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Oxidative Stress Studied in Intact Mammalian Cells [and Discussion]

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Oxidative stress studied in intact mammalian cells

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Exposure of isolated rat hepatocytes to toxic doses of menadione (2-methyl-1,4-naphthoquinone) results in enhanced formation of active oxygen species, depletion of cellular glutathione and protein thiols, and perturbation of intracellular calcium ion homeostasis. An increase in cytosolic Ca^{2+} concentration, resulting from inhibition of the plasma membrane Ca^{2+} translocase by menadione metabolism, appears to be critically involved in the development of cytotoxicity.

Introduction

The toxicological implications of the formation of active oxygen species have attracted growing interest in recent years. Under aerobic conditions oxygen radicals are normal cellular metabolites that are inactivated by various enzyme systems, including superoxide dismutases, catalase, and glutathione peroxidase. The production of oxygen radicals may, however, be greatly stimulated by the presence of various redox-active compounds. Under certain conditions, this stimulation of oxygen radical formation may be so great as to overwhelm the cellular defence systems and cause oxidative stress and toxicity.

In a series of studies we have used freshly isolated rat hepatocytes incubated with menadione (2-methyl-1,4-naphthoquinone) to investigate the sequence of events involved in the development of oxidative cell injury. The results of our studies, which are briefly summarized in this presentation, suggest that depletion of glutathione (GSH) and protein thiols and perturbation of calcium ion homeostasis are important early events in the development of cytotoxicity.

Metabolism of quinones

The metabolism of quinones by flavoenzymes can occur by either one- or two-electron reduction routes, which often differ greatly in cytotoxicity. As illustrated schematically in figure 1, one-electron reduction results in the formation of semiquinone radicals which can rapidly reduce dioxygen, forming the superoxide anion radical, O_2^-, and regenerating the quinone. Dismutation of O_2^- and production of other highly reactive species of oxygen quickly lead to conditions of oxidative stress and toxicity as redox cycling of the quinone continues (Hassan & Fridovich 1979). However, quinones can also undergo two-electron reduction, forming hydroquinones without production of free semiquinone intermediates. This reaction is catalysed by DT-diaphorase (Ernster 1967), and may serve an important protective function for the cell by competing with the single-electron pathway, because hydroquinones are often less reactive, and more easily excreted by the cell, than semiquinone radicals.

Although menadione can be released from liver cells as the free hydroquinone (Thor *et al.*, 1982), conjugates with glucuronic acid, sulphate and glutathione appear to constitute the predominant metabolites. Whereas conjugation with glucuronic acid and sulphate occurs with

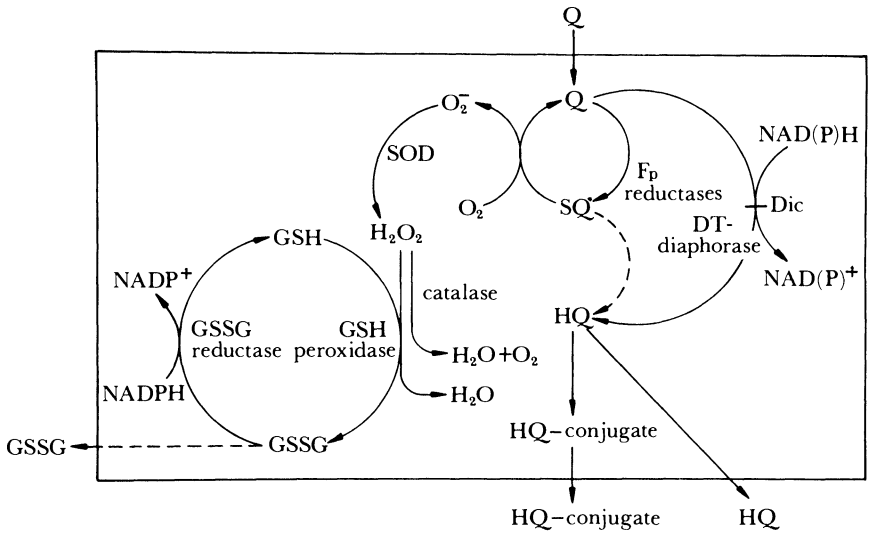

FIGURE 1. Schematic representation of quinone metabolism in isolated hepatocytes. Q, quinone; SQ·, semiquinone radical; HQ, hydroquinone; Fp, flavoprotein; Dic, dicoumarol; SOD, superoxide dismutase; GSH, reduced glutathione; GSSG, glutathione disulphide.

menadiol, it is not yet clear to what extent the formation of glutathione conjugates also is preceded by reduction of the quinone. Quinones are able to form thiol conjugates non-enzymatically, and a glutathione conjugate of menadione has been reported to contribute to the overall redox cycling of the quinone in the perfused liver (Wefers & Sies 1983).

METABOLISM AND TOXICITY OF MENADIONE IN INTACT HEPATOCYTES

In isolated liver cells, menadione metabolism involves both one- and two-electron reduction pathways (Thor *et al.* 1982). The relative contribution of the two routes depends on menadione concentration, and can be manipulated by selective induction of either NADPH–cytochrome-P_{450} reductase or DT-diaphorase. Thus, hepatocytes isolated from phenobarbital-treated rats exhibit increased redox cycling and toxicity when exposed to menadione, whereas the reverse is true for hepatocytes from 3-methylcholanthrene-treated rats.

As mentioned above, the cytotoxicity of menadione and other quinones has been related to the oxidative stress caused by the redox cycling of these agents in their target cells (Hassan & Fridovich 1979; Thor *et al.* 1982). O_2^- is produced during the one-electron reduction of menadione to the semiquinone radical and its subsequent reoxidation by molecular oxygen, as well as by the direct interaction of menadione with intracellular thiols (Thor *et al.* 1982; Di Monte *et al.* 1984*b*). Dismutation of O_2^- yields H_2O_2 which can be further metabolized to H_2O by the glutathione peroxidase system at the expense of GSH and NADPH (Wendel 1980).

Exposure of hepatocytes to toxic concentrations of menadione is associated with extensive formation of O_2^- and H_2O_2, and the oxidation of glutathione and pyridine nucleotides (Thor *et al.* 1982). Treatments which promote one-electron reduction and redox cycling of the quinone potentiate menadione toxicity. Conversely, toxicity is diminished when resynthesis of glutathione is facilitated, and in hepatocytes from selenium-deficient rats in which menadione-induced GSH depletion occurs more slowly than in normal cells because of the decreased glutathione peroxidase activity (Orrenius *et al.* 1984). It thus appears that glutathione depletion is a critical

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event in the initiation of menadione toxicity in isolated hepatocytes, and that the metabolism of large amounts of H_2O_2 by glutathione peroxidase is responsible for the loss of the major fraction of reduced glutathione.

A recent analysis has shown that menadione metabolism by isolated hepatocytes affects the status of both soluble (GSH) and protein thiols (Di Monte *et al.* 1984*b*). In addition to oxidation of GSH to GSSG, formation of mixed disulphides and glutathione conjugate contribute to GSH depletion during menadione metabolism. Following the decrease in soluble thiols there is also a time- and dose-dependent loss of protein sulphydryl groups which is a result of both oxidation and arylation of the protein thiols, oxidation being the quantitatively more important mechanism (Di Monte *et al.* 1984*a*). This loss of protein thiols correlates very closely with the onset of toxicity and invariably precedes cell death. It therefore seems that the depletion of protein thiols, chiefly through oxidative mechanisms, is a critical event which follows GSH depletion in oxidative cell injury caused by menadione.

Perturbation of intracellular Ca^{2+} homeostasis during oxidative stress

We have previously reported that menadione and several other toxic agents cause the formation of numerous small blebs on the surface of isolated hepatocytes (Thor *et al.* 1982; Jewell *et al.* 1982). The same type of surface blebbing can be induced by the calcium ionophore A23187, suggesting that the alterations in surface structure caused by menadione and other toxins are a result of a redistribution of intracellular Ca^{2+} (Jewell *et al.* 1982). This hypothesis is further supported by the observation that GSH depletion is followed by a loss of cell Ca^{2+} from hepatocytes incubated with menadione (Thor *et al.* 1982). Detailed analysis has shown that this effect is due to inhibition of Ca^{2+} sequestration in both the mitochondria and endoplasmic reticulum as result of menadione metabolism (Thor *et al.* 1982: Jewell *et al.* 1982). Whereas the effect of menadione on mitochondrial Ca^{2+} sequestration seems to be related to the oxidation of pyridine nucleotides (Bellomo *et al.* 1982), the inhibitory effect on endoplasmic reticular Ca^{2+} sequestration appears to be a result of oxidation–arylation of thiol group(s) critical for Ca^{2+}-ATPase activity (Jones *et al.* 1983). The effects of menadione metabolism on regulation of Ca^{2+} compartmentation in hepatocytes are illustrated schematically in figure 2.

Because of the inhibition of Ca^{2+} sequestration in the endoplasmic reticulum and mitochondria, incubation of hepatocytes with menadione results in a release of Ca^{2+} sequestered in these compartments into the cytosol. Normally, this would probably only cause a transient rise in the cytosolic Ca^{2+} concentration, because the plasma membrane Ca^{2+} pump would remove this Ca^{2+} from the cell, and the cytosolic Ca^{2+} concentration would return to its usual very low level. Recent studies in our laboratories have shown, however, that the hepatic plasma membrane Ca^{2+}-ATPase is inhibited by agents which oxidize protein thiol groups, including menadione (Bellomo *et al.* 1983).

The redox cycling of menadione in hepatocytes could therefore inhibit all three Ca^{2+} translocases present in the mitochondria, endoplasmic reticulum and plasma membrane. This would undoubtedly lead to a sustained rise in cytosolic Ca^{2+} level, which could cause surface blebbing by altering microfilament organization. Obviously, measurements of cytosolic Ca^{2+} concentration following exposure of hepatocytes to menadione are required to substantiate this hypothesis, but this is very difficult in practice. We have therefore used phosphorylase *a* activity to monitor alterations in cytosolic free-Ca^{2+} concentration in hepatocytes subjected to oxidative

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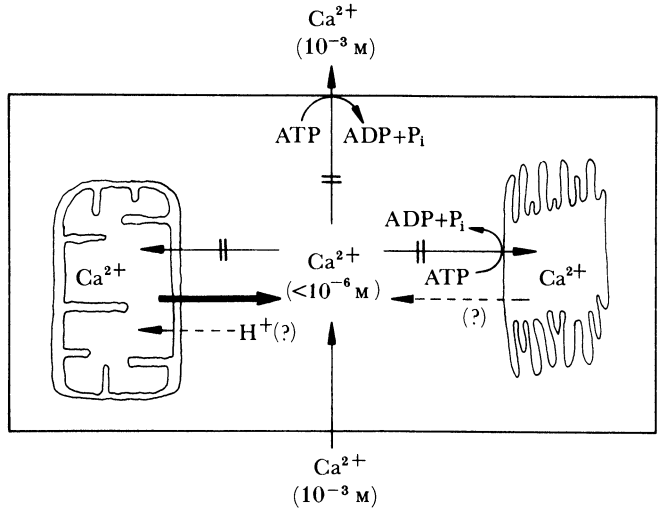

FIGURE 2. Schematic representation of regulation of Ca^{2+} compartmentation in hepatocytes. Effects of menadione metabolism. This illustrates how menadione metabolism causes the release of Ca^{2+} sequestered in the mitochondria and inhibition of the Ca^{2+} translocases located in the endoplasmic reticular and plasma membranes.

stress. Phosphorylase *a* has previously been demonstrated to be a valid indicator of fluctuations in cytosolic Ca^{2+} level because, under appropriate experimental conditions, its activity is strictly dependent on a Ca^{2+}-requiring phosphorylase kinase (Malencik & Fisher 1982). Using this approach we have found that exposure of hepatocytes to toxic levels of menadione causes prolonged phosphorylase activation (Bellomo *et al.* 1984; Di Monte *et al.* 1984*a*). Thus, it appears that menadione-induced oxidative stress in hepatocytes can cause depletion of GSH and protein thiols, and a perturbation of Ca^{2+} homeostasis that may lead to an uncontrollable rise in cytosolic free-Ca^{2+} concentration, resulting in cell death. A schematic representation of these events is given in figure 3.

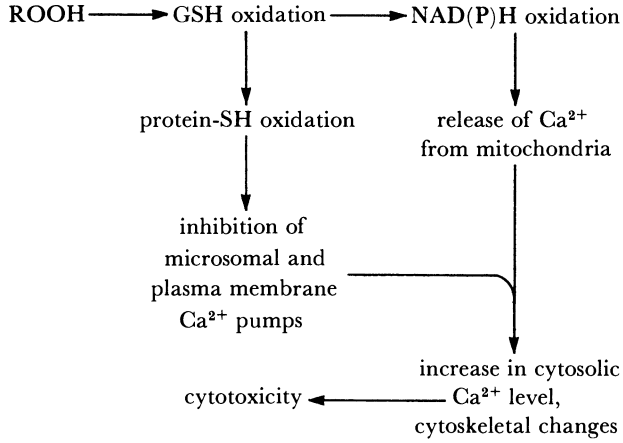

FIGURE 3. Schematic representation of the possible relation between hydrogen peroxide formation, glutathione and pyridine nucleotide oxidation, perturbation of Ca^{2+} homeostasis and cell toxicity during menadione metabolism in isolated hepatocytes.

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Concluding remarks

Our studies have shown that exposure of hepatocytes to toxic levels of menadione results in extensive production of oxygen radicals, oxidation of cellular thiols and pyridine nucleotides, and perturbation of intracellular Ca^{2+} homeostasis. The latter is associated with characteristic blebbing of the plasma membrane, which appears to be an early morphologic sign of cell injury. Agents that promote one-electron reduction of menadione, and thereby its redox cycling with molecular oxygen, potentiate menadione toxicity.

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Discussion

H. Sies (*Institut für Physiologische Chemie I, Universität Düsseldorf, F.R.G.*). There were several interesting points in Professor Orrenius's lecture. Just for one: does he think that the thioether formation of menadione with protein-bound thiols might be crucial for the expression of quinone toxicity?

S. Orrenius. Although this mechanism may contribute to the toxicity, our present results suggest that the oxidation of protein thiols is more important.

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