Epidermal Growth Factor Receptor Is a Common Mediator of Quinone-induced Signaling Leading to Phosphorylation of Connexin-43

ROLE OF GLUTATHIONE AND TYROSINE PHOSPHATASES*

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Rat liver epithelial cells were exposed to three quinones with different properties: menadione (2-methyl-1,4-naphthoquinone, vitamin K₃), an alkylating as well as redox-cycling quinone, the strongly alkylating p-benzquinone (BQ), and the non-arylation redox-cycler, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ). All three quinones induced the activation of extracellular signal-regulated kinase (ERK) 1 and ERK 2 via the activation of epidermal growth factor receptor (EGFR) and MAPK/ERK kinases (MEK) 1/2. ERK activation resulted in phosphorylation at Ser-279 and Ser-282 of the gap junctional proteins, connexin-43, known to result in the loss of gap junctional intercellular communication. Another EGFR-dependent pathway was stimulated, leading to the activation of the antiapoptotic kinase Akt via phosphoinositide 3-kinase (PI3K)/Akt cascade, and others (7, 8). In summary, EGFR-dependent signaling was mediated by protein-tyrosine phosphatase regulating the EGFR, as concluded from an EGFR dephosphorylation assay; (ii) although menadione-induced activation of ERK was unimpaired by pretreatment of cells with N-acetyl cysteine, activation by BQ and DMNQ was prevented; (iii) cellular glutathione (GSH) levels were strongly depleted by BQ. The mere depletion of GSH by application of diethyl maleate EGFR-dependently activated ERK and Akt, thus mimicking BQ effects. GSH levels were only moderately decreased by menadione and not affected by DMNQ. Not only are intracellular signaling pathways affected that regulate the survival and proliferation of the respective cell harboring these signaling cascades, but a stress response may also consist of disconnecting a cell from its environment, e.g. by down-regulation of gap junctional intercellular communication (GJC) to prevent diffusion of xenobiotics or toxic metabolites thereof within a tissue. Gap junctions allow the diffusion of compounds of low molecular mass (up to about 1 kDa) between the cytoplasm of adjacent cells (9), a process known to be regulated by phosphorylation of the gap junctional proteins, the connexins (Cx) (10, 11). Recently, the exposure of rat liver epithelial cells to menadione (2-methyl-1,4-naphthoquinone, vitamin K₃), which is both a redox-cycling and an alkylating quinone, was shown to lead to the activation of extracellular signal-regulated kinase (ERK) 1 and ERK 2, which are well known for their prominent role in the regulation of cellular proliferation (12). ERK activation resulted in phosphorylation of the gap junctional channel protein Cx43 and down-regulation of GJC (8). The activation of ERK was blocked by inhibitors of the direct upstream kinases of ERK 1/2, MAPK/ERK kinase (MEK 1), and MEK 2 and by inhibitors of the epidermal growth factor receptor by boxylation of blood coagulation factors involving the K vitamins, or redox cycling as a result of quinone reduction followed by reoxidation with molecular oxygen, concomitantly generating the superoxide anion (2, 3). Further, a variety of alklylation reactions are observed, including Michael-type additions of sulfhydryl groups to quinones (4) or DNA alklylation (5). This biochemistry of quinones is exploited in cancer chemotherapy, such as with mitomycin c or certain anthraquinone derivatives including doxorubicin, yet the exact contributions of both redox and alkylation reactions to either the desired or adverse effects of these compounds are not fully elucidated.

Cells respond to stimuli such as those imposed by xenobiotic quinones by activating stress-responsive signaling cascades regulating cellular proliferation and survival. These include pathways activated by DNA damage, such as p53-related events (6), and general stress-responsive cascades, such as mitogen-activated protein kinase (MAPK)¹ cascades, the phosphoinositide 3-kinase (PI3K/Akt cascade, and others (7, 8).

Quinones are involved in a number of biochemical reactions (1), including redox reactions such as the transport of electrons by ubiquinol in the mitochondrial respiratory chain, the car

* This work was supported by Deutsche Forschungsgemeinschaft, Bonn, Germany (Grants SFB 575/B4; SFB 503/B1). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† In partial fulfillment of the requirements for a Ph.D. (Dr. rer. nat.) degree at Heinrich-Heine-Universität Düsseldorf.

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¹ The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; EGFR, epidermal growth factor receptor; EGF, EGFR receptor; NAC, N-acetyl cysteine; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; PTPase, protein-tyrosine phosphatase; PBS, phosphate-buffered saline; DEM, diethyl maleate; PI3K, phosphoinositide 3-kinase; Cx, connexins; GJC, gap junctional intercellular communication; DAPI, 4',6-diamino-2-phenylindole; BQ, p-benzquinone; MQ, menadione; IP, immunoprecipitation.
We here demonstrate that quinones of different structures and reactivities all activate the same signaling pathway, resulting in Cx43 phosphorylation. Both a strongly alkylating quinone, \( p \)-benzoquinone (BQ), as well as an exclusive redox-cycler, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), cause an EGFR-dependent activation of ERK and the PI3K/Akt cascade, as does menadione, an alkylating and redox-cycling compound. Regarding the mechanism of activation, protein-tyrosine phosphatase (PTPase) inhibition, glutathione depletion, and redox cycling are proposed to be responsible for the activation of the pathway by menadione, BQ, and DMNQ, respectively. It thus appears that different modes of action of the various quinones are similarly interpreted and integrated by the cell and funneled into the same signaling pathway that regulates cellular proliferation and intercellular communication.

EXPERIMENTAL PROCEDURES

Cell Culture—WB-F344 rat liver epithelial cells (13) with stem cell-like properties (14) were a kind gift of Dr. James E. Trosko, East Lansing, MI. They were held in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with (final concentrations) 10% (v/v) fetal calf serum (BioWest, Frickenhausen, Germany), 2 mM l-glutamine, and penicillin/streptomycin. HeLa cells (European Collection of Cell Cultures, Salisbury, UK) were grown under identical conditions. Quinones were from Sigma (menadione), Merck (BQ), and Calbiochem (DMNQ), and diethyl maleate (DEM) was from Sigma. Stocks of these compounds were in MeSO and stored frozen in the dark. Cells grown to confluence in 7-cm² dishes were exposed to quinones diluted in serum-free medium for the times indicated. In experiments with inhibitors of signaling cascades (U0126, compound 56, AG1478, wortmannin, LY294002, all from Alexis, Lausen, Switzerland, or Calbiochem) culture medium was removed, and cells were briefly washed with PBS prior to preincubation for 30 min with either MeSO (control) or the respective inhibitor (diluted from stock solutions in MeSO) diluted in serum-free medium. Inhibitors were also present in the medium during exposure to the respective quinone tested.

Western Blotting, Dot Blotting, and Immunohistochemistry—Cells were grown to 90–100% confluence and exposed to the respective quinone or to diethyl maleate. Cells were washed once with PBS and lysed in 2× SDS-PAGE buffer (125 mM Tris/HCl, 4% (w/v) SDS, 20% (w/v) glycerol, 100 mM dithiothreitol, 0.2% (w/v) bromphenol blue, pH 6.8) followed by brief sonication. Samples were applied to SDS-polyacrylamide gels of 10% (w/v) acrylamide followed by electrophoresis and by blotting onto nitrocellulose. Membranes were blocked in 5% (w/v) non-fat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST), except for incubations with anti-phosphotyrosine (see below). Detection of antibody binding to the membrane was with secondary antibodies coupled to horseradish peroxidase and detection thereof with an enhanced chemiluminescent substrate (SuperSignal, Pierce/Perbio). For immunodetection of connexin-43, dot blotting (A) and immunohistochemistry (B). In A, loading control was by detection of total Cx43. In B, nuclei were stained with DAPI for orientation. Data are representative of at least three independent experiments.

FIG. 1. ERK activation by quinones. WB-F344 rat liver epithelial cells were exposed to menadione (MQ, 50 \( \mu \)M), BQ (100 \( \mu \)M), or DMNQ (100 \( \mu \)M) for the times indicated (A) or for 15 min (B) followed by determination of ERK phosphorylation and total ERK levels by Western blotting. The MEK inhibitor U0126 was used at 10 \( \mu \)M. MeSO was taken as vehicle control “C” for the quinones and for U0126. Data are representative of at least three independent experiments.

FIG. 2. Induction of connexin phosphorylation by quinones. WB-F344 rat liver epithelial cells were exposed to MeSO (vehicle control, C), MQ (50 \( \mu \)M), BQ (50 \( \mu \)M), or DMNQ (100 \( \mu \)M) for 30 min. Phosphorylation of Cx43 at Ser-279 and Ser-282 was then analyzed by dot blotting (A) and immunohistochemistry (B). In A, loading control was by detection of total Cx43. In B, nuclei were stained with DAPI for orientation. Data are representative of at least three independent experiments.

(EGFR) tyrosine kinase, leading to the hypothesis that ligand-independent activation of the EGFR by menadione was responsible for the above described effects (8).
Quinone-induced Signaling via EGF Receptor

Fig. 3. Role of EGFR in menadione-induced ERK activation. In A, WB-F344 cells were exposed to menadione (50 μM) for 15 min with or without pre- and coinoculation with inhibitors of the EGF tyrosine kinase (AG1478 and compound 56, c56; 10 μM each) followed by analysis of ERK phosphorylation by Western blotting. Exposure to inhibitors alone (not shown) did not yield results different from control (−). Me2SO served as vehicle control for menadione and for the inhibitors. In B, cells were treated with EGF at 400 ng/ml (+) or control treated (−) for 1 h followed by washing and incubation in serum-free medium for 2 h. Cells were then exposed to menadione at the indicated concentrations or EGF (100 ng/ml) for 15 min followed by lysis and Western blotting. In C, cells were exposed to menadione at the concentrations given for 15 min followed by lysis and IP of the EGFR and determination of tyrosine phosphorylation by Western blotting. Detection of EGFR in the precipitates served as control for successful IP and equal loading; EGF (100 ng/ml for 15 min) was used as positive control for tyrosine phosphorylation. Data are representative of at least three independent experiments.

Fig. 4. Quinone-induced activation of EGFR-dependent signaling. In A, WB-F344 cells were exposed to Me2SO (vehicle control, C), BQ (50 μM), or DMNQ (100 μM) for 30 min with or without pre- and coinoculation with inhibitors of the EGF tyrosine kinase (AG1478 and compound 56; c56; 10 μM each) followed by analysis of ERK phosphorylation by Western blotting. Exposure to inhibitors alone (not shown) did not yield results different from control (−). Me2SO served as vehicle control both for menadione and for the inhibitors. In B, cells were exposed to MQ (50 μM), BQ, or DMNQ (100 μM each) in the absence or presence of inhibitors of phosphoinositide 3-kinase, wortmannin (100 nM), and LY294002 (20 μM). Phosphorylation of Akt at Ser-473 was detected by Western blotting. Data are representative of at least three independent experiments.
µl of 4× SDS-PAGE sample buffer to the washed protein A pellets, samples were boiled, and the proteins were resolved on 8% Tris-glycine gels followed by blotting onto polyvinylidene difluoride membranes. Detection of phosphorylated tyrosines was with monoclonal anti-phosphotyrosine antibody (4G10; Upstate Biotechnology). Membranes were blocked for 1 h in 1% (w/v) bovine serum albumin in TBST, briefly washed with TBST, and incubated with the primary antibody diluted 1:1,000 in 1% bovine serum albumin in TBST at 4 °C overnight. The secondary antibody was diluted in TBST. Human recombinant EGF (R&D Systems, Minneapolis, MN) was taken as positive control.

**Assay of EGF Receptor Dephosphorylation**—The assay was essentially performed as described by Knebel et al. (17). Briefly, HeLa cells were grown to 80–100% confluency and serum-starved overnight. EGF receptor tyrosine phosphorylation was stimulated by incubation in the presence of EGF (100 ng/ml) for 5 min. The cells were washed with PBS, treated with the respective quinone (100 µM) or Me3SO (as vehicle control) for 15 min. The quinone was removed, and fresh serum-free medium containing the EGFR tyrosine kinase inhibitor compound 56 (Calbiochem; 10 µM) was added to prevent any further autophosphorylation of the receptor. After 30 s, medium was quickly removed, and cells were lysed in 2× SDS-PAGE sample buffer followed by SDS-PAGE on a gel of 8% (w/v) acrylamide and Western blotting with detection of phosphorylated tyrosine residues (4G10 monoclonal antibody, Upstate Biotechnology).

**Glutathione Determination**—Total glutathione and glutathione disulfide were determined enzymatically according to ref. 18 with minor modifications. Briefly, cells on 7-cm2 culture dishes were lysed by scraping them in 250 µl of ice-cold HCl (10 mM) followed by one freeze/thaw cycle, brief sonication on ice, and centrifugation at 20,000 × g for 10 min to remove cell debris. Aliquots of the supernatants were kept for protein determination according to Bradford. For glutathione determination, protein was precipitated from the supernatant with 5% (w/v) sulfosalicylic acid on ice. Samples were vortexed and centrifuged at 20,000 × g for 10 min. Total glutathione (GSH plus GSSG) and, after blocking thiols with 2-vinylpyridine, GSSG were determined from the supernatant using 5,5'-dithiobis(2-nitrobenzoic acid in the presence of NADPH and glutathione reductase (18).

**RESULTS**

**Activation of ERK 1/2 and Connexin-43 Phosphorylation after Exposure to Quinones**—Exposure of cells to menadione is known to result in the activation of ERK 1/2 and ERK-dependent phosphorylation of connexin-43 (Cx43), resulting in a decreased intercellular communication (8). Two other quinones of different reactivities were tested for ERK activation and Cx43 phosphorylation: BQ, a strong alkylator, and DMNQ, an exclusive redox-cycler, were compared with menadione, which is known both to undergo redox cycling and to alkylate. Exposure of cells to any of the three quinones resulted in the strong dual phosphorylation of ERK 1/2 (Fig. 1A), which is mediated by MEK 1 and MEK 2, the kinases directly upstream of ERK 1/2, as U0126, a specific inhibitor of MEK 1/2 activation, completely (BQ, DMNQ) or largely (menadione) blocked ERK phosphorylation (Fig. 1B). When cells were exposed to any of the quinones for 15 min followed by aspiration of quinone-containing media and postincubation in serum-free medium, the activation of ERK 1/2 remained equally strong with slight decreases observable starting after 2 h of postincubation (data not shown).

As was demonstrated for menadione (8), phosphorylation of Cx43 at Ser-279 and Ser-282, sites specifically recognized by ERK 1/2 (10, 11), was also induced in cells exposed to BQ or DMNQ (Fig. 2). Phosphorylation of Cx43 at ERK 1/2 sites was demonstrated both by dot blotting (Fig. 2A) and by immunohistochemistry (Fig. 2B) using two different polyclonal phospho-specific antibodies.

**Role of the Epidermal Growth Factor Receptor in Quinone-induced Signaling**—Based on inhibitor studies, it was proposed that menadione-induced activation of ERK 1/2 is mediated by the activation of the EGFR (8). As demonstrated in Fig. 3A, AG1478 and compound 56, two specific inhibitors of the EGFR tyrosine kinase, largely blocked menadione-induced ERK activation. Another strategy used to demonstrate the dependence of ERK activation by menadione on EGFR was based upon the fact that permanent exposure of cells to high concentrations of growth factors may result in an enhanced internalization of the respective receptors (19–21). Exposure of WB-F344 cells to high concentrations of EGF (400 ng/ml) for 1 h followed by washing and 2 h of incubation with serum-free medium rendered cells refractory for further activation by EGF (100 ng/ml) or menadione (10, 25 µM), as seen in Fig. 3B. Taken together, menadione-induced ERK activation is dependent on EGF stimulation. The activation of the EGFR upon exposure to menadione was demonstrated by immunoprecipitation and detection of tyrosine-phosphorylated receptor molecules (Fig. 3C).

Like menadione, BQ- and DMNQ-induced ERK activation relies on EGFR activation, as demonstrated using AG1478 and compound 56 (Fig. 4A), both of which abrogated ERK phosphorylation induced by exposure of cells to these quinones. If the EGFR is activated in cells exposed to menadione, BQ, or DMNQ, downstream signaling pathways other than the MEK/ERK pathway should also be stimulated. In fact, the PI3K/Akt cascade was activated in WB-F344 cells as serine 473 phosphorylation of Akt was strongly enhanced after treatment with menadione, BQ, or DMNQ and abrogated in the presence of either of two structurally unrelated inhibitors of PI3K, wortmannin or LY294002 (Fig. 4B). Taken together, menadione, BQ, and DMNQ all activate the EGFR as well as downstream signaling pathways.

**Role of Protein-tyrosine Phosphatase Inactivation in EGFR Activation by Quinones**—One possible way of activating a receptor tyrosine kinase such as the EGFR in the absence of ligand would be to disrupt negative regulation of the receptor. It was demonstrated that the activation of receptor tyrosine kinases by ultraviolet irradiation and reactive oxygen species relies on the inactivation of a PT-Pase regulating the respective receptor (22). To test for the involvement of a PT-Pase in the activation of the EGFR upon exposure to quinones, cells were treated with EGF followed by addition of the EGFR tyrosine kinase inhibitor, compound 56. HeLa cells were used for these

![Fig. 5. Protein-tyrosine phosphatase inhibition by quinones.](image-url)
experiments because they responded better to EGF treatment, yielding more intense tyrosine phosphorylation than WB-F344 cells, which is crucial for the assay. Stimulation of the cells with EGF resulted in tyrosine phosphorylation of the receptor (Fig. 5A, lane 2), which disappeared after addition of compound 56 (Fig. 5A, lane 3). This can be explained only by the existence of a tyrosine phosphatase that dephosphorylates the activated receptor (Fig. 5B). In the presence of menadione, however, dephosphorylation was blocked, resulting both in an enhanced responsiveness of the cells to EGF (Fig. 5A, lane 4 versus lane 2) and in the maintenance of tyrosine phosphorylation of the EGFR even in the presence of the EGFR tyrosine kinase inhibitor (Fig. 5A, lane 5). On the contrary, neither BQ nor DMNQ had the same effect (Fig. 5A, lanes 7 and 9), pointing to mechanisms other than FTPase inactivation being responsible for EGFR activation by these quinones.

**Fig. 6. Interaction of quinones with glutathione.** A, UV-visible spectra of a mixture of GSH (0.4 mM) with MQ, BQ, or DMNQ. The final concentration of all quinones was 0.25 mM. In each of the panels, “A” represents calculated sum spectrum of the GSH spectrum plus the quinone spectrum and is expected if no interaction between GSH and quinone occurs, and “B” shows the measured spectrum of GSH plus quinone mixtures. B, relative changes in total glutathione (GSH + 0.5 GSSG; top) and GSSG (bottom) levels in rat liver epithelial cells exposed for 15 min to MeSO (as vehicle control, C), menadione (50 μM), BQ (100 μM), DMNQ (100 μM), or DEM (1 mM). Data are means of at least three independent experiments performed in triplicate ± S.D.

**Fig. 7. Effect of NAC on quinone-induced ERK activation.** In A, rat liver epithelial cells were exposed to menadione (50 μM), BQ (100 μM), DMNQ (100 μM), or DEM (1 mM) in the presence of 30 mM NAC for 15 min followed by lysis and analysis of ERK phosphorylation and total ERK levels by Western blotting. In B, cells were exposed to NAC (30 mM) for 3 h followed by washing and exposure to the quinones for 15 min. Data are representative of three independent experiments.
On the Role of Glutathione in Quinone-induced EGFR and ERK Activation—The results obtained from experiments on PTPase inhibition render it highly likely that menadione-induced EGFR activation is due to inactivation of a PTPase negatively regulating the receptor. Menadione both is an alkylator and undergoes redox cycling in the cell. Both the alkylation of a PTPase and its oxidation by reactive oxygen species generated upon redox cycling are feasible mechanisms of inactivating the enzyme. All PTPases known so far harbor an essential cysteine thiolate at their active site that is prone to alkylation and undergoes redox cycling. In the cell, both the alkylating properties of menadione, it interacts with GSH, as can be seen from the difference in UV-visible spectra of menadione before and after reaction with GSH: the calculated sum spectrum of GSH plus menadione significantly differs from the spectrum measured after mixing the two compounds (Fig. 6A, top). Even more dramatic spectral changes were observed with BQ, whereas no differences between calculated and measured spectra were seen for DMNQ (Fig. 6A, middle and bottom). This is in line (i) with BQ being a strong alkylator that is known to easily react with GSH, not only forming monogluthathionylated hydroquinone but also di-, tri-, and tetra-gluthathionyl)-hydroquinone in cells (4, 24); and (ii) with DMNQ being a non-alkylating quinone: both the C2 and middle C3 positions are occupied. In accordance with the spectra in Fig. 6A, about 35% of the cellular GSH was depleted in cells exposed to menadione already after 15 min, whereas about 90% was lost after exposure to BQ, and no more than a tendency toward GSH depletion was observed with DMNQ (Fig. 6B, top).

Significant accumulation of glutathione disulfide was found only in cells exposed to menadione; a tendency to accumulate GSSG was seen with DMNQ (Fig. 6B, bottom). The extensive accumulation of GSSG in menadione-treated cells may be explained by redox cycling of menadione in combination with its known inhibitory effect on glutathione reductase (25): superoxide derived from redox cycling would yield hydrogen peroxide that is reduced by glutathione peroxidase at the expense of GSH, which in turn, ends up as GSSG that cannot be reduced by glutathione reductase if menadione is present.

To delineate the role of GSH depletion in the activation of ERK 1/2 by quinones, cells were exposed to N-acetyl cysteine (NAC), a cell-permeant thiol employed to deliver antioxidant capacity to cells that serves as a precursor of cysteine which is utilized for GSH synthesis. ERK activation by menadione and BQ, but not by DMNQ, was prevented by the concomitant presence of NAC with the quinones (Fig. 7A). This was probably due to the direct interaction of NAC, which was applied in a 300–600-fold molar excess, with menadione or BQ, respectively. No such interaction with the non-alkylating DMNQ is to be expected. Indeed, spectral changes of the quinones in the presence of NAC were very similar to those observed with GSH (Fig. 6A, bottom). In accordance with Fig. 5A, which demonstrates that inhibition of a PTPase regulating the EGFR was exclusively brought about by menadione.

Glutathione Depletion Leads to Activation of EGFR-dependent Signaling—To test whether the depletion of GSH might cause the activation of EGFR-dependent signaling, cells were exposed to DEM, a glutathione S-transferase substrate that is coupled to GSH, thus depleting the thiol. Exposure of WB-F344 cells to various concentrations of DEM resulted in a concentration-dependent activation of ERK 1/2 and of Akt (Fig. 8A). Eighty-three percent (83 ± 3%, means ± S.D., n = 3) of cellular...
glutathione was depleted with 1 mM DEM under these conditions. Interestingly, GSH depletion had to be rapid, i.e. within minutes (as with BQ), to result in the activation of ERK: exposure of cells to buthionine sulfoximine (4 mM), an inhibitor of GSH biosynthesis, for 24 h decreased total glutathione by 71 ± 9% (means ± S.D., n = 3) of control, but no ERK activation was seen (data not shown).

ERK and Akt activation was blocked in the presence of inhibitors of the EGFR, AG1478, and compound 56. Interestingly, this activation was also partly prevented by AG1295, an inhibitor of the platelet-derived growth factor receptor tyrosine kinase (Fig. 8B), pointing to a minor role of another receptor tyrosine kinase in addition to EGFR. As with menadione or BQ, the depletion of GSH by the application of DEM entails phosphorylation of the ERK 1/2-specific sites of Cx43, Ser-279 and Ser-282, as demonstrated by dot blotting (Fig. 9A) and immunocytochemistry (Fig. 9B) using two different antibodies.

**DISCUSSION**

**Significance of ERK Activation by Quinones**—The activation of ERK 1/2 is usually connected with cell proliferation, which is due to the substrates of the kinases, including transcription factors as well as key enzymes involved in nucleotide and protein synthesis (see Ref. 12 for review). Connexin-43 is also phosphorylated by ERK 1/2, resulting in an attenuation of GJC (10, 11, 26). Three Cx43 sites were demonstrated to be phosphorylated by ERK 1/2, Ser-255 as well as Ser-279/Ser-282, all of which are located in the C-terminal cytoplasmic domain of the protein (10, 11). Recently, ERK 5 was implicated in Cx43 phosphorylation at Ser-255 and impairment of GJC (27).

Menadione as well as BQ and DMNQ all activate ERK 1/2 (Fig. 1), resulting in the phosphorylation of Cx43 (Fig. 2), which is known to result in an attenuation of GJC (8). This is of significance for cancer chemotherapy approaches exploiting the so-called bystander effect that is based upon the direct diffu-
sion of active drug between the cytoplasmas of adjacent cells. Many chemotherapeutics are quinones, such as mitomycin c or the anthraquinone derivatives doxorubicin and daunorubicin. Exposure of cancerous tissue to these drugs is aimed at killing the respective cells. If, however, the very agent employed induces Cx phosphorylation, thus impairing GJC, the outcome of chemotherapy might be suboptimal because diffusion between cytoplasmas is impaired. We here devise a way of sustaining GJC in the presence of quinones: the concomitant addition of inhibitors of the EGFR-MEK-ERK pathway would prevent Cx phosphorylation and reestablish GJC. 

Different Modes of Activation of EGFR-dependent Signaling by Quinones, Role of PTases and GSH—Receptor tyrosine kinases such as the EGFR or the platelet-derived growth factor receptor forms appear to be activated by a variety of stressful stimuli, mediating the activation of downstream signaling events in the absence of the respective ligands. Such stimuli include several different reactive oxygen and nitrogen species, such as hydrogen peroxide (21) or peroxynitrite (28), as well as heavy metal ions (29) or anticancer agents such as cisplatin (30).

How should menadione and other quinones lead to the activation of the EGFR and of downstream signaling? A hypothesis widely accepted for the activation of EGFR signaling by reactive oxygen species is that of the inactivation of a phosphotyrosine phosphatase (or PTase) negatively regulating the EGFR (31), and indeed, isolated PTases are inhibited by menadione (8) as well as other naphthoquinone derivatives (32). It is demonstrated in Fig. 5 that in a cellular environment, menadione (but not BQ or DMNQ) also blocks PTase activity. The identity of the PTase inhibited is yet unknown. However, the same pathways activated by menadione, leading to EGFR, MEK, and ERK activation as well as Cx phosphorylation and GJC down-regulation, are also activated by an inhibitor of Cdc25A,2 a dual specificity PTase involved in cell cycle regulation that is known to interact with the EGFR (33). In the case of menadione, we propose that direct alkylation of a PTase is the major mechanism for PTase inactivation and EGFR stimulation. First, menadione-induced ERK activation was demonstrated to be independent of NAPDH:quinone oxidoreductase I (8), which reduces menadione to the corresponding hydroquinone that may then undergo redox cycling. Secondly, menadione may directly interact with isolated PTases (see above). Thirdly, incubation of cells with NAC prior to exposure to menadione did not impair ERK activation by the quinone (different from BQ and DMNQ; Fig. 7).

PTase inactivation does not necessarily need to be by direct interaction of the enzyme with the quinone. Rather, an indirect mechanism might also be considered that, at the expense of GSH, relies on the regeneration of PTases that are oxidized during cellular metabolism. A depletion of cellular GSH such as by BQ or DEM might therefore render PTases and other oxidant-sensitive signaling proteins prone to oxidative inactivation. As PTase inactivation was not seen for BQ, either other target proteins have to be considered or only a slight PTase inactivation occurred that escaped detection. The latter, however, is questionable because of the strong effects on kinase activation of BQ. Thioredoxins or peroxiredoxins are examples for proteins known for their regulatory role in redox signaling. Like PTases, they harbor thiolates at their reactive sites and can be oxidized to sulfenic acids by metabolically generated H2O2 (34). The sulfenic acids can be reduced by GSH, leading to the reactivation of the proteins. If, however, cellular GSH levels are strongly diminished, such as with BQ and DEM (Fig. 6B), reduction and reactivation are impaired. DMNQ, undergoing redox cycling, does neither inactivate a PTase (Fig. 5) nor significantly deplete GSH levels in the cell within 15 min (Fig. 6), the time required to see the activation of the signaling pathways investigated. It may be speculated that superoxide and hydrogen peroxide formation oxidize signaling proteins such as the aforementioned, entailing effects identical to those seen under conditions with depleted GSH levels. In summary, three different quinones, the alkylating and redox cycling menadione, the strong alkylator BQ, and the exclusive redox cycler DMNQ, activate distinct signaling mechanisms converging at the level of EGFR activation and leading to the phosphorylation of Cx (Fig. 10).

Acknowledgment—We thank Elisabeth Sauerbier for expert technical assistance.

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