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Cellular Thiols as a Determinant of Responsiveness to Menadione in Cardiomyocytes

Woan-Fang Tzeng*, Tzeon-Jye Chiou†, Chin-Peng Wang*, Jia-Luen Lee* and Yee-Hsiung Chen‡

*Department of Biology, Fu Jen University, Taipei, R.O.C., †Section of Medical Oncology, Department of Medicine, Veterans General Hospital-Taipei, Taiwan, R.O.C. and ‡Institute of Biochemical Sciences, College of Sciences, National Taiwan University, and Institute of Biological Chemistry, Academia Sinica, Taipei, R.O.C.

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W.-F. TZENG, T.-J. CHIOU, C.-P. WANG, J.-L. LEE AND Y.-H. CHEN. Cellular Thiols as a Determinant of Responsiveness to Menadione in Cardiomyocytes. *Journal of Molecular and Cellular Cardiology* (1994) **26**, 889–897. The role of intracellular thiols in menadione-mediated toxicity was studied in neonatal rat cardiomyocytes. The sensitivity of cardiomyocytes to menadione was greater than that of skeletal muscle cells and 3T3 fibroblasts. Before cell degeneration, menadione induced marked depletion of intracellular thiols and an increase of oxidized glutathione. The sensitivity of these cells to menadione correlated with the level of depletion of intracellular thiols. After incubation of cardiomyocytes with menadione, glutathione reductase activity was inhibited and lipid peroxidation was increased. Both dicumarol (an inhibitor of DT-diaphorase) and diethyldithiocarbamate (an inhibitor of superoxide dismutase) enhanced the capacity of menadione to induce cellular damage and to cause depletion of intracellular glutathione. Decreasing intracellular glutathione by pretreatment of cells with N-ethylmaleimide or buthionine sulphoximine also increased menadione-induced cell degeneration. Preincubation with cysteine or dithiothreitol suppressed the capacity of menadione to damage the cells. Menadione-induced lipid peroxidation was also suppressed by the same treatment. These results show that the oxidative stress induced by menadione in cardiomyocytes results in the depletion of glutathione and protein thiols. Both DT-diaphorase and superoxide dismutase can protect cells from the toxicity of menadione. Cellular thiols are determinants of the responsiveness to menadione.

KEY WORDS: Cardiomyocytes; Glutathione; Menadione.

Introduction

Menadione (2-methyl-1,4-naphthoquinone, vitamin K_3) is a synthetic derivative of phylloquinone (vitamin K_1). It has a quinone structure and has been reported to inhibit the growth of tumour cells *in vitro* (Prasad *et al.*, 1981). Quinones may undergo either one- or two-electron reduction. The one-electron reduction of a quinone results in the formation of a semiquinone radical. In the presence of O_2, this radical can be re-oxidized to the parent quinone with the concomitant formation of superoxide anion $O_2^{\cdot-}$. Cellular superoxide dismutase can dismutate $O_2^{\cdot-}$ to O_2 and H_2O_2. Hydrogen peroxide can be further metabolized to H_2O by the glutathione peroxidase system at the expense of glutathione (GSH). Quinones can be reduced through two-electron reduction to the corresponding hydroquinones by DT-diaphorase [NAD(P)H: (quinone acceptor) oxidoreductase] without the formation of semiquinone free radical imtermediates. Dicumarol, an inhibitor of DT diaphorase, prevents the two-electron reduction of certain quinones and makes more quinones available for single-electron reduc-

Please address all correspondence to: Dr Woan-Fang Tzeng, Department of Biology, Fu Jen University, Hsinchuang, Taipei, Taiwan, ROC.

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tion, which can produce reactive oxygen species (Jewell *et al.*, 1982; Di Monte *et al.*, 1984b).

Glutathione is an intracellular sulphydryl-containing tripeptide that is known to detoxify free radicals and the damage that they produce. Most studies of the oxidative stress produced by menadione were made on hepatocytes. Relatively less is known about the oxidative stress of menadione in cardiomyocytes. In the present study, we have evaluated the effect of GSH-modulation on the toxicity of menadione in a neonatal rat cardiomyocyte culture system and we have compared the sensitivity of cardiomyocytes to that of skeletal muscle cells and 3T3 fibroblasts. We found that menadione is toxic to cardiomyocytes: cellular thiols were depleted, glutathione reductase activity was impaired and oxidized glutathione (GSSG) and lipid peroxidation were increased before cell degeneration developed. The protective role of both DT-diaphorase and superoxide dismutase in preventing menadione toxicity was demonstrated. GSH depletion enhanced the sensitivity of cardiomyocytes to menadione. The level of depletion of cellular thiols in cardiomyocytes, skeletal muscle cells and 3T3 fibroblasts is closely related to their sensitivity to menadione.

Materials and Methods

Materials

Neonatal Wistar rats were obtained from the animal centre of National Taiwan University. NIH 3T3 fibroblasts were obtained from the cell bank of Taipei Veterans General Hospital. GSH, GSSG, glutathione reductase, menadione sodium bisulphate, 2-thiobarbituric acid, NADPH, dicumarol, diethyldithiocarbamate, DL-buthionine-S,R-sulphoximine (BSO), 5-5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and N-ethylmaleimide (NEM) were obtained from Sigma. F10, DMEM, horse serum and fetal bovine serum were obtained from Gibco. Nucleotide releasing reagent and luciferase were obtained from Lumac company. All other chemicals were of reagent grade.

Culture of cells

Cardiomyocytes of neonatal Wistar rats (0–3 days) were prepared according to the method of Tzeng and Chen (1988). Cells were cultured in a medium composed of 80% (v/v) F10/10% (v/v) horse serum/10% (v/v) fetal bovine serum/14 mM $NaHCO_3$/20 mM Hepes, pH 7.4. For the various experiments the cells were plated at different densities. The cells were plated at a density of 10^5 cells per Petri dish of 35 mm × 10 mm, or at a density of 10^6 cells per Petri dish of 60 mm × 15 mm. At the third day of culture, 90% of the cells were cardiomyocytes. Skeletal muscle cells of the hind limbs were isolated using the same method described for cardiomyocytes. 3T3 fibroblasts were cultured in a medium composed of 90% (v/v) DMEM/10% (v/v) fetal bovine serum, pH 7.4. Throughout the study, F10 medium without serum was used as the bathing medium. The incubation of cells with menadione sodium bisulphate or other agents was at 37°C unless stated otherwise.

Observations of cells on morphology and contraction

The morphology and contraction of cells were observed with a phase-contrast microscope equipped with a temperature controlled stage. The number of contractions of cardiomyocytes was usually counted for 30 s. Cell degeneration was indicated by distortion of cell morphology and was confirmed using the Trypan Blue-exclusion test (Glick *et al.*, 1974; Tzeng *et al.*, 1992).

Measurement of ATP and lactate dehydrogenase

Cellular ATP was determined after the cells were disrupted with a nucleotide releasing reagent, through the transformation of luciferin to decarboxyluciferin by luciferase. Luminescence was measured in a Lumac biocounter M2010. Lactate dehydrogenase (LDH) activity was measured according to the method of Lejohn and Stevenson (1975).

Determinations of glutathione and protein thiols

Cells in Petri dishes (1×10^6 each) were rinsed three times with a bathing medium, scraped from the dish and then lysed with ice-cold 6.5% trichloroacetic acid (TCA). The samples were centrifuged at 13 500 × *g* for 10 min. The supernatant fractions were analysed for GSH and GSSG by the method of Griffith (1980). The pellet was washed twice with ice cold 6.5% TCA and suspended in 0.5 M Tris-HCl containing 100 μM DTNB, pH 8.3, and the protein thiols content were determined according to the method of Sedlak and Lindsay (1968). The concentration of GSH and GSSG were

expressed as nmol/10^6 cells and the data of protein thiol was expressed as nmol GSH equivalents/10^6 cells, calculated on the basis of a GSH calibration curve.

Glutathione reductase measurement

Glutathione reductase activity was determined by monitoring the oxidation of NADPH at 340 nm using the method of Worthington and Rosemeyer (1974). The assay mixture contained 0.2 M KCl, 1 mM EDTA, 1 mM GSSG, 0.1 M potassium phosphate buffer, pH 7.0 and 1×10^6 cells lysate. The reaction was initiated by the addition of NADPH to a final concentration of 0.1 mM.

Lipid peroxidation assay

Lipid peroxidation was analysed using a modification of the method of Buege and Aust (1978). Malondialdehyde, formed by the breakdown of polyunsaturated fatty acids, served as an index for determining the extent of lipid peroxidation. Malondialdehyde reacted with thiobarbituric acid to give a red colour absorbing at 535 nm. Cells were rinsed three times with a bathing medium, scraped from the dish and then lysed with 6.5% TCA. Reagents containing 15% TCA, 0.375% thiobarbituric acid, and 0.25 N hydrochloric acid were added. Mixed thoroughly, the solution was then heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 $\times g$ for 10 min. The absorbance of the supernatant was determined at 535 nm against a blank that contained all the reagents except the cells.

Statistical analysis

Data were expressed as mean ± S.E. and analysed according to Student's *t*-test. Results were considered to have reached statistical significance when the *P* value was < 0.05.

Results

Relationship between intracellular thiols and sensitivity to menadione

In a previous study (Tzeng *et al.*, 1992) we have presented the dose response curve of the toxicity of menadione in cardiomyocytes. There is a period during which cell morphology was not distorted but contraction of cardiomyocytes was slowed or stopped. Fura-2 in the bath medium was unable to enter the cells, as determined by fluorescence microscopy. Neither LDH nor ATP was found in the bath medium. Apparently, the cells remained intact; note that LDH should not leak from and fura-2 should not penetrate into intact cells (Glick *et al.*, 1974; Higgins *et al.*, 1980). Increasing the menadione dosage shortened this period and accelerated cell degeneration. No cell degeneration occurred within 60 or 90 min at a dose of 80 or 40 μM menadione, respectively. For convenience, we use a dose of 80 μM menadione to investigate the relationship between the toxicity of this agent and the depletion of cellular thiols. We measured GSH and protein thiols after the addition of 80 μM menadione to cells. As shown in Figure 1, cardiomyocytes were more sensitive to menadione than skeletal muscle cells and 3T3 fibroblasts. Within 60 min, cardiomyocytes stopped beating and a marked decrease of intracellular GSH level was observed. Concomitant with GSH oxidation, GSSG was formed (Fig. 2). Since GSH is believed to be important for the maintenance of intracellular protein thiols (Reed, 1986), the status of cellular protein

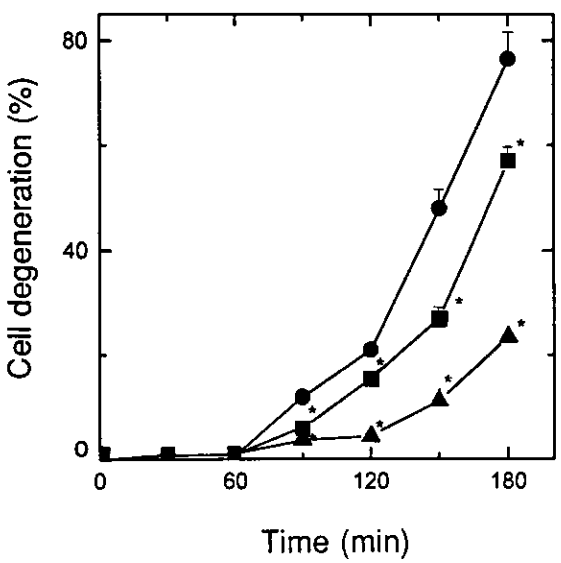

Figure 1 Sensitivity of a variety of cells to menadione. Cells were incubated with 80 μM menadione at 37°C. Cell degeneration was indicated by distortion of cell morphology and confirmed by the Trypan Blue-exclusion test. Symbols: ●, cardiomyocyte; ■, skeletal muscle cell; ▲, 3T3 fibroblast. Each point represents the mean ± S.E. of six separate determinations. Points without a vertical bar indicate that the standard error is within the size of the symbol. *Sensitivity is significantly different from that of the cardiomyocyte.

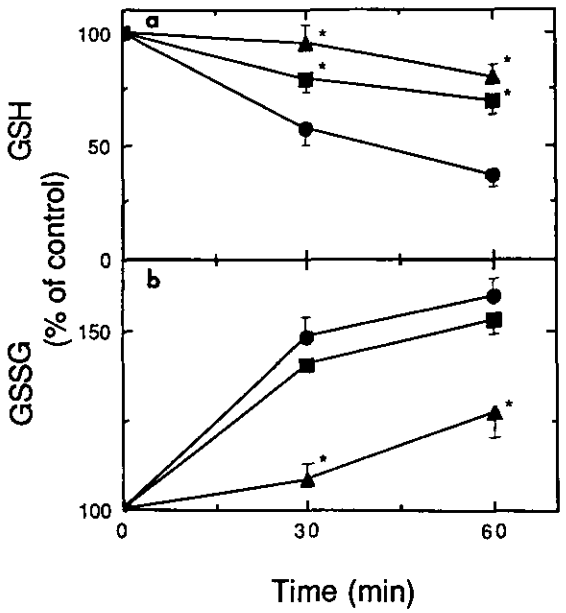

Figure 2 Depletion of GSH and increase of GSSG during treatment with menadione. Cells were incubated with 80 μM menadione at 37°C. The concentrations of GSH and GSSG are expressed as the percentage of the corresponding values obtained in control (untreated) cells. Symbols: ●, cardiomyocyte; ■, skeletal muscle cell; ▲, 3T3 fibroblast. Each point represents the mean ± S.E. of four separate determinations. Points without a vertical bar indicate that the standard error is within the size of the symbol. *Concentration is significantly different from that in the cardiomyocyte ($P < 0.05$).

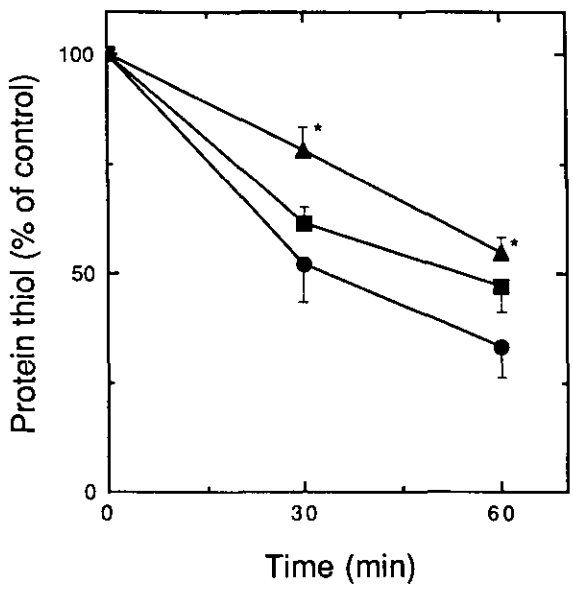

Figure 3 Depletion of protein thiols during treatment with menadione. Cells were incubated with 80 μM menadione at 37°C. The concentrations of protein thiols are expressed as the percentage of the corresponding values obtained in control (untreated) cells. Symbols: ●, cardiomyocyte; ■, skeletal muscle cell, ▲, 3T3 fibroblast. Each point represents the mean ± S.E. of three separate determinations. Points without a vertical bar indicate that the standard error is within the size of the symbol. *Concentration is significantly different from that in cardiomyocyte ($P < 0.05$).

thiols was examined. Protein thiol levels in menadione-treated cells were also reduced within 60 min (Fig. 3), by which time the protective effect of GSH had been virtually eliminated. After 60 min of incubation with 80 μM menadione, LDH and ATP were released from the cells into the bath medium. The extent of the release of LDH and ATP correlated with the percentage of cell degeneration observed by the distortion of cell morphology or by the Trypan-Blue exclusion test. All of the cardiomyocytes, skeletal muscle cells and 3T3 fibroblasts had similar response patterns. A comparison between cell sensitivity and thiols depletion showed a relationship between depletion of thiols and responsiveness to menadione. The greater resistance of skeletal muscle cells and 3T3 cells to menadione treatment, compared to that of cardiomyocytes, is not due to a difference in the basal level of intracellular GSH or protein thiols. The basal level of intracellular GSH, expressed as nmol/10^6 cells, was 12.2 for cardiomyocytes, 10.9 for skeletal muscle cells, and 7.3 for 3T3 fibroblasts. The basal level of GSSG, expressed as nmol/10^6 cells, was 0.55 for cardiomyocytes, 0.59 for skeletal muscle cells and 1.01 for 3T3 fibroblasts. The basal level of protein thiols, expressed as GSH equivalents/10^6 cells, was 26.2 for cardiomyocytes, 24.9 for skeletal muscle cells, and 20.4 for 3T3 fibroblasts. After incubation of cardiomyocytes with menadione, glutathione reductase activity was inhibited (Fig. 4), impairing the regeneration of GSH from GSSG.

Effect of dicumarol and diethyldithiocarbamate on menadione's toxicity

Diethyldithiocarbamate is an inhibitor of superoxide dismutase. Dicumarol is an inhibitor of DT-diaphorase. Cardiomyocytes did not degenerate when they were incubated in 25 mM diethyldithiocarbamate or 30 μM dicumarol for 3 h. The time courses of degeneration induced by 80 μM menadione in cells pretreated with dicumarol and diethyldithiocarbamate and in a control sample are compared in Fig. 5(a). After 2 h of incubation, the extent of cell degeneration was 25.1 ± 2.2% for the

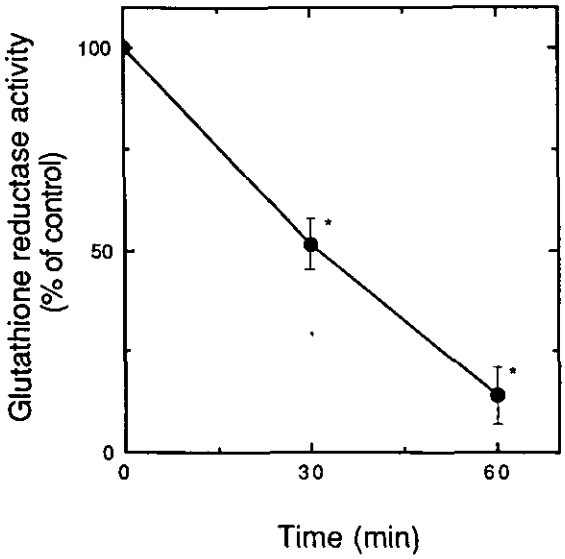

Figure 4 Inhibition of glutathione reductase activity during treatment with menadione. Cardiomyocytes were incubated with 80 μM menadione at 37°C. Each point represents the mean ± S.E. of three separate determinations, *, indicates that the activity is significantly different from that in control (untreated) cells ($P < 0.05$). The activity of glutathione reductase in control (untreated) cardiomyocytes was 24.1 nmoles of NADPH oxidized/min/10^6 cells.

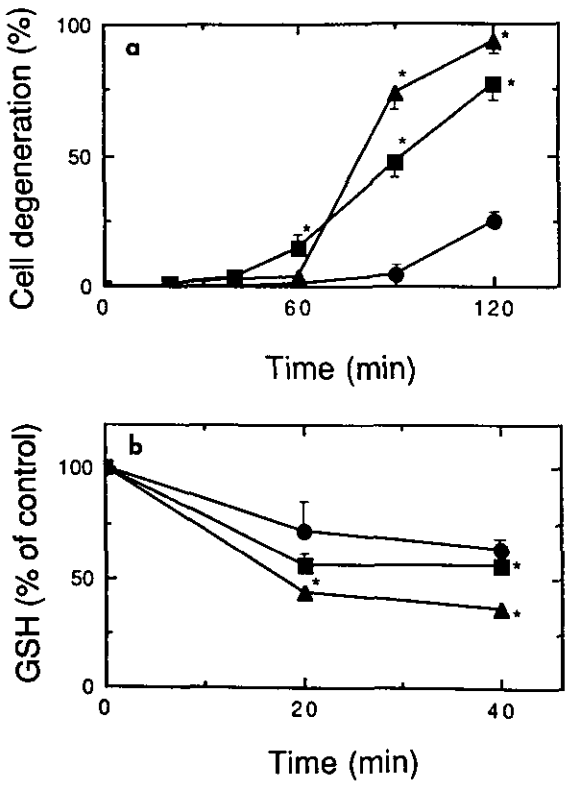

Figure 5 Effects of diethyldithiocarbamate and dicumarol on menadione-induced cell degeneration and depletion of GSH in cardiomyocytes. Cardiomyocytes were incubated with the agents at 37°C. Symbols: ●, cells treated with 80 μM menadione; ▲, cells treated with 80 μM menadione and 25 μM diethyldithiocarbamate; ■, cells treated with 80 μM menadione and 30 μM dicumarol. Each point represents the mean ± S.E. of nine separate determinations. *, indicates that the sensitivity to menadione or the concentration of GSH is significantly different from that in cells treated with menadione alone ($P < 0.05$).

control cells, 93.2 ± 3.0% for the diethyldithiocarbamate-treated cells and 77.3 ± 5.2% for the dicumarol-treated cells. Since inhibition of superoxide dismutase or DT-diaphorase could enhance the menadione-induced degeneration of cells, these data reveal that superoxide anions were produced during exposure to menadione and that menadione can undergo both one-electron and two-electron reduction within cardiomyocytes. As Fig. 5(b) shows, we found that cellular GSH was more seriously depleted in cells treated with diethyldithiocarbamate and menadione or dicumarol and menadione than in those treated with menadione alone. After 40 min of incubation, GSH was 63.0 ± 3.7% for the control cells, 55.7 ± 2.8% for the dicumarol-treated cells and 35.7 ± 2.1% for the diethyldithiocarbamate-treated cells. The depletion of GSH correlated with the extent of cell degeneration. More O_2^- and H_2O_2 may be produced in cells cotreated with dicumarol and menadione. The depleted GSH may be consumed to remove H_2O_2 by glutathione peroxidase. In the presence of diethyldithiocarbamate, superoxide radicals can decompose to yield hydroxyl radicals. These are known to induce lipid peroxidation. Much more GSH than usual were consumed to protect cells against lipid peroxidation. Because of this the level of GSH decreased after this treatment.

Menadione toxicity after cellular glutathione depletion

To investigate further the role of GSH in menadione-induced toxicity, cardiomyocytes were pretreated with NEM or BSO to lower the level of GSH. NEM forms a complex with free GSH and thus depletes the available GSH. Treatment of cells with 100 μM NEM at 37°C for 4 h did not affect the viability of the cells, but cellular GSH was undetectable after 10 min of this treatment. BSO is an irreversible inhibitor of gamma glutamylcysteine

synthetase. Treatment of cells with BSO resulted in a gradual decrease in GSH. At a concentration of 200 μM, BSO did not have any influence on the viability of cardiomyocytes over the 24 h period. After 6 h of incubation, the GSH level decreased to 65.1 ± 4.2% of the control value. The time courses of degeneration induced by 80 μM menadione of NEM or BSO-pretreated cells and control cells are shown in Fig. 6. NEM or BSO-pretreated cells were more sensitive to menadione. When cells were coincubated with menadione and either 2 mM dithiothreitol or 2 mM cysteine, the menadione-induced cell degeneration was clearly suppressed.

Lipid peroxidation after treatment with menadione

The expected loss of antioxidant protection due to GSH depletion by menadione is reflected in the formation of thiobarbituric acid-reactive substances, a measurement of lipid peroxidation. Figure 7 shows that lipid peroxidation was increased when cardiomyocytes were treated with 80 μM menadione. When cells were coincubated with menadione and either 2 mM dithiothreitol or 2 mM cysteine, the menadione-induced lipid peroxidation was suppressed.

Discussion

The importance of oxidative stress in the cytotoxicity of menadione has been demonstrated in rat hepatocytes and human platelets (Di Monte *et al.*, 1984a, b). One- and two-electron reduction of menadione, resulting in the formation of $O_2^{\cdot-}$ and H_2O_2, are responsible for the loss of GSH. Thus, cellular GSH and the activities of GSH-metabolizing enzymes play important roles in the ultimate efficiency of several chemotherapeutic agents (Townsend and Cowan, 1989; Russo and Mitchell, 1985).

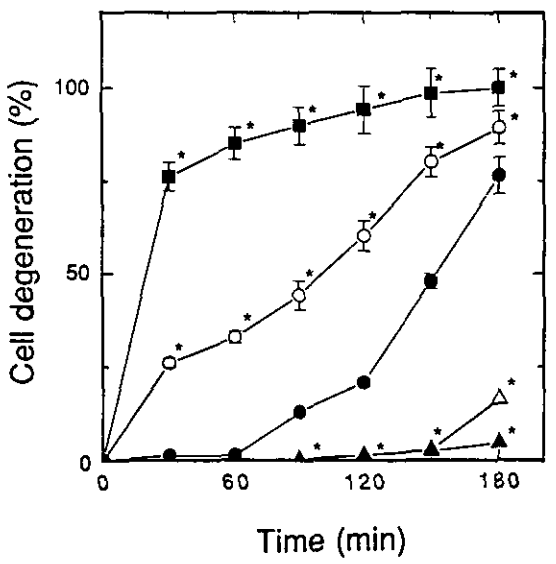

Figure 6 Effects of several agents on cardiomyocyte degeneration induced by menadione. Cardiomyocytes were treated with 80 μM menadione alone or pretreated with agents and then treated with 80 μM menadione and agents at 37°C. Symbols: ●, 80 μM menadione; ■, cells pretreated with 100 μM NEM for 10 min; ○, cells pretreated with 200 μM BSO for 6 h; △, cells pretreated with 2 mM dithiothreitol for 10 min; ▲, cells pretreated with 2 mM cysteine for 10 min. Each point is the mean ± S.E. of seven determinations. Points without a vertical bar indicate that the standard error is within the size of the symbol. *Sensitivity is significantly different from that in cells treated with menadione alone ($P < 0.05$).

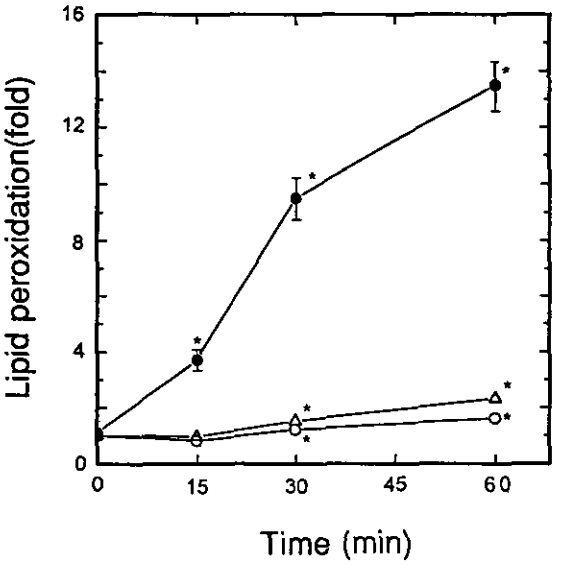

Figure 7 Lipid peoxidation during treatment with menadione. Cardiomyocytes were incubated with agents at 37°C. Symbols: ●, cells treated with 80 μM menadione; △, cells pretreated with 2 mM dithiothreitol for 10 min and then treated with 80 μM menadione; ○, cells pretreated with 2 mM cysteine for 10 min and then treated with 80 μM menadione. Each point represents the mean ± S.E. of six separate determinations. Points without a vertical bar indicate that the standard error is within the size of the symbol. *Concentration is significantly different from the corresponding values obtained in control (untreated) cells ($P < 0.05$). The optical density at 535 nm was 0.018/10^6 cardiomyocytes. Pretreatment of cells with dithiothreitol or cysteine did not significantly change the basal level of lipid peroxide.

In this study, we have shown that menadione is toxic to neonatal rat cardiomyocytes, skeletal muscle cells and 3T3 fibroblasts. A large portion of GSH and protein thiols was depleted while GSSG was increased before the cells degenerated. The depletion of GSH and the increase in GSSG may have been due to the severe inhibition of glutathione reductase activity in cardiomyocytes. Whether or not GSH depletion can promote the toxic effect of an endogenous oxidative stress is dependent on the type of cell. Lewko (1987) demonstrated that depletion of GSH and GSSG with 10^{-4} M BSO did not appear to be toxic to the primary cultures of cardiomyocytes over a 5-day period. Under similar conditions, BSO was more effective in depressing the growth of human MCF-7 and MDA-231 cells. Russo and Mitchell (1985) also demonstrated that there was no appreciable effect on the plating efficiency of Chinese hamster V79 cells treated with 10 mM BSO for 4 h. The level of GSH was 5% of control value after 4 h of exposure to BSO. In our culture system, at a concentration of 200 μM, BSO did not have any influence on the viability of cardiomyocytes over a 24 h period. However, GSH depletion sensitized the cells to an imposed oxidative stress. Depletion of GSH via inhibition of GSH synthesis with BSO or NEM promoted the menadione's toxicity. Inhibition of superoxide dismutase or DT-diaphorase depleted intracellular GSH and increased the toxicity of menadione, indicating that this agent could be metabolized by DT-diaphorase and that both DT-diaphorase and superoxide dismutase provide a protective mechanism in the cells. Pretreatment of cells with cysteine or dithiothreitol suppressed the menadione-induced cell degeneration. It seems very likely that these two thiol-containing compounds exert their effect via a thiol-disulphide exchange. Perturbation of GSH homeostasis may represent a crucial step in menadione-induced toxicity in cardiomyocytes. Babson *et al.* reported that when the level of GSH was reduced to less than 20% in the presence of a specific inhibitor of the GSH regenerating enzyme glutathione reductase, doxorubicin cytotoxicity was increased in hepatocytes (Babson *et al.*, 1981). The use of the general sulphydryl trap, diethyl maleate, has been shown to increase the cardiotoxicity of doxorubicin in an *in vivo* system (Olson *et al.*, 1977). The metabolism of menadione by isolated hepatocytes greatly affects the status of both GSH and protein thiols. Oxidation of GSH to GSSG, mixed disulphide formation, and conjugation with GSH are the mechanisms responsible for depletion of GSH during the metabolism of menadione in hepatocytes (Thor *et al.*, 1982; Mirabelli *et al.*, 1989). All of these data emphasize the importance of thiols homeostasis.

Thiol depletion and elevation of intracellular Ca^{2+} are common cytopathologic responses that often follow toxic exposure to a wide variety of drugs and chemicals. Considerable experimental evidence supports the concept that Ca^{2+} is a primary mediator of lethal cell injury during chemical intoxication (Nicotera *et al.*, 1990). An elevation of intracellular Ca^{2+} results in the stimulation of Ca^{2+}-dependent processes that ultimately cause cell degeneration. However, equally convincing evidence has been presented that argues for the primacy of Ca^{2+}-independent cytotoxic mechanisms in response to a number of chemical challenges (Farber, 1990). In the present and the previous study (Tzeng *et al.*, 1992) we have shown that menadione alters intracellular thiol status and Ca^{2+} homeostasis, and that both of these changes precede cell degeneration. A rapid and extensive depletion of cellular thiols, in response to menadione treatment, preceded a modest elevation of cytosolic Ca^{2+} prior to loss of cell viability. Some studies suggest that the depletion of GSH is linked to an elevation in intracellular Ca^{2+} concentration. Ca^{2+}-dependent ATPase is highly sensitive to changes in the cellular thiols status, which serves as a membrane-bound Ca^{2+} pump to maintain cytoplasmic Ca^{2+} at low concentration (Bellomo *et al.*, 1983; Bellomo and Orrenius, 1985; Miccadei *et al.*, 1988). Tzeng *et al.* (1992) showed that menadione induces formation of blebs in cardiomyocytes as the intracellular Ca^{2+} concentration increases. Surface blebbing has also been observed in hepatocytes under conditions of altered ionized calcium flux (Jewell *et al.*, 1982). In addition to cardiomyocytes, changes in transmembrane calcium flux due to menadione have been observed in hepatocytes, pancreatic islet cells and polymorphonuclear leucocytes (Bachur *et al.*, 1979). Loss of GSH also may result in impaired protection against lipid peroxidation or disruption of vital macromolecules via thioether formation (Babson *et al.*, 1981). Previous investigators have shown that superoxide radical ions can decompose to yield hydroxyl radicals, peroxy radicals and hydrogen peroxide. These are known to initiate free radical-mediated chain reactions which result in conversion of unsaturated fatty acids in membranes to lipid peroxides (Myers *et al.*, 1977). Superoxide generation leading to lipid peroxidation has been implicated in membrane damage that may lead to cell lysis. Thus, lipid peroxidation has also been suggested as one of the major mechanisms for the toxicity induced by anthracyclines (Bachur *et al.*, 1979). Awasthi *et al.* (1975) pointed

out that lipid peroxides may inhibit the activities of selected enzymes, such as glutathione peroxidase, probably by oxidation of reduced thiol groups. Glutathione peroxidase is known to contain reduced thiols. Because of the relative lack of superoxide dismutase, catalase and non-selenium-dependent glutathione peroxidase, selenium-dependent glutathione peroxidase provides a major enzymatic pathway for removal of reactive oxygen metabolites from the heart. Since lowering lipid peroxidation also decreases cell degeneration (Fig. 7), it appears that lipid peroxidative damage may be another cause of degeneration of cardiomyocytes. This problem needs further investigation.

Cardiomyocytes are more sensitive to menadione than are skeletal muscle cells and 3T3 fibroblasts. The responsiveness of a cell to menadione is dependent on the following. (1) The concentration of menadione in the cell. (2) The concentration of reactive oxygen species produced in the cell; this depends on the activities of flavin-enzyme systems and DT-diaphorase. (3) The activities of antioxidant enzymes, including catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase, to decompose reactive oxygen species. Bachur *et al.* reported that the activities of these antioxidant enzymes, which detoxify activated oxygen species, are much lower in the heart than in the liver (Bachur *et al.*, 1979). However, the activities of these enzymes are higher in cardiomyocytes than in skeletal muscle cells (Hien *et al.*, 1975; Tappel *et al.*, 1982; Higuchi *et al.*, 1985; Monsalve *et al.*, 1991). Whether the relative concentration of menadione in these cells is different remains unanswered. The susceptibility of cells to menadione was not predictable from the basal values of cellular thiols or from the activities of antioxidant enzymes. Mitochondria, which are more abundant in cardiomyocytes than in skeletal muscle cells and 3T3 fibroblasts, represent a potential source of oxyradical generation. This could be the main reason why cardiomyocytes are more sensitive to menadione than are skeletal muscle cells and 3T3 fibroblasts.

In summary, menadione is more toxic to cultured cardiomyocytes than to cultured skeletal muscle cells and 3T3 fibroblasts. It increases GSSG, and depletes GSH and protein thiols. The extent of depletion of cellular thiols correlates with the severity of menadione's toxicity in cardiomyocytes.

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