A variety of environmental stresses stimulate the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) cascade (ERK) > stress-activated protein kinase (SAPK). SAPK > ERK kinase (MEKK) > stress-activated protein kinase (SAPK) > ERK kinase (MEKK). Mechanisms of stress activation upstream of MEKK1 have not been precisely determined. Redox mechanisms involving sulfhydryls are likely because N-acetyl-cysteine at millimolar concentrations blocks stress signals. Because intracellular sulfhydryl concentrations can be regulated through redox cycling involving reactive quinones (1), we tested the ability of dicoumarol, a coumarin derivative. Dicoumarol prevented SAPK activation in vitro by chemical cell stressors and also prevented SAPK activation induced by expression of the tumor necrosis factor α (TNFα) receptor-associated protein (TRAF2) but not by expression of truncated active MEKK1. Other coumarin derivatives failed to block SAPK activation, but other inhibitors of quinone reductases, particularly menadione, similarly blocked SAPK activation. Cells deficient in a major quinone reductase, NQO1, displayed hypersensitivity to dicoumarol stress inhibition, whereas SAPK in cells reconstituted with the NQO1 gene displayed relative dicoumarol resistance. Consistent with the proposed role of overlapping upstream signaling cascades in activation of NFκB, dicoumarol also blocked NFκB activation in primary macrophages stimulated with either lipopolysaccharide or TNFα. In addition, dicoumarol strongly potentiated TNFα-induced apoptosis in HeLa cells, probably by blocking the anti-apoptotic effect of NFκB. The ability of dicoumarol to simultaneously inhibit SAPK and NFκB activation and to potentiate apoptotic cell death suggests that SAPK is not an obligate participant in apoptosis. Dicoumarol, currently in clinical use as an oral anticoagulant, represents a potential therapeutic inhibitor of the SAPK and NFκB response.

The MEKK1 protein kinase (2) is a proximate activator of the stress-activated protein kinase (SAPK), alternatively known as JNK) stress-signaling pathway (3). A variety of environmental stresses, including inflammatory cytokines, hyperosmotic shock, and UV light, stimulate the cascade (4). MEKK1 is implicated in apoptotic cell death (5, 6), but perhaps surprisingly MEKK1 activates NFκB pathways (7, 8) that induce expression of genes to counteract the apoptotic death response (9–11).

Stress activators upstream of MEKK1 remain obscure. The small G-proteins Rac and Rho activate stress signaling (12, 13), but their placement in relation to MEKK1 is unclear. TNFα and the TNFα receptor-associated effector protein TRAF2 activate the SAPK pathway (14, 15). Reactive oxygen species are implicated in activation of stress kinase pathways in response to TNFα and UV irradiation. Reducing agents such as N-acetyl-cysteine at millimolar concentrations can block stress signals (15), suggesting that redox mechanisms are required for an undefined and probably early event in transmission of stress signals.

Intracellular thiols can be consumed by a process termed “redox cycling” in which reactive quinones catalyze the oxidation of sulfhydryls to disulfides. In the cell, reactive quinones are regulated by the enzymatic activity NAD(P)H dehydrogenase (formerly DT-diaphorase) (E.C. 1.6.99.2) (16), representing a family of cytoplasmic flavo-enzymes responsible for two-electron reduction of quinones (17), using NADPH or NADH as electron donor. NADP/H dehydrogenase, also termed “quinone reductase,” is inhibited by dicoumarol, a coumarin derivative (18). Like other coumarins, dicoumarol is used clinically to inhibit blood coagulation processes dependent on vitamin K, a biological quinone.

Here we report evidence that inhibitors of quinone reductases can inhibit SAPK and NFκB signaling. In addition, dicoumarol potentiates the apoptotic effect of TNFα, probably by preventing anti-apoptotic events dependent upon NFκB.

MATERIALS AND METHODS

SAPK Activation Assays—Human embryonic kidney 293 cells, or other cells as indicated, were seeded at 104 cells/55-mm plastic dish and transferred to serum-free medium 18 h before stimulation as indicated in the figure legends. 20 min following stimulation with 400 mM sorbitol (or as indicated) cells were assayed for SAPK/JNK activity as described (3) using rabbit anti-holo-SAPK-βI (p54) antisemur and glutathione

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The abbreviations used are: SAPK, stress-activated protein kinase; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MEKK, MAPK/extracellular signal-regulated kinase; CMV, cytomegalovirus; LPS, lipopolysaccharide; TPA, 12-O-tetradecanoylphorbol-13-acetate; CHO, Chinese hamster ovary; PHA, phytohemagglutinin.
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**RESULTS**

To determine whether quinone reductase inhibitors affect transmission of stress signals, we pretreated cultures of human embryonic kidney 293 cells with dicoumarol before stimulation with hyperosmotic sorbitol or anisomycin. Dicoumarol alone did not affect SAPK but completely blocked activation by osmotic shock, which is unrelated to stress signaling.

Inhibition of SAPK activation by dicoumarol was dose-dependent (Fig. 2), with an IC₅₀ between 19 and 38 μM in 293 cells. Dicoumarol blocked SAPK activation in response to expression of TRAF2, a TNFα receptor interacting protein that activates SAPK (15) (Fig. 3A). In contrast, dicoumarol was unable to block SAPK activation in response to expression of truncated active MEKK1, indicating that the point of dicoumarol inhibition of SAPK activation lies downstream of TRAF2 but upstream of MEKK1 in a poorly characterized segment of the stress-signaling cascade.

In contrast to its ability to inhibit activation of SAPK by agents such as sorbitol and anisomycin, dicoumarol failed to inhibit activation of SAPK in response to heat shock (Fig. 3B), which is believed to proceed by an alternate pathway (19). The specificity of dicoumarol inhibition of stress signaling was demonstrated by its failure to inhibit activation of MAPK in CV1 cells in response to TPA (Fig. 3C). However dicoumarol did block MAPK activation resulting from osmotic shock, which likely proceeds via upstream pathways that overlap the SAPK signaling pathway, including the involvement of MEKK1 (20). Thus, dicoumarol inhibition is specific for stress-activated pathways and does not affect mitogenic signaling.

To verify the importance of quinone reductases in stress signaling, we tested a subline of CHO fibroblasts (clone 77254) deficient in NQO1, one of the major cellular quinone reductases (21). Although these cells are still capable of activating SAPK in response to hyperosmotic sorbitol, they have a reduced IC₅₀ for dicoumarol inhibition of SAPK activation, about 5 μM (Fig. 4A). Reconstitution of NQO1 by stable expression of the NQO1 gene (21) reduced the sensitivity of these cells to dicoumarol (Fig. 4B). Thus, inhibition of SAPK activation by dicoumarol can be partly abrogated by expression of NQO1, genetically supporting a role for NQO1 and other quinone reductases in stress signaling.

We tested whether other metabolic toxins and redox inhibi-
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FIG. 2. Concentration dependence of dicoumarol inhibition of SAPK. 293 cells were treated with serial dilutions of dicoumarol, and stimulated with sorbitol as described in Fig. 1. The IC₅₀ of dicoumarol for SAPK inhibition is approximately 19–33 μM.

FIG. 3. A, dicoumarol blocks SAPK activation at a point upstream of MEKK1 and downstream of TRAF2. 293 cells were transfected with empty pCMV plasmid (lanes 1 and 2), TRAF2-CMV (lanes 3 and 4) or ΔMEKK-EE-CMV plasmid (lanes 5 and 6). Endogenous SAPK was assayed by immunoprecipitation and in vitro kinase assay. Dicoumarol reduced basal SAPK activity and TRAF2-stimulated activity but did not block SAPK activation in MEKK1 transfected cells. B, dicoumarol does not inhibit activation of SAPK by heat shock. Cells were stimulated with sorbitol or anisomycin as above or by incubating at 45 °C for 45 min (heat shock) The heat shock results represent a longer exposure of the same gel, because stimulation of SAPK by heat shock is comparatively weaker than the other stimuli. Dicoumarol failed to inhibit activation of SAPK by heat shock. C, dicoumarol does not block TPA activation of MAPK. HeLa cells (used because TPA poorly stimulated MAPK in 293 cells) were treated with 400 mM sorbitol (lanes 1 and 2), TPA (lanes 3 and 4) or with 1 μg/ml TPA (lanes 5 and 6) for 20 min. Extracts were divided and assayed for SAPK activation (top) and MAPK activation using immune complex kinase assays and the indicated substrates. Dicoumarol reduced activation of both SAPK and MAPK by hypertonic sorbitol treatment (lane 4) but did not block stimulation of MAPK by TPA (lane 5).

FIG. 4. Expression of the NQO1 gene reduces sensitivity to SAPK inhibition by dicoumarol. A, an NQO1-deficient subline of CHO cells is hypersensitive to dicoumarol inhibition of SAPK relative to 293 cells. Cultures received a 10-min pretreatment with varying concentrations of dicoumarol before 400 mM sorbitol treatment. SAPK was assayed as in Fig. 1. B, CHO-77254 cells stably transfected with a plasmid encoding NQO1 regained resistance to higher levels of dicoumarol, assayed as in A. The IC₅₀ of CHO-77254 was below 5 μM, whereas in the reconstituted cells the IC₅₀ was between 10 and 20 μM.

FIG. 5. Addition of hydroquinone overcomes dicoumarol inhibition of SAPK activation. CV-1 cells were pretreated for 10 min with dicoumarol and hydroquinone at the indicated concentrations, and then stimulated with 400 mM sorbitol for 20 min. SAPK activity was measured as in Fig. 1. Addition of hydroquinone restored SAPK activation in the presence of dicoumarol.

Dicoumarol, assayed as in Fig. 1. Addition of hydroquinone restored SAPK activation.

These data suggest that dicoumarol inhibition of SAPK signaling may be related to a change in the state of quinone reduction in the cell. Specifically, inhibition of quinone reductases by dicoumarol would result in a decrease in the levels of reduced quinones in the cell. We examined whether replacing the reduced quinones would restore the ability of the cells to signal to SAPK (Fig. 5). Although dicoumarol completely inhibited activation of SAPK in response to sorbitol (lane 4), the addition of increasing amounts of hydroquinone restored the SAPK activation in a dose-responsive manner (lanes 5–8). Treating the cells with hydroquinone alone had no effect on SAPK activity (lanes 9–12). These data suggest that the inhibition of SAPK activation by dicoumarol is due in large part to the decrease in reduced quinones that results from the inhibition of quinone reductases, supporting a role for quinone reduction in establishing an intracellular environment that is permissive for stress signaling.

We examined consequences of interfering with stress signaling using dicoumarol. Many stress stimuli, for example TNFα and LPS, activate both SAPK and the NFkB transcription factor. Recent findings have suggested that the upstream pathways leading to NFkB activation may significantly overlap with those for SAPK activation, including a role for MEKK1 in both signaling cascades (7, 8). Dicoumarol blocks NFkB activation, indicated by the loss of NFkB-DNA binding activity...
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FIG. 6. A, dicoumarol inhibits activation of NFκB by TNFα or LPS. Primary human pulmonary macrophages were tested immediately upon isolation (lane 1) or after culture for 30 min (lanes 2–9). Mobility shifted complexes appeared following stimulation with LPS (1 μg/ml) (lane 3) or TNFα (30 ng/ml) (lane 7). The shifted complexes were inhibited by pre-incubation with dicoumarol (lanes 4 and 8). Participation of p50 and p65 NFκB proteins in the shifted complexes was demonstrated in LPS-stimulated extracts using anti-p50 and anti-p65 polyclonal antibodies (lanes 5 and 6). A 30-fold excess of unlabeled oligonucleotide competitor blocked specific complexes (lane 9). B, dicoumarol synergizes with TNFα to induce apoptosis. HeLa cells were pretreated with 25 μM dicoumarol followed by 30 ng/ml TNFα for 8 h or individually as indicated. Cells were fixed with methanol, stained with propidium iodide, and assayed using flow cytometry for DNA content. The ordinate shows cell number normalized to the mode, the abscissa relative fluorescence. Apoptotic cells with lower than 2N DNA content were enumerated, as indicated near each panel. Dicoumarol increased the fraction of apoptotic cells in synergy with TNFα, as did the protein synthesis inhibitor cycloheximide (CHX).

Like cycloheximide, dicoumarol also potentiated apoptosis of HeLa cells treated with TNFα (Fig. 6B). This synergism is likely a result of the inhibition of NFκB activation by dicoumarol and points to possible therapeutic uses for dicoumarol in synergism with this or other apoptosis-inducing agents. Importantly, the simultaneous inhibition of SAPK/JNK activation and potentiation of apoptosis demonstrates that SAPK/JNK activation is not a required event in the apoptotic response.

Peripheral blood lymphocytes undergo DNA synthesis in response to the mitogen PHA. Pretreatment of human peripheral blood lymphocytes with hypertonic NaCl stimulated SAPK and blocked DNA synthesis in response to PHA (Table II). Pretreatment of peripheral blood lymphocytes with dicoumarol before hypertonic shock prevented SAPK activation and restored PHA-stimulated DNA synthesis. Thus, inhibition of PHA-induced cell cycle entry in response to hypertonic shock is

TABLE II

| Pretreatment | Incorporation of 3H-thymidine | SAPK activity |
|--------------|-----------------------------|---------------|
|              | mean cpm ± S.D. | With PHA | (Imager cpm) |
| None         | 597 ± 80                | 25,158 ± 2,165 | 3.82 |
| Hypertonic NaCl | 422 ± 18              | 12,120 ± 992  | 34.78 |
| Dicoumarol + Hypertonic NaCl | 344 ± 37 | 24,847 ± 1,813 | 3.07 |

Fig. 7. Model for regulation of SAPK and NFκB activation by dicoumarol. Cytokine and physical stresses simultaneously activate apoptotic and survival pathways, resulting in variable cell response (death or survival) depending on context-specific variables. Proximal events in the “survival” pathway depend upon a regulated redox environment for an uncharacterized event distal to the function of TRAF2 but proximal to MEKK1. This event is regulated by disulfides (15) and is also targeted by dicoumarol. The well established role of dicoumarol in inhibiting quinone reductases as well as the role of quinones in disulfide regulation via redox cycling suggest an important role for quinone reductases in maintaining the overall redox environment critical for transmission of stress signals. The “survival” pathway almost certainly involves new gene expression controlled by NFκB. The simultaneous inhibition of NFκB and SAPK activation pathways together with synergism with TNFα to produce apoptosis demonstrates that SAPK activation is not obligately required for apoptosis.

Dicoumarol reverses osmotic shock blockade of PHA activation of peripheral blood mononuclear cells

Resting peripheral blood mononuclear cells were pre-treated either with hypertonic saline (100 mM NaCl) for 30 min or with 300 μM dicoumarol for 10 min followed by 30 min in hypertonic saline. Lymphocytes were washed to remove stimulators and replaced in medium containing PHA. DNA synthesis during a period 24–48 h following PHA exposure was monitored by incorporation of 3H-thymidine, and the results of triplicate samples are shown. SAPK activity was assayed as in Fig. 1. Dicoumarol blocks both the osmotic shock activation of SAPK and prevents the reduction of PHA activation observed following osmotic shock. Similar results were observed in two other experiments.

Dicoumarol inhibits TNFα-induced gene expression by cycloheximide or actinomycin D synergizes with TNFα-induced apoptosis.

Primary human pulmonary macrophages were tested immediately upon isolation (lane 1) or after culture for 30 min (lanes 2–9). Mobility shifted complexes appeared following stimulation with LPS (1 μg/ml) (lane 3) or TNFα (30 ng/ml) (lane 7). The shifted complexes were inhibited by pre-incubation with dicoumarol (lanes 4 and 8). Participation of p50 and p65 NFκB proteins in the shifted complexes was demonstrated in LPS-stimulated extracts using anti-p50 and anti-p65 polyclonal antibodies (lanes 5 and 6). A 30-fold excess of unlabeled oligonucleotide competitor blocked specific complexes (lane 9). B, dicoumarol synergizes with TNFα to induce apoptosis. HeLa cells were pretreated with 25 μM dicoumarol followed by 30 ng/ml TNFα for 8 h or individually as indicated. Cells were fixed with methanol, stained with propidium iodide, and assayed using flow cytometry for DNA content. The ordinate shows cell number normalized to the mode, the abscissa relative fluorescence. Apoptotic cells with lower than 2N DNA content were enumerated, as indicated near each panel. Dicoumarol increased the fraction of apoptotic cells in synergy with TNFα, as did the protein synthesis inhibitor cycloheximide (CHX).
a specific stress-induced effect rather than a nonspecific toxic effect. In future experiments, dicoumarol should similarly be useful for identifying other biologic consequences of stress signaling.

**DISCUSSION**

Our data demonstrate an obligate role for a dicoumarol-inhibitable activity in stress signaling at a point upstream of MEKK1, and downstream of an osmotic shock, LPS, anisomycin, TNFα, and the TNFα receptor-associated protein TRAF2 (Fig. 7). In contrast, this dicoumarol-sensitive activity appears dispensable for activation of SAPK by heat shock, which is thought to proceed via a distinct pathway. Our data support the hypothesis that heat shock acts via an alternate pathway which does not share the apparent dependence on quinone reductase function.

Quinone reductases likely provide an intracellular environment permissive to stress signaling rather than themselves being activated during the signaling. Inhibition of these quinone reductases by dicoumarol would interfere with this environment, and inhibit the ability of the cells to transmit stress signals. In support of this, replacing reduced quinones that are lost as a result of dicoumarol inhibition of quinone reductases restores the ability of the cells to signal to SAPK. These data suggest a role for quinone reduction in generating this permissive intracellular environment. Interestingly, very high concentrations of hydroquinone inhibit activation of SAPK in response to sorbitol, suggesting that there is a tightly regulated balance, where either too high or low levels of reduced quinones are detrimental to stress signal transmission.

Cells lacking NQO1 display increased sensitivity to dicoumarol treatment. The observation that these cells continue to signal to SAPK supports our model in which NQO1 is involved in establishing an intracellular environment permissive for stress signaling, rather than fulfilling an obligate role in a linear pathway. In the absence of NQO1, other less abundant dicoumarol-sensitive quinone reductases assume responsibility for establishing the homeostatic quinone balance. When these NQO1-deficient cells are treated with dicoumarol, this balance is more easily upset, as reflected in the decreased IC50 for SAPK signaling when compared with the cells that have been reconstituted by stable transfection of NQO1.

Inhibition of stress signaling by dicoumarol synergizes with TNF-α to promote apoptosis, suggesting a role for quinone reductases in the regulation of apoptotic events. Other evidence similarly supports this theory. First, using the SAGE technique, it was shown that expression of the apoptotic regulator p53 strongly activated expression of a quinone reductase among several other redox enzymes (22). In a separate study, induction of the p53-induced cell cycle inhibitor p21cip1/whf1 by aziridinylbenzoquinones was also inhibited by dicoumarol (23), demonstrating a requirement for quinone reductases in this induction.

NFκB is a central participant in pathologic inflammation (24). SAPK is also activated in pathologic inflammation, and in other pathologies such as hepatic regeneration and cirrhosis (25), and in reperfusion following cardiac and cerebral vascular occlusion (26). Dicoumarol or similar quinone reductase inhibitors might serve clinically to inhibit stress and inflammatory processes. Dicoumarol has been used as an oral anticoagulant in patients for many years. Although it is a poison, the known toxic effects of its administration in animals are related solely to anticoagulation, which can be reversed by simultaneous administration of vitamin K. Inhibition of SAPK and NFκB by dicoumarol or related compounds might thus prove doubly valuable; first for identifying the function of stress signals in experimental situations, and also for translation into therapeutic utility in the clinical setting.

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