final concentration of 100 µg/ml to stop further protein synthesis. β-Galactosidase activity was determined in air as described previously (11).

4NQO Sensitivity Experiments. Overnight cultures were diluted 200-fold into LB broth and incubated at 37°C for 60 min to reach 1 × 107 cells/ml. Sample aliquots were treated with 30 µg 4NQO at 37°C with vigorous shaking at 200 rpm. At the indicated times, samples were removed and the cells diluted and plated on LB agar to measure colony-forming ability.

Results and Discussion

The induction of soxS transcription by 4NQO assayed in strains carrying a soxS::lacZ fusion was rapid and tightly dependent on a functional soxR gene (Fig. 2A), as found previously for several well characterized redox-cycling agents (11). The extent of soxS induction by 4NQO was concentration dependent, with >10-fold induction occurring at ~5 µM (Fig. 3). Two well known redox-cycling agents that were tested required higher concentrations for 10-fold induction of soxS::lacZ (Fig. 3): (a) ~40 µM for PQ; and (b) 400–500 µM for menadione bisulfite. Only phenazine methosulfate induced soxS at concentrations lower (2- to 5-fold) than required for 4NQO (Fig. 3).
4NQO, a compound related to 4NQO but lacking the benzene ring (Fig. 1), induced soxS with only slightly less potency than found for 4NQO (Fig. 3). The reduced 4NQO metabolite 4HAQO (Fig. 1) was also a significant soxS inducer, but 10-fold induction required 100–200 μM 4HAQO (Fig. 3). The varying potency of 4NQO, 4NPO, and 4HAQO might be due to differences in reduction of these compounds by cellular enzymes or to differences in uptake into the bacteria. The carcinogenic potency of these compounds in animals also varies (1), but it is unclear how these differences are related to differences in uptake or metabolism in animal cells.

Redox-cycling agents require molecular oxygen in order to impose oxidative stress: their catalytic effect depends on oxygen as an electron acceptor and the source of damaging radicals (17). When bacteria were exposed to 4NQO under near anaerobic conditions, almost no induction of soxS was observed (Fig. 2B), as previously seen for PQ and phenazine methosulfate (11). This result suggests that 4NQO participates in a redox cycle to generate the signal that switches on the soxRS regulon.

Nitro anion radicals of nitroaromatic and nitroheterocyclic compounds (Ar-NO₂⁻) are intermediates in the enzymatic reduction of these compounds to hydroxylamines or amines (18–20). Superoxide might therefore be generated during the oxidation of these radicals by molecular oxygen. Nitroxide radical (Ar-NHO) may also support a redox cycle involving oxygen (17).

Biaglow et al. (21) found that 4NQO, nitrofurans, and menadione bisulfite stimulate oxygen consumption of Ehrlich ascites cells in suspension in the presence of KCN, which suggested redox cycling by 4NQO (17). The production of superoxide and hydrogen peroxide was also demonstrated with isolated microsomes in the presence of these (21), by electron spin spectroscopy to convert to a radical compound in a manner dependent on oxygen, again suggesting superoxide generation. Consistent with this conclusion, a marked production of hydrogen peroxide (the dismutation product of O₂⁻) by 4HAQO, but not by 4NQO, was observed under aeration in vitro (7). This production may occur during the oxidation of 4HAQO to 4-nitrosoquinoline-N-oxide (Fig. 1) via a nitroxide radical (4-NHO⁻QO) (Fig. 1) (7).

Further evidence for the production of reactive oxygen in vivo by 4NQO was obtained from examination of strains with various defects in antioxidant defense. Bacteria lacking SOD (by deletion of both bacterial sod genes), which are hypersensitive to redox-cycling agents such as paraquat (15), also exhibited hypersensitivity to 4NQO (Fig. 4A). Significant production of oxygen radicals in vivo causes oxidative damage to DNA, which requires specific repair pathways (8, 14, 22). An E. coli mutant lacking two oxidative DNA repair enzymes (Δthr hflp-1::kan; deficient in exonuclease III and endonuclease IV) was hypersensitive to 4NQO compared to its repair-proficient counterpart (Fig. 4B). Moreover, an excision repair-deficient (uvrA⁻) mu-

![Fig. 1. Structures of 4NQO and its derivatives.](image1)

![Fig. 2. Induction of the soxS gene by 4NQO. A, dependence of soxS induction by 4NQO on a functional soxR gene. Cultures of E. coli TN521 (○, DJ901 ΔsoxR sox5':lacZ) and TN531 (□, DJ901 ΔsoxR sox5':lacZ) were treated with 10 μM 4NQO at 37°C for the indicated times. β-Galactosidase activity was then determined (10). B, oxygen dependence of soxS induction by 4NQO and PQ. Cultures of TN521 were maintained either aerobically or anaerobically, with or without exposure to 4NQO (10 μM) or PQ (50 μM). After a 60-min incubation, β-galactosidase activity was determined (10).](image2)

![Fig. 3. Potency of soxS induction by 4NQO and several redox-cycling agents. Cultures of TN521 were treated with the indicated agents for 60 min, and the β-galactosidase activity determined (10). The basal level of β-galactosidase in TN521 was 225 units. The decrease in β-galactosidase observed at 50 μM 4NQO was accompanied by inhibition of cell growth. A similar decrease in β-galactosidase activity was also observed at 100 μM compared to 10 μM 4NPO (data not shown).](image3)

![Fig. 4. Sensitivity of mutant strains to killing by 4NQO. Cultures of mutant strains were exposed to 50 μM of 4NQO at 37°C with vigorous shaking at 200 rpm. At the indicated times, the samples were removed and the cells were diluted and plated on LB agar to measure colony-forming ability. (A) GC4468, DJ901, and QC909 devoid of superoxide dismutase. (B) AB1157, BW528, and AB1886, wild type.](image4)
tant was by far the most 4NQO-sensitive strain (Fig. 3B), which corroborates the well known formation of DNA adducts by 4NQO.

It seemed likely that the hypersensitivity of SOD-lacking cells to 4NQO was due to intracellular oxidative stress imposed by the com-

ponent was by far the most 4NQO-sensitive strain (Fig. 3B), which

adducts in DNA. Consistent with this hypothesis, the SOD-

lacking strain exposed to 4NQO in phosphate buffer exhibited wild-
type resistance (data not shown); under these circumstances, aerobic

respiration (the likely source of electrons for redox cycling; Ref. 17)

would be eliminated. In contrast, the uvrA mutant in phosphate buffer

retained its hypersensitivity to 4NQO (data not shown).

A soxRS-deletion strain was only slightly more sensitive to killing by 4NQO than was wild-type E. coli (Fig. 4A). Although this strain fails to increase various antioxidant functions (e.g., Mn-SOD and endonuclease IV), the mutant still expresses normal basal levels of the regulated enzymes (9). Together with the induction of the soxRS-

independent defenses (23), the basal expression of soxRS-regulated functions evidently affords substantial protection against the redox toxicity of 4NQO.

The intracellular signal that activates SoxR has not yet been estab-

lished. Superoxide is an attractive candidate for the signal, but this conclusion is open to debate (9, 11, 12). For example, several studies (24–26) have detected no effect of high basal expression of SOD on the induction of soxRS-controlled proteins or genes by PQ; a high level of SOD would be expected to lower intracellular superoxide levels and interfere with induction by superoxide. Quinones that in-

duce the soxRS response, such as plumbagin and menadione, may
directly inhibit the respiratory NADH dehydrogenase and cause a redox imbalance (27), such as a significant change in the NAD(P)⁺/NAD(P)H ratio (26). The effect of 4NQO on NADH dehydrogenase or other respiratory enzymes has not yet been explored.

Kohda et al. (28) have reported that 8-oxoguanine residues are

formed in the DNA of Ehrlich cells exposed to 4NQO. According to

their proposed mechanism, 4NQO forms 8-oxoguanine by metabolic

reduction and the formation of an adduct to N7, which decomposes to

release 4-aminoquinoline-N-oxide and 8-oxoguanine (28). Our experiments indicate an alternative explanation that 4NQO undergoes redox
cycling to generate superoxide, which can be converted to H₂O₂ and forms various oxidative DNA damages including 8-oxoguanine. Consistent with this hypothesis, the hypersensitivity to 4NQO of E. coli deficient in exonuclease III and endonuclease IV indicates the oxidative formation of DNA strand breaks which require these enzymes for efficient repair (29).

Although some previous reports connected 4NQO to the production of reactive oxygen, the potency of 4NQO as an oxidative stress agent was not anticipated. The potent carcinogenicity of 4NQO might therefore depend on the exertion of oxidative stress in target cells, in addition to the formation of DNA adducts involving this agent. This oxidative stress could have significant epigenetic effects, e.g., by inducing cell proliferation (30, 31) or by producing additional muta-
tagenic DNA damages that might arise from 4NQO-generated oxygen radicals (22). However, the ability to exert the type of oxidative stress examined here may be insufficient for carcinogenesis; 4NPO, shown here to be a strong inducer of soxRS, was not detectably carcinogenic in experimental animals (1).

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