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Original Research Communication

Reactive Quinones Differentially Regulate SAPK/JNK and p38/mHOG Stress Kinases

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ABSTRACT

The stress-activated protein kinases SAPK/JNK and p38/mHOG are activated by diverse classes of stress stimuli, many of which induce redox perturbations. We studied the effects of reactive quinones on stress signaling pathways. Menadione (2-methyl-1,4-naphthoquinone), which undergoes both one- and two-electron reduction, completely inhibited SAPK activity at high concentrations while activating SAPK at lower concentrations. Menadione activated p38/mHOG dose responsively. 2,3-Dimethyl-1,4-naphthoquinone (DMNQ), which preferentially undergoes two-electron reduction, had similar effects. In contrast, 1,4-naphthoquinone, which preferentially undergoes one-electron reduction, inhibited SAPK at high concentrations, but failed to activate SAPK at any concentration tested. In addition, this quinone activated p38 only at lower concentrations; high concentrations inhibited p38 activity. These activity profiles correlated with the activation state of the upstream kinase, indicating that the effects were mediated by an upstream step in the kinase pathway. The quinone reductase inhibitor dicoumarol blocked activation of SAPK by menadione and DMNQ, suggesting that two-electron reduction is important. Finally, addition of increasing amounts of hydrogen peroxide mimicked the effects of menadione and DMNQ, suggesting that hydrogen peroxide may be the relevant mediator. Differential activation of stress kinases by reactive quinones demonstrates that the cellular redox environment independently modulates these pathways. *Antioxid. Redox Signal.* 5, 103–113.

INTRODUCTION

MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) cascades mediate the transduction of extracellular stimuli to the nucleus. The MAPKs are serine/threonine protein kinases that control proliferation, differentiation, development, and survival in many cell types (for review, see 8). The MAPKs are divided into three subfamilies: ERK, (extracellular signal regulated kinase), SAPK/JNK, and p38/mHOG (mammalian high osmolarity glycerol). The stress-activated protein kinase, SAPK (also known as c-Jun N-terminal kinase, or JNK) responds to stresses such as UV light, osmotic shock, and inflammatory cytokines. A distinct stress-activated MAPK, p38/mHOG, is activated by many of the same agents, including inflammatory cytokines, hydrogen peroxide, and osmotic shock. Phosphorylation of specific threonine and tyrosine residues by upstream MAPK kinases, MKK4 and MKK7, results in the activation of SAPK. p38/mHOG is activated in a similar manner by its upstream kinases, MKK3 and MKK6. These events are preceded by the action of MAPK-kinase kinases, such as MEKK1, ASK (apoptosis signaling kinase), or TAK1 (transforming growth factor-βactivated kinase), and culminate in the phosphorylation of transcription factors such as c-*jun* or ATF-2 by SAPK or p38/mHOG, bringing about new gene transcription (for review, see 12).

SAPK and p38/mHOG are regulated by redox stresses such as hydrogen peroxide, nitric oxide, and other reactive oxygen species (ROS) (2, 10, 13). We have previously shown that

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dicoumarol, an inhibitor of the two-electron NAD(P)H quinone oxidoreductase 1 (NQO1), can inhibit SAPK activation (6). We have continued this work to examine the role of reactive quinones in controlling stress-activated protein kinases.

ROS are produced by all aerobic cells, and the levels of intracellular ROS can influence cellular processes such as proliferation (4), apoptosis (19), and development (3). Endogenous antioxidants, such as glutathione, maintain the reduced oxidation state of cells under normal conditions. There is an extensive body of literature describing the effects of ROS on cell signaling in general (21), and protein kinase signaling pathways are targets of ROS. For example, thioredoxin, a redox regulatory protein, binds ASK1 in a redox-dependent mechanism (18), resulting in inhibition of ASK1 activity. Glutathione *S*-transferase (GST), isoform pi, which functions as a glutathione (GSH) conjugating enzyme and can modulate intracellular GSH levels through this activity, has been shown to bind SAPK and directly modulate its basal activity (1).

Quinones exist both naturally and as environmental toxins and therapeutic drugs that can undergo one- or two-electron reduction to form a variety of harmless and harmful by-products. Reactive quinones produce a variety of ROS as a by-product of reduction to quinols. Additionally, unsubstituted and partially substituted quinones may covalently modify proteins, potentially altering protein function (14). Menadione, a naphthoquinone, may induce cellular stress through the depletion of intracellular glutathione (14), depletion of nucleotides (15), and phosphorylation of *hsp*27 (11) by MAPK-activated protein kinase-2, a kinase downstream of p38/mHOG kinase (7, 17).

We describe here a role for stress kinase activation in response to reactive quinones. We show that reactive quinones differentially activate SAPK and p38/mHOG, depending on the way that they are metabolized within cells. Blockade of two-electron reduction of quinones by dicoumarol prevents activation of SAPK and partially blocks the activation of p38/mHOG by quinones that undergo two-electron reduction. Finally, hydrogen peroxide mimics the effects of the quinones that undergo two-electron reduction, suggesting a mechanism for the effects of these quinones on stress signaling pathways.

MATERIALS AND METHODS

Reagents, cell culture, and cell treatments

Reagents were obtained from Sigma–Aldrich unless otherwise noted. NIH 3T3 cells were stably transfected with either pEBG SAPK or pEBG p38/mHOG encoding GST-tagged forms of the protein kinases. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were plated (105 per 35-mm plate) and serum-starved (0.5% serum) overnight, then pretreated in some experiments with dicoumarol or 10 mM diethyl ester GSH (eeGSH; Bachem) for the indicated time periods. Cells were then treated with quinones dissolved in dimethyl sulfoxide (DMSO) for the indicated time period in medium with 0.5% serum.

Immunoprecipitation and immunoblotting

Cells were lysed on ice for 10 minutes in MLB (50 m*M* MOPS, pH 7.0, 250 m*M* NaCl, 5 m*M* EDTA, 0.1% NP-40) containing 1 m*M* dithiothreitol, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 50 μg/ml phenylmethylsulfonyl fluoride, 10 m*M* sodium fluoride, 5 m*M* sodium pyrophosphate, 1 m*M* sodium orthovanadate, and 10 m*M* β-glycerophosphate. Extracts were clarified at 15,000 *g* for 10 min before precipitation or immunoblotting. SAPK and p38/mHOG proteins were precipitated by using GSH-conjugated Sepharose beads following mixing at 4°C for 1 h. Beads were washed three times with 1 ml of MLB and additionally in 1 ml of 50 m*M* Tris, pH 7.4, with 1 m*M* dithiothreitol before use in kinase assays.

Precipitates or whole-cell extracts for immunoblots were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to Immobilon-P (Millipore). GST-tagged proteins were detected using polyclonal antibody 29.3 recognizing GST, generated in our lab. Anti-phospho-SAPK antibodies (Promega) and anti-phospho-p38/mHOG and MKK3/6 (New England Biolabs) were used, and bands visualized using either alkaline-phosphatase or horseradish perioxidase conjugated secondary antibodies.

SAPK and p38/mHOG kinase assays

Precipitates were incubated for 30 minutes at 20°C in 20 μl of kinase buffer (25 m*M* Tris-HCl, pH 7.4, 10 m*M* $MgCl_2$, 500 μ*M* dithiothreitol, and 7.5 μ*M* ATP) containing 10 μCi of [γ-^{32}P] ATP, and 1 μg of either GST-jun (for SAPK) or myelin basic protein (MBP; for p38/mHOG). Radiolabeled GST-jun or MBP was quantified with a Packard Instant Imager.

Measurement of hydrogen peroxide generation

SAPK 3T3 cells were loaded with 5 (and 6)-carboxy-2′,7′-dihydrodichlorofluorescein diacetate (H_2DCFDA; Molecular Probes) by incubation for 45 min in serum-free Dulbecco's modified Eagle's medium containing 100 μ*M* dye. The cells were detached in HEPES-buffered saline containing 3 m*M* EDTA, collected by centrifugation, and treated in suspension with either 10 m*M* hydrogen peroxide or 250 μ*M* menadione. The H_2DCFDA fluorescence was measured by flow cytometry.

RESULTS

Three isoforms of the p38/mHOG and six of the SAPK proteins are present within cells. To examine specifically the redox effects on single examples of each of these kinases, we engineered NIH 3T3 cells that stably express GST-epitope-tagged forms of SAPK (isoform α1, Genbank ID L27111) and p38/mHOG (isoform β, Genbank ID D83073). These are referred to as SAPK 3T3 and p38/mHOG 3T3 cells.

Menadione differentially activates SAPK and p38/mHOG

We treated cultures of SAPK 3T3 or p38/mHOG 3T3 cells with varying concentrations of menadione (2-methyl-1,4-naphthoquinone) for 20 min. Menadione (Fig. 1A) inhibited

SAPK activity below basal levels at 250 and 25 μ*M* (Fig. 1B, lanes 4 and 5). However, at 2.5 μ*M*, menadione strongly activated SAPK (lane 6). In contrast, p38/mHOG (Fig. 1C) was activated very strongly by higher concentrations (250 and 25 μ*M*) of menadione (lanes 4 and 5), but this activation decreased in a dose-dependent manner as the concentration of menadione decreased to 2.5 μ*M* (lane 6). Thus, menadione activates SAPK at lower concentrations, but activates p38/mHOG in a dose-dependent manner, demonstrating distinct regulation of these two kinases by redox stress.

Effects of other naphthoquinones

To determine if the ability of menadione to activate stress kinases is a general property of reactive quinones, we tested 1,4-naphthoquinone (1,4-NQ) and 2,3-dimethyl-1,4-naphthoquinone (DMNQ), which differ structurally from menadione only by the absence or addition of a methyl group. These quinones also differ in the means by which they are metabolized by the cells (see below). DMNQ (Fig. 2) activated SAPK and p38/mHOG in a pattern very similar to menadione (Fig. 1). SAPK activity was inhibited at the 250 μ*M* concentration (Fig. 2E, lane 3), but slightly activated at the 2.5 μ*M* concentration (lane 5). p38/mHOG (Fig. 2F) was strongly activated at all concentrations, with the activation decreasing with decreasing concentrations of DMNQ (lanes 3–5).

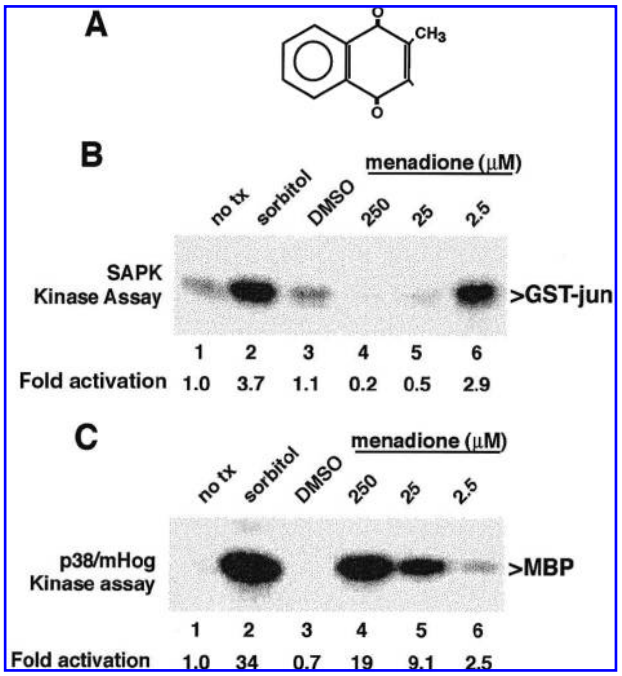

FIG. 1. Menadione differentially activates SAPK and p38/mHOG. SAPK (**B**) or p38/mHOG 3T3 cells (**C**) were treated for 20 min with increasing concentrations of menadione (**A**). GST-tagged proteins were precipitated with GSH beads, and *in vitro* kinase assays were performed. (B) High concentrations (250 and 25 μ*M*) of menadione inhibit basal activity of SAPK (lanes 4 and 5), whereas 2.5 μ*M* menadione is a modest activator (lane 6). (C) In contrast, menadione activates p38/mHOG in a dose-dependent manner. The structure of menadione is shown (A).

In contrast to menadione and DMNQ, 1,4-NQ did not activate SAPK, even at 2.5 and 0.25 μ*M*, the lowest concentration tested (Fig. 2B, lane 5 and not shown, see Fig. 7B, lane 6). The higher concentrations of 1,4-NQ (250 and 25 μ*M*) inhibited SAPK activity similar to menadione treatment (Fig. 2B, lanes 3 and 4). In the case of p38/mHOG, 1,4-NQ activated only at the lower concentrations (25 and 2.5 μ*M*) (Fig. 2C, lanes 4 and 5), in contrast to the dose-dependent activation seen with menadione and DMNQ. These data suggest that the presence of an open C3 carbon position on the molecule, such as found in both 1,4-NQ and menadione, is unrelated to the activation of these stress kinases. This makes both nonenzymatic redox cycling and covalent adduct formation unlikely mechanisms of activation, as both of these processes require the availability of an open C3 carbon on the quinone (14).

Activation of upstream kinases by quinones

We next looked at kinases upstream of SAPK and p38/mHOG to determine if activation of these MAPKs by reactive quinones was a direct effect or was mediated through effects on their upstream activators. 3T3 cells were treated for 20 min with increasing concentrations of the three quinones. Whole-cell extracts were subjected to immunoblotting with phospho-specific antibodies that reflect the activation state of these kinases. An immunoblot probed with a phospho-specific MKK3/6 antibody showed that 250 μ*M* 1,4-NQ inhibited phosphorylation (Fig. 3, lane 3), whereas treatment with the lower concentrations of 1,4-NQ (Fig. 3, lanes 4–6) resulted in increased phosphorylation of the endogenous MKK3/6. These results correlate with the p38/mHOG activation measured by *in vitro* kinase assay. The highest concentration of menadione, 250 μ*M*, inhibited phosphorylation of MKK3/6 (Fig. 3, lane 7), although this concentration of menadione activates p38/mHOG (Fig. 1, lane 4). Lower concentrations, 0.25–25 μ*M*, of menadione increased phosphorylation of endogenous MKK3/6 in concordance with activation of p38/mHOG (Fig. 3, lanes 8–10). One would expect to see direct correlation between the activation of p38/mHOG and its upstream activators. However, it is possible that these upstream activators are susceptible to inhibition by alkylation of the quinone to the protein. All concentrations of DMNQ resulted in increased phosphorylation of MKK3/6, which corresponds to the activation of p38/mHOG seen in Fig, 2. In a similar experiment looking at the phosphorylation of the upstream activator of the SAPK pathway, SEK1, an immunoblot showed no phosphorylation of SEK1 after treatment with 1,4-NQ, menadione, or DMNQ (data not shown). It is possible that activation of SAPK is through MKK7, which has been shown to activate SAPK in response to tumor necrosis factor-α and interleukin-1, both of which generate ROS upon binding to their receptors (23). Reagents to examine phosphorylation of MKK7 are not currently available.

Reactive quinones alter stimulation of stress-activated kinases by osmotic shock

In order to determine whether pretreatment with reactive quinones could inhibit subsequent activation of SAPK and p38/mHOG by osmotic shock, we pretreated SAPK and p38/mHOG 3T3 cells with varying concentrations of 1,4-NQ,

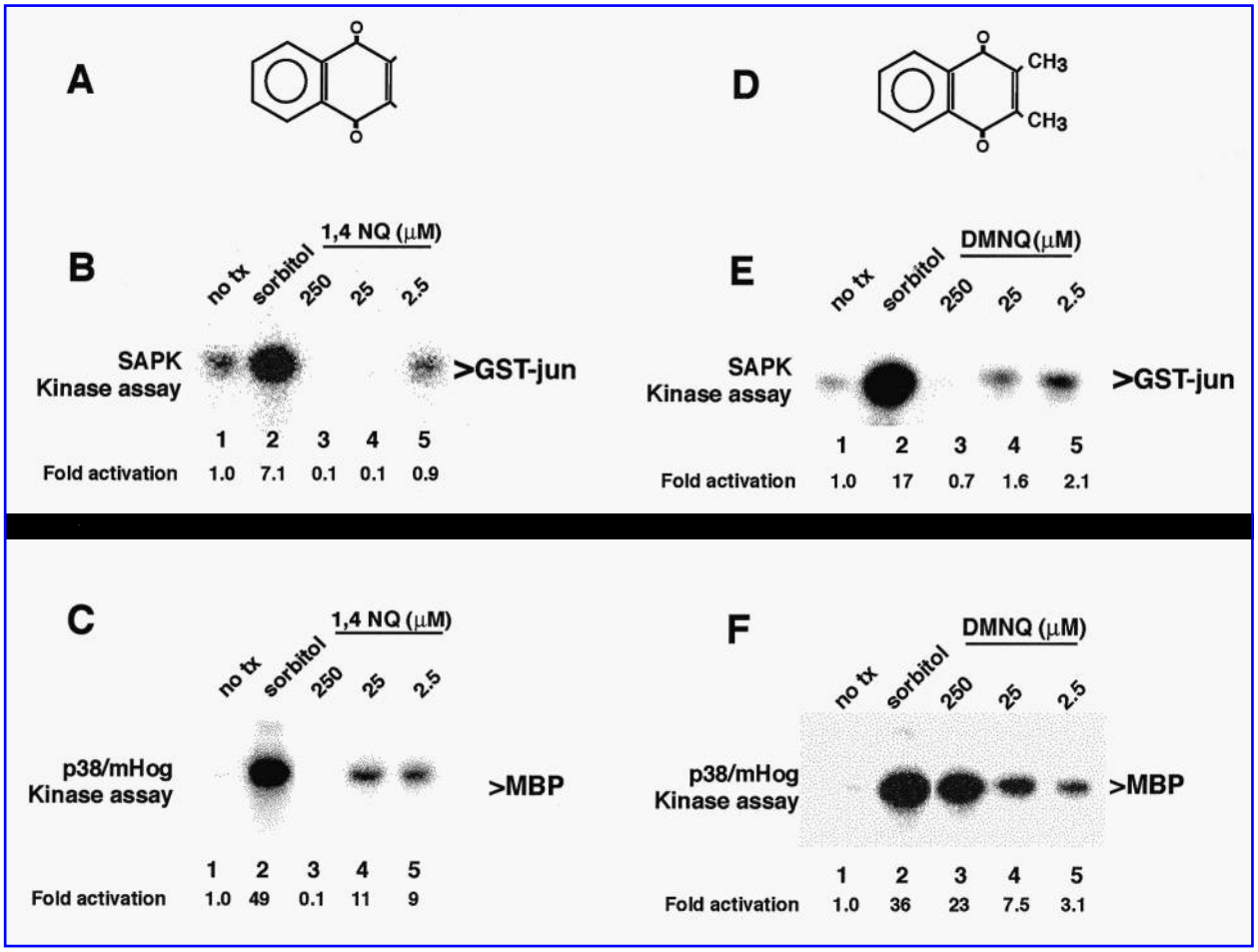

FIG. 2. High concentrations of 1,4-NQ inhibit SAPK and p38/mHOG, whereas DMNQ activates stress kinases similarly to menadione. SAPK (**B** and **E**) or p38/mHOG 3T3 cells (**C** and **F**) were treated for 20 min with increasing concentrations of either 1,4-NQ (B and C) or DMNQ (E and F). GST-tagged SAPK and p38/mHOG were precipitated with GSH beads, and *in vitro* kinase assays were performed. 1,4-NQ strongly inhibits SAPK at 250 and 25 μ*M* concentrations, but activates p38/mHOG strongly at 25 and 2.5 μ*M*. In contrast, DMNQ activates both kinases similarly to menadione. SAPK (E) is inhibited at 250 μ*M* and activated at 25 and 2.5 μ*M*, whereas p38/mHOG (F) is activated in a dose-dependent manner. The structures of 1,4-NQ (**A**) and DMNQ (**D**) are shown.

menadione, and DMNQ. The cells were then stimulated with 400 m*M* sorbitol. Pretreatment with high concentrations (250 μ*M*) of any one of these quinones inhibited subsequent activation of SAPK (Fig. 4A, lanes 3, 7, and 13) and p38/mHOG (Fig. 4B, lanes 3, 7, and 13) by osmotic shock, as evidenced by diminished phosphorylation of their respective substrates in *in vitro* kinase assays. Importantly, this inhibition of activity correlated with a loss of phosphorylation as seen by phospho-SAPK (Fig. 4C) and phospho-p38 (Fig. 4D) immunoblots, indicating inhibition of upstream kinases that activate SAPK and p38/mHOG by phosphorylation. Lower concentrations of all three quinones synergized to some extent with osmotic shock treatment to activate SAPK (lanes 5, 6, 9, 10, and 15). Only 1,4-NQ synergized with osmotic shock to activate p38/mHOG. Menadione and DMNQ both inhibited osmotic shock-induced p38/mHOG activity in a dose-dependent manner. These data suggest that both inhibition and activation of SAPK and p38/mHOG by reactive quinones take place through effects at a step upstream in their respective signaling pathways.

Reactive quinones inhibit SAPK and p38/mHOG activity in vitro

We also determined the ability of the reactive quinones to directly inhibit stress kinases *in vitro*. In order to accomplish this, cells stably transfected with GST-SAPK or GST-p38/mHOG were stimulated with sorbitol and then lysed. The GST-tagged kinases were then pulled down with GSH beads and washed. Finally, these partially purified active kinases were treated with the quinones *in vitro*. Activated GST-SAPK (Fig. 5A) or GST-p38/mHOG (Fig. 5B) precipitates were treated with increasing concentrations of reactive quinones, and the kinase activity was assessed by *in vitro* kinase assay. All three reactive quinones inhibited SAPK and p38/mHOG kinase activity in a dose-dependent manner. These *in vitro* re-

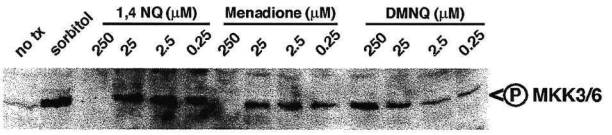

FIG. 3. Reactive quinones activate MKK3/6. NIH 3T3 cells were treated with increasing concentrations of quinones for 20 min. The cells were lysed, the lysate was clarified, and 25 μg of total protein was subjected to western blotting using a phospho-specific anti-MKK3/6 antibody. 1,4-NQ, menadione, and DMNQ activate MKK3/6 similarly to their activation of p38/mHOG, except for 250 μ*M* menadione, which inhibits MKK3/6 phosphorylation.

sults suggest that reactive quinones at very high concentrations may interfere directly with SAPK or p38/mHOG kinases and perhaps other kinases within the cell.

Properties of reactive quinones may explain differential activation

To elucidate possible mechanisms of activation and explore the differences between the quinones, we consulted literature comparing various properties of reactive quinones (Table 1). As expected by their structure, 1,4-NQ and menadione are known to covalently modify proteins. This activity is dependent on the presence of an open C3 position on the molecules. Therefore, DMNQ is unable to covalently modify proteins (14). Additionally, it has been shown that treatment with either 1,4-NQ or menadione resulted in dramatic decreases in intracellular GSH levels, whereas DMNQ only slightly decreased GSH levels (14). Redox cycling of quinones generates ROS that can be detoxified by several processes that result in the oxidation of GSH (16). In our experiments, menadione and DMNQ exert similar effects on stress signaling, which differ substantially from the effects of 1,4-NQ (Table 2). As menadione and 1,4-NQ have similar abilities to induce GSH depletion, whereas DMNQ does not, it is unlikely that GSH depletion is the mediator of these stress signaling effects. However, the possibility that a slight decrease in GSH activates stress kinases cannot be ruled out.

To determine if the depletion of intracellular GSH plays a role in the mechanism of stress kinase activation or inhibition by reactive quinones, we added exogenous eeGSH to cells treated with menadione. The addition of 10 m*M* eeGSH had no effect on the ability of menadione to either activate or inhibit SAPK (Fig. 6A, compare lanes 4–7 with lanes 9–12) or p38/mHOG (Fig. 6B, compare lanes 4–7 with lanes 9–12).

TABLE 1. PROPERTIES OF STRUCTURALLY RELATED REACTIVE QUINONES

	1,4-NQ	*Menadione*	*DMNQ*
Alkylate	Yes	Yes	No
Decrease GSH	Yes	Yes	Yes, slight
Reduction of cytochrome *c*	Yes	Yes	Yes (less than menadione)
Cytochrome *c* reduction SOD-dependent		Yes (50%)	Slight
Cytochrome *c* reduction blocked by dicoumarol		Yes (50%)	Yes (~90%)
Toxicity	Most	Moderate	Least
Dicoumarol potentiates toxicity	Yes	Yes	Yes

Reactive quinones that differ in their degree of substitution have differential abilities to alkylate proteins, decrease intracellular GSH levels, and redox-cycle. Their ability to redox-cycle through one- or two-electron reduction is determined by the use of inhibitors of either one- or two-electron reduction. Data are from references 14 and 16. SOD, superoxide dismutase.

Therefore, it is unlikely that the GSH depletion by these reactive quinones is responsible for either the activation or inhibition of SAPK or p38/mHOG.

Dicoumarol blocks activation of SAPK by menadione and DMNQ

Cytochrome *c* reduction assays described in the literature demonstrate that DMNQ preferentially undergoes two-electron reduction (16). Menadione is known to undergo both one- and two-electron reduction, whereas 1,4 NQ preferentially undergoes one-electron reduction. Our data suggest that menadione and DMNQ act similarly with regard to activation of stress signaling cascades, correlating with their ability to undergo two-electron reduction. This correlation is further supported by our finding that 1,4-NQ, which preferentially undergoes one-electron reduction, has substantially different effects on stress signaling.

The literature describes the ability of dicoumarol, an inhibitor of two-electron reduction, to inhibit the reduction of cytochrome *c* by either DMNQ or menadione (16). Previous studies in our lab (6) suggest a potential role for two-electron

TABLE 2. SUMMARY OF THE OBSERVED EFFECTS OF REACTIVE QUINONES ON STRESS SIGNALING

	SAPK		*p38/mHOG*	
	High concentration	*Low concentration*	*High concentration*	*Low concentration*
1,4-NQ	−	=	−	++
Menadione	−	+	+++	+
DMNQ	−	+	+++	+

−, inhibition; =, no effect; +, weak activation; +++, strong activation.

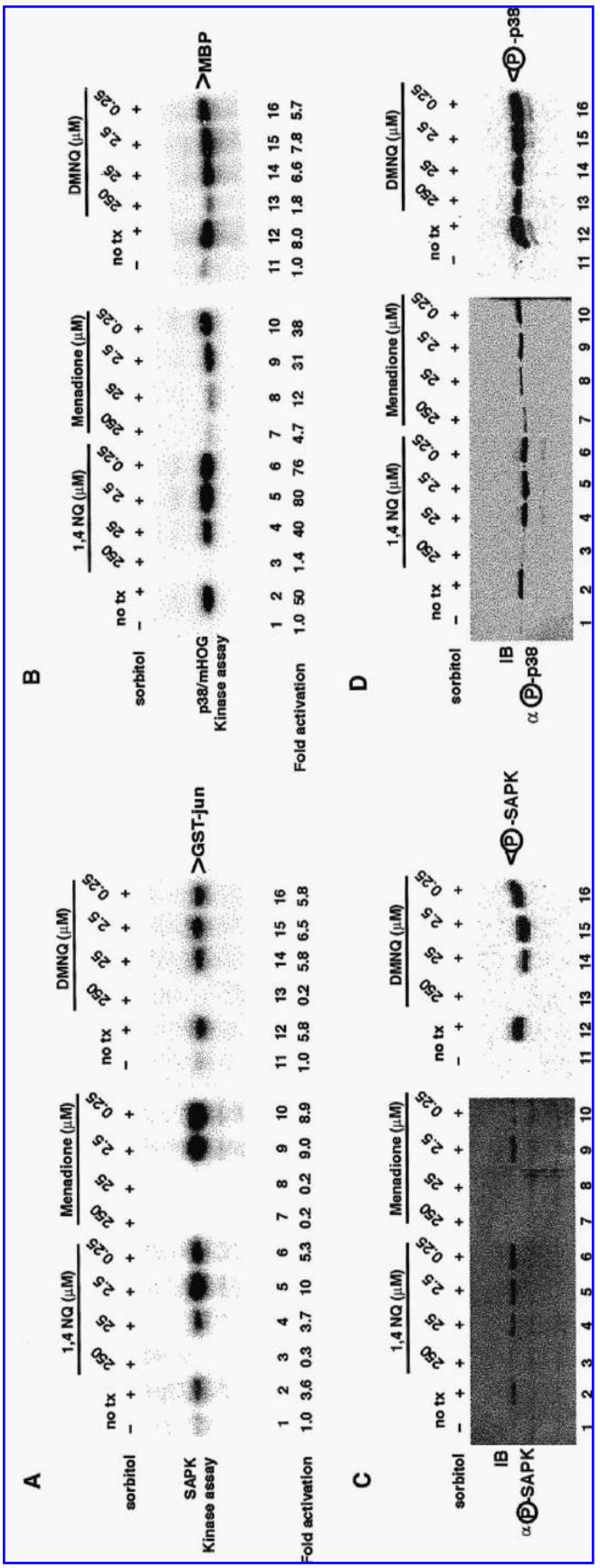

FIG. 4. Pretreatment with quinones inhibits subsequent activation of SAPK and p38/mHOG by osmotic shock. SAPK (**A** and **C**) and p38/mHOG (**B** and **D**) 3T3 cells were pretreated with increasing concentrations of menadione, 1,4-NQ, or DMNQ for 20 min, and then treated with 400 m*M* sorbitol for 20 min. GST-tagged proteins were precipitated with GSH beads, and an *in vitro* kinase assay was performed. All quinones inhibited SAPK activity at high concentrations (250 μ*M*, lanes 3, 7, and 13), but synergized with sorbitol activation at lower concentrations (2.5 and 0.25 μ*M*, lanes 5, 6, 9, 10, and 15 in A). p38/mHOG is also inhibited at the 250 and 25 μ*M* concentrations of 1,4-NQ (lanes 3 and 4), but at lower concentratons, the 1,4-NQ synergizes with sorbitol activation (lanes 5 and 6 in B). In contrast, menadione and DMNQ inhibit p38/mHOG activation in a dose-dependent manner (lanes 7–10 and 13–16). This inhibition of kinase activity correlated with loss of phosphorylation as seen on an immunoblot probed with an antibody to phospho-SAPK (B) or phospho-p38/mHOG (D).

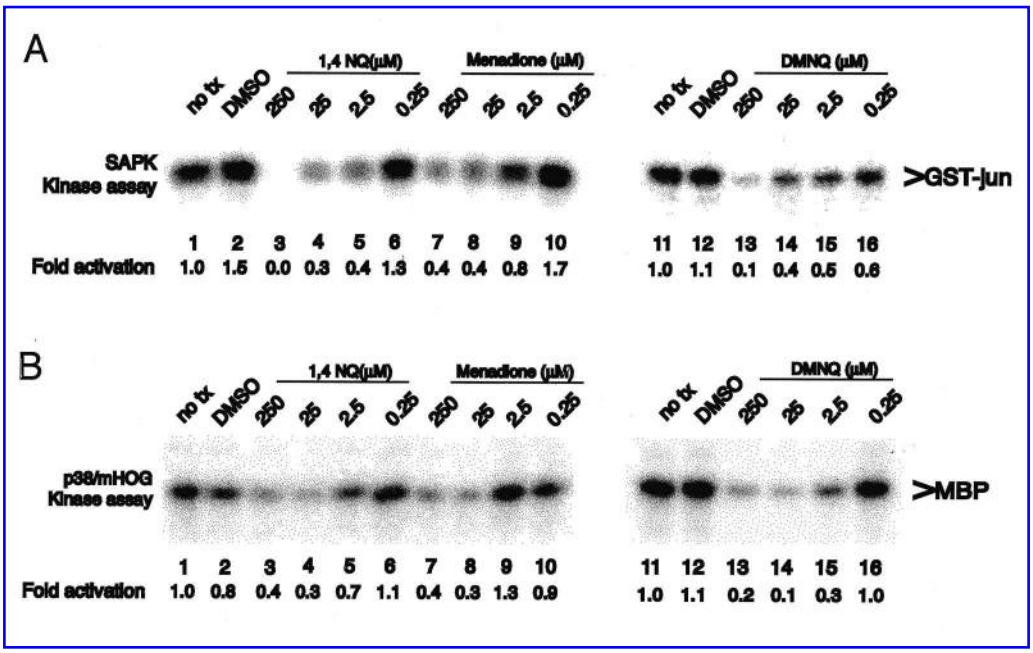

FIG. 5. ***In vitro*** **treatment with reactive quinones inhibits preactivated SAPK and p38/mHOG**. SAPK (**A**) or p38/mHOG (**B**) 3T3 cells were treated with 400 m*M* sorbitol for 20 min. The GST-tagged proteins were precipitated with GSH beads. The beads were then washed three times. Increasing concentrations of quinone were added to phosphate-buffered saline on the beads, and this was incubated for 20 min at room temperature. The beads were washed an additional three times, and an *in vitro* kinase assay was performed. All three quinones tested inhibited both SAPK and p38/mHOG activity in a dose-dependent manner.

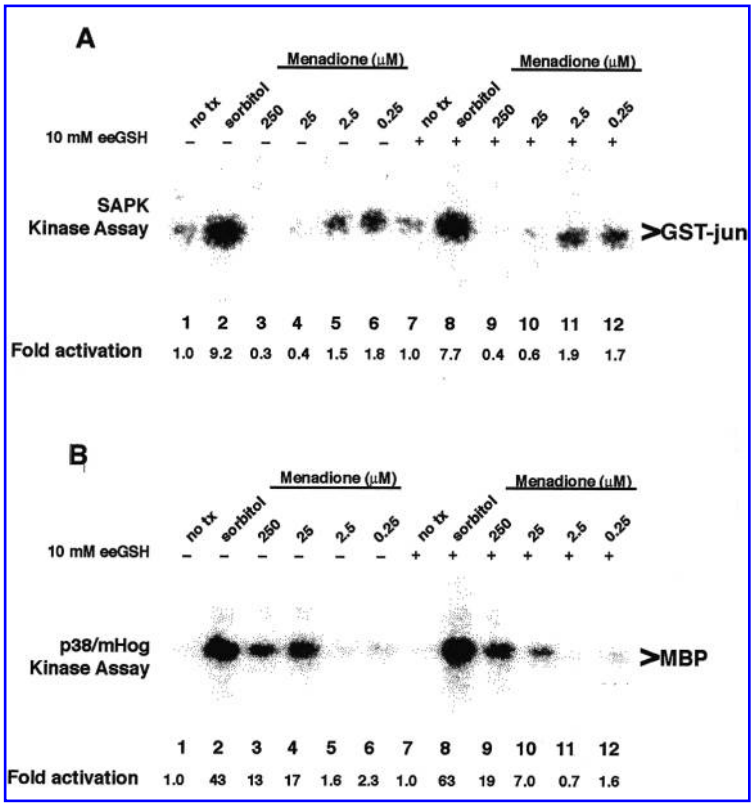

FIG. 6. Addition of eeGSH has no effect on menadione inhibition or activation of SAPK or p38/mHOG. SAPK (**A**) or p38/mHOG (**B**) 3T3 cells were pretreated for 20 min with 10 m*M* GSH. The cells were washed with conditioned media, and menadione was added in increasing concentrations. The GST-tagged proteins were precipitated with GSH beads, and *in vitro* kinase assays were performed. Addition of GSH to the cells had no effect on the ability of menadione to inhibit SAPK activity at higher concentrations or activate at the lower concentrations. Addition of GSH did not inhibit the dose-dependent activation of p38/mHOG.

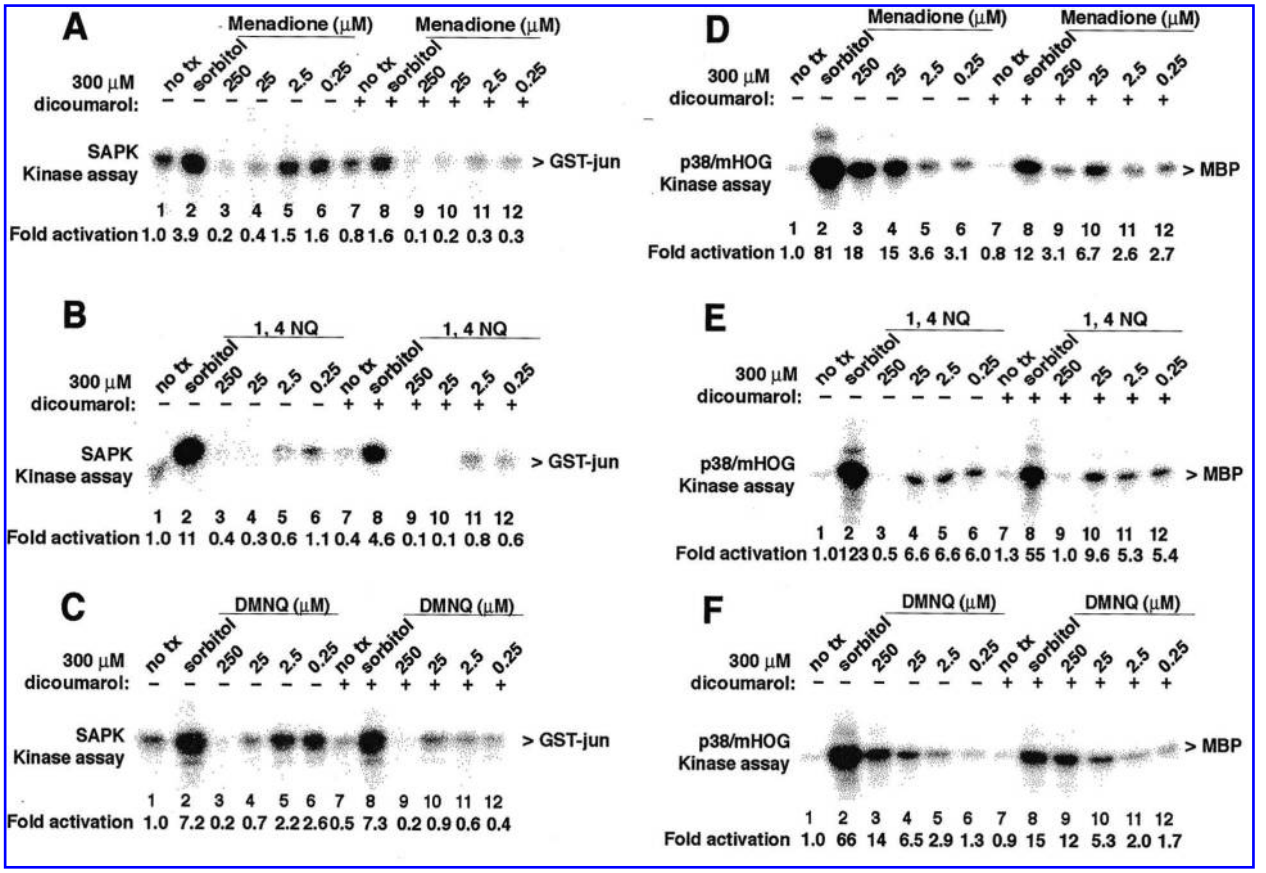

FIG. 7. Dicoumarol blocks activation of SAPK induced by two-electron reduction of quinones. SAPK (**A–C**) or p38/mHOG (**D–F**) 3T3 cells were pretreated with 300 μ*M* dicoumarol for 20 min, and then treated with increasing concentrations of menadione (A and D), 1,4-NQ (B and E), or DMNQ (C and F) for 20 min. GST-tagged proteins were pulled down with GSH beads, and *in vitro* kinase assays were performed. Dicoumarol treatment completely blocked the modest activation of SAPK by menadione (A, compare lanes 5 and 6 with lanes 11 and 12) and DMNQ (C, compare lanes 5 and 6 with lanes 11 and 12). Dicoumarol also partially inhibited menadione-induced activation of p38/mHOG (D, compare lanes 3–6 with lanes 9–12). In contrast, dicoumarol had no effect on 1,4-NQ or DMNQ activation of p38/mHOG (E and F).

reduction in the activation of SAPK. Therefore, we considered whether two-electron reduction was required for the activation of stress signaling by reactive quinones.

To test whether two-electron reduction is involved in the activation of SAPK and p38/mHOG, we pretreated SAPK 3T3 or p38/mHOG 3T3 cells for 20 min with 300 μ*M* dicoumarol and then stimulated as before with increasing concentrations of menadione. As shown previously, dicoumarol inhibited both basal activity of SAPK and sorbitol stimulation (Fig. 7A, compare lanes 7 and 8 with lanes 1 and 2). Dicoumarol completely inhibited menadione-induced SAPK activity (Fig. 7A, compare lanes 11 and 12 to lanes 5 and 6). Additionally, dicoumarol also inhibited activation of p38/mHOG by menadione (Fig. 7D, compare lanes 3–6 with lanes 9–12), although the inhibition of p38/mHOG by dicoumarol was not as complete as the inhibition of SAPK. In contrast, dicoumarol did not inhibit activation of p38/mHOG by 1,4-NQ (Fig. 7E). DMNQ activation of SAPK (Fig. 7C) was blocked by dicoumarol treatment, but DMNQ activation of p38/mHOG (Fig. 7F) was not inhibited by dicoumarol pretreatment.

These data support the hypothesis that the two-electron reduction of reactive quinones is necessary for menadione- and DMNQ-mediated activation of SAPK, but plays a lesser role in the activation of p38/mHOG by menadione and DMNQ.

Hydrogen peroxide mimics the effects of menadione on stress signaling

Two-electron oxidation is required for the formation of hydrogen peroxide. As it appears that two-electron reduction of menadione is important for inhibition of SAPK and the activation p38, we hypothesized that *in situ* production of hydrogen peroxide may be part of the mechanism of action of the reactive quinones. This hypothesis predicted that hydrogen peroxide added exogenously to cells might mimic the effects of menadione and DMNQ on the activation of SAPK and p38/mHOG.

To test this hypothesis, we added increasing concentrations of hydrogen peroxide to SAPK or p38/mHOG 3T3 cells and then measured the activity of the kinases by *in vitro* kinase assay. As shown in Fig. 8, we found that low concentrations of

hydrogen peroxide activated SAPK (0.62 and 1.25 m*M*), whereas high concentrations (5 and 10 m*M*) inhibited SAPK below basal levels. These results parallel the pattern of SAPK activation and inhibition by menadione. Conversely, p38 was activated by hydrogen peroxide in a dose responsive manner. These results show that, in support of our hypothesis, exogenous hydrogen peroxide mimics the complex effects of menadione and DMNQ on SAPK and p38.

A second prediction of our hypothesis is that cells treated with menadione should produce measurable hydrogen peroxide. To measure hydrogen peroxide, we used H_2DCFDA, a stain that becomes fluorescent when oxidized by hydrogen peroxide. As shown in Fig. 8B, cells treated with either 10 m*M* hydrogen peroxide or 250 μ*M* menadione and analyzed by flow cytometry showed high levels of (oxidized) DCFDA fluorescence, whereas untreated cells showed weak fluorescence. For unknown reasons, a subset of the untreated cells also manifested elevated levels of fluorescence, perhaps a result of stress associated with detachment from growth substrate. This result supports the hypothesis that the divergent pattern of SAPK versus p38 activation is a result of induction of hydrogen peroxide within cells.

DISCUSSION

Our data demonstrate the differential effects of reactive quinones on cellular stress signaling kinase pathways. Although most stress stimuli, such as osmotic shock, UV irradiation, and inflammatory cytokines activate both SAPK and p38/mHOG, some reactive quinones (specifically menadione and DMNQ at 250 μ*M*) can activate p38/mHOG, yet strongly inhibit SAPK activity. We are unaware of any other physical stimulus that distinguishes between these two pathways besides these reactive quinones and their apparent mediator hydrogen peroxide. The activation of SAPK by reactive quinones is directly tied to the ability of the compound to undergo two-electron reduction, whereas the activation of p38/mHOG is not related to this effect. Our results suggest that these quinones may represent prototypes for pharmacologic agents that could discriminate between these closely related and otherwise coordinately regulated pathways.

Quinone activation and inhibition of SAPK and p38/mHOG occur at a proximal point in the kinase cascades. This conclusion stems from the close correlation between activation of the upstream kinases, MKK3/6, and the activation of

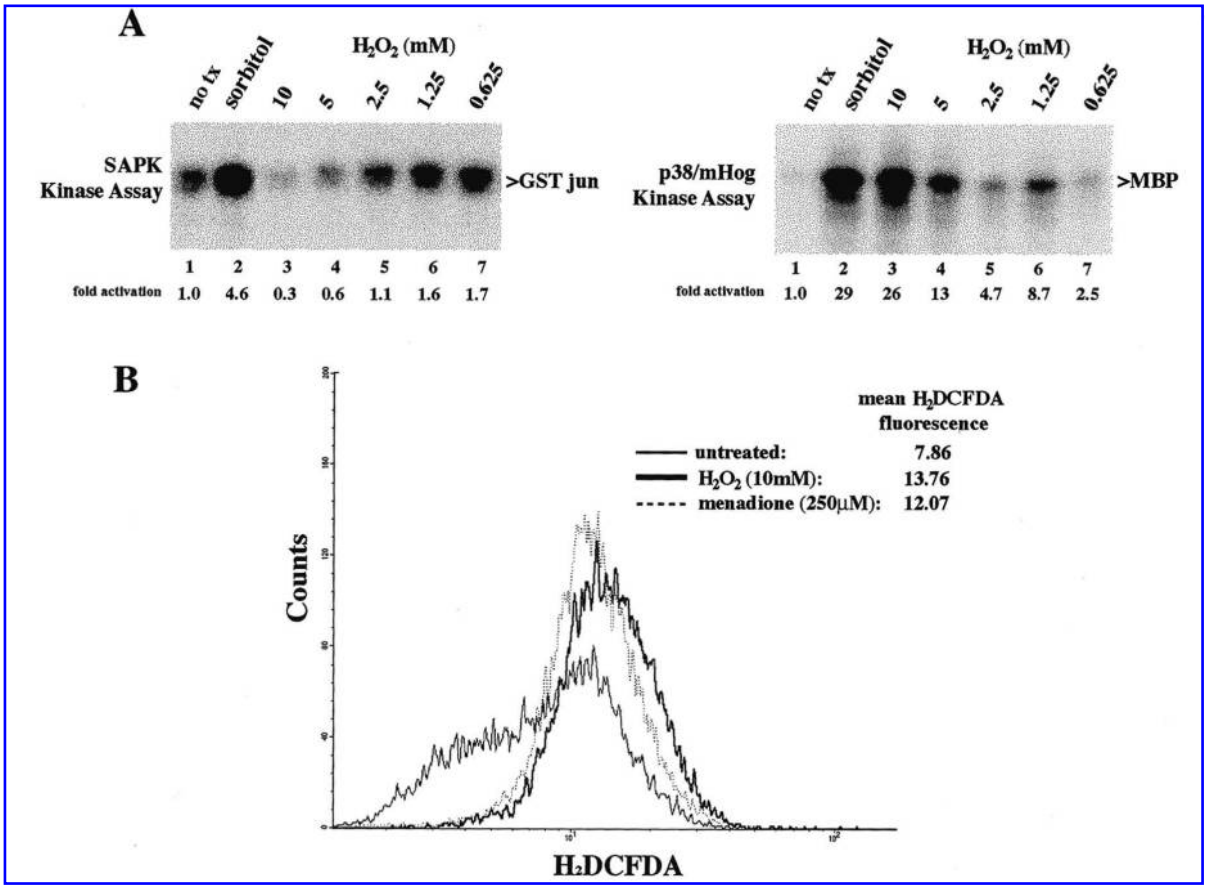

FIG. 8. Hydrogen peroxide mimics the effect of menadione on stress signaling pathways. (**A**) SAPK (**left**) or p38/mHOG (**right**) 3T3 cells were treated with the indicated doses of hydrogen peroxide for 20 min. GST-tagged proteins were pulled down with GSH beads, and *in vitro* kinase assays were performed. Hydrogen peroxide at high concentrations inhibits SAPK activity (lanes 3 and 4), whereas lower concentrations mildly activate SAPK (lanes 6 and 7). In contrast, hydrogen peroxide dose-responsively activates p38/mHOG. This activation pattern is similar to that seen with menadione, which induces hydrogen peroxide generation as reflected in increased H_2DCFDA fluorescence (**B**). SAPK 3T3 cells were loaded with H_2DCFDA and then treated with either 10 m*M* hydrogen peroxide or 250 μ*M* menadione for 20 min. H_2DCFDA fluorescence was measure by flow cytometery.

p38/mHOG (Figs. 1, 2, and 6). Thus, redox control of stress signaling pathways is likely to be at the relevant MAPK kinase kinase (or higher), rather than through direct effects on SAPK or p38/mHOG. However, based on *in vitro* treatment studies, at the highest doses of quinones there also appears to be direct inhibitory effects on SAPK itself, possibly through nonspecific oxidative means.

We considered the mechanism by which reactive quinones regulate the stress-activated protein kinase pathways. Our data argue against direct quinone covalent modification of stress kinases, based on the finding that DMNQ, which is fully substituted and therefore incapable of covalently modifying proteins, still inhibits SAPK and p38/mHOG. In addition, DMNQ and menadione have similar effects on stress signaling, and they do not share the ability to covalently modify proteins. Secondly, the activation and inhibition by quinones are unlikely to be caused by loss of cellular glutathione because addition of eeGSH (Fig. 6) as well as treatment with *N*-acetylcysteine (data not shown), which have both been shown to increase the available intracellular GSH, did not change the pattern of activation and inhibition of SAPK seen with menadione treatment.

Our data suggest that p38/mHOG is primarily activated in response to treatment with high concentrations (250 μ*M*) of quinones (menadione and DMNQ) and the massive production of ROS that accompanies it. In contrast, high concentrations (250 μ*M*) of menadione and DMNQ inhibit SAPK activity, whereas lower concentrations (2.5 μ*M*) of menadione and DMNQ are activating. The activation and inhibition of stress kinases can also be differentiated by the potential of the quinone for redox cycling through one- or two-electron reduction. p38/mHOG is activated by quinones that undergo both one- and two-electron reduction, whereas SAPK is only activated by quinones that undergo two-electron reduction. The differential activation of stress kinases may be important for a finely tuned cell stress response.

Although we have not yet identified a specific protein that serves as a "receptor" that initially detects the presence of menadione, our experiments point to the type of reaction that might serve as this sensor. Specifically, we believe that hydrogen peroxide generated *in situ* in menadione-treated cells is an intermediate reactant. Menadione has previously been shown to result in hydrogen peroxide formation (21), a finding that we confirmed in Fig. 8B. Indeed, we show that the effects of exogenous addition of hydrogen peroxide to the cells parallel the effects of treatment of cells with menadione, supporting the conclusion that hydrogen peroxide generation may mediate the effects of this quinone on stress signaling.

We have shown that the regulatory effects of reactive quinones appear to function through upstream elements in the protein kinase cascade. However, we cannot yet conclude that the sensor of the ROS response to quinones (*e.g.,* hydrogen peroxide) is itself a protein kinase. It is also possible that a protein phosphatase (many of which have cysteine or transition metals at their catalytic cores) could be regulated by the ROS response. A response by a protein phosphatase, though, might be viewed as less likely only because of the rapidity (minutes) of the protein kinase response.

Quinones are also widely found in nature. Coenzyme Q_{10} is a critical component of the mitochondrial electron transport chain and is also found in the plasma membrane. It has been hypothesized that coenzyme Q_{10} in the plasma membrane may contribute to the regulation of membrane tyrosine kinases, with this regulation being dependent on the redox state of coenzyme Q_{10} (5). Additionally, many chemotherapeutic drugs have a quinone backbone, and their metabolism by one- or two-electron reduction results in activation of the compound. Menadione has been used as an anticancer therapeutic with mitomycin C in the treatment of lung cancer (20). Treatment of cells with menadione also leads to both double- and single-strand DNA breakage and apoptosis (22). NQO1 has been suggested to be important in the generation of cancer because its expression is elevated in many tumors. By utilizing the high expression of NQO1 in cancer cells, various quinone chemotherapeutic agents can selectively kill tumor cells (9). Our work suggests a role for stress kinase pathways in the response to production of quinones and the by-products of quinone metabolism such as hydrogen peroxide. Selective use of quinones that undergo one- or two-electron reduction could prove valuable in the selection of chemotherapeutic agents that elicit a specific response by stress kinases.

AUTHORS' CONTRIBUTIONS

M.Y. made initial observations and performed pilot experiments.

K.L.S. performed most experiments and drafted the early version of the manuscript.

J.V.C. repeated critical experiments, performed experiments using hydrogen peroxide and drafted the final version of the manuscript.

S.M.N. assisted in the hydrogen peroxide experiments.

D.J.T. coordinated the project and oversaw the analysis of results and drafting of the manuscript.

All authors approved the final manuscript.

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ABBREVIATIONS

ASK, apoptosis signaling kinase; DMNQ, 2,3-dimethyl-1,4-naphthoquinone; DMSO, dimethyl sulfoxide; eeGSH, diethyl ester GSH; GSH, glutathione; GST, glutathione *S*-transferase; H_2DCFDA, 5(and 6)-carboxy-2′,7′-dihydrodichlorofluorescein diacetate; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEKK1, mitogen-activated protein kinase kinase kinase; mHOG, mammalian high osmolarity glycerol; MKK, mitogen-activated protein kinase kinase; NQO1, 1,4-NQ, 1,4-naphthoquinone; NAD(P)H quinone oxidoreductase 1; ROS, reactive oxygen species; SAPK, stress-activated protein kinase.

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