Menadione Toxicity in Cultured Rat Cortical Astrocytes

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ABSTRACT—We examined the effect of menadione on the morphology and viability of cultured astrocytes. Exposure of astrocytes to menadione induced a progressive cell death. The toxic effect of menadione was concentration- and time-dependent. Menadione toxicity was blocked by antioxidants, superoxide dismutase, catalase and iron chelators, indicating that superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$) are all involved in menadione toxicity. Menadione cytotoxicity should be useful as a model for studying the mechanisms underlying oxidative injury in the brain and for the screening of drugs that may attenuate cell damage caused by oxidative stress.

Keywords: Menadione, Toxicity, Reactive oxygen species, Astrocyte, Culture

Recent evidence suggests that increased formation of reactive oxygen species is responsible for a variety of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis, and for pathological conditions such as ischemia and excitotoxicity (1–3). Reactive oxygen species involved in cytotoxicity are mainly superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$). Although neurotoxic effects of reactive oxygen species have been extensively studied, the impact of oxidative stress on astrocytes is not well-understood. Since astrocytes are supposed to promote neuronal regeneration by producing neurotrophic factors around the locus of brain injuries (4–6), it is important to study astrocytic responses to oxidative stress.

As an *in vitro* model of oxidative stress, some investigators have employed an exogenous free-radical-generating system such as hypoxanthine/xanthine oxidase (7, 8). However, the effect of reactive oxygen species generated outside the cells should be different from that inside the cells. Menadione (2-methyl-1,4-naphthoquinone), which is also designated vitamin K_3 , has been reported to enter the cells and generate O_2^- via one-electron-transfer reactions (9–12). This agent could be useful for studying the effect of reactive oxygen species generated endogenously as a result of stress. In the present study, we therefore investigated the effect of menadione on the morphology and viability of cultured astrocytes, in comparison with the effect of exogenously applied H_2O_2 .

MATERIALS AND METHODS

Basal culture medium used in the present study was a modified Eagle's medium containing 30 mM glucose, 2 mM glutamine and 1 mM pyruvate. Primary cultures of astrocytes were prepared from the cerebral cortices of 18-day-old embryos of Wistar rats. After removal of the meninges, the cortices were dissociated by trypsinization and pipetting as described previously (13). Cells were suspended in a medium supplemented with 10% fetal bovine serum and plated on uncoated 25 cm² flasks at a density of 600,000 cells/cm². The culture medium was changed 12 hr after the plating and then every 3–4 days. The cells grew and became confluent after 10–14 days. At this point, more than 90% of the adherent cells exhibited the flattened, polygonal appearance typical of type I astrocytes. Non-astrocytes were detached from the flasks by shaking and removed by changing the medium. The remaining cells were dissociated by trypsinization (0.1% trypsin – 0.04% EGTA) and plated on uncoated 96-well plates at a density of 10,000 cells/cm². After the cells became confluent (5–6 days after the plating), the medium was switched to serum-free medium, and experiments were initiated 24 hr later. Test reagents were added to the culture medium.

Changes in cell morphology were assessed by microscopic examination, and cell viability was quantitatively measured by the modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay (14). For the MTT assay, the culture medium was

replaced by serum-free medium containing 0.5 mg/ml MTT and then the incubation continued for 1 hr at 37°C. The dye MTT is converted into a purple formazan by the redox activity of living cells. After the medium was removed, the cells were solubilized by adding a solution containing 50% dimethylformamide and 20% sodium

dodecyl sulfate (pH 4.8), and the amount of formazan product was determined by measuring its absorbances at 570 and 630 nm. By microscopic examination, we confirmed that decrease in MTT reduction was parallel to cell death. In all assays, triplicate wells were used for each condition, and the averaged value was obtained. Each

Fig. 1. Effect of menadione on morphology of cultured astrocytes. Menadione was added at a concentration of 100 μ M or 1 mM, and cells in the same field were photographed at the indicated time after addition of menadione.

experiment was repeated in five separate cultures, and the data are finally presented as the mean $\pm$ S.E.M. of five separate observations.

To check the purity of cultures, cells were fixed with 4% paraformaldehyde and employed for immunocytochemical examination. After overnight incubation at 4°C with monoclonal antibodies to glial fibrillary acidic protein (GFAP; Amersham, Little Chalfont, UK), cells were stained with a Vectastain Elite ABC kit (Vector, Burlingame, CA, USA), according to the manufacturer's instructions. More than 95% of the cells used for assays were identified as type I astrocytes by positivity for GFAP and by morphological characteristics.

Since menadione is insoluble in water, we used menadione sodium bisulphite, the water-soluble form of menadione. In preliminary experiments, we checked the effect of sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$), which yields sodium bisulfite (NaHSO_3) when dissolved in water. Sodium metabisulfite (1–1000 μM) had no effect on morphology and viability of astrocytes. Thus the influence of the sodium bisulfite moiety is negligible in the present study. H_2O_2 , propyl gallate, superoxide dismutase from bovine erythrocyte and N,N,N',N' -tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) were purchased from Wako

Fig. 2. Effect of exogenously applied H_2O_2 on morphology of cultured astrocytes. H_2O_2 (200 or 400 μM) was added to the culture medium, and cells were photographed 8 hr later.

Fig. 3. Time course of toxicity of menadione (A: $\circ$, 10 μM ; $\square$, 100 μM ; $\triangle$, 1 mM) and exogenously applied H_2O_2 (B: $\circ$, 200 μM ; $\square$, 400 μM ; $\triangle$, 800 μM). The abscissa indicates time after addition of menadione or H_2O_2 . Cell viability was determined by the MTT reduction assay and expressed as a percentage of the control. The data represent the mean $\pm$ S.E.M. of the determinations on five separate cultures.

Fig. 4. Effects of antioxidants, propyl gallate (A: $\circ$, control; $\blacksquare$, 0.5 μM ; $\blacktriangle$, 5 μM ; $\blacktriangledown$, 50 μM) and N -acetylcysteine (B: $\circ$, control; $\bullet$, 500 μM), on menadione toxicity. Antioxidants were added 30 min prior to addition of menadione, and the cells were exposed to menadione (10–1000 μM) for 8 hr. Cell viability was determined with the MTT reduction assay and represent the mean $\pm$ S.E.M. of five separate observations. Asterisks indicate significant differences from the control: * $P < 0.01$ (Duncan's multiple range test).

Pure Chemical Industries, Ltd. (Osaka). *N*-Acetylcysteine, catalase from bovine liver, *tert*-butyl hydroperoxide and deferoxamine were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

RESULTS

Exposure of astrocytes to menadione caused a progressive cell death. Figure 1 shows menadione-induced morphological changes of cultured astrocytes. When cells were exposed to 100 μ M menadione, apparent morphological changes were observed 2 hr later. Cells gradually atrophied and were finally detached from the culture dishes. All cells were killed within 12 hr. Such morphological changes occurred earlier when higher concentrations were applied. Menadione at 1 mM killed all cells within 6 hr.

Addition of H_2O_2 (400 μ M or more) also induced a progressive cell death, but morphological change induced by exogenous H_2O_2 was considerably different from that by menadione (Fig. 2). Cells seemed to have burst, and flattened cell membranes were left on the culture dishes. Another characteristic of exogenous H_2O_2 toxicity was that its concentration-effect relationship was very steep. More than 90% of the astrocytes were viable in the presence of 200 μ M H_2O_2 , but all cells were killed by 400

μ M H_2O_2 . This means that twofold increase of H_2O_2 level is sufficient to induce cell death.

The effects of menadione and H_2O_2 on cell viability were quantitated with the MTT reduction assay, and the results are shown in Fig. 3. Menadione toxicity was seen at concentrations of more than 10 μ M. The time course of menadione toxicity was slower than that of exogenous H_2O_2 .

To determine if menadione toxicity is caused by oxidative stress, the effects of antioxidants on menadione toxicity were investigated. Antioxidants were added 30 min prior to addition of menadione, and the cells were exposed to menadione (10 μ M–1 mM) for 8 hr. The antioxidant propyl gallate alone had no effect on cell viability, but significantly attenuated menadione toxicity (Fig. 4A). Similarly, another antioxidant, *N*-acetylcysteine, was effective in attenuating menadione toxicity (Fig. 4B).

To determine which oxygen species (O_2^- , H_2O_2 or $\cdot\text{OH}$) are involved in menadione toxicity, we investigated the effects of scavengers for these reactive oxygens. First, O_2^- dismutates into H_2O_2 via a reaction catalyzed by the enzyme superoxide dismutase (SOD). If O_2^- is involved in menadione toxicity, then addition of SOD should attenuate the toxicity. As shown in Figs. 5 and 6A, menadione toxicity was attenuated by SOD. H_2O_2 toxicity was not affected by SOD (Fig. 6B), supporting that SOD

Fig. 5. Effects of menadione and SOD on morphology of cultured astrocytes. SOD (50 U/ml) was added 30 min prior to menadione, and cells were exposed to menadione (100 μ M) for 8 hr.

specifically blocked O_2^- -mediated toxicity. Second, H_2O_2 is decomposed into H_2O and O_2 by the action of catalase. If H_2O_2 is involved in menadione toxicity, it should be attenuated by catalase. As shown in Figs. 7 and 8A, menadione toxicity was markedly attenuated by catalase. We confirmed that catalase was effective in attenuating exogenous H_2O_2 toxicity (Fig. 8B). It should be noted that catalase protected the cells against exogenous H_2O_2 more potently than against menadione. As shown in Fig. 8C, catalase had no effect on toxicity induced by the lipid peroxidizing agent *tert*-butyl hydroperoxide (15, 16), supporting that catalase specifically blocked H_2O_2 -mediated toxicity. Third, $\cdot OH$ is generated through the breakdown of H_2O_2 in the presence of ferric ions ($H_2O_2 + Fe^{2+} \rightarrow OH^- + \cdot OH + Fe^{3+}$, the Fenton reaction; 17, 18). If $\cdot OH$ production is involved in menadione

Fig. 6. Effects of SOD (○, control; ■, 0.05 U/ml; ▲, 0.5 U/ml; ▼, 5 U/ml; ◆, 50 U/ml) on astrocytic cell death induced by menadione (A) or exogenously applied H_2O_2 (B). Cells were exposed to menadione or H_2O_2 for 8 hr, and then cell viability was determined by the MTT reduction assay. The data represent the mean $\pm$ S.E.M. of five separate observations. * $P < 0.01$ vs control (Duncan's multiple range test).

Fig. 7. Effects of menadione and catalase on morphology of cultured astrocytes. Catalase (200 U/ml) was added 30 min prior to addition of menadione, and cells were exposed to menadione (100 μM) for 8 hr.

Fig. 8. Effects of catalase (○, control; ■, 2 U/ml; ▲, 20 U/ml; ▼, 200 U/ml; ◆, 500 U/ml) on astrocytic cell death induced by menadione (A), H_2O_2 (B) or *tert*-butyl hydroperoxide (C). After the cells were exposed to test reagents for 8 hr, cell viability was determined by the MTT reduction assay. The data represent the mean $\pm$ S.E.M. of five separate observations. * $P < 0.01$ vs control (Duncan's multiple range test). The effect of catalase against H_2O_2 was significant, but asterisks are not shown for clarity.

toxicity, then iron chelators should attenuate the toxicity. The iron chelator deferoxamine significantly attenuated menadione toxicity (Figs. 9 and 10A). Similarly, another iron chelator TPEN was effective in attenuating menadione toxicity (Fig. 11A). Deferoxamine and TPEN were also effective in attenuating exogenous H_2O_2 toxicity (Figs. 10B and 11B), but their effects against exogenous H_2O_2 were relatively smaller than those against menadione.

DISCUSSION

In the present study, we found that menadione can be used as a pharmacological tool to induce astrocytic cell death via formation of reactive oxygen species. The menadione toxicity on astrocytes was blocked by antioxidants, SOD, catalase and iron chelators, indicating that O_2^- , H_2O_2 and $\cdot OH$ are all involved in this toxicity. Since among the tested scavengers catalase was most effective in protecting cells against menadione, H_2O_2 may play a major role in menadione toxicity.

Morphological changes induced by menadione were considerably different from that by extracellularly applied H_2O_2 . One possible explanation for this difference is that different types of oxygen species are involved in menadione toxicity and exogenous H_2O_2 toxicity. In fact, experiments with SOD demonstrate that O_2^- is involved in menadione toxicity but not in exogenous H_2O_2 toxicity. Another possibility is that intracellularly generated H_2O_2 has different impact on the cell than extracellular H_2O_2 . For example, intracellular H_2O_2 may preferentially damage intracellular organelle.

Catalase, which is membrane impermeable like most proteins, is supposed to scavenge excessive H_2O_2 outside the cell. Since H_2O_2 is freely diffusible across the membrane, reduction of extracellular H_2O_2 by catalase would lower H_2O_2 within cells. Catalase protected cells against extracellularly applied H_2O_2 much more potently than against menadione toxicity. On the other hand, deferoxamine and TPEN, which are supposed to chelate cellular ferric ions, were effective in attenuating menadione toxicity rather than extracellular H_2O_2 toxicity. These results also suggest that menadione generates H_2O_2 inside the cell.

Our present study has focused on astrocytes, but it is also interesting to compare menadione effects on other types of cells. Recently, Husain and Juurlink (19) have reported that oligodendrocyte precursor cells were more readily killed by menadione than astrocytes. However, there are several problems with their study. First of all, they employed mixed glial cultures, in which possible interactions between oligodendrocyte precursors and astrocytes must be considered. Second, they examined the effect of menadione in serum-containing medium, which includes undefined concentrations of substances that may potentiate or attenuate the menadione effect. Third, they assayed menadione effects only at concentrations of 50–80 μM . We are planning to compare menadione effects on astrocytes, oligodendrocytes and microglia by using cell cultures with higher purity.

In conclusion, menadione toxicity is a valuable model for the study of mechanisms underlying oxidative injury in the brain, and it can be used for the screening of drugs that may protect cells from reactive oxygen species.

Fig. 9. Effects of menadione and deferoxamine on morphology of cultured astrocytes. Deferoxamine (10 μ M) was added 30 min prior to menadione, and cells were exposed to menadione (30 μ M) for 8 hr.

Fig. 10. Effects of deferoxamine (○, control; ■, 0.1 μ M; ▲, 1 μ M; ◆, 100 μ M) on astrocytic cell death induced by menadione (A) or H₂O₂ (B). The data represent the mean $\pm$ S.E.M. of five separate observations. * P < 0.01 vs control (Duncan's multiple range test).

Fig. 11. Effects of TPEN (○, control; ■, 0.1 μ M; ▲, 1 μ M; ▼, 10 μ M) on astrocytic cell death induced by menadione (A) or H₂O₂ (B). Experimental protocol and data representation are the same as in Fig. 10.

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