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MENADIONE-INDUCED DNA FRAGMENTATION WITHOUT 8-OXO-2′-DEOXYGUANOSINE FORMATION IN ISOLATED RAT HEPATOCYTES

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Abstract—Menadione (2-methyl-1,4-naphthoquinone) induces oxidative stress in cells causing perturbations in the cytoplasm as well as nicking of DNA. The mechanisms by which DNA damage occurs are still unclear, but a widely discussed issue is whether menadione-generated reactive oxygen species (ROS) directly damage DNA. In the present study, we measured the effect of menadione on formation of 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG), an index of oxidative DNA base modifications, and on DNA fragmentation. Isolated hepatocytes from phenobarbital-pretreated rats were exposed to menadione, 25–400 μM, for 15, 90 or 180 min with or without prior depletion of reduced glutathione (GSH) by diethyl maleate. Menadione caused profound GSH depletion and internucleosomal DNA fragmentation, which was demonstrated by a prominent fragmentation ladder on agarose gel electrophoresis. We found no oxidative modification of DNA in terms of increased 8-oxodG formation. In contrast, the positive control of sunlamp light increased 8-oxodG 5-fold in rat hepatocytes. We conclude that oxidative modification of DNA bases is unlikely to be important in menadione-induced DNA damage.

Key words: menadione; rat hepatocytes; oxidative DNA damage; 8-oxodeoxyguanosine; DNA fragmentation; glutathione

Reactive oxygen species (ROS§) are generated throughout the life of aerobic organisms and may damage cellular molecules and structures. ROS have been implicated as a pathogenetic factor in a great number of disease processes in which DNA is considered an important target, including ageing and carcinogenesis [1–5]. A well-known source of ROS involves redox cycling of foreign compounds, such as quinones, which are both widely distributed in nature and extensively used as therapeutic agents. Consequently, a prototype quinone, menadione (2-methyl-1,4-naphthoquinone), has been extensively used as a model compound for the study of cellular oxidative stress [6–12]. After one-electron transfer by the enzyme NADPH cytochrome P450 reductase, menadione is reoxidized by molecular oxygen generating large amounts of the superoxide anion (O_2^-). Dismutation of O_2^- generates H_2O_2 and in turn hydroxyl radicals ($OH^\cdot$), which are considered the most deleterious ROS.

Marked effects have been observed in cells exposed to menadione, including alterations in thiols [8, 9] and perturbation of intracellular calcium homeostasis [9, 10]. In addition, different forms of DNA damage such as fragmentation [13] and single strand breaks [11, 12, 14–16] have been reported. The suggested mechanisms of DNA damage include activation of a Ca^{2+}-dependent endonuclease [13], binding of semiquinone radicals to DNA [14], and direct oxidative modification to DNA bases and/or sugars [11, 12, 14]. Strand breaks may be caused by any of these mechanisms. ROS are also known to induce modifications of bases in DNA [17]. However, the relation of such modifications to menadione toxicity has not been investigated *in vitro* in parallel with DNA fragmentation. An extensively studied marker of oxidative DNA damage is the product from C-8 hydroxylation of guanine, 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG) [17–19]. The ratio of 8-oxodG to deoxyguanosine (dG) is a direct measure of ROS-mediated DNA base modification and, as such, could help to distinguish between some of the above-mentioned mechanisms.

In the present study, we investigated DNA damage, as measured by 8-oxodG formation and DNA fragmentation, in freshly isolated rat hepatocytes exposed to menadione.

MATERIALS AND METHODS

Chemicals

Agarose, BSA, standard calf thymus DNA, menadione, diethylmaleate (DEM), ethidium bromide, Hoechst dye B-33258, *N*-methylsarcosine, and Nuclease P_1 were purchased from Sigma (St.

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§ Abbreviations: ROS, reactive oxygen species; 8-oxodG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; dG, deoxyguanosine; O_2^-, superoxide anion; $OH^\cdot$, hydroxyl radical; DEM, diethylmaleate; GSH, reduced glutathione; GSSG, oxidized glutathione; AGE, agarose gel electrophoresis.

Louis, MO, U.S.A.). Collagenase was from Worthington (Freehold, NJ, U.S.A.), alkaline phosphatase and Proteinase K from Boehringer Mannheim (Mannheim, Germany), and monobromobimane from Calbiochem (La Jolla, CA, U.S.A.).

Experimental procedure

Isolation and exposure of hepatocytes to menadione. Male Sprague–Dawley rats, 200–300 g, were kept under constant temperature and humidity and fed stock pellets and water *ad lib*. For one week prior to hepatocyte isolation, phenobarbital (1 mg/mL) was included in the drinking water in order to induce the enzyme NADPH cytochrome P450 reductase. Hepatocytes were isolated using the collagenase method [20]. Cell viability exceeded 90% as determined by Trypan blue exclusion. In some experiments the hepatocytes were pretreated with DEM, 0.7 mM, for 30 min, to induce depletion of reduced glutathione (GSH). Hepatocytes were incubated in Krebs–Ringer buffer, pH 7.4, in 50 mL round-bottom flasks containing 4, 6.5 or 7.5 mL cell suspension (10^6 or 2×10^6 viable cells/mL) depending on the assay performed. Flasks were mounted on a rotary evaporator adaptor with humidified 95% O_2/5% CO_2 and rotated at 20 rpm through a 37° water bath [20]. Menadione was dissolved in DMSO, the final concentration of which was 0.25% (v/v) in all cell incubations including controls (without menadione).

In the first set of experiments for 8-oxodG determination, the cell suspension from one rat was divided and half the cells were pretreated with DEM. Each cell suspension was transferred to six round-bottom flasks (6.5 mL/flask, 2×10^6 cells/mL) and exposed to menadione concentrations of 0, 25, 50, 100, 200, and 400 μM. After 15, 90, or 180 min exposure, samples of 2 mL were taken from each flask for 8-oxodG determination. This procedure was repeated for a total of five rats on five separate days.

In the second set of experiments for 8-oxodG determination, the entire cell suspension from one rat was pretreated with DEM and transferred to 12 flasks (4 mL/flask, 2×10^6 cells/mL). Cells in six of the flasks were controls, i.e. not exposed to menadione, whereas cells in the remaining six flasks were exposed to 100 μM of menadione. The cells from three control flasks and from three flasks containing menadione-exposed cells were separately prepared for 8-oxodG determination after 15 or 90 min exposure. This procedure was repeated for a total of five rats on five separate days.

Light exposure. DEM-pretreated hepatocytes were resuspended (10^6 cells/mL) in PBS, pH 7.4, and 6 mL of this suspension was placed in a Petri dish (d = 8 cm) and exposed to light from a sunlamp 15 cm from the surface for 10 min (Höhensonne 1000 Impuls, without IR bulbs, emitting light from 240 to 580 nm with major peaks at 240–320, 365, 405, 435, 550 and 580 nm). This exposure was equivalent to 76 mW/cm^2 at the 15 cm distance.

Determination of intracellular GSH. Reduced glutathione was determined by the method described by Fahey and Newton [21] with modifications [22]. Briefly, 1 mL of hepatocyte suspension was placed on ice in pre-chilled tubes, centrifugated for 2 min at 1000 rpm, washed with PBS, and the cell pellet resuspended in 1 mL of distilled water. The cells were lysed by sonification and 100 μL of the lysate was derivatized with monobromobimane. From each sample, 10 μL was injected onto a reverse-phase C18 Beckmann Ultrasphere ODS 5 μ column, eluted by gradient elution, and GSH quantitated by fluorescent detection (415 nm excitation and 490 nm emission).

DNA isolation and digestion for subsequent 8-oxodG determination. Cells were centrifugated at 1000 rpm for 2 min and washed twice in PBS. In the first set of menadione experiments, DNA was isolated by using the simple salting out procedure described by Miller *et al.* [23]. In order to achieve higher purity in the second set of experiments, we isolated DNA from the cell pellet by means of chloroform/isoamyl alcohol extraction as described previously [24], with the sole modification that extraction in the present study was performed twice. The isolated and dried DNA was dissolved in 200 μL 20 mM sodium acetate (pH 4.8) and digested to nucleoside level with Nuclease P_1 and alkaline phosphatase [24]. Liver homogenate from phenobarbital-treated rats was handled the same way as the centrifugation pellet from isolated hepatocytes.

Determination of 8-oxodG and dG. HPLC with electrochemical detection was followed as originally described by Floyd *et al.* [25] with modifications as detailed elsewhere [26]. Briefly, 50 μL of the nucleoside mixture was injected onto a reverse-phase C18 Nucleosil 5 μ column, and the dG and 8-oxodG were measured by means of ultraviolet absorption and electrochemical detection (Coulochem II, ESA, Inc., Bedford, MA, U.S.A.), respectively. Quantitation of 8-oxodG was performed by injection of known amounts of 8-oxodG standard.

Determination of DNA fragmentation by agarose gel electrophoresis (AGE). Samples were centrifuged (2 min at 400 rpm) and the cell pellet resuspended in incubation medium supplemented with 5% DMSO, 20% fetal calf serum and 20 mM EDTA. Cells were slowly frozen in isopropanol/dry ice, and stored at −70° until analysis. Thawed cell suspension was treated with sarkosyl lysis buffer (0.5% *N*-methylsarcosine, 0.1 M Tris–HCl, 5 mM EDTA, pH 8) for 20 min at 0–4°. Proteinase K (100 μg/mL) was added and the suspension incubated at 48° for 6 hr, followed by digestion with RNase A (1 μg/mL, 30 min, 37°). DNA was extracted 3× with ethanol and sodium acetate [27]. DNA samples of 8 μg (quantitated fluorometrically using Hoechst dye B-33258 and standard calf thymus DNA) were resolved on a 1.6% agarose gel containing ethidium bromide (0.4 μg/mL) using a Bio-Rad electrophoresis apparatus (Richmond, CA, U.S.A.). A *Hin*dIII digest of lambda DNA served as molecular size standards.

Statistical analysis. Data were analysed with Student's *t*-test and one-way analysis of variance with individual groups compared by the method of least significant difference (LSD). P values less than 0.05 were considered statistically significant.

RESULTS

As an indicator of oxidative stress, we measured

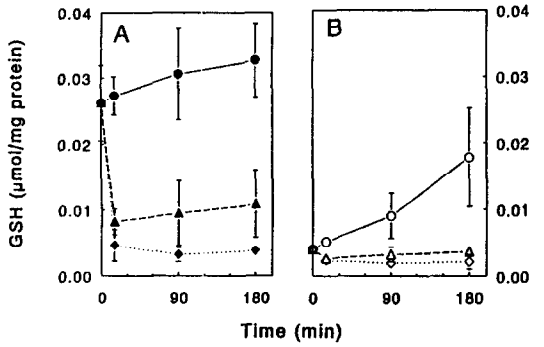

Fig. 1. Intracellular GSH concentration was measured in control rat hepatocytes (●, ○) and in hepatocytes exposed to 100 μM (▲, △) or 200 μM (◆, ◇) menadione. (A): cells received no pretreatment; (B) cells were pretreated with DEM. Values are given as mean ± SD of three separate experiments.

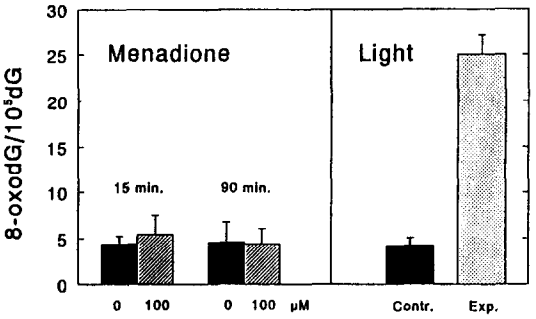

Fig. 2. Intracellular 8-oxodG formation in control rat hepatocytes (■) and hepatocytes exposed to menadione (▨) or light from a sunlamp (▩). Light-exposed cells are significantly different from control ($P < 0.05$) whereas menadione-exposed cells are not. Values are given as mean ± SD of five experiments performed in triplicate or of two experiments performed in duplicate, as described in Materials and Methods.

Table 1. Formation of 8-oxodG in isolated rat hepatocytes exposed to menadione as described in Materials and Methods

	8-oxodG/10^5 dG					
	Menadione concentration					
	0 μM	25 μM	50 μM	100 μM	200 μM	400 μM
Without DEM						
15 min	4.3 ± 1.0	3.9 ± 1.2	4.2 ± 1.3	6.0 ± 2.0	6.0 ± 1.6	3.7 ± 1.1
90 min	4.5 ± 2.1	5.5 ± 1.1	7.8 ± 0.7	5.7 ± 2.7	5.0 ± 3.0	4.7 ± 3.2
180 min	6.0 ± 0.4	5.6 ± 1.5	5.4 ± 1.8	5.1 ± 1.7	7.4 ± 3.8	7.0 ± 3.0
With DEM						
15 min	4.3 ± 2.5	5.3 ± 3.3	6.5 ± 4.0	5.7 ± 4.8	5.1 ± 3.2	4.9 ± 2.4
90 min	4.5 ± 1.8	6.4 ± 5.7	5.7 ± 1.8	7.5 ± 2.4	6.5 ± 4.5	4.8 ± 3.6
180 min	6.9 ± 5.6	4.2 ± 1.2	3.5 ± 0.8	6.9 ± 0.9	5.0 ± 1.5	4.1 ± 1.7

Values are given as mean ± SD of five separate experiments. No values are significantly different from corresponding controls.

intracellular GSH which, as expected, was considerably decreased in the menadione-exposed hepatocytes compared to controls. Following 15 min exposure of cells to 100 or 200 μM menadione, GSH decreased to about 30% and 15% of the amount in controls, respectively ($P < 0.05$; Fig. 1a). No further decrease was observed over 3 hr of exposure. In cells pretreated with DEM, GSH was reduced to about 15% of values in untreated cells and increased continuously over 3 hr to a level of about 50% of untreated cells (Fig. 1b). When DEM-pretreated cells were exposed to menadione, GSH concentration was further decreased only very slightly and failed to show any recovery over the 3 hr period.

In the first set of experiments on 8-oxodG formation, menadione exposure (25–400 μM) for 15 to 180 min, with or without prior GSH depletion, did not induce statistically significant changes in 8-oxodG formation (Table 1). On the other hand, we could not entirely exclude an increased 8-oxodG formation in cells pretreated with DEM and exposed to 100 μM menadione for 15 or 90 min. A second set of experiments focusing on these conditions demonstrated that menadione had no such effect (Fig. 2). For a positive control, we used a sunlamp as an alternative ROS generator to induce 8-oxodG formation. In DEM-pretreated rat hepatocytes exposed to light from this sunlamp for 10 min (≈45 J/cm^2), we found a 5-fold increase in 8-oxodG in cellular DNA ($P < 0.05$; Fig. 2). The 8-oxodG level in unexposed liver homogenate from phenobarbital-treated rats was 4.8 ± 1.9 8-oxodG/10^5 dG ($N = 2$).

In contrast to the lack of menadione-induced 8-oxodG formation, menadione in both low (25 μM) and high concentrations (200 μM) caused significant internucleosomal DNA fragmentation, as shown by a prominent fragmentation ladder on AGE analysis (Fig. 3). DNA fragmentation present after 90 min of menadione exposure was apparently unaffected by DEM pretreatment of hepatocytes.

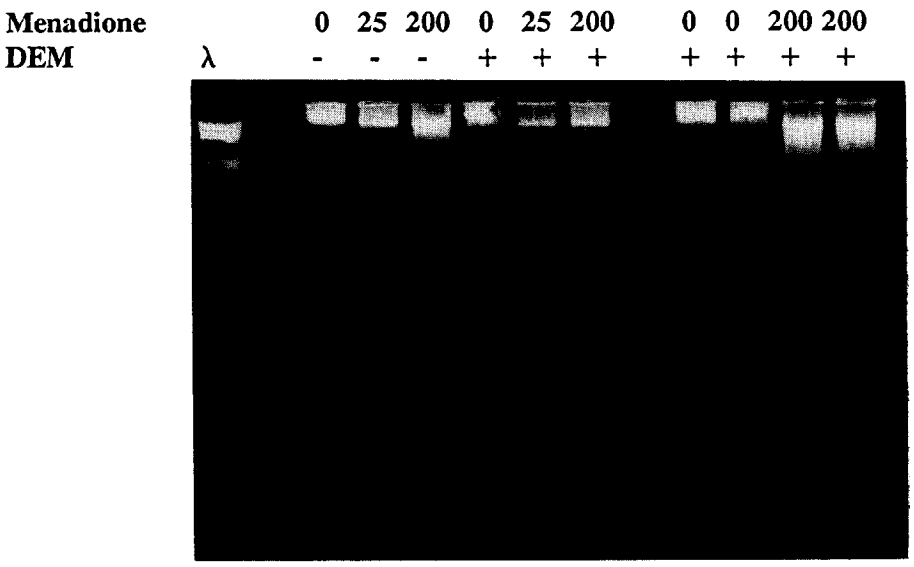

Fig. 3. Electrophoretogram demonstrating ladder pattern of DNA fragmentation in rat hepatocytes exposed to 25 μM or 200 μM menadione for 90 min, with or without DEM pretreatment.

DISCUSSION

In the present study, oxidative DNA modification measured as increased 8-oxodG content was not found in freshly isolated rat hepatocytes exposed to menadione over a range of concentrations and experimental conditions. In contrast, we found DNA fragmentation and GSH depletion consistent with oxidative stress.

Several studies have demonstrated various effects of menadione on isolated rat hepatocytes, but to our knowledge there are no previous reports examining 8-oxodG formation in this system. An *in vivo* study by Denda *et al.* [28] reports 8-oxodG formation in livers from rats exposed to redox enzyme modulators including menadione. These investigators found that the hepatic 8-oxodG/dG ratio in rats was not affected by exposure to menadione alone. On the other hand, when menadione treatment was combined with exposure to phenobarbital, phorone (depleting GSH), and/or dicumarol (inhibition of the two-electron transferring enzyme DT-diaphorase), the ratio of 8-oxodG to dG increased to 40–75% above controls. However, the combination of phenobarbital + phorone + dicumarol without menadione increased 8-oxodG similarly, showing that the indicated oxidative DNA modification was not specific to menadione redox cycling.

Damage to DNA, including 8-oxodG formation, has been studied by Winyard *et al.* [29] in isolated rat and human hepatocytes exposed to bleomycin. This drug forms a complex with transition metals such as Fe(II), which results in increased 8-oxodG content of isolated calf thymus DNA. Like menadione, the bleomycin–Fe(II) complex possibly generates $OH^{\cdot}$ via a one-electron reduction. However, in isolated hepatocytes, bleomycin did not induce 8-oxodG formation whereas unscheduled DNA synthesis (UDS), a measure of DNA repair, was increased [29]. These published results, together with the present findings, show that two probable ROS generators, menadione and bleomycin, cause strand breaks but no 8-oxodG formation in intact cells. In contrast, many other sources of intra- or extracellular oxidative stress have been demonstrated to induce 8-oxodG formation in different cell types. Examples include H_2O_2 in P388 D1 cells [30], tobacco smoke in human lung cells [31], the carcinogen 4-nitroquinoline 1-oxide in Ehrlich ascites cells [32], X-rays in HeLa cells [33], UV light in V79 cells [24] and in the present rat hepatocytes, and activation of polymorphonuclear granulocytes [34]. The UV exposure needed for 8-oxodG formation was higher in hepatocytes than in V79 cells, possibly due to better protective mechanisms in the former, including higher pigment levels.

In the present study, isolation of rat hepatocytes and exposure to 95% O_2 could be suspected of increasing the level of oxidative damage, including 8-oxodG formation. However, the basal level of 8-oxodG in the isolated control cells was equivalent to that in fresh liver homogenate from phenobarbital-treated rats. Phenobarbital treatment alone does not seem to influence 8-oxodG levels in rat liver [28].

In menadione-exposed hepatocytes, $OH^{\cdot}$ has most often been suggested as the ultimate DNA damaging agent introducing strand breaks. In support of this hypothesis, various types of DNA damage have been diminished by specific radical scavengers and iron-chelating agents that prevent $OH^{\cdot}$ formation [11, 12, 15]. However, these interventions would curtail both direct oxidative attack on DNA and secondary ROS-related effects on DNA. Indeed, the lack of oxidative DNA base modifications observed in menadione-treated hepatocytes suggests that any oxidative stress reflected by GSH depletion

was restricted from the nucleus. Possibly, oxidative stress confined to the cytoplasm could trigger secondary events leading to DNA fragmentation.

Internucleosomal DNA fragmentation, supposedly caused by activation of Ca^{2+}-dependent endonucleases, is a common but non-specific characteristic of apoptosis [35]. This form of cell death has been described as a physiological process that occurs during embryonic development, later in a variety of normal adult human tissues with high cell turnover, and in clonal deletion of auto-reactive thymocytes [36]. In addition, DNA damage has been correlated with toxic cell killing. For example, Ray *et al.* [27] report that early Ca^{2+}-dependent DNA fragmentation in livers of mice treated with acetaminophen correlates with toxin-induced necrosis. In keeping with the present study, McConkey *et al.* [13] found DNA fragmentation after exposure to menadione concentrations of 50 and 100 μM, but not 25 and 200 μM. The latter were concentrations we used to demonstrate DNA fragmentation. The experimental designs of the two studies are comparable, except that we used phenobarbital to induce NADPH cytochrome P450 reductase. Apart from inducing this enzyme and perhaps favouring redox cycling and oxidative stress, phenobarbital has also been reported to inhibit apoptosis [37]. The net effect of these mechanisms is uncertain. However, the results presented show that internucleosomal DNA fragmentation was induced by both low and high concentrations of menadione.

As expected, intracellular GSH was depleted, an observation indicative of cellular oxidative stress and consistent with previous reports [8, 9]. Although GSH depletion may also reflect some non-oxidative reactions with menadione, including adduct generation [7, 8], studies have revealed that the formation of oxidized glutathione (GSSG) accounts for the major decrease in GSH [8]. DEM pretreatment caused GSH to decline to a low level, which was not significantly changed by subsequent menadione exposure. Because similar GSH depletion was obtained within 15 min of 100 or 200 μM menadione exposure alone, DEM would appear to decrease resistance to menadione toxicity at the very start of the exposure period.

In conclusion, we have demonstrated that exposure of rat hepatocytes to menadione caused extensive internucleosomal DNA fragmentation but no significant 8-oxodG formation. Accordingly, oxidative modification of DNA bases is unlikely to be important in menadione-induced DNA damage.

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