Biological effects of menadione photochemistry: effects of menadione on biological systems may not involve classical oxidant production

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Because cell-mediated reduction of menadione leads to the generation of reactive oxygen species (ROS), this quinone is widely used to investigate the effects of ROS on cellular functions. We report that A549 human lung epithelial cells exposed to menadione demonstrate a dose-dependent increase in both intracellular calcium ($[Ca^{2+}]_i$) and ROS formation. The concentrations of menadione required to initiate these two events are markedly different, with ROS detection requiring higher levels of menadione. Modulators of antioxidant defences (e.g. buthionine sulphoximine, 3-amino-1,2,4-triazole) have little effect on the $[Ca^{2+}]_i$ response to menadione, suggesting that ROS formation does not account for menadione-dependent alterations in $[Ca^{2+}]_i$. Additional evidence suggests that menadione photochemistry may be responsible for the observed $[Ca^{2+}]_i$ effects. Specifically: (a) EPR studies with the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) show that light exposure (maximum effect at 340 nm) stimulates menadione-dependent formation of the DMPO/$^{\cdot}$OH spin adduct that was not sensitive to antioxidant interventions; (b) DMPO inhibits menadione and light-dependent increases in $[Ca^{2+}]_i$; and (c) light (maximum effect at 340 nm) augments the deleterious effects of menadione on cell viability as determined by ^{51}Cr release. These photo effects do not appear to involve formation of singlet oxygen by menadione, but rather are the result of the oxidizing chemistry initiated by menadione in the triplet state. This work demonstrates that menadione species generated by photo-irradiation can exert biological effects on cellular functions and points to the potential importance of photochemistry in studies of menadione-mediated cell damage.

Key words: A549 cells, electron paramagnetic resonance, free radical, intracellular calcium, singlet oxygen.

INTRODUCTION

Exposure of cells to reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) has been linked to a variety of physiological and pathological events [1,2]. The cellular consequences of oxidant exposure, as well as the relative importance of different cellular antioxidant systems in protecting cells from such exposures, varies with both the nature of the oxidant species generated and the intracellular and/or extracellular location of generation [2,3]. To study *in vitro* the effects of increased cellular exposure to $O_2^{\cdot-}$ and H_2O_2, investigators have employed a variety of redox-active compounds such as menadione, methyl viologen (paraquat), pyocyanine and streptonigrin [4–7]. Each of these compounds can undergo cell-mediated one-electron reduction, with subsequent electron transfer to oxygen, generating $O_2^{\cdot-}$ and H_2O_2 [4–9].

Among the effects resulting from cellular oxidant exposure is a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) [10–15]. Depending on the nature and site of oxidant exposure this can result from either release of Ca^{2+} from intracellular stores or influx of extracellular Ca^{2+}. $[Ca^{2+}]_i$ levels play an important regulatory role in a variety of cellular events due to the $[Ca^{2+}]_i$ dependence of many cellular signalling pathways [10–15]. Additionally, oxidant-mediated increases in $[Ca^{2+}]_i$ have been implicated in the deleterious effects of oxidants on a variety of cell types [10–15].

Recent work from our laboratories demonstrates that pyocyanine, a redox-active secretory product of *Pseudomonas aeruginosa*, causes an increase in $[Ca^{2+}]_i$ in lung epithelial cells by inducing release of Ca^{2+} from intracellular stores via an oxidant-dependent mechanism [14]. To understand this process further, we initiated studies to compare the known effect of pyocyanine on epithelial-cell Ca^{2+} metabolism with that of the redox-active compound, menadione. In the course of this work, we identified unique menadione effects on cellular Ca^{2+} that were distinct from those of pyocyanine. More importantly, these appeared to arise from photochemical products of the interaction of light with menadione rather than through $O_2^{\cdot-}$ and H_2O_2 generated via cell-mediated redox cycling of the compound. Although the photochemistry of menadione has been described by others [16–20], the results of the work we now report emphasize the potential biological impact of these events and highlight the need for investigators using menadione to consider the photochemistry of menadione when analysing their data.

EXPERIMENTAL

Cell culture

The human alveolar type-II cell line A549 (A.T.C.C. CL-185) [21] was cultured (37 °C, 5% CO_2) in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, U.S.A.) with 10% heat-inactivated calf serum, 2 mM L-glutamine and 500 units/ml penicillin and streptomycin. For all experiments, cells

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+}; BSO, buthionine sulphoximine; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; HBS/G/BSA, Hepes-buffered saline/glucose/BSA; HBSS, Hank's balanced salt solution; NAC, *N*-acetyl cysteine; $^1O_2$, singlet oxygen; $O_2^{\cdot-}$, superoxide radical; ROS, reactive oxygen species; SOD, superoxide dismutase.

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were used between passages 80 and 120. Cells were grown to near confluence (80–90 %) in either 24-well (EPR studies) or 48-well (^{51}Cr release) plates, or on collagen-coated glass coverslips (fluorescence studies). To alter the cellular antioxidant profile, cells were exposed for 24 h to 100 μM buthionine sulphoximine (BSO), or for 1 h to 5 mM 3-amino-1,2,4-triazole or 10 mM *N*-acetyl cysteine (NAC; all from Sigma, St. Louis, MO, U.S.A.).

Measurement of $[Ca^{2+}]_i$

Cells were cultured on collagen-coated 25 mm round glass coverslips (for fura 2) or 24-well tissue-culture plates (for Calcium Orange) and loaded with 5 μM of the acetoxy methylester derivatives of the calcium-sensitive fluorescent probes fura 2 or Calcium Orange (Molecular Probes, Eugene, OR, U.S.A.), for 30 min at 37 °C. Cells were then washed 4–5 times over a 5-min period with HBS/G/BSA [Hepes-buffered saline (135 mM NaCl, 5 mM KOH, 10 mM Hepes, 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$) supplemented with 10 mM glucose and 0.1 % BSA]. Fura 2-based measurements of cytosolic [Ca^{2+}] were performed using a Photoscan II spectrofluorometer (Photon Technologies International, New Brunswick, NJ, U.S.A.) with a Nikon microscope (Nikon, Niles, IL, U.S.A.) [14]. Baseline fluorescence was measured for 2 min, then agonists (menadione, thapsigargin, ionomycin) were added and fluorescence measurements were taken over a 20-min period. Background was subtracted and final Ca^{2+} concentrations were determined from the ratio of emission intensities ($\lambda_{em} = 510$ nm) for excitation wavelengths (λ_{exc}) of 340 and 380 nm [14,22]. Maximum and minimum ratios, as well as the K_d of the fura 2–Ca^{2+} complex, were empirically derived from Ca^{2+} concentration curves generated using the instrument. Calcium Orange-based measurements ($\lambda_{exc} =$ 530 nm; $\lambda_{em} = 575$ nm) were performed using a BMG Fluostar microplate spectrofluorometer (BMG Labtechnologies, Durham, NC, U.S.A.).

For experiments using ^{45}Ca, cells were seeded into 6- or 12-well culture dishes and grown to 70–80 % confluence. Cultures were washed three times with warm low-calcium buffer (135 mM NaCl, 10 mM Hepes, 5 mM KOH, 1.0 mM $MgCl_2$ and 0.1 mM $CaCl_2$, pH 7.4) supplemented with 0.1 % BSA and incubated subsequently in 1 ml of low-calcium buffer for 10 min at room temperature. ^{45}Ca (20 μCi/ml in low-calcium buffer) with or without menadione was added and cells were incubated for 5 min at room temperature in the presence or absence of light. At the end of the incubation period, the isotope was removed and the cells were washed rapidly three times with 1 ml of ice-cold Hank's balanced salt solution (HBSS) without Phenol Red. Finally, the cells were solubilized with 1 ml of 1 % SDS, the extract was transferred to a scintillation vial, 4 ml of scintillation cocktail was added, and the radioactivity was measured using instrument settings for ^{35}S.

Fluorescence measurement of intracellular oxidant formation

Cells were washed with HBS/G/BSA and incubated at 37 °C for 30 min in HBS/G/BSA containing 20 μM dihydrofluorescein diacetate (Molecular Probes). Menadione was then added, and the cells incubated for 3 h at 37 °C. To measure cell-associated fluorescence, the cells were washed twice with ice-cold PBS, and incubated for 10 min on ice with PBS containing 0.2 % Triton X-100. The cell extract was removed from the cells, and the fluorescence intensity of the extract ($\lambda_{exc} = 485$ nm; $\lambda_{em} =$ 535 nm) was determined using a Gilford Fluoro IV spectrofluorimeter.

Spin trapping

Experiments were performed using a 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, Sigma; additionally purified with charcoal [23]) spin-trapping system containing 0.1 mM diethylenetriaminepenta-acetic acid (DTPA, Aldrich Chemical Co., Milwaukee, WI, U.S.A.). All spectra are the result of seven signal-averaged scans, and were obtained at room temperature using a Bruker ESP 300 spectrometer. The magnitude of the EPR signal observed is directly proportional to the concentration of spin adduct in the sample. Instrument settings were: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 0.94 G; time constant, 82 ms; scan rate, 80 G/42 s.

In the cell-free studies, DMPO-containing samples were exposed to relevant wavelengths using a Perkin-Elmer luminescence spectrometer with an excitation slit width of 15 nm in a standard quartz fluorescence cuvette for 10 min with mixing before transfer to an EPR flat cell in the dark. For studies involving A549 cell monolayers, cells in multi-well plates were exposed to DMPO under various conditions of menadione and light exposure. The supernatants from each well were removed and flash-frozen in liquid nitrogen for subsequent assay. In some cases, cell monolayers in multi-well plates were kept in near dark until exposure to light.

Photo-production of singlet oxygen ($^1O_2$) by menadione

$^1O_2$ phosphorescence in the infra red was recorded on a steady-state $^1O_2$ spectrophotometer [24]. Aerated solutions of menadione in acetonitrile, methanol and ^2H_2O were irradiated (200 W mercury lamp) at its absorption maximum through a broad-band interference filter (see Figure 5 below, inset, spectrum t). In some experiments menadione solubilized in SDS micelles or β-cyclodextrin was also used. The quantum yield of $^1O_2$ formation was calculated relative to the perinaphthenone standard by comparing the integrated steady-state intensities of $^1O_2$ phosphorescence, normalized to the same number of absorbed photons by both menadione and perinaphthenone in the same solvent. All measurements were conducted using freshly prepared solutions and were carried out at room temperature. Absorption spectra were measured using an HP diode array spectrophotometer model 8452A.

^{51}Cr-release assay

Cell injury was quantified with a standard ^{51}Cr-release assay [25]. Briefly, cells were plated at 5×10^4 cells per well in 48-well tissue-culture plates (CoStar, Cambridge, MA, U.S.A.) and incubated for 4–6 days. When the cells were at near-confluence, they were loaded with ^{51}Cr by incubation for 18 h in Dulbecco's minimal essential medium containing 2.5 μCi/ml ^{51}Cr (1 mCi/ml sodium chromate; Amersham, Arlington Heights, IL, U.S.A.). After multiple washes in HBSS, desired compounds were added to each well in HBSS (final volume 0.25 ml), except for maximum-release controls which contained HBSS with 10 % Triton X-100. The wells were then incubated for the desired time period under the specified conditions, after which the supernatants were removed and radioactivity determined by gamma counting using a Packard Cobra II Gamma Counter (Packard, Meriden, CT, U.S.A.). For UV exposure, 48-well plates were placed at room temperature with lids removed under an inverted UV-light box. Additional experiments utilized long-pass glass filters transmitting wavelengths greater than 305 and 360 nm. The temperature of the culture plate was essentially unchanged (< 2 °C) during UV exposure. Each experimental data point was the mean of triplicate wells, and each experiment was repeated

three times. Results of each experiment are expressed as the mean (±S.E.M.) specific ^{51}Cr release, where specific ^{51}Cr release is defined as (test-well ^{51}Cr c.p.m. − spontaneous-release ^{51}Cr c.p.m.)/(maximum-release ^{51}Cr c.p.m. − spontaneous-release ^{51}Cr c.p.m.).

Statistics

Statistical analysis was performed using a two-tailed Student's *t* test.

RESULTS

Effect of menadione on $[Ca^{2+}]_i$ in A549 cells

Previous work from our laboratories demonstrated that the redox-active compound pyocyanine, a secretory product of the human pathogen *P. aeruginosa*, causes a rapid rise in $[Ca^{2+}]_i$ levels in airway epithelial cells by inducing the release of Ca^{2+} from intracellular stores and subsequent influx of extracellular Ca^{2+} [14]. To further investigate this pyocyanine-mediated effect, we sought to compare the effect of pyocyanine with that of another redox-active compound, menadione. As with pyocyanine, exposure of A549 cells to menadione caused a rise in $[Ca^{2+}]_i$ as detected by fura 2 fluorescence. Figure 1 shows a typical tracing (trace A), with basal $[Ca^{2+}]_i$ levels of approx. 100 nM and a steady increase in $[Ca^{2+}]_i$ following menadione addition (10 μM, added at the arrow) up to a $[Ca^{2+}]_i$ level of approx. 250 nM, or about a 2.5-fold increase. This menadione-associated increase in $[Ca^{2+}]_i$ was markedly diminished in Ca^{2+}-free buffer containing 2 mM EDTA (Figure 1, trace B). In contrast to earlier studies with pyocyanine [14], depletion of endoplasmic reticulum $[Ca^{2+}]_i$ stores by pretreatment with 2 μM thapsigargin did not abrogate the response to menadione (Figure 1, trace C). In fact, thapsigargin treatment reproducibly enhanced the menadione-mediated effect. These data suggest that menadione exposure increases $[Ca^{2+}]_i$ in A549 cells predominately by stimulating influx of extracellular Ca^{2+} [26].

Role of oxidant production in the menadione-induced rise in $[Ca^{2+}]$

It is well established that exposure of cells to menadione increases intracellular levels of ROS such as $O_2^{\bullet-}$ and H_2O_2 [27]. As a first step towards defining the one or more oxidant species responsible for the effect of menadione on A549 Ca^{2+} homoeostasis, we compared the ability of menadione to induce a rise in $[Ca^{2+}]_i$ with its ability to induce cellular production of oxidants in these cells using a dihydrofluorescein oxidation assay [14,28]. Although $[Ca^{2+}]_i$ increases were consistently observed in response to 10 μM menadione (Figure 2A, ●), detection of intracellular ROS production required 50 μM or higher doses of the compound (Figure 2B, ●). These results were surprising given that in our previous work with pyocyanine we found a close correlation between the amount of pyocyanine required to induce a rise in $[Ca^{2+}]_i$ and that required for oxidation of the dihydrofluorescein probe [14].

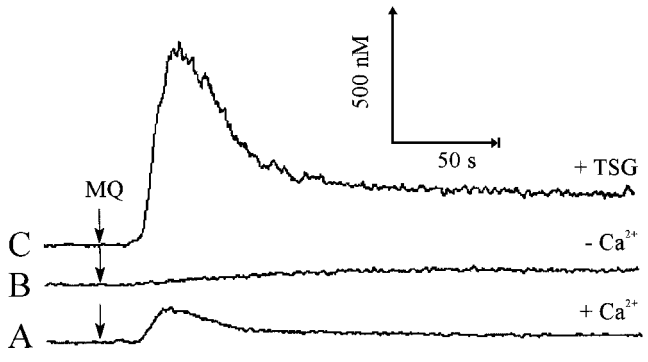

Figure 1 Menadione-dependent increases in cytosolic $[Ca^{2+}]_i$ in A549 cells

Trace A, A549 cells loaded with fura 2 and stimulated with 10 μM menadione (MQ) in the presence of extracellular Ca^{2+}; trace B, as trace A but stimulated in the absence of extracellular Ca^{2+}; trace C, as trace A but cells pretreated for 40 min with 2 μM thapsigargin (TSG) before fura 2 loading. Ca^{2+} measurements were performed as described in the Experimental section. Similar responses were observed in two other independent experiments.

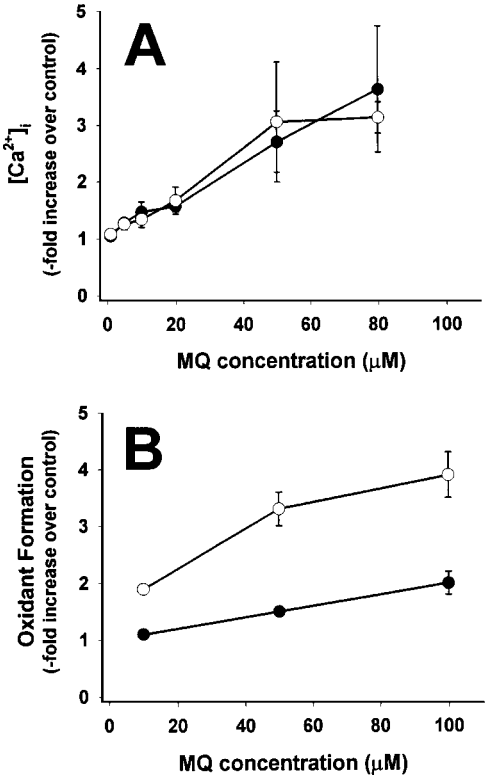

Figure 2 Effect of BSO pretreatment on menadione-dependent changes in $[Ca^{2+}]_i$ (A) and ROS (B) in A549 cells

(**A**) Increase in $[Ca^{2+}]_i$ levels over basal (100 nM), determined by fura 2 fluorescence as a function of increasing menadione (MQ) exposure, and (**B**) increase in ROS formation, determined by dihydrofluorescein fluorescence as a function of increasing menadione exposure, in control (●) and BSO-pretreated (○) A549 cells. Each data point is the mean ± S.E.M. of triplicate determinations from three independent experiments.

DMPO/$^{\bullet}$OH formation by UV-light exposure

To explore further the discrepancy between the menadione concentrations required to increase $[Ca^{2+}]_i$ relative to ROS production, we employed an alternative technique for detecting cellular free-radical production: spin trapping. When A549 cell monolayers were exposed to menadione in the presence of the spin-trap DMPO (100 mM), the concentrations of menadione required for detectable DMPO/$^{\bullet}$OH production (results not shown) were similar to those which led to an increase in cytosolic Ca^{2+} (Figure 2A), i.e. 5–10-fold lower than those required to produce detectable oxidation of the dihydrofluorescein probe. This initially suggested to us that spin trapping might be more

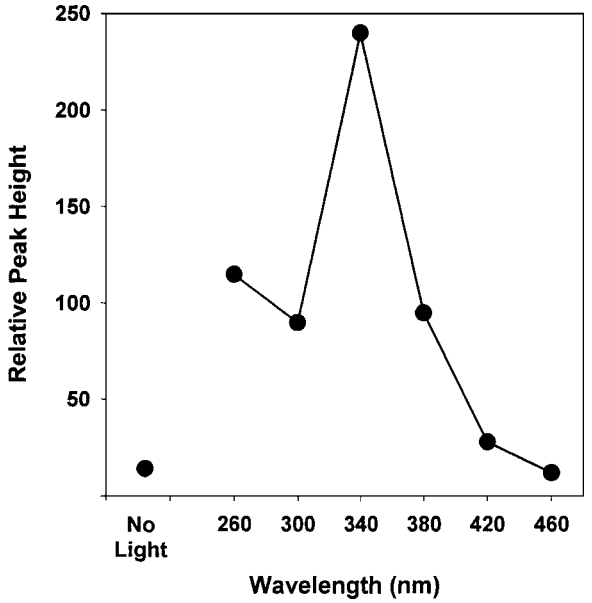

Figure 3 DMPO/•OH formation by menadione; dependence on photo-irradiation wavelength

Cell-free buffer solutions containing 100 μM menadione and 100 mM DMPO were exposed to 260–460 nm light (slit width 15 nm) for 10 min with mixing before transfer to an EPR flat cell in the dark. Relative DMPO/•OH peak height is plotted as a function of wavelength. 'No Light' represents DMPO/•OH levels due to ambient light exposure in the lab with subdued lighting.

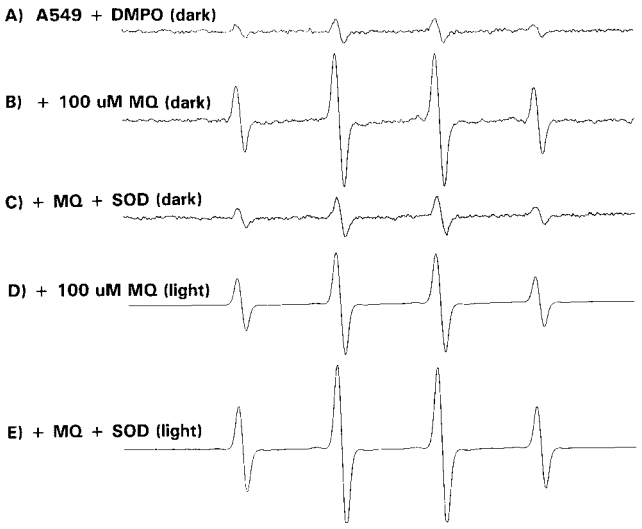

Figure 4 EPR spectra of DMPO/•OH spin adducts formed by menadione-treated A549 cells

A549 cell monolayers exposed to 100 μM menadione (MQ) in near-darkness or room (ambient) light in the presence of 100 mM DMPO. (**A**) Cells without menadione in the dark; (**B**) as (**A**), but cells exposed to 100 μM menadione; (**C**) as (**B**) but cells treated with 300 unit/ml SOD; (**D**) as (**B**) but cells exposed to room light for 10 min; (**E**) as (**C**), but cells exposed to room light for 10 min. Because spectra (**D**) and (**E**) were approx. 5–20-fold more intense than (**A**–**C**), these two spectra have been reduced (y axis) by a factor of 8 for presentation.

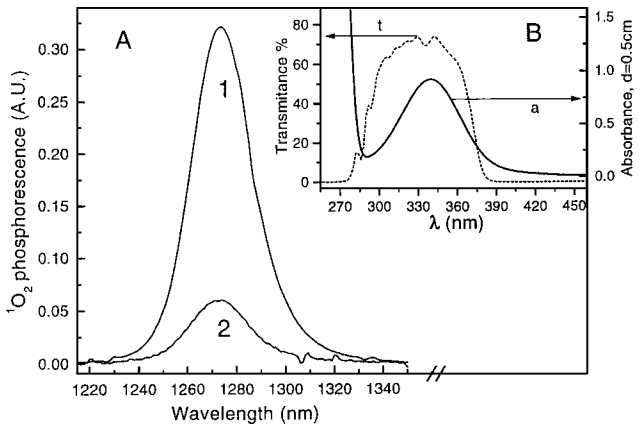

Figure 5 Photo-production of $^1O_2$ by menadione in acetonitrile and ^2H_2O

Spectra represent $^1O_2$ phosphorescence in acetonitrile (trace 1) and in ^2H_2O (trace2) observed upon irradiation of menadione through a broad-band interference filter (inset, trace t). The inset also shows the absorption spectrum for menadione in ^2H_2O (trace a) measured in a UV cell with 0.5 cm light path.

sensitive than dihydrofluorescein oxidation for detecting $O_2^{\bullet-}$ formation and that this might explain our lack of correlation in dose–response curves for changes in $[Ca^{2+}]_i$ and ROS.

Since we routinely detect DMPO/•OH formation as a consequence of pyocyanine-induced $O_2^{\bullet-}$ generation in A549 cells [25,29,30], we hypothesized that the formation of DMPO/•OH in A549 cells exposed to menadione resulted from $O_2^{\bullet-}$ formation by similar cell-associated pathways. However, in contrast to our previous experience with pyocyanine or paraquat [14,25,29–31], we observed that exogenous addition of superoxide dismutase (SOD) was apparently ineffective at inhibiting DMPO/•OH generation resulting from the addition of menadione to samples containing A549 cells (results not shown). These results suggested that a mechanism other than $O_2^{\bullet-}$ formation could be responsible for the DMPO/•OH detected in response to menadione. In a series of control experiments associated with the above studies, we observed that a large amount of DMPO/•OH could be detected following the addition of menadione to a solution of DMPO in HBSS in the absence of cells, and that the magnitude of the DMPO/•OH signal correlated with the extent of exposure of the solution to ambient light in the laboratory. These findings are consistent with those recently reported by Monroe and Eaton [20].

To determine the wavelength of light responsible for the menadione-dependent DMPO/•OH formation, cell-free solutions of menadione and DMPO were exposed to defined wavelengths of light in 40-nm increments from 260 to 460 nm. The resulting DMPO/•OH peak amplitudes were then measured. As shown in Figure 3, wavelength-dependent DMPO/•OH formation was observed with a maximum yield at 340 nm, which is in accordance with the absorption maximum of menadione [19], suggesting that menadione photochemistry is involved. This finding is particularly significant since the methodology used for fluorometric detection of $[Ca^{2+}]_i$ levels by fura 2 uses an excitation wavelength of 340 nm (see the Experimental section).

To determine the extent to which menadione photochemistry was responsible for DMPO/•OH formation, A549 cell monolayers were exposed to menadione in the presence of DMPO under conditions that carefully controlled the exposure of the cells to light. In the absence of menadione, A549 cells exposed to DMPO formed a small amount of DMPO/•OH (Figure 4A), probably due to endogenous formation of $O_2^{\bullet-}$ by mitochondrial

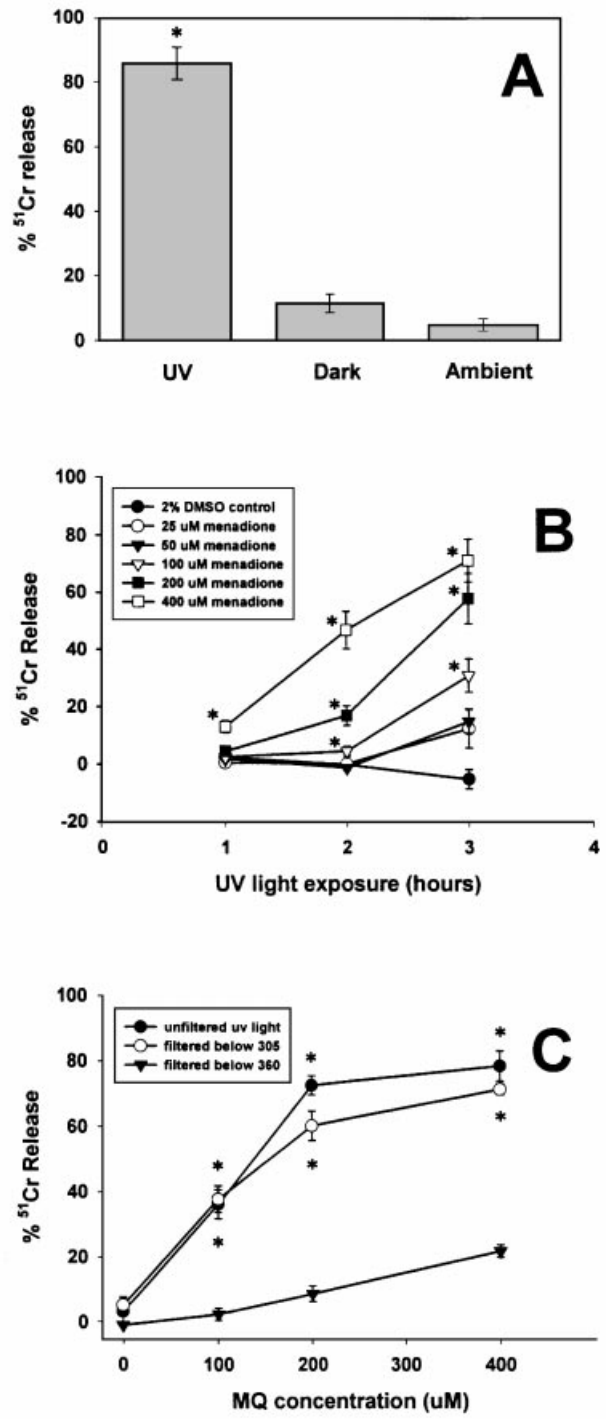

Figure 6 Effect of UV light exposure on menadione-dependent A549 cell injury using a ^{51}Cr-release assay

(**A**) A549 cells treated with 400 μM menadione, with ^{51}Cr release plotted as a function of various light-exposures. *, Significantly greater than either dark or ambient, $P < 0.01$. (**B**) A549 cells treated with a range of menadione concentrations, with ^{51}Cr release plotted as a function of time of UV-light exposure after menadione treatment. *, Significantly greater than corresponding 2% DMSO control time point, $P < 0.01$. (**C**) A549 cells treated with 400 μM menadione (MQ), with ^{51}Cr release plotted as a function of menadione concentration for cells exposed to UV light in the presence of no filter (●), a long-pass glass filter transmitting above 305 nm (○), or a long-pass glass filter transmitting above 360 nm (▼). *, Significantly greater than corresponding menadione-treated cells exposed to UV light filtered below 360 nm, $P < 0.01$. Each point in (**A**–**C**) represents the mean ± S.E.M. of triplicate determinations from three independent experiments.

respiration. When cells were exposed to DMPO and 100 μM menadione in the dark, there was a modest but reproducible increase in the amount of DMPO/$^{\bullet}$OH spin adduct formed (Figure 4B). This signal was greatly reduced by the inclusion of SOD (Figure 4C), implying that the DMPO/$^{\bullet}$OH signal seen in these dark-treated cells was likely to be the result of $O_2^{\bullet-}$ formation by menadione, possibly via a cellular bio-reductive mechanism at or near the plasma membrane. However, when the cells were exposed to menadione in the presence of 340-nm light, the magnitude of the resulting DMPO/$^{\bullet}$OH signal was far greater (Figure 4D). Irradiation of these samples in the presence of SOD (Figure 4E) did not decrease the magnitude of the DMPO/$^{\bullet}$OH signal, implying that the light-dependent formation of DMPO/$^{\bullet}$OH proceeds via a mechanism that does not directly involve $O_2^{\bullet-}$ formation. This is consistent with our earlier results showing an inability of SOD to inhibit menadione-mediated DMPO/$^{\bullet}$OH formation under ambient light conditions. Thus, A549 cells treated with menadione and exposed to light demonstrate the formation of greater amounts of the DMPO/$^{\bullet}$OH adduct than did cells treated with menadione in the dark.

Role of $^1O_2$ in DMPO/$^{\bullet}$OH formation resulting from menadione photochemistry

The above data suggested that a majority of the DMPO/$^{\bullet}$OH signal seen in the presence of menadione is light-dependent rather than resulting from the cellular reduction of menadione to form $O_2^{\bullet-}$ or other ROS. Singlet oxygen ($^1O_2$) is a well-recognized product of many photochemical reactions [32] and has been shown previously to oxidize DMPO to DMPO/$^{\bullet}$OH [33–35].

To find out whether $^1O_2$ was involved in menadione- and light-dependent DMPO/$^{\bullet}$OH formation we first determined the ability of menadione to photosensitize $^1O_2$ formation using a highly sensitive steady-state infra red detection system [24]. Figure 5 shows the luminescence spectra observed during irradiation of menadione in acetonitrile (trace 1) and ^2H_2O (trace 2). The spectra show a maximum at 1270 nm that strongly suggests that they might be due to $^1O_2$ phosphorescence, consistent with $^1O_2$ generation. The quantum yield of $^1O_2$ generation depends strongly on the type of solvent used; for example, the yields determined in acetonitrile and ^2H_2O were 0.32 and 0.06 respectively. In methanol, only a trace of emission was seen. Generation of $^1O_2$ by menadione incorporated into SDS micelles in ^2H_2O or in β-cyclodextrin was below the limits of detection.

The inability of menadione to form significant amounts of $^1O_2$ in aqueous solution suggested that the photo-induced DMPO/$^{\bullet}$OH formation in the cell-free buffered systems described above is unlikely to occur via $^1O_2$ formation. This conclusion is further corroborated by the observation that when cell-free solutions containing menadione and DMPO were photo-irradiated in the presence of known $^1O_2$ scavengers (histidine and azide; 100 mM), the levels of DMPO/$^{\bullet}$OH formation were similar to those observed in the absence of these quenchers (results not shown), implying that menadione-photosenzitized $^1O_2$ formation was not responsible for production of this spin adduct by light-exposed menadione.

Role of photo-irradiation in menadione-associated increases in A549 $[Ca^{2+}]_i$

The above observations suggested that the menadione-mediated increases in A549 $[Ca^{2+}]_i$ levels observed with the fura 2 fluor-

escence assay might occur through cellular interactions with unique products resulting from the 340-nm photo-irradiation of menadione rather than through menadione metabolic reduction and redox cycling. To further examine the relationship between menadione-mediated ROS production, menadione photochemistry and increase of $[Ca^{2+}]_i$, we treated A549 cells with various agents known to either enhance or diminish ROS formation and determined their effect on the ability of menadione to modulate $[Ca^{2+}]_i$.

Previous studies have shown that A549 cells pretreated for 48 h with 100 μM BSO, an inhibitor of γ-glutamyl synthetase, demonstrate an 80 % decrease in GSH levels [14]. As expected, BSO-treated cells showed a significant increase in oxidant formation both before and following menadione exposure, as detected by dihydrofluorescein oxidation (Figure 2B, ○). However, this was not paralleled by a shift in basal $[Ca^{2+}]_i$ levels or in the dose–response curve of menadione-dependent increases in $[Ca^{2+}]_i$ (Figure 2A, ○). Similar findings were seen with cells pretreated with 5 mM 3-amino-1,2,4-triazole to inhibit catalase activity (results not shown).

Treatment of A549 cells with 10 mM NAC (a thiol reagent that increases GSH levels and scavenges many ROS) in the presence and absence of menadione resulted in the predicted decrease in oxidant formation, decreasing menadione-dependent dihydrofluorescein oxidation by 50 %. Paradoxically, 10 mM NAC treatment led to a 2-fold enhancement of the menadione-dependent rise in $[Ca^{2+}]_i$. These data strongly suggest that menadione-mediated production of oxidants such as $O_2^{\bullet -}$ and H_2O_2 was not responsible for menadione-dependent increases in $[Ca^{2+}]_i$.

Given that DMPO can interact with some reaction products resulting from menadione photo-irradiation to form the DMPO/$^{\bullet}$OH spin adduct, we performed experiments to assess the ability of DMPO to modulate menadione-mediated changes in $[Ca^{2+}]_i$ levels. If photochemical products of menadione were responsible for the rise in $[Ca^{2+}]_i$, and the same products could be trapped by DMPO, we hypothesized that the rise in $[Ca^{2+}]_i$ would be inhibited by the presence of DMPO. In contrast to the other antioxidant interventions, DMPO (2 mM) completely blocked the increase in $[Ca^{2+}]_i$ resulting from exposure of the cells to menadione (50 μM) and light. It is important to note that exogenous DMPO had no effect on the fluorescence of preformed fura 2–Ca^{2+} complexes (results not shown). Thus DMPO was the only compound found to react with the photo-irradiation products of menadione and was the only compound able to block the effect of menadione on $[Ca^{2+}]_i$.

Role of photo-irradiation in biological effects of menadione on A549 cells

The above studies implicated a photochemical product(s) of menadione as the mediator of increased $[Ca^{2+}]_i$. Because fura 2 requires an excitation wavelength of 340 nm, we could not directly assess the effect of photo-irradiation on menadione-modulated $[Ca^{2+}]_i$ using this probe. In an attempt to overcome this limitation, we tried two alternative approaches: (i) the Ca^{2+}-sensitive fluorescent probe, Calcium Orange, whose excitation and emission wavelengths ($\lambda_{exc} = 530$ nm; $\lambda_{em} = 575$ nm) should not contribute to photo-activation of menadione; and (ii) ^{45}Ca uptake. In both cases, calcium determinations were attempted in the presence and absence of menadione and in the presence and absence of 340-nm photo-irradiation. Unfortunately, changes in neither Calcium Orange fluorescence nor ^{45}Ca uptake proved sensitive enough to reliably detect $[Ca^{2+}]_i$ changes in A549 cells in response to either menadione or other agonists (ionomycin, thapsigargin) that were effective in our fura 2 studies (results not shown).

Given the limitations of the above Ca^{2+}-detection methodologies, we turned to alternative indices of cellular function that could be used to assess the potential biological implications of menadione photochemistry. Release of ^{51}Cr from previously labelled intracellular pools is a commonly employed method for assessing cellular injury [25]. Accordingly, specific ^{51}Cr release from A549 monolayers was determined over time in the presence of varying concentrations of menadione as a function of light exposure. As shown in Figure 6(A), specific ^{51}Cr release from A549 cells treated with 400 μM menadione was below 10 % under conditions of darkness or ambient light exposure; however, if the same menadione treatment was performed in the presence of UV light, the specific ^{51}Cr release was over 80 %. The UV-dependent specific ^{51}Cr release was both time- and menadione-concentration- dependent, as seen in Figure 6(B), with increasing specific ^{51}Cr release over a 3-h period at menadione concentrations from 25 to 400 μM. UV exposure by itself yielded essentially no specific ^{51}Cr release (Figure 6B, 2 % DMSO control). The impact of UV light exposure was not attributable to alterations in temperature of the monolayer resulting from UV exposure (results not shown). Through the use of long-pass glass filters, the critical wavelength required to observe menadione-associated specific ^{51}Cr release from A549 cells was determined to be between 360 and 305 nm (Figure 6C), in agreement with both the known absorption maximum for menadione (340 nm) and the DMPO/$^{\bullet}$OH formation studies (Figure 4).

DISCUSSION

Exposure of cells to menadione is commonly employed to study the effects of intracellular $O_2^{\bullet -}$ and H_2O_2 formation [4–9]. Using a fluorescence-based assay with the Ca^{2+}-sensitive probe fura 2, we found that exposure of the human alveolar type-II cell line A549 to menadione resulted in a rapid rise in $[Ca^{2+}]_i$, which was the result of an influx of extracellular Ca^{2+}. These results were considerably different from our recent data in which another redox-active compound, pyocyanine, increased $[Ca^{2+}]_i$ predominantly though the release of Ca^{2+} from intracellular stores.

Previously we found a close correlation between the pyocyanine concentrations needed to increase $[Ca^{2+}]_i$ and those needed to cause intracellular oxidant production as assessed by oxidant-sensitive fluorescent probes [14]. Furthermore, the effects of pyocyanine on Ca^{2+} metabolism in A549 cells were influenced by increasing or decreasing cellular antioxidant defences. This suggested that the pyocyanine effects on Ca^{2+} metabolism were mediated via intracellular production of $O_2^{\bullet -}$ and H_2O_2. Using the same fluorescent-probe methodology, we were surprised to find that the menadione concentrations required to produce detectable intracellular-oxidant production were much greater than those required to increase $[Ca^{2+}]_i$ in A549 cells. Furthermore, several treatments that modulated intracellular antioxidant levels in these cells had no impact on the ability of menadione to increase $[Ca^{2+}]_i$. These data raised the possibility that menadione might be working via a mechanism that is alternative to the one usually attributed to it, i.e. formation of $O_2^{\bullet -}$ and H_2O_2.

Consistent with this possibility, we found that exposure of menadione to light in the presence of the spin trap DMPO generated the DMPO/$^{\bullet}$OH spin adduct. Formation of this spin adduct was detectable at wavelengths up to 420 nm, with a maximal effect at 340 nm. The lack of effect of SOD and catalase

suggests that DMPO/$^{\bullet}$OH is not produced through the formation of either $O_2^{\bullet-}$ or $^{\bullet}$OH. Although we confirmed that formation of $^1O_2$ (light exposure of rose bengal) can lead to generation of DMPO/$^{\bullet}$OH, we found no evidence that this mechanism was responsible for production of this spin adduct by light-exposed menadione. As assessed by chemiluminescence methodology, we were unable to detect the production of $^1O_2$ under experimental conditions similar to those leading to generation of DMPO/$^{\bullet}$OH, and $^1O_2$ scavengers failed to inhibit formation of DMPO/$^{\bullet}$OH by menadione and light.

Due to its lipophilic nature, menadione could potentially concentrate in the lipid bilayer of the plasma membrane. $^1O_2$ was detectable with menadione photo-irradiation under aprotic conditions, raising the possibility that within a biological membrane this species might be generated: however, experiments in which menadione was photo-irradiated in the presence of SDS micelles appear to make this unlikely. The inability of menadione to photosensitize $^1O_2$ formation in a micellar/aqueous environment may be surprising considering that $^1O_2$ generation occurs *via* triplet quenching, and the yield of menadione triplet in water is high, 0.66 [17]. Menadione may not produce $^1O_2$ in an aqueous environment due to efficient quenching of the menadione triplet by SDS molecules [36,37]. Irradiation of menadione in SDS micelles generates SDS-derived radicals, which points at hydrogen abstraction as a possible mechanism of triplet quenching [36,37].

These data suggest that DMPO/$^{\bullet}$OH formation occurring via the light exposure of menadione is not the result of $^1O_2$ formation. While our work was under way, Monroe and Eaton similarly reported that photo-irradiation of menadione results in DMPO/$^{\bullet}$OH production [20]. They proposed a mechanism in which photo-irradiation leads to formation of the excited triplet state of menadione that reacts directly with DMPO to form DMPO/$^{\bullet}$OH [20].

Although earlier studies clearly demonstrated that photochemical products of menadione can be generated [16–20], their impact on biological functions has been examined to only a limited extent [35,38]. Based on: (i) the inability of classical oxidant scavengers to block the menadione-induced rise in cytosolic Ca^{2+}; (ii) the different dose–response curves for menadione-associated Ca^{2+} effects and intracellular oxidant production; and (iii) the coincidental fact that menadione-dependent detection of $[Ca^{2+}]_i$ using fura 2 requires illumination of the cell monolayer with 340-nm light (absorbance maximum of menadione), we hypothesized that photochemical products of menadione were responsible for the ability of the compound at low concentrations to induce an increase in A549 cell $[Ca^{2+}]_i$. Consistent with this hypothesis, only DMPO, which the spin-trapping data indicate reacts with menadione-photoactivated products (i.e. DMPO/$^{\bullet}$OH is generated), inhibits the ability of menadione to raise A549 $[Ca^{2+}]_i$. Unfortunately, due to their apparent lesser sensitivity, this hypothesis could not be confirmed using alternative techniques for monitoring $[Ca^{2+}]_i$.

Nevertheless, another means of assessing cellular cytotoxicity, specific release of ^{51}Cr from pre-labelled cytosolic pools, revealed a UV-light-dependent increase in cell injury that was also dependent on the time and concentration of menadione exposure. The relative lack of effect of ambient light on ^{51}Cr release (Figure 6A) compared with DMPO/$^{\bullet}$OH formation (Figures 3 and 4) in the presence of menadione is probably related to the higher sensitivity of the chemical system for detecting menadione-dependent reactions. These data provide definitive evidence that photochemical products of menadione can have profound effects on cellular function. The mechanism for this is not clear but it could be related to the ability of menadione to form a highly reactive triplet state following photo-irradiation. In this excited state, menadione is capable of abstracting hydrogen from SDS molecules [36,37] and oxidizing DNA bases [19,39].

In summary, our data confirm and extend the observations of others that photo-irradiation of menadione leads to products that directly or indirectly react with the spin trap DMPO to form the DMPO/$^{\bullet}$OH spin adduct. Thus detection of this spin adduct in an experimental system containing menadione does not necessarily indicate that $^{\bullet}$OH or $O_2^{\bullet-}$ has been spin trapped. It may, however, be of greater importance that menadione is among the redox-active compounds commonly used to study the effects of intracellular $O_2^{\bullet-}$ production in biological systems. Our data indicate that photochemical products of the reaction of light with menadione can have significant effects on cellular biochemistry. The exact chemical product responsible requires further delineation. Nevertheless, investigators employing menadione as a means to study oxidant stress must be cognizant of the photochemistry of menadione and realize that consequences of cellular exposure to menadione may not solely be the result of production of $O_2^{\bullet-}$ or products derived from it.

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