[CANCER RESEARCH 62, 4922–4928, September 1, 2002]

2-Methyl-1,4-naphthoquinone, Vitamin K_3, Decreases Gap-Junctional Intercellular Communication via Activation of the Epidermal Growth Factor Receptor/Extracellular Signal-regulated Kinase Cascade[1]

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ABSTRACT

2-Methyl-1,4-naphthoquinone, vitamin K_3 (menadione), which is frequently used as a model quinone in cell culture and *in vivo* studies, was tested for its effects on gap-junctional intercellular communication (GJC). Exposure of WB-F344 rat liver epithelial cells to menadione (50–100 μM) led to a 50–75% decrease in GJIC. Different from the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate, menadione did not induce internalization of gap junctions. Rather, the decreased GJIC was found to be because of phosphorylation of connexin 43, the major connexin in the used cell line, which was mediated by MAPK/ERK kinase (MEK) 1 and MEK 2 as well as by activation of their direct substrates, extracellular signal-regulated kinase (ERK) 1 and ERK 2. Activation of ERK 1/2 was demonstrated to be independent of NAD(P)H:quinone oxidoreductase using the inhibitor dicoumarol, thus excluding redox cycling as the major mechanism causing these menadione effects. A substantial increase in tyrosine phosphorylation was detected in the cell membrane immunocytochemically upon exposure to menadione, consistent with arylation by menadione bearing the responsibility for the signaling events induced and consistent with the fact that protein tyrosine phosphatases are known targets of arylation reactions. ERK activation was attenuated using specific inhibitors of the epidermal growth factor receptor tyrosine kinase. Similarly, these inhibitors as well as inhibitors of MEK 1/2 counteracted the loss in gap-junctional communication elicited by menadione. This is of interest for chemotherapeutic approaches exploiting the bystander-effect, which is based upon intact GJIC.

INTRODUCTION

Various quinones, such as the mitomycins or daunorubicin/doxorubicin, are in use clinically in the therapy of solid cancers. Menadione[3] is frequently used as a model quinone in cell culture and *in vivo* studies. It undergoes both redox cycling as well as arylation reactions, performing the two major reactions common to quinones of biological relevance (for review, see Refs. 1, 2; see also Fig. 3*A*). The potential of menadione to induce cancer cell death has been extensively described (3, 4) and has been demonstrated to be correlated with the activation of a MAPK family subgroup, the ERK 1 and ERK 2 (5). Inhibition of activation of the latter prevented menadione-induced growth inhibition in the stomach cancer cells used for these experiments (5).

ERK activation is known to not only result in enhanced proliferation but also in growth arrest, and which effect outbalances the other appears to depend on intensity and duration of ERK activation and the consecutively induced expression of cyclin D1 and/or the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$ (for review, see Ref. 6). Both effects, however, are on the level of transcriptional regulation and result from ERKs phosphorylating and thereby activating transcription factors such as ternary complex factors or other kinases (such as MAPK-activated protein kinase 1) that, in turn, phosphorylate transcription factors, including c-Fos (see Ref. 7 for review). ERK 1/2 substrates other than transcription factors are carbamoyl phosphate synthetase II (8) or the Cxs (9, 10). The latter are the building blocks for gap-junctional channels and are thus essential for intercellular communication.

GJIC has been hypothesized to play a crucial role in the regulation of carcinogenesis. Gap junctions are clusters of gap junction channels that connect the cytoplasms of two adjacent cells and consist of two hemi-channels provided by each of the adjacent cells. Hemi-channels, in turn, are hexamers of gap junction proteins, the Cxs (11). It appears that a diminished capacity of cells to communicate intercellularly relates to a loss of control over these cells, favoring a carcinogenic degeneration of the latter. As has been pointed out by Trosko *et al.* (12), this occurs at different levels: GJC is low or even absent in many tumor cells, and deficiency in certain Cxs renders cells prone to carcinogenic changes, as has been demonstrated for Cx32-knockout mice, which tend to develop liver cancer more likely than their control counterparts (13). Furthermore, various tumor promoters and carcinogens such as TPA (14, 15) lindane (16), phenobarbital (17), and others decrease GJC, either by suppressing Cx expression, by impairing intracellular Cx trafficking, or by inducing posttranslational modifications such as phosphorylation, entailing a decreased gap junction channel conductance (for review, see Ref. 10). Known Cx kinases in addition to ERKs include protein kinase C and the tyrosine kinase v-Src (10).

We demonstrate that exposure of rat liver epithelial cells to menadione decreases GJC by ERK-mediated Cx phosphorylation. Activation of ERK is demonstrated to be initiated at the level of the EGFR that we propose to be attributable to arylation rather than redox-cycling reactions of menadione. Inhibition of the EGFR attenuates the decrease in GJC induced by menadione.

The results presented outline a pathway for the action of arylating chemotherapeutics leading to impaired intercellular communication. This diminished GJC could impair cancer treatment approaches that exploit the GJC-based bystander-effect such as suicide gene therapy plus prodrug applications (18). The inhibition of EGFR may be a possible means of avoiding this dilemma.

MATERIALS AND METHODS

Cell Culture. WB-F344 rat liver epithelial cells were a kind gift from Dr. John Trosko (East Lansing, MI). Cells were grown at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in DMEM (Sigma-Aldrich, Deisenhofen,

Received 3/4/02; accepted 6/26/02.

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[1] This study was supported by Deutsche Forschungsgemeinschaft, Bonn, Germany (SFB 575/B4 and SFB503/B1). H. S. is a Fellow of the National Foundation for Cancer Research, Bethesda, MD.

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[3] menadione, 2-methyl-1,4-naphthoquinone, vitamin K_3; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; GJIC, gap-junctional intercellular communication; GJC, gap-junctional communication; Cx, connexin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; BQ, p-benzoquinone; EGFR, epidermal growth factor receptor; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; pNPP, *p*-nitrophenyl phosphate; NQOR, NAD(P)H:quinone oxidoreductase; PTPase, protein tyrosine phosphatase.

Germany), supplemented with 10% (v/v) FCS (Greiner Labortechnik, Frickenhausen, Germany), 2 mM L-glutamine, and with penicillin/streptomycin.

Treatments with menadione (Sigma-Aldrich), BQ (Sigma-Aldrich), or DMNQ (Calbiochem, San Diego, CA) as well as with TPA were in fresh serum-free medium. Enzyme inhibitors such as the EGFR tyrosine kinase inhibitors AG1478 (Alexis Biochemicals, San Diego, CA) and compound 56 (Calbiochem), the MEK 1/2 inhibitors PD98059 (Alexis Biochemicals), and U0126 (Alexis Biochemicals) were applied in serum-free medium 30 min before treatment with menadione. The NQOR inhibitor dicoumarol was from ICN Biomedicals, Eschwege, Germany and was applied together with the respective quinone.

Cell viability was determined using the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) to the corresponding blue formazan as in Ref. 19.

Sucrose Gradient Cell Fractionation. WB-F344 cells were grown to confluence in 90-mm cell culture dishes. After treatment with menadione or TPA, cells were washed with PBS and collected in 1 ml of STED10 buffer [10% (w/v) sucrose, 10 mM Tris-HCl, 10 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin (pH 7.6)] with a cell scraper. Samples were sonicated on ice three times for 20 s each and centrifuged at 500 $\times$ *g* for 5 min. One ml of the supernatant was applied to the sucrose gradient prepared as follows.

Four ml of STED53 buffer [53% (w/v) sucrose, 10 mM Tris-HCl, 10 mM EDTA, 1 mM DTT (pH 7.6)] were pipetted into an SW40 centrifugation tube (Beckman), followed by the careful overlay of 4 ml of STED36 buffer [like STED53 but 36% (w/v) sucrose]. The tube was then filled up with STED20 buffer [like STED53, but 20% (w/v) sucrose] and sealed with parafilm, avoiding any air bubbles, and carefully turned into a horizontal position. After incubation at 4°C for 3 h, the gradient mixture was carefully moved back into a vertical position. One ml of the sucrose gradient was replaced by 1 ml of sample prepared as above. After 14 h of centrifugation at 4°C and 160,000 $\times$ *g*, 800-μl fractions were taken from top to bottom of the tube and analyzed by Western blotting. As verified refractometrically using these aliquots, the resulting sucrose gradient was nearly linear from 10 to 53% sucrose.

Western Blotting and Immunohistochemistry. For Western blotting, cells were lysed in 2$\times$ SDS-PAGE buffer [125 mM Tris-HCl, 4% (w/v) SDS, 20% (w/v) glycerol, 100 mM DTT, 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 blotting. Immunodetections were performed using the following antibodies at dilution recommended by the suppliers, respectively: rabbit polyclonal anti-Cx43 (Zymed Laboratories, San Francisco, CA); monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (Chemicon, Temecula, CA); anti-phospho-ERK (Thr^{202}/Tyr^{204}), anti-total ERK; and anti-phospho-p38 (Thr^{180}/Tyr^{182}; all from Cell Signaling Technology, Beverly, MA). Densitometric analyses were performed using Scion Image software (Scion Corporation, Frederick, MD).

For immunohistochemistry, WB-F344 cells were grown on coverslips in 35-mm plastic dishes until they reached ~90% confluence. Cells were then washed and kept in serum-free medium overnight before treatment with TPA or menadione. After treatment, cells were washed with PBS and fixed with methanol for 10 min at −20°C. After additional washing with PBS, nonspecific binding sites were blocked with 3% (v/v) normal goat serum (Life Technologies, Inc., Rockville, MD) in PBS containing 0.3% (v/v) Triton X-100 for 45 min at room temperature. For detection of Cx43, the abovementioned rabbit polyclonal anti-Cx43 antibody was diluted 1:1500 in PBS containing 1% (v/v) goat serum, and cells were incubated at 4°C overnight under slight agitation. Cells were then washed with PBS and incubated with an Alexa 546-coupled goat antirabbit IgG (H+L) antibody (Molecular Probes, Eugene, OR) for 45 min at 37°C. After washing and embedding, images were taken with a Zeiss Axiovert fluorescent microscope coupled to a charge-coupled device camera (ORCA II, Hamamatsu, Japan). For detection of phosphotyrosine, a monoclonal antiphosphotyrosine antibody (4G10, Upstate) was used at a final concentration of 0.6 μg/ml, and an Alexa 488-coupled goat antimouse IgG (H+L) antibody (Molecular Probes) was used as a secondary antibody. Nuclear staining was performed after immunodetection of Cx43 or phosphotyrosine. Cells were washed with PBS three times and treated with 4′,6-diamidino-2-phenylindole (0.2 μg/ml final concentration) dissolved in citric acid (40 mM)/Na_2HPO_4 (140 mM) buffer (pH 5.5) for 5–10 min, followed by washing and fluorescent detection.

Determination of GJIC. Cells were kept in serum-free medium overnight before treatment with menadione or TPA. After treatment, GJC was determined by microinjection of the fluorescent dye Lucifer Yellow CH [10% (w/v) in 0.33 M LiCl] into selected cells by a micromanipulator and a microinjector system (Eppendorf, Hamburg, Germany). One min after injection, the number of fluorescent cells surrounding the cells loaded with the dye were counted. Per dish 10 individual cells were loaded with dye, and means of the numbers of fluorescent neighboring cells were calculated.

Determination of CD45 Tyrosine Phosphatase Activity. Tyrosine phosphatase activity of human recombinant CD45 (Calbiochem) was measured using pNPP as substrate. CD45 [0.1 μM in 50 mM HEPES buffer (pH 6.8)] was preincubated with either DMSO or menadione at the given concentrations for 15 min in a volume of 50 μl and then added to 750 μl of pNPP/HEPES [2 mM pNPP in 50 mM HEPES buffer (pH 6.8)]. The subsequent increase in absorbance at 405 nm (associated with the formation of *p*-nitrophenol) was monitored, and the formation of *p*-nitrophenol/min calculated using the absorption coefficient of 18,000 M^{-1} cm^{-1} corrected for p-nitrophenol ionisation.

Statistics. Results are reported as means ± SD ($n \geq 3$). ANOVA with Student-Newman-Keuls test was used for the determination of statistical significance between treatment groups. $P < 0.05$ was selected before the study as the level of significance.

RESULTS

Menadione Decreases GJIC in Rat Liver Epithelial Cells. Exposure of WB-F344 rat liver epithelial cells to menadione (50 and 100 μM) rapidly decreases GJIC as demonstrated in dye transfer assays using Lucifer Yellow CH, a fluorescent dye permeating gap-junctional channels (Ref. 20; Fig. 1). The number of communicating cells is decreased within 15 min to 49 and 25% of the control by treatment of the cells with 50 and 100 μM of menadione, respectively. At this time point, none of the used menadione concentrations impaired cell viability as seen from their unchanged capability of reducing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to the corre-

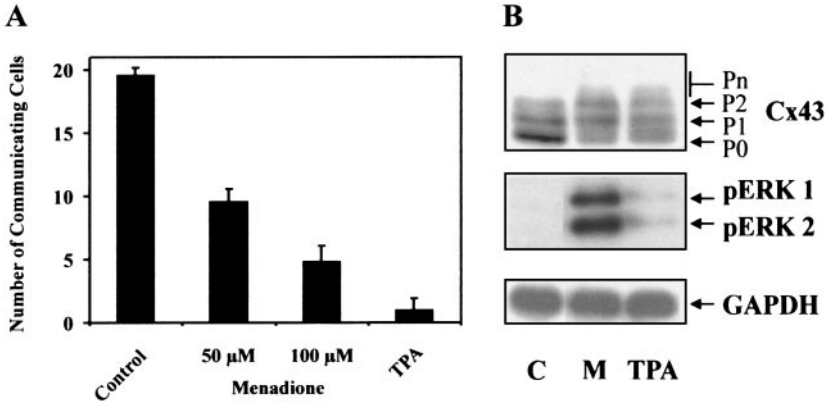

Fig. 1. Effect of menadione on GJIC, Cx43 phosphorylation, and ERK activation. *A*, WB-F344 rat liver epithelial cells were exposed to menadione at the given concentrations or to TPA (100 nM) for 15 min before analysis of GJC with the dye transfer assay using Lucifer Yellow CH. Controls were treated with DMSO, 0.1% (v/v). Data are means of at least three different experiments performed in duplicate ± SD. *B*, cells were exposed to DMSO (*C*), menadione (*M*, 100 μM for 15 min), or TPA (100 nM for 30 min), lysed and phosphorylation of Cx43, ERKs 1 and 2 as well as expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; used as gel-loading control) expression analyzed by Western blotting. *P0*, unphosphorylated Cx43; *P1/P2/Pn*, singly, doubly, and hyperphosphorylated Cx43, respectively.

sponding formazan (data not shown). The early attenuation of dye transfer and loss of communication point to a posttranslational, rather than transcriptional, regulatory mechanism that is activated by menadione. It is known that GJC may be regulated by phosphorylation of Cxs, including Cx43, the prominent Cx in WB-F344 cells. Such a phosphorylation is known to occur after treatment of WB-F344 cells with the phorbol ester TPA (10). Menadione treatment, like TPA, phosphorylates Cx43, leading to a shift in electrophoretic mobility as seen in Western blots (Fig. 1*B*). ERK 1 and ERK 2 were identified as Cx kinases (9) and both are indeed activated by menadione and, albeit much weaker, by TPA (Fig. 1*B*). The strong Cx43 phosphorylation with only a weak ERK activation by TPA may be explained by the fact that not only ERKs but mainly protein kinase C act as Cx kinases here (10).

Activation of MEK 1/2 and ERK 1/2 Is Responsible for Induced Cx Phosphorylation by Menadione. The increased phosphorylation of Cx43 after exposure to menadione (50 μM) for 30 min, as seen from shifts in electrophoretic mobility and a decrease in amount of the unphosphorylated protein (P0) band, can be almost completely prevented by inhibitors of MEKs 1 and 2, the kinases directly upstream of ERKs 1 and 2 (Fig. 2*A*). The structurally unrelated MEK inhibitors PD98059 and U0126 were used at 50 and 10 μM, respectively. The electrophoretic mobility shift and its reversion is also demonstrated in densitometric scans (Fig. 2*A*, bottom panel). In line with this apparent role of MEK 1/2 in menadione-induced hyperphosphorylation of Cx43, the activation of ERK 1 and ERK 2 is also attenuated in the presence of the MEK inhibitors as demonstrated using antibodies specific for the dually phosphorylated (and thus active) forms of ERK 1 and 2 (Fig. 2*B*), as well as in Western blot analysis of the electrophoretic shift of ERK 1/2 because of phosphorylation, which is reversed by the MEK inhibitors (Fig. 2*C*). Interestingly, U0126 appears to be more efficient in suppressing the menadione effects than PD98059 (Fig. 2, *B* and *C*), which is in accordance with the at least 40-fold higher affinity for MEK of U0126 (21).

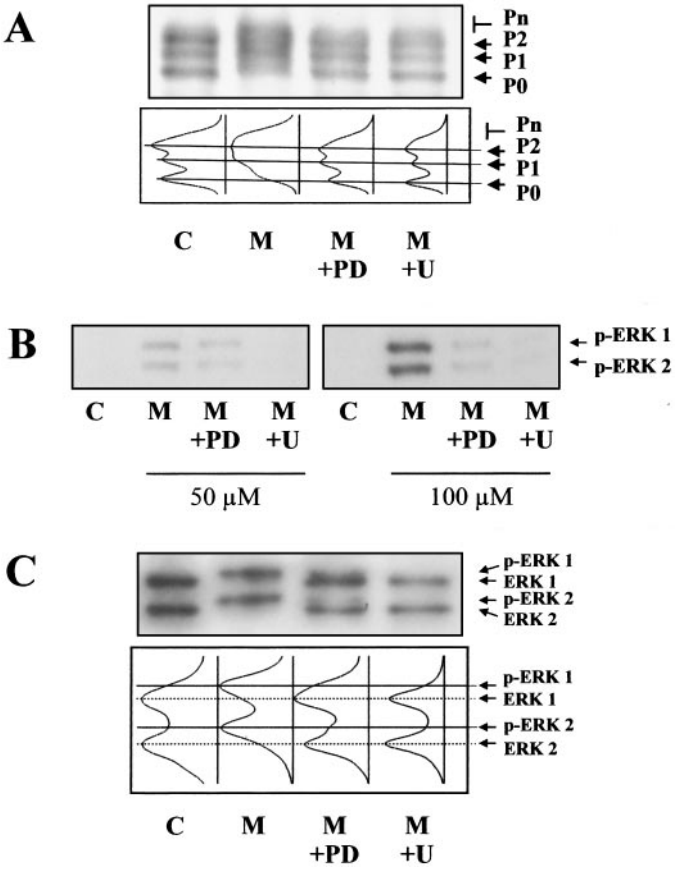

Fig. 2. Cx43 hyperphosphorylation and ERK activation by menadione are dependent on MEK activation. *A*, WB-F344 cells were exposed to menadione (50 μM) for 30 min in the absence or presence of inhibitors of MEKs 1 and 2, PD98059 (*PD*, 50 μM), and U0126 (*U*, 10 μM), respectively. Controls (*C*) were with vehicle (DMSO) treatment instead. Phosphorylation status of Cx43 was analyzed by Western blotting and image analysis. Nonphosphorylated and phosphorylated forms are identified (see Fig. 1 legend for nomenclature). Cells were pretreated with the respective inhibitors or DMSO for 30 min before menadione was added. *B*, exposure of cells to menadione in the given concentrations for 30 min in the absence or presence of MEK inhibitors was followed by Western blot analysis of ERK phosphorylation. *C*, analysis of electrophoretic mobility of phosphorylated and nonphosphorylated ERK 1 and ERK 2 using a pan-ERK antibody. Treatment as in *B* (*left panel*). Data are representative of at least three separate experiments. In any case, the inhibitor-only controls, which are not shown for reasons of clarity, yielded results not different from vehicle controls (*C*).

ERK Activation by Menadione Is Attributable to Arylation Reactions. Menadione may react intracellularly in two ways (Fig. 3*A*), *i.e.,* by arylating nucleophilic compounds such as thiols, or by undergoing redox cycling. The latter may be expedited by NQOR ("DT-diaphorase"), which reduces menadione and other quinones to the corresponding hydroquinone at the expense of NAD(P)H. The hydroquinone, in turn, may be oxidized by molecular oxygen present in high micromolar concentrations in biological systems, thus generating superoxide and other reactive oxygen species derived from it.

To test for the involvement of redox cycling in activation of ERKs by menadione, WB-F344 cells were exposed to menadione in the presence of dicoumarol, an inhibitor of NQOR. As controls, two other quinones were examined for their capability of activating ERKs and for inhibition of the respective effects by dicoumarol. BQ is a very strongly arylating quinone, whereas DMNQ is a pure redox cycler. Attenuation of redox cycling by blockade of NQOR should therefore inhibit effects of DMNQ but not BQ. Exposure of WB-F344 cells to both BQ and DMNQ leads to activation of ERK 1/2 (Fig. 3*B*), but dicoumarol indeed completely blocks the DMNQ effect only. Activation of ERK 1/2 by menadione is not impaired by dicoumarol, suggesting that this effect is largely because of the arylating effects of menadione.

Role of the EGF Receptor in Menadione-induced ERK Activation. Experiments with arylating menadione analogs suggest that a possible point of an arylating attack is the cysteine residues found in the active sites of all known PTPases and that are crucial for activity in that they are the nucleophiles attacking the phosphate moiety, forming a transient phosphocysteine (22, 23). Such an arylation and inactivation of a phosphatase would entail a net increase in tyrosine phosphorylation.

Indeed, menadione is capable of directly inactivating tyrosine phosphatases as demonstrated *in vitro* with isolated human CD45 PTPase (Fig. 4*A*): enzyme activity is strongly lowered within 15 min of exposure to menadione.

The subcellular localization of an increased tyrosine phosphorylation in liver epithelial cells treated with menadione was analyzed immunocytochemically. The data show that after exposure to menadione there is a strong tyrosine phosphorylation signal in the cell membrane (Fig. 4*B*). Inhibiting the EGFR tyrosine kinase using two different inhibitors of the EGFR tyrosine kinase, AG1478 and compound 56, strongly attenuated ERK phosphorylation (Fig. 5*A*) and shift in electrophoretic mobility (Fig. 5*B*) induced by menadione. The specificity of the inhibitors was proven by the fact that activation by menadione of a kinase the activation of which is usually largely independent of the EGFR, the stress kinase p38, is not prevented by AG1478 (Fig. 5*C*).

Inhibition of EGFR or of MEK 1/2 Prevents Decrease in GJC after Menadione Treatment. If the EGFR and MEK/ERK are involved in Cx hyperphosphorylation, the inhibition of either of these kinases should prevent the decrease in GJC after exposure to mena-

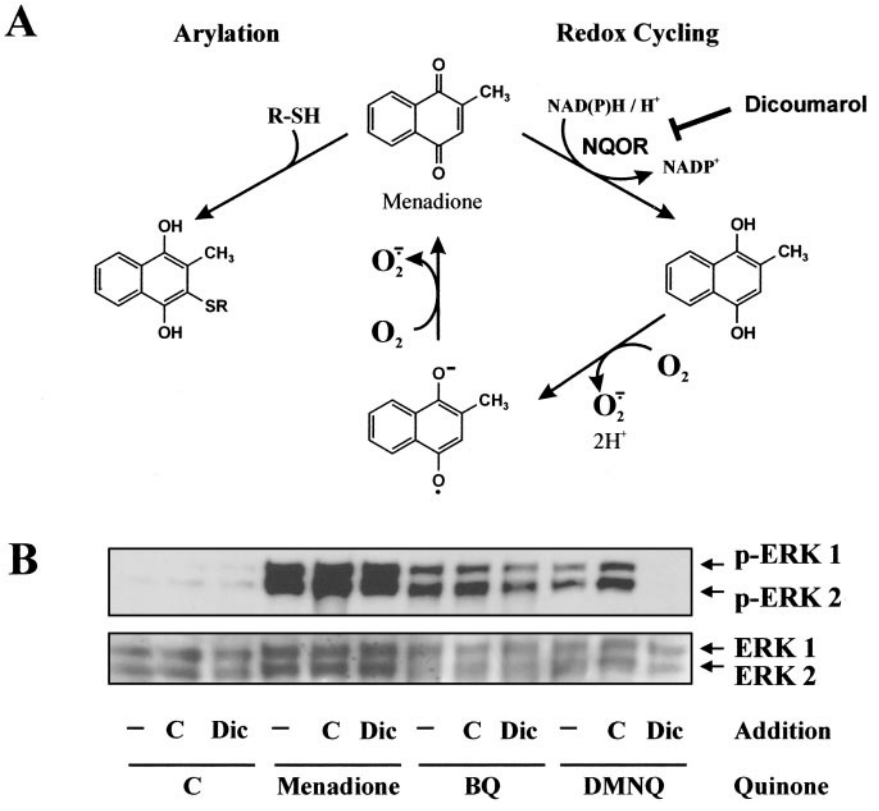

Fig. 3. Menadione action: arylation *versus* redox cycling. *A*, scheme depicting the two major reaction pathways of menadione in mammalian cells and the role of NQOR. *B*, WB-F344 cells were exposed to menadione (50 μM), BQ (100 μM), DMNQ (100 μM), or vehicle (DMSO, *C*) in the absence or presence of the NQOR inhibitor dicoumarol (*Dic*, 100 μM) or additional vehicle (DMSO, *C*) for 60 min. Cells were lysed, and ERK phosphorylation was analyzed by Western blotting. Results are representative of at least three independent experiments. A pan-ERK antibody was used to control for the presence of total ERK.

dione. Fig. 6 shows that this is indeed the case. After exposure to 50 μM menadione, the number of communicating cells is 49% of vehicle (DMSO)-treated control cells. In the presence of the MEK inhibitor U0126, the number of communicating cells is up to 95% of control, whereas 82% of communication is regained in the presence of PD98059, which is consistent with the weaker effect of the PD inhibitor on menadione-induced ERK activation (Fig. 2, *B* and *C*). A similar prevention of a decrease in GJC upon exposure to menadione

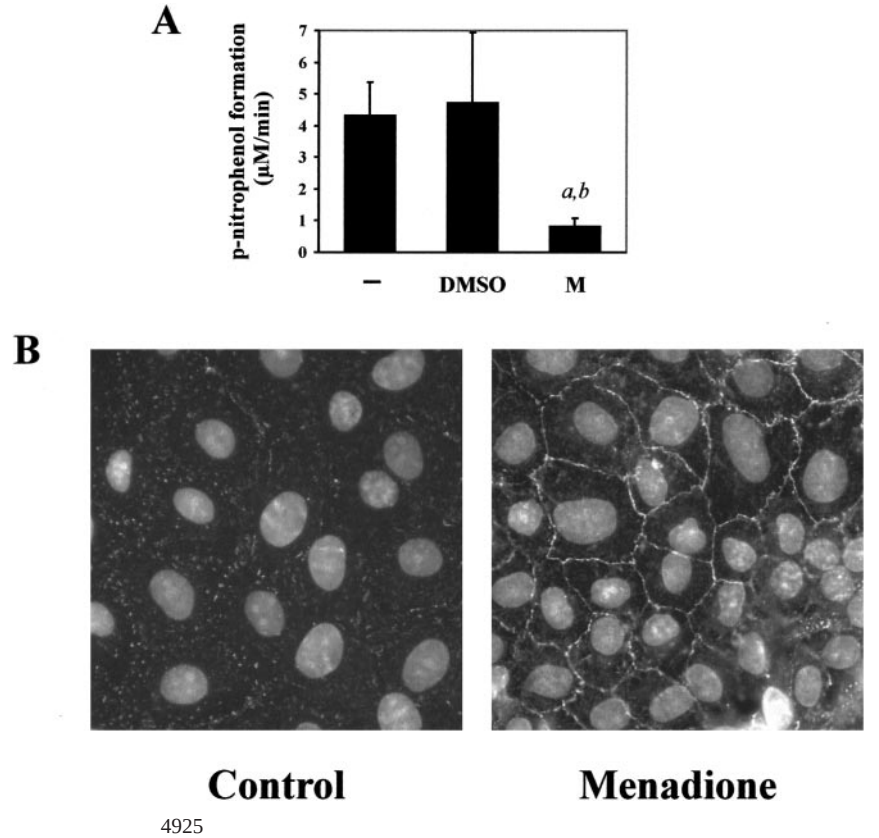

Fig. 4. Phosphatase inhibition and tyrosine phosphorylation induced by menadione. *A*, inhibition of recombinant human CD45 PTPase by menadione. Phosphatase activity of the isolated enzyme was analyzed without (−) or with prior exposure for 15 min to DMSO (as vehicle control; 0.05%, v/v) or menadione (*M*, 50 μM). Data are means of three independent experiments ± SD. *a*, significantly different from −; *b*, significantly different from DMSO control ($P < 0.05$ as determined by ANOVA with Student-Newman-Keuls test). *B*, immunocytochemistry analysis of tyrosine phosphorylation of control- (DMSO as vehicle) and menadione-treated (50 μM for 30 min) WB-F344 cells. Nuclei were stained with 4′,6-diamidino-2-phenylindole. The photographs are representative of two separate experiments performed in duplicate.

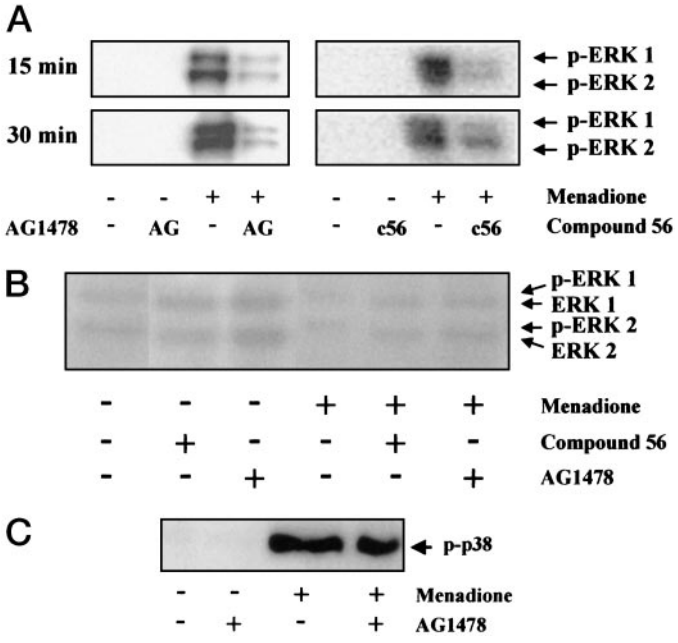

Fig. 5. Role of EGF receptor in menadione-induced ERK activation. *A*, cells were exposed to menadione (50 μM) for the given times in the absence or presence of inhibitors of the EGF receptor tyrosine kinase AG1478 (*AG*, 10 μM) and compound 56 (*c56*, 10 μM), followed by analysis of ERK phosphorylation by Western blotting using phosphospecific ERK antibodies. *B*, analysis of electrophoretic mobility of phosphorylated and nonphosphorylated ERK 1 and ERK 2 using a pan-ERK antibody. Treatment as in *A* with exposure to menadione for 15 min. *C*, analysis of p38 activation after menadione treatment (50 μM for 15 min) in the absence or presence of AG1478 by Western blotting.

is seen in cells treated with the EGFR tyrosine kinase inhibitors AG1478 (92%) and compound 56 (81%; Fig. 6). At 100 μM menadione, the number of communicating cells is only 25%. In the presence of MEK and EGFR inhibitors, these numbers are restored to up to 84% with U0126, 51% with PD98059, 67% with AG1478, and 51% with compound 56 (Fig. 6, right panel).

Changes in Subcellular Distribution of Cx43 after Menadione Treatment. Exposure of liver epithelial cells to certain tumor promotors, including the phorbol ester TPA, is known to induce Cx hyperphosphorylation and a decrease in GJC, which is paralleled by an internalization of Cx molecules (15, 24). Although mechanism and significance of this internalization are yet poorly defined, it can be speculated that it is a second mechanism of regulation of GJC. To assess the effect of menadione on Cx localization, WB-F344 cells were exposed to either TPA (100 ng/ml) or menadione (50 μM), and the localization of Cx43 was analyzed by immunocytochemistry. Cells exposed to TPA, but not menadione-treated cells, experienced a strong decrease of Cx43 concentration in the membrane (Fig. 7*A*). Interestingly, there was a tendency toward an aggregation of Cx molecules in the membrane after exposure to menadione as can be seen in the right panel of Fig. 7*A*. To more clearly discern this aggregation under the influence of menadione from Cx43 localization patterns under control conditions, cells were fractionated on a sucrose density gradient after treatment. In Fig. 7*B*, a representative result is shown, demonstrating that there is a clear shift of Cx43 distribution in cells treated with TPA to fractions of lower density, concomitant with a decrease of Cx43 concentrations in high-density regions, including the cell membrane. Clearly different from that, exposure to menadione induces an accumulation of Cx43 in regions of higher density, and this accumulation is indeed distinguishable from control conditions. In summary, menadione induces an accumulation of Cx43 molecules in the cell membrane rather than an internalization. It can thus further be concluded that internalization of Cx molecules is not a necessary condition for a decrease in GJC.

DISCUSSION

It is demonstrated here that exposure of rat liver epithelial cells to menadione (vitamin K_3) leads to a decrease in intercellular communication via gap junctions. As summarized in Fig. 8, this is attributable to a phosphorylation of Cx43, the major Cx in the used cell line, which is mediated by MEK 1 and MEK 2 as well as their direct substrates ERK 1 and ERK 2. Activation of these MAPKs was demonstrated to be independent of NQOR using the inhibitor dicoumarol, thus excluding redox cycling as the major mechanism responsible for these menadione effects. Menadione not only undergoes redox cycling but is also capable of arylating nucleophilic compounds such as thiols (Fig. 3*A*). Possible menadione targets the arylation of which results in activation of signaling pathways include tyrosine phosphatases, all of which are known to have an essential cysteine at their active site (25, 26). This is in line with data on arylating vitamin K analogs, which were demonstrated to block cellular PTPase activity in breast cancer cells, concomitantly activating the EGFR as well as ERK 1 and ERK 2, and inducing growth inhibition (22, 23). It may thus be hypothesized that the activation of ERK 1/2 is because of the inhibition of tyrosine phosphatases, entailing a net increase in tyrosine phosphorylation and the activation of kinases such as the EGFR tyrosine kinase. Indeed, exposure to menadione strongly inhibits a model tyrosine phosphatase, CD45 (Fig. 4*A*), and leads to increased tyrosine phosphorylation in the cell (Fig. 4*B*). Furthermore, both ERK

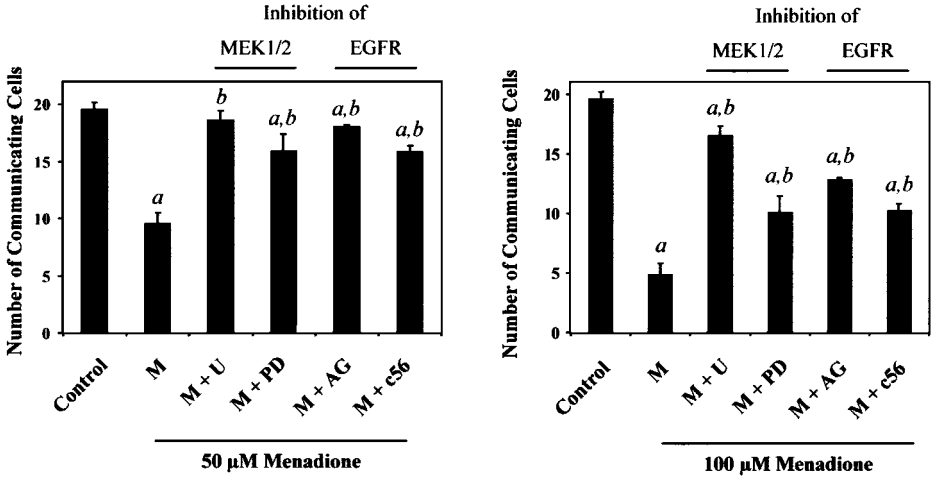

Fig. 6. GJC after exposure to menadione. WB-F344 rat liver epithelial cells were exposed to DMSO (control) or menadione (*M*) in the given concentrations for 15 min in the absence or presence of U0126 (*U*, 10 μM), PD98059 (*PD*, 50 μM), AG1478 (*AG*, 10 μM), or compound 56 (*c56*, 10 μM), followed by analysis of GJIC by dye transfer analysis. Cells were pretreated with inhibitors or vehicle (DMSO) for 30 min before the addition of menadione. Data are means of three to six separate experiments $\pm$ SD. *a*, significantly different from control; *b*, significantly different from *M* ($P < 0.05$ as determined by ANOVA, followed by Student-Newman-Keuls test).

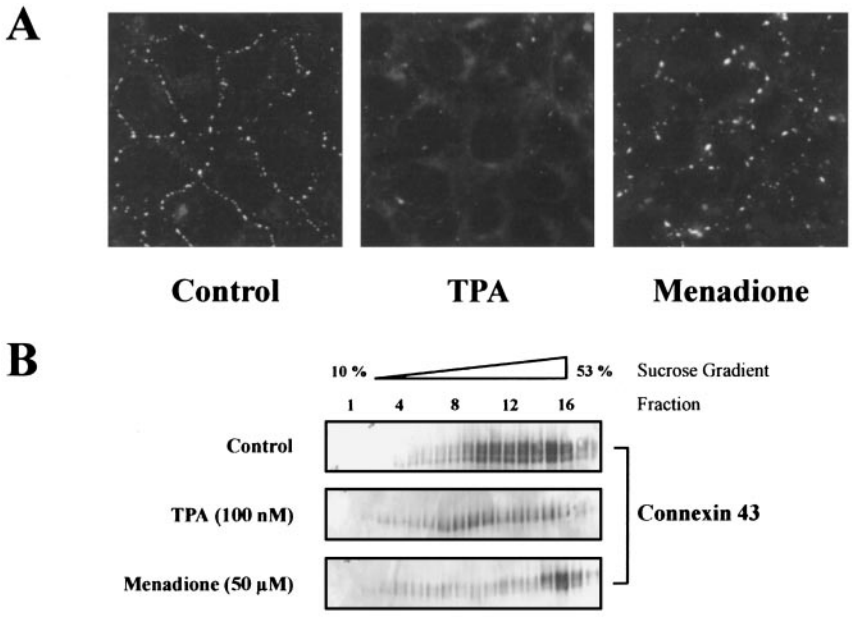

Fig. 7. Subcellular localization of Cx43 after exposure to menadione. *A*, immunocytochemical analysis of Cx43 distribution in WB-F344 cells treated with DMSO (control), TPA (100 nM), or menadione (50 μM) for 30 min. *B*, Western blot analysis of subcellular Cx43 distribution in cells exposed to DMSO (control), TPA, or menadione for 30 min after separation on a sucrose density gradient. Data are representative of three independent experiments.

activation and decrease in GJC as induced by menadione are prevented by EGFR inhibitors, the tyrphostins AG1478 and compound 56 (Figs. 5 and 6).

From the data presented (Fig. 7), it additionally appears that Cx phosphorylation rather than Cx internalization, which has been known to occur after exposure to tumor promotors for years (24), is responsible for the menadione-induced decrease in GJC.

As for the significance of a diminished intercellular communication after treatment with quinones, it may appear paradoxical that a compound potently killing cancer cells and additional standing for a series of clinically used anticancer quinones should decrease GJC, thus diminishing tissue control over the targeted cells and thus enhancing the danger of the formation of uncontrollably growing cancer cell populations possibly resistant to the respective quinone. However, a decrease in GJC may not only be regarded as a carcinogenic event (loss of control), but this closure of cytoplasmic contacts may also prevent the uncontrolled transfer of carcinogens from cell to cell: certain quinones, in addition to alkylating and/or redox cycling, interact with and modify DNA, and cells attached to and communicating with target cells could receive quinones at concentrations not sufficient for cell killing but inducing cell transformation. For example, the spreading of a carcinogenic signal via gap junctions was described for the exposure of cells to ionizing radiation with α particles (27).

Certain cancer therapy strategies rely on the so-called bystander effect that exploits the diffusion of drug through gap junction channels such as suicide gene therapy using thymidine kinase plus application of nucleoside analogue prodrugs (18). The effects of menadione and of EGFR or MEK inhibitors regarding GJC described here could serve as a basis for improvements in chemotherapy using quinones or other agents leading to a decreased intercellular communication in a similar manner. A restoration of the bystander effect in chemotherapy, *e.g.*, by use of EGFR or MEK inhibitors (Fig. 6), could result in an increased efficiency in chemotherapy because of avoidance of resistance formation. In line with this, tumorigenic cells are frequently characterized by the expression of EGFR mutants that are constitutively active (28). Such a condition should, according to the data shown here, entail a diminished cell-cell communication capacity in these cells. Indeed, overexpression of a constitutively active EGFR mutant renders human glioblastoma cells resistant toward treatment with *cis*-platinum, but in the presence of the EGFR tyrosine kinase inhibitor AG1478, sensitivity of the cells toward *cis*-platinum treatment is enhanced (29).

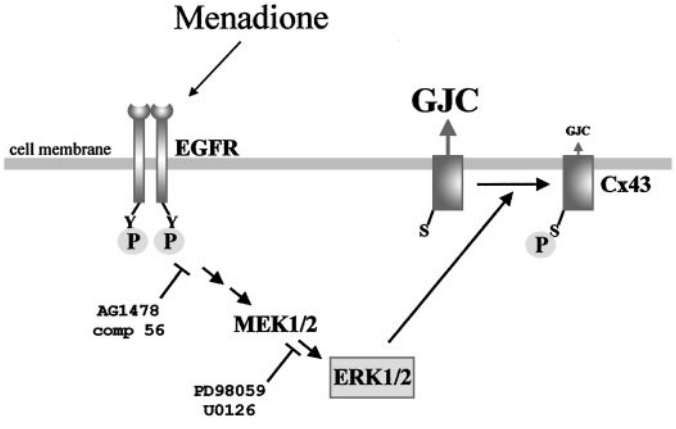

Fig. 8. Summary of the pathway leading to decreased GJC after exposure to menadione.

ACKNOWLEDGMENTS

We thank Elisabeth Sauerbier for expert technical assistance. We also thank Dr. H. Possel for help with immunofluorescence studies and Professor W. Stahl for fruitful discussions. Dedicated to Professor Waldemar Adam on the occasion of his 65th birthday.

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