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Lipid peroxidation in electroporated hepatocytes occurs much more readily than does hydroxyl-radical formation

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1. Rat hepatocytes suspended in 0.25 M-sucrose were electropermeabilized. This completely disrupted their plasma-membrane permeability barrier. 2. The endoplasmic reticulum in electroporated hepatocytes appeared morphologically preserved and maintained its permeability barrier as evidenced by electron-microscopic examination and latency measurements on luminal reticular enzymes. 3. Upon aerobic incubation with an NADPH-generating system and iron/ADP, porated hepatocytes peroxidized their membrane lipids at rates similar to those of matched microsomal preparations. 4. When hepatocytes were incubated with iron/EDTA and azide, radical formation detectable with dimethyl sulphoxide (DMSO) was only 10–20% that shown by microsomes. Omitting azide abolished hepatocyte reactivity with DMSO completely. Effects of hydroxyl-radical (·OH) scavengers and of added catalase suggest that the radical detected by DMSO is ·OH. 5. Cytosolic inhibitor(s) from hepatocytes seemed to be a major factor limiting ·OH formation. These were macromolecular, but showed a degree of heat-stability. Dialysis largely abolished inhibition, but this could be restored again by adding GSH. 6. Since ·OH formation in hepatocytes seems to be much more stringently prevented than lipid peroxidation, free-radical damage originating from intracellular redox systems seems more likely to take the form of lipid peroxidation.

INTRODUCTION

It is well established that liver microsomes (microsomal fractions) incubated aerobically with NADPH release substantial amounts of superoxide (see, e.g., Aust *et al.*, 1972) and H_2O_2 (see e.g., Ernster *et al.*, 1982), whereas, if suitably chelated transition-metal ions such as Fe are present, microsomes rapidly peroxidize their membrane phospholipids and efficiently generate hydroxyl radical (·OH) or radicals with comparable oxidative power (reviewed by Halliwell & Gutteridge, 1985). By contrast, indirect assessments on perfused liver or isolated hepatocytes indicate that these more intact systems form only small quantities of H_2O_2 used as a general indicator for the formation of reactive oxygen species (see, e.g., Jones *et al.*, 1978; Chance *et al.*, 1979; Sies & Graf, 1982; Halliwell & Gutteridge, 1985), and ·OH formation has not been detected with certainty. Nevertheless, hepatocytes incubated with Fe/ADP readily autoxidize their membrane lipids (see e.g., Högberg *et al.*, 1975; Poli *et al.*, 1985).

Sies & Graf (1982) and Halliwell & Gutteridge (1985) have speculated that the large apparent difference in formation of reactive oxygen species between liver microsomes and intact cell preparations may be due to 'artefactually' increased electron flow to dioxygen in microsomes caused by alterations in the intramembranous arrangement of the cytochrome *P*-450 system occurring when the endoplasmic reticulum (ER) is fragmented and vesiculated.

This explanation clearly implies that microsomes may be unsuitable models for studies on oxygen-radical formation, in which they are, nevertheless, extensively used. It seemed of interest, therefore, to compare free-radical reactions in electroporated hepatocytes, which we knew contained arrays of undisrupted lamellar ER (Stanley *et al.*, 1987), with the same reactions in rat liver microsomes.

MATERIALS AND METHODS

Chemicals were purchased from Sigma Chemical Co., Boehringer-Mannheim or BDH and were analytical reagents or the best quality available.

Preparation of the hepatocytes and microsomes

Hepatocytes and microsomes were prepared from 300–450 g male Sprague–Dawley rats fed on standard laboratory chow. Hepatocytes were prepared by collagenase perfusion (Seglen, 1976) with additions of 50 mg of trypsin inhibitor type 2S and 20 mg of DNAase I to the collagenase medium. Perfusates were propelled and continuously gassed with O_2/CO_2 (19:1). Viability, assessed by measuring the expression of cellular lactate dehydrogenase, averaged $89 \pm 3\%$ ($n = 9$).

Microsomes were prepared from a lobe immediately excised from the liver after commencing perfusion with EGTA buffer (Seglen, 1976) or from a separate rat killed by cervical dislocation. Microsomes were isolated by a rapid calcium-aggregation method (Kamath & Rubin, 1972) from 20% (w/v) post-mitochondrial supernatant in 0.25 M-sucrose.

Hepatocytes washed twice in 0.25 M-sucrose were resuspended at 27×10^6/ml in sucrose. They were electroporated by two 2000 V discharges in a 1 cm × 1 cm cell identical with that used by Gordon & Seglen (1982) and immediately adjusted to 25–37.5 mM-KCl and 5–10 mM-Mops, pH 6.8 or 7.4. Electroporation expressed $96 \pm 5\%$ of the lactate dehydrogenase ($n = 7$).

Measurement of lipid peroxidation

Lipid peroxidation was assayed at 37 °C, generally for 3–30 min, in 2 ml incubations containing 0.3–0.5 mg of microsomal protein or $(0.8–1.2) \times 10^6$ hepatocytes, with 18 μM-$FeSO_4$,

Abbreviations used: ·OH, hydroxyl radical; DMSO, dimethyl sulphoxide; ER, endoplasmic reticulum; TBARS, thiobarbituric-acid-reactive substances; UDP-GT, UDP-glucuronyltransferase; G-6-Pase, glucose 6-phosphatase.

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1.6 mM-ADP and an NADPH-generating system consisting of 0.18 μM-$NADP^+$, 4 mM-glucose 6-phosphate and glucose-6-phosphate dehydrogenase (0.19 unit/ml) (Esterbauer *et al.*, 1982).

Activity was assayed at 532 nm by measuring the rate of formation of thiobarbituric-acid-reactive substances (TBARS) (Suematsu & Abe, 1982). Butylated hydroxytoluene (1 mM) was added to the colour-development mixture, which was heated at 80 °C and butanol-extracted.

Measurement of hydroxyl-radical formation

Formation of ·OH or its oxidative equivalent was assayed by measuring formaldehyde formed from the high-affinity ·OH scavenger dimethyl sulphoxide (DMSO) at 33 mM (Cederbaum & Cohen, 1985). Generally, microsomes (1.5–2.0 mg of protein) or (3–4) × 10^6 hepatocytes were incubated at 37 °C with 50 μM-$FeSO_4$, 100 μM-EDTA, 1 mM-NaN_3, 0.4 mM-$NADP^+$, 4 mM-glucose 6-phosphate and glucose-6-phosphate dehydrogenase (0.8 unit/ml) in 1.5 ml. The reaction was stopped with 0.5 ml of 20% (w/v) trichloroacetic acid, and formaldehyde was assayed by the Nash (1953) method at 415 nm. To verify that the reactive species reacting with DMSO is likely to be ·OH, some assays were run with NaN_3 omitted and catalase (33–2000 i.u./ml) added, or in the presence of any of five documented ·OH scavengers, added with DMSO and NaN_3.

Additional methods

Latent or fully expressed UDP-glucuronyltransferase activity was assayed at 37 °C with 0.5 mM-2-[^{14}C]naphthol and 2 mM-UDP-glucuronic acid at pH 7.4. Triton X-100 (0.03–0.08%) was used to abolish latency (Hallinan, 1983).

Latent or fully expressed glucose-6-phosphatase activities were assayed at 37 °C using 10 mM-sugar phosphates in 50 mM-cacodylate buffer, pH 6.5. Sodium taurocholate (0.05–0.2%) was used to abolish latency (Arion, 1989).

RESULTS

Latency of ER enzymes in permeabilized hepatocytes

To obtain evidence about the state of preservation of the ER membranes in electroporated hepatocytes, two enzyme systems were examined. The latency of these systems, UDP-glucuronyltransferase (UDP-GT) and glucose-6-phosphatase (G-6-Pase) assayed with mannose 6-phosphate or glucosamine 6-phosphate, is a sensitive marker for the integrity of the ER membrane permeability barrier (Hallinan & De Brito, 1981; Arion, 1989). In electropermeabilized cells assayed directly that expressed 95–100% of their lactate dehydrogenase and therefore had fully leaky plasma membranes, these enzymes exhibited only 6–7% of their total activity and were thus both highly latent. Total activity was made manifest when detergent was added to assay mixtures to permeabilize ER membranes additionally and abolish enzyme latency; this increased activity 14–17-fold (Table 1). As expected, G-6-Pase assayed with glucose 6-phosphate as substrate showed only modest latency at 37 °C.

These results indicate that the ER membranes in electroporated hepatocytes still maintained their normal permeability barrier.

Electron-microscopic examination of permeabilized hepatocytes

Examination of fixed sections of permeabilized hepatocytes show that these contain a full repertoire of intracellular organelles, including ER membranes, which appeared morphologically intact and in normal continguity to one another (Fig. 1). This is consistent with the evidence obtained from measurements of ER-enzyme latency. Gordon & Seglen (1982) have shown that lysosomes and endosomes are likewise well preserved in electroporated hepatocytes.

Rates of lipid peroxidation by microsomes or permeabilized hepatocytes

Clearly, to compare validly rates of lipid peroxidation or ·OH formation in hepatocytes with those in microsomes, it is necessary that the comparison be done on some common basis, since the two preparations differ markedly in structure and therefore in composition.

Two different bases were chosen for comparing rates of lipid peroxidation by microsomes and hepatocytes. First, since normal rat liver yields approx. 40 mg of microsomal protein/g wet weight if fully homogenized (Wibo *et al.*, 1971) and contains approx. 10^8 hepatocytes/g wet weight (Bock *et al.*, 1976; DeClercq *et al.*, 1982), then 2.5 × 10^6 hepatocytes will contain ≈ 1 mg of microsomal protein. Microsomal rates per mg of protein are therefore compared with hepatocyte rates per 2.5 × 10^6 cells in Table 2.

Table 1. Latency of UDP-GT and of G-6-Pase in permeabilized hepatocytes

Hepatocytes (~ 0.5 × 10^6 cells/assay) were incubated at 37 °C for 3–5 min plus taurocholate with 10 mM-sugar 6-phosphate or plus Triton X-100 with 0.5 mM-2-[^{14}C]naphthol (220000 d.p.m.) + 2 mM-UDP-glucuronic acid to measure latent (−surfactant) or fully expressed G-6-Pase or UDP-GT activities. Abbreviations: Gln6*P*, glucosamine 6-phosphate; Man6*P*, mannose 6-phosphate; Glc6*P*, glucose 6-phosphate.

Enzyme system	Enzyme activity (nmol/min per 10^6 hepatocytes) −Detergent	Enzyme activity (nmol/min per 10^6 hepatocytes) +Detergent	Latency (%)
UDP-GT	1.37±0.5 (5)	21.4±3.9 (5)	94.0
G-6-Pase			
with Gln6*P*	7.3±3.2 (3)	98±25.7 (3)	92.6
with Man6*P*	8.0	112.0	92.9
with Glc6*P*	84.0±8.5 (3)	117±22.6 (3)	28.2

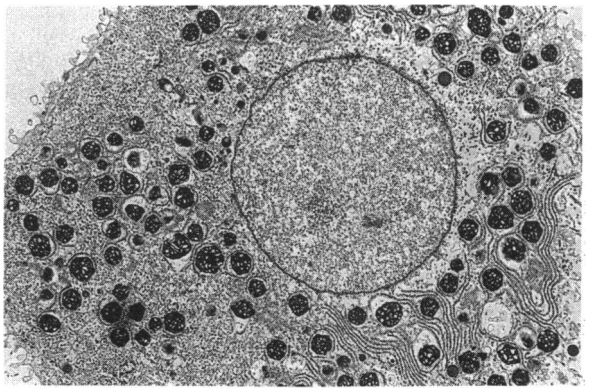

Fig. 1. An electroporated hepatocyte

A fixed section of an electroporated hepatocyte (×4250). Note the intact organelles, including lamellar ER arrays. The dense shrunken appearance of the mitochondria, with the inner membranes withdrawn from the surrounding outer membrane, is the expected conformation for intact coupled organelles in the absence of substrate, ADP and P_i, owing to the leakage of these metabolites from the permeabilized cells (Burgess *et al.*, 1983).

Table 2 shows that microsomes and hepatocytes gave broadly comparable rates of lipid peroxidation. Addition of the cytosolic components leaked from $(2.2–2.8) \times 10^6$ hepatocytes failed to inhibit microsomal peroxidation (results not shown).

Rates for both preparations were also compared per unit activity of an ER marker enzyme, UDP-GT. Expressed on this basis, they again showed similar time course for lipid peroxidation from 6 to 15 min (Figs. 2*a* and 2*b*). Similar time courses were also seen per mg of microsomal protein compared with rates per 2.5×10^6 hepatocytes over this incubation period (Figs. 2*c* and 2*d*), though the presence or absence of a lag at 3 min varied.

Formation of ·OH or its oxidative equivalent

Microsomes incubated aerobically with NADPH and Fe/EDTA, in the presence of NaN_3 to prevent the destruction of H_2O_2 by catalase, generated ·OH or compound(s) with comparable oxidative power at a linear rate for 15 min at pH 7.4 (Fig. 3). Omission of NaN_3 decreased formation approx. 4-fold. By contrast, permeabilized hepatocytes exhibited only 10–20% of the microsomal rate, and omission of NaN_3 abolished their activity completely.

Inhibition of formation of ·OH or its oxidative equivalent by soluble components from permeabilized hepatocytes

A major reason why permeabilized hepatocytes form ·OH or its oxidative equivalent less well than microsomes is the presence of inhibitors in the particle-free cytosol from hepatocytes. This was shown by adding the ultracentrifuged supernatant leaked from permeabilized hepatocytes to microsomes, where it inhibited ·OH formation (Fig. 4). At a cytosol/microsomal protein ratio of 1.5–2.0, which is approximately the physiological ratio, ·OH formation was almost abolished.

Heating for 5 min at 85 °C moderately diminished the inhibitory potency of the supernatant, though results were variable (Fig. 4). Therefore supernatant was fractionated into low-

Table 2. Rates of lipid peroxidation by microsomes or by permeabilized hepatocytes

Permeabilized hepatocytes ($0.8–1.2 \times 10^6$ cells) or 0.3–0.5 mg of microsomal protein were incubated aerobically at 37 °C with Fe/ADP and an NADPH-generating system at pH 6.8 or 8.0 in 0.15 M-KCl/20 mM-Mops, pH 6.8–7.4, or in 83.5 mM-KCl/37 mM-Tris, pH 8.0.

Hepatocyte rate (nmol/min per 2.5×10^6 cells)		Microsomal rate (nmol/min per mg of protein)	
pH 6.8	pH 8.0	pH 6.8	pH 8.0
1.86±0.9 (6)	3.7±0.95 (6)	1.6±0.4 (7)	2.1±0.4 (7)

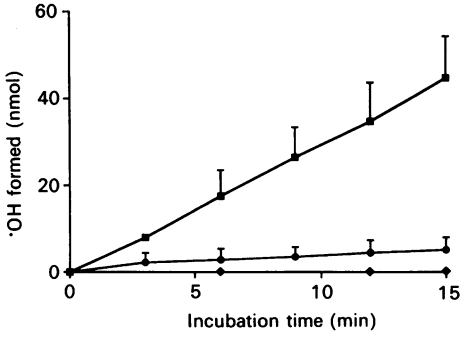

Fig. 3. Formation of ·OH by permeabilized hepatocytes (●) or microsomes (■)

Rates of ·OH formation per 2.5×10^6 hepatocytes (●) are compared with microsomal rates (■) per mg of protein ($n = 4–6$). Zero-time values have been subtracted for clarity. In the absence of NaN_3, hepatocytes formed no detectable ·OH in two experiments (◆).

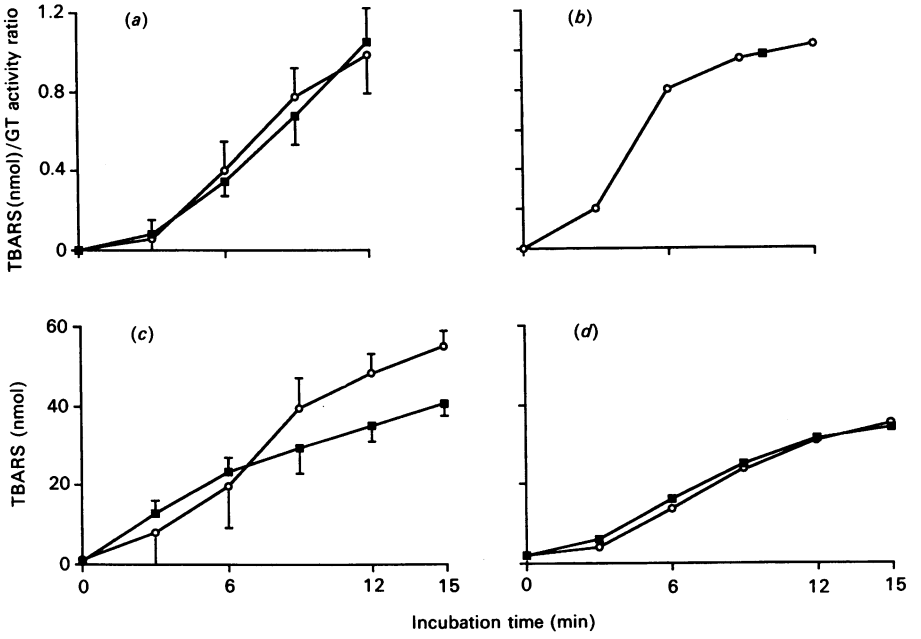

Fig. 2. Lipid peroxidation by permeabilized hepatocytes or microsomes

Formation of TBARS by hepatocytes (○) or microsomes (■) incubated aerobically at 37 °C with NADPH and Fe/ADP is shown. Rates per unit activity of UDP-GT are compared in (*a*) at pH 6.8 ($n = 3$ or 4) and in (*b*) at pH 8.0 ($n = 1$). In (*c*) and (*d*), rates per 2.5×10^6 hepatocytes are compared with microsomal rates/mg of protein in (*c*) at pH 6.8 ($n = 3$ or 4) and in (*d*) at pH 7.4 ($n = 1$). In (*a*), hepatocytes were adjusted to 37.5 mM-KCl and 10 mM-Mops, pH 6.8.

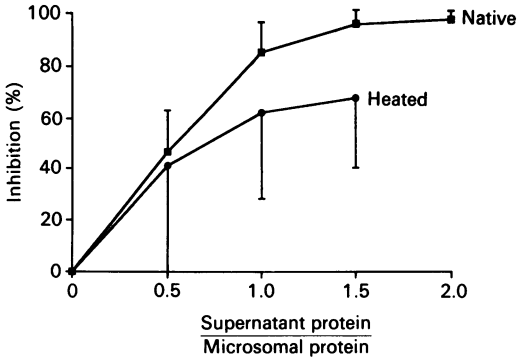

Fig. 4. Inhibition of microsomal 'OH formation by native or heated hepatocyte cytosol

The Figure shows inhibition of microsomal 'OH formation by the soluble cytosolic components leaked from electroporated hepatocytes and frozen at −70 °C for 2 days. Hepatocyte supernatant was added untreated (■) or after (●) heating for 5 min at 85 °C at a range of supernatant/microsomal protein ratios ($n = 3$ or 4).

Table 3. Inhibition of microsomal 'OH formation by GSH or by hepatocyte cytosol

The soluble components from electroporated hepatocytes were treated in various ways and added at cytosol/microsomal protein ratios of 0.6–0.9. Dialysis was for 15 h at 5 °C against 0.15 M-KCl. Cytosol filtrate was prepared by using YM 10 centrifugal filter cones.

	Inhibitor	Inhibition (%) [mean ± S.D. (*n*)]
A	Cytosol frozen < 4 days	81 ± 15 (6)
B	Cytosol frozen 5–12 days	26 ± 14 (9)
C	Cytosol B + 0.15 mM-GSH	82 ± 12 (3)
D	Cytosol A, dialysed	20 ± 8 (3)
E	Dialysed cytosol + 0.15 mM-GSH	90 ± 5 (3)
F	0.15 mM-GSH	16 ± 13 (3)
G	Membrane filtrate of cytosol A	4 ± 3.5 (3)

Table 4. Inhibition of microsomal 'OH formation

With added catalase, NaN_3 was omitted, and resultant inhibitions are calculated relative to controls without NaN_3. In A–E, 'OH-radical-scavenger concentrations were 33 mM; second-order rate constants (K_2) for their reaction with 'OH ($M^{-1} \cdot s^{-1}$) are from Halliwell & Gutteridge (1985) (pp. 26 and 44).

	Inhibitor	Inhibition (%) [mean ± S.D. (*n*)]
	NaN_3 omitted	79 ± 16 (16)
	Catalase ($-NaN_3$)	
	1000–2000 units/ml	90 ± 2 (3)
	133 units/ml	46
	66 units/ml	31
	33 units/ml	13
A	Thiourea ($K_2 = 4.7 \times 10^9$)	84,77
B	Sodium benzoate ($K_2 = 4.3 \times 10^9$)	32,32
C	Sodium formate ($K_2 = 2.7 \times 10^9$)	29 ± 7 (3)
D	n-Butanol ($K_2 = 2.2 \times 10^9$)	23,26
E	Mannitol ($K_2 = 1.0 \times 10^9$)	15 ± 2 (3)

molecular-mass and macromolecular components by centrifugal filtration through a YM10 membrane. The YM10 filtrate ($\leqslant 10$ kDa) was virtually inactive, showing also that sucrose entrained from electroporation did not cause the inhibition (Table 3). However, the retained macromolecules, plus small molecules which did not pass through the filter, still inhibited 'OH formation.

While investigating this inhibition, we found that storing the supernatant for > 4 days even at −70 °C decreased inhibitory potency. However, activity could be fully restored by supplementation with 0.15 mM-GSH (Table 3). Similarly overnight dialysis markedly impaired inhibition, which again was restored with 0.15 mM-GSH (Table 3).

Washed versus unwashed hepatocytes

In support of an important effect of soluble component from permeabilized cells restraining their ability to form 'OH or its equivalent, washing these cells twice to deplete soluble components increased cellular activity 2–3-fold (results not shown).

Evidence that the reactive species detected with DMSO is likely to be 'OH

Table 4 shows that formation of formaldehyde from DMSO by microsomes incubated aerobically with NADPH and Fe/EDTA is strongly NaN_3-dependent, whereas exogenous thymol-free catalase potently inhibited. This is consistent with H_2O_2 being an intermediate, as it is in the iron-catalysed Haber–Weiss reaction. Finally, five documented 'OH scavengers decreased formaldehyde formation from DMSO in the order of their published rate constants for reaction with 'OH. This suggests that the assay indeed measures 'OH, as maintained by Cederbaum & Cohen (1985).

DISCUSSION

The present study illustrates the utility of electroporated hepatocytes for investigating free-radical reactions originating on the ER under more physiological conditions than is possible with microsomes. Permeabilized hepatocytes contain substantially intact ER membranes, whereas this organelle is totally fragmented into resealed vesicles in microsomes. Hepatocytes also retain all of the cytosolic antioxidant protective enzymes and their cofactors, though these will be present at well below physiological concentrations, owing to leakage, unless they are supplemented.

Hepatocytes incubated aerobically with iron/ADP and NADPH showed similar initial rates of lipid peroxidation to microsomes isolated from the same livers. By contrast, despite the presence of a non-physiological iron chelate (Fe/EDTA) and of NaN_3, both of which tend to optimize 'OH formation (Cederbaum & Cohen, 1985), electroporated hepatocytes formed this radical considerably more slowly than did microsomes. In the absence of NaN_3, 'OH could not be demonstrated in hepatocyte incubations over 30 min.

Whether structurally induced increases in electron leakage in microsomes accounts for their greater observable formation of reactive oxygen species is unclear. However, it appears in permeabilized hepatocytes that cytosolic antioxidant protective factors considerably damp 'OH formation, despite being diluted about 38–50-fold in assays containing (3–4) × 10^6 hepatocytes in a volume of 1.5 ml.

Crude fractionation of the components in centrifugal supernatants from permeabilized hepatocytes shows that both macromolecules and small molecules are required for full 'OH inhibitory potency. Depletion of small molecules by dialysis shows that the macromolecular fraction then loses most of its inhibitory capacity, which is restored with GSH, suggesting that GSH-dependent enzyme(s) may account for much of the effect. The

weight of the evidence from the relative thermal stability of the inhibitor(s) in contraindicating an enzymic basis is lessened by the fact that the most plausible candidate enzyme, glutathione peroxidase, is fairly thermostable (Sun *et al.*, 1988).

If glutathione peroxidase were to account for much of the inhibitory effect of the hepatocyte supernatant in damping $^{\cdot}OH$ formation, it would presumably interrupt the following sequence of events:

$$O_2 + e^- \xrightarrow{\text{ER cytochrome } P\text{-450 system}} O_2^{\cdot} \quad (1)$$

$$2O_2^{\cdot} + 2H^+ \xrightarrow{\text{Superoxide dismutase}} H_2O_2 + O_2 \quad (2)$$

$$H_2O_2 + Fe^{2+}/EDTA \longrightarrow {}^{\cdot}OH + OH^- + Fe^{3+}/EDTA \quad (3)$$

$$Fe^{3+}/EDTA \xrightarrow{\text{ER cytochrome } P\text{-450 reductase}} Fe^{2+}/EDTA \quad (4)$$

By interdicting H_2O_2, the peroxidase could then effectively prevent formation of $^{\cdot}OH$.

Some consideration needs to be given to whether the radical reactions in permeabilized hepatocytes incubated aerobically with NADPH are necessarily exclusively of ER origin. It has been assumed in the present study that reducing equivalents channelled into the ER cytochrome *P*-450 system electron-transfer chain have been used to initiate the free-radical reactions investigated. The only other electron-transfer pathway which might be able to use NADPH to initiate free-radical reactions, namely mitochondrial NADPH/NADH transhydrogenase, faces the matrix space and is therefore inaccessible, in sealed mitochondria, to externally added NADPH (Harold, 1986). During lipid peroxidation, however, it may be that amphipathic reactive intermediates formed during the peroxidation of ER membrane lipids could diffuse to other membranes within hepatocytes and initiate lipid peroxidation.

Microsomes were isolated in 5–10 min at $\approx 2000\,g$ by the Ca^{2+}-aggregation technique (Kamath & Rubin, 1972). These gave rates of lipid peroxidation and $^{\cdot}OH$ formation identical with those of microsomes conventionally isolated at $105000\,g$ (results not shown).

In conclusion, electroporated hepatocytes and microsomes appeared comparably susceptible to lipid peroxidation when incubated aerobically with NADPH and Fe/ADP. Insignificant amounts of $^{\cdot}OH$ ($\sim 3\%$ of that with Fe/EDTA) were formed with Fe/ADP. By contrast, permeabilized hepatocytes incubated aerobically with NADPH and Fe/EDTA formed no detectable $^{\cdot}OH$ radical unless azide was also present. Even in the presence of azide, cytosolic antioxidants inhibited $^{\cdot}OH$ formation 80–90%, despite their dilution 38–50-fold in assays. In intact cells, therefore, with cytosolic antioxidants at their physiological concentration and catalase fully active, $^{\cdot}OH$ formation in the presence of decompartmentalized iron must be very stringently inhibited indeed compared with lipid peroxidation. Therefore, if hepatocytes are to be subject to oxidative toxicity caused by intracellular free-radical reactions, damage appears much more likely to arise from lipid peroxidation and less likely to be caused by $^{\cdot}OH$. Reactive oxygen species originating outside hepatocytes, such as from phagocytes, might, however, produce quite a different pattern of damage.

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