Effects of 4-hydroxynonenal on isolated hepatocytes

Studies on chemiluminescence response, alkane production and glutathione status

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The effect of 4-hydroxy-2,3-*trans*-nonenal, a diffusible product of lipid peroxidation, on isolated hepatocytes was evaluated with two non-invasive techniques measuring low-level chemiluminescence and alkane evolution. Oxygen-induced low-level chemiluminescence and ethane and n-pentane formation by hepatocytes is enhanced over 7-fold in the presence of 4-hydroxynonenal (2 mM). Glutathione-depleted hepatocytes show a higher increase than controls in both low-level chemiluminescence and alkane formation upon supplementation with 4-hydroxynonenal. The effects on both parameters are diminished by vitamin E pretreatment of rats and are absent under anaerobiosis. At variance with chemiluminescence and alkane formation, 4-hydroxynonenal does not elicit a concomitant increase in malonaldehyde or diene-conjugate formation. Addition of 4-hydroxynonenal to a suspension of hepatocytes causes a rapid loss of cellular glutathione in the form of a glutathione conjugate with the alkenal as observed with high-pressure liquid-chromatographic analysis. The reaction between glutathione and 4-hydroxynonenal proceeds also spontaneously *in vitro* at 1:1 stoichiometry. The cellular effects of 4-hydroxynonenal evaluated by low-level chemiluminescence and alkane formation are independent of the formation of a glutathione conjugate and seem to rely on the remaining not-bound 4-hydroxynonenal. The sensitivity of 4-hydroxynonenal-enhanced chemiluminescence and alkane formation to free-radical quenchers suggests the participation of a free-radical propagation process.

The process of lipid peroxidation involves free-radical reactions that lead to the formation of diverse lipid hydroperoxides; biological membrane disturbance is generally quoted as the basis for the pathology of lipid peroxidation. Free radicals formed during lipid peroxidation are short-lived, and their effect on targets at a distance from the lipid-peroxidation loci seems unlikely. On the other hand, the non-radical hypothesis for dissemination of toxic substances by lipid peroxidation has received support in recent reports on the formation of diffusible products of peroxidation (see Esterbauer, 1982). These products could induce cytopathological effects over sizeable distances, and are mainly aldehydes, of which 4-hydroxy-2,3-*trans*-

nonenal has probably major importance because it is formed in larger amounts than the other alkenals and because of its potency in exerting cytopathological effects.

The fact that diffusible products can originate from the peroxidative breakdown of membrane fatty acids is supported by several lines of research (Rodgers *et al.*, 1978; Benedetti *et al.*, 1979, 1982). However, the identification of 4-hydroxyalkenals as products of unsaturated fatty acid oxidation goes back to over a decade ago with the discovery that they could inhibit growth of transplanted tumours (Schauenstein & Esterbauer, 1967).

The effects of the hydroxyalkenals on cellular functions are multiple, and they involve the binding of thiol groups either of low-molecular-weight thiol compounds such as glutathione, cysteine, CoA (Esterbauer *et al.*, 1975) or enzymes (Esterbauer *et al.*, 1981), inhibition of cytochrome *P*-450 or microsomal glucose-6-phosphate phosphatase

Abbreviations used: 4-hydroxynonenal, 4-hydroxy-2,3-*trans*-nonenal; h.p.l.c., high-pressure liquid chromatography; GSH, reduced glutathione; GSSG, oxidized glutathione.

(Benedetti *et al.*, 1979), and red-cell haemolysis (Benedetti *et al.*, 1977; Roders *et al.*, 1978).

The effects of synthetic 4-hydroxynonenal on several cell target molecules were similar to those observed with 4-hydroxynonenal isolated from microsomes which underwent lipid peroxidation, and similar aldehydic products were found during carbon tetrachloride and bromotrichloromethane poisoning (Benedetti *et al.*, 1982).

In order to gain more insight on 4-hydroxynonenal-induced cytopathological effects, we have made use of two non-invasive methods that reflect free-radical oxidative reactions, low-level chemiluminescence and alkane evolution. The effect of 4-hydroxynonenal on hepatocytes from normal, glutathione-depleted- and vitamin E-pretreated rats is analysed here by using the information afforded by such methods. Large doses of 4-hydroxynonenal lead to an irreversible loss of cellular glutathione in the form of a glutathione *S*-conjugate.

Materials and methods

Isolation of hepatocytes

Hepatocytes were isolated from rat liver perfused with collagenase (Sies & Grosskopf, 1975). Livers were perfused *in situ* for 15 min with a solution containing 114 mM-NaCl/25 mM-NaHCO₃/5.9 mM-KCl/1.18 mM-MgCl₂/1.2 mM-Na₂SO₄/1.24 mM-NaH₂PO₄/10 mM-glucose/0.5 mM-EGTA (medium I). Perfusate flow was 2.3–4.0 ml/min per g of liver. Effluent perfusate was discarded during the perfusion. The system was closed and liver perfused with clostridial collagenase (60 mg) with a medium as above but which contained in addition 1.24 mM-CaCl₂, 2.1 mM-lactate and 0.3 mM-pyruvate (medium II). After 15 min perfusion the liver was transferred into a beaker and cells dispersed gently with a plastic spatula. About 95% of the cells were intact as judged by Trypan Blue exclusion.

Depletion of hepatic glutathione was obtained by pretreatment of rats with phorone (2,6-dimethyl-hepta-2,5-dien-4-one) at a dose of 250 mg/kg body weight injected into the animal 2 h before preparation of the hepatocytes (Cadenas *et al.*, 1981b). Pretreatment of rats with vitamin E (α -tocopherol) was carried out as described by Pfeifer & McCay (1971); vitamin E was dissolved in 1 vol. of ethanol, followed by the addition of 9 vol. of 16% (v/v) Tween 80 in 0.9% NaCl. Portions (12.5, 37.5 or 55 mg per kg body weight) of α -tocopherol were injected intraperitoneally 15 h before the preparation of hepatocytes.

Low-level chemiluminescence

Low-level chemiluminescence was measured as described previously (Boveris *et al.*, 1981; Cadenas & Sies, 1983). An EMI-9658 photomultiplier,

sensitive in the 350–800 nm range and cooled to –25°C by a thermoelectric cooler, was used. The photomultiplier was connected to an amplifier–discriminator (model 1121A; Princeton Applied Research, Princeton, NJ, U.S.A.) and adjusted for single-photon counting and connected to a recorder. Chemiluminescence was assayed in a 35 mm × 5 mm × 56 mm cuvette maintained at 37°C, and constant stirring was applied during the measuring period. Desired oxygen concentrations in the cuvette were obtained by gassing with a gas mixture of oxygen and nitrogen through a gas proportioner. Additions to the hepatocyte suspension were made from outside of a light-tight box through thin polyethylene tubing.

Alkane evolution

The experiments were carried out in 43 ml sealed flasks which were capped with a PVC [poly(vinyl chloride)] septum impermeable to alkanes. Isolated hepatocytes were preincubated for 5 min in order to remove contaminating alkanes in the incubation medium. Samples (5 ml) were withdrawn with gas-tight Hamilton syringes every 30 min from the head space above the hepatocyte suspension and rapidly substituted with 5 ml of alkane-free oxygen. Ethane and n-pentane determination in the samples was analysed by gas chromatography with a Porasil C column at 80°C (Müller *et al.*, 1981; Wendel & Dumelin, 1981). The amounts of alkanes were calculated by means of a calibration gas mixture (Müller *et al.*, 1981) and expressed as pmol of alkane/10⁸ cells.

H.p.l.c.

H.p.l.c. analysis samples were deproteinized with equal volumes of 1 M-HClO₄ and a 0.5 ml sample was used for derivatization. Samples were treated with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene to form the *S*-carboxymethyl and *N*-dinitrophenyl derivatives respectively from thiol-group-containing amino acids and peptides as described by Reed *et al.* (1980). Samples ranging between 20 and 100 μ l were applied to a μ -Bondapak-NH₂ column. The analyses were carried out with a Waters h.p.l.c. system (model 720; automatic sample processor WISP 710B; two pumps, M45 and 6000 A). Elution solvents were: 200 ml of solvent A (154 g of sodium acetate, 122 ml of water and 378 ml of acetic acid) and 800 ml of solvent B (methanol/water, 4:1, v/v). Elution was started with an isocratic period of 80% B and 20% A for 5 min. Then a hyperbolic gradient until 100% A (completed within 15 min) was started; 100% A was held for a further 15 min. Elution was finished after 35 min and the column was equilibrated under starting conditions. Total flow was 3 ml/min during the whole time of chromatography. Glutathione in hepatocytes was identified and

quantified by comparing the retention times as well as the integrator response with those of standards of known concentrations.

[glycine- ^3H]GSH was reduced with 0.2% NaBH_4 before use (see Table 2 below).

Spectrophotometric assays

Total glutathione, i.e. the sum of GSH + 2GSSG expressed as GSH equivalents, was estimated with the oxidation of 5,5'-dithiobis(2-nitrobenzoic acid) in a reaction mixture containing 0.25 mM-NADPH, 0.1 unit of glutathione reductase/ml, and hepatocyte suspension in an Eppendorf photometer at 405 nm (Akerboom & Sies, 1981). GSH was measured by monitoring the glutathione transferase-catalysed formation of *S*-(2,4-dinitrophenyl)glutathione conjugate with 1-chloro-2,4-dinitrobenzene (Brigelius *et al.*, 1983). The reaction was monitored at 340–400 nm ($\Delta\epsilon = 6.9 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).

Malonaldehyde formation was measured by the thiobarbituric acid assay (Bernheim *et al.*, 1948) at 535–570 nm ($\Delta\epsilon = 156 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). Diene-conjugate formation was detected as described by Buege & Aust (1978); the increase in A_{233} was recorded and an absorption coefficient of $25.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ was used.

Assay conditions

For alkane evolution and low-level chemiluminescence experiments, hepatocytes (2.1×10^6 cells/ml) were incubated in medium II (as described above, but containing no EGTA) under constant stirring and oxygenation. The temperature was 37°C . In some cases h.p.l.c. analysis, glutathione and chemiluminescence measurements were performed in parallel; in these experiments, samples were withdrawn from the hepatocyte suspension in the photon-counter cuvette and processed.

Chemicals

Chemicals were from Merck (Darmstadt, Germany) and biochemicals from Boehringer (Mannheim, Germany). A solution of 4-hydroxynonenal in chloroform was evaporated at 30°C under vacuum and constant rotation (Rotavapor-R; Büchi, Flawil, Switzerland). The residue was diluted in water and kept in ice until use. The concentration of 4-hydroxynonenal in water was measured at 224 nm ($\epsilon = 13\,750 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

Results and discussion

4-Hydroxynonenal-induced low-level chemiluminescence

We have previously reported that isolated hepatocytes (Cadenas *et al.*, 1981b) or tissue homogenates (Cadenas *et al.*, 1981a) under constant oxygenation yield low-level chemiluminescence, which is reflect-

ing lipid peroxidation (as detected by malonaldehyde accumulation). The increase of the rate of chemiluminescence is preceded in both cases by a lag phase, apparently related to the presence of cellular defences that have to be overcome. When hepatocytes under these conditions are supplemented with 4-hydroxynonenal, a decrease of duration of the lag phase and an enhancement in chemiluminescence intensity over control levels are observed (Fig. 1). Total light intensity is linearly related to the concentration of 4-hydroxynonenal (Fig. 1, inset), and at 2 mM-4-hydroxynonenal a 7–8-fold increase over control levels is observed. Depletion of cellular glutathione by pretreatment of rats with phorone produces a larger chemiluminescence signal in controls (Fig. 2) as well as malonaldehyde formation (Cadenas *et al.*, 1981b). Supplementation of glutathione-depleted hepatocytes with 4-hydroxynonenal also enhances light emission (about 4.2-fold increase over controls with 2 mM-4-hydroxynonenal). The time course of the reaction did not significantly differ whether the hepatocytes were depleted of glutathione or not (Figs. 1 and 2).

Fig. 1. Effect of 4-hydroxynonenal on oxygen-induced low-level chemiluminescence of isolated hepatocytes. Assay conditions: isolated hepatocytes (2.1×10^6 cells/ml) were incubated under constant oxygenation in the absence (control) and presence of different concentrations (shown against the curves) of 4-hydroxynonenal. Inset: dependence of chemiluminescence on 4-hydroxynonenal concentration; values correspond to 50 min incubation.

Fig. 2. Effect of 4-hydroxynonenal on oxygen-induced low-level chemiluminescence of glutathione-depleted hepatocytes

Assay conditions were as in Fig. 1.

Pretreatment of rats with vitamin E 15 h before the preparation of hepatocytes decreased chemiluminescence in both controls and 4-hydroxynonenal-supplemented hepatocytes. This inhibition was about 50% when rats were injected with 12.5 mg of vitamin E/kg (Fig. 3a), whereas inhibition was almost complete with 56 mg of vitamin E/kg (results not shown). This inhibited chemiluminescence might be attributed to the known free radical quenching effect of vitamin E. Furthermore, addition of a free-radical quencher, cyanidanol, to a hepatocyte incubation, causes an inhibition of the 4-hydroxynonenal-enhanced chemiluminescence (half-maximal inhibitory effect at about 1.4 μ M-cyanidanol) (Fig. 3b).

The 4-hydroxynonenal-induced chemiluminescence is an oxygen-dependent process. Under anaerobiosis there was no chemiluminescent signal, whereas substituting oxygen for nitrogen re-established the light emission.

Alkane evolution

Generation of hydrocarbons has long since been related to lipid peroxidation, on the basis of evidence arising from either autooxidation of fatty acids (Riely *et al.*, 1974; Dumelin & Tappel, 1978), or microsomal or cellular preparations under oxidative stress (Kapus & Muliawan, 1982; Smith *et al.*, 1982), or during

the perfusion of organs (Müller *et al.*, 1981) as well as *in vivo* (Riely *et al.*, 1974; Burk & Lane, 1978). During the oxygenation of hepatocytes in control experiments, modest amounts of ethane production can be observed. Ethane formation increases 7–8-fold over controls upon supplementation with 4-hydroxynonenal (Fig. 4). 4-Hydroxynonenal-induced n-pentane formation is 2.5-fold higher than that of ethane.

In agreement with the observations on chemiluminescence, pretreatment of rats with phorone in order to deplete cellular glutathione induces an enhancement of ethane formation in control cells (Fig. 5). 4-Hydroxynonenal-induced ethane and n-pentane formation by glutathione-depleted cells is about 4.5-fold higher than control values (similar to what was observed with low-level chemiluminescence). Controls from glutathione-depleted hepatocytes did not show any n-pentane either, whereas it appeared on supplementation of these cells with the alkenal.

Finally, pretreatment of rats with vitamin E leads to a significant decrease in ethane and n-pentane formation in 4-hydroxynonenal-supplemented hepatocytes (Fig. 6).

Effect of 4-hydroxynonenal on malonaldehyde and diene conjugate formation by hepatocytes

Chemiluminescence observed with either glutathione-depleted- or control hepatocytes in the absence of 4-hydroxynonenal correlates well with the formation of malonaldehyde (Wright *et al.*, 1979; Cadenas *et al.*, 1981a,b; Smith *et al.*, 1982). A relationship between generation of ethane and n-pentane and lipid peroxidation has also been established. However, the higher chemiluminescence intensity and alkane evolution observed in the presence of 4-hydroxynonenal under both conditions do not correlate with a parallel increase in malonaldehyde accumulation, neither do they with an increase in diene-conjugate formation (Table 1).

Effect of 4-hydroxynonenal on the glutathione status of the cell

In the absence of 4-hydroxynonenal, hepatocytes incubated under oxygenation show a decrease of reduced glutathione (results not shown) which is recovered in the form of GSSG, as judged by the unmodified total cellular glutathione level (Fig. 7). In the presence of 4-hydroxynonenal, reduced glutathione is rapidly decreased, but it is not recovered in the form of GSSG (Fig. 7). This rapid decrease of total glutathione cannot be prevented by either pretreatment of rats with vitamin E or under anaerobiosis, two conditions which exerted a strong inhibitory effect on chemiluminescence (Fig. 3a) and alkane evolution (Fig. 6).

The fact that cellular glutathione seems to be completely depleted by 4-hydroxynonenal at these

Fig. 3. Effect of vitamin E (α -tocopherol) pretreatment of rats and cyanidanol on 4-hydroxynonenal-enhanced chemiluminescence of isolated hepatocytes

Assay conditions: (a) hepatocytes (2.1×10^6 cells/ml) from control or vitamin E-pretreated rats were incubated under constant oxygenation in the absence and presence of 2 mM 4-hydroxynonenal. (b) Assay conditions were as in (a). Control hepatocytes were incubated with various amounts of cyanidanol.

Fig. 4. Alkane evolution of isolated hepatocytes incubated under aerobiosis in the absence and presence of 4-hydroxynonenal

Assay conditions were as in Fig. 1 and the Materials and methods section.

Fig. 5. Alkane evolution by glutathione-depleted hepatocytes in the absence and presence of 2 mM 4-hydroxynonenal.

Assay conditions were as in Figs. 1 and 2 and the Materials and methods section.

Fig. 6. Alkane evolution by isolated hepatocytes from vitamin E-pretreated rats in the absence and presence of 2 mM-4-hydroxynonenal. Assay conditions were as in Figs. 1 and 3.

Fig. 7. Effect of 4-hydroxynonenal on total cellular glutathione (2GSSG + GSH).

Assay conditions: hepatocytes (2.1×10^6 cells/ml) were incubated under constant oxygenation in the absence and presence of 1 mM-4-hydroxynonenal. Samples for glutathione (the sum of 2GSSG + GSH) determination were withdrawn at the time points indicated. Other assay conditions were as described in the Materials and methods section.

Fig. 8. H.p.l.c. traces of free glutathione (GSH) and glutathione conjugate.

Retention times of 8.6 and 17.0 min correspond to derivatized samples of glutathione conjugate and free glutathione respectively. (a) 4-Hydroxynonenal (1 mM) in water, injection volume 100 μ l; (b) 60 μ M-GSH in water, injection volume 100 μ l; (c) mixtures of 0.5 mM-GSH and 0.4 mM-4-hydroxynonenal in 0.1 M-potassium phosphate buffer, pH 7.4; injection volume 20 μ l; (d, e) extract obtained from a suspension of 2.1×10^6 hepatocytes/ml in the absence (d) and presence (e) of 120 μ M-4-hydroxynonenal.

concentrations, and without the mediation of glutathione oxidation, as it takes place under different conditions of oxidative stress [e.g. redox cycling of paraquat (Brigelius *et al.*, 1982); oxidative metabolism of organic hydroperoxides (Akerboom *et al.*, 1982)], points to the formation of a glutathione conjugate with 4-hydroxynonenal. Antecedents favouring this possibility rely on the high affinity of the compound for thiol groups, ranging from soluble low-molecular-weight thiol compounds to thiol-group-containing proteins (Esterbauer *et al.*, 1981).

Conjugate formation between GSH and 4-hydroxynonenal was studied by using the h.p.l.c. method developed by Reed *et al.* (1980). 4-Hydroxynonenal alone did not show a peak in the chromatogram (Fig. 8a), whereas GSH showed a distinct peak at 17 min retention time (Fig. 8b). Incubation of glutathione with 4-hydroxynonenal resulted in a decrease of GSH and in the occurrence of a new peak eluting at a retention time expected for a glutathione conjugate (Fig. 8c).

Injection of a portion of derivatized supernatant

of acid-treated hepatocytes showed a distinct glutathione peak corresponding to a 70 μM solution, with only minor amounts of endogenous conjugates (Fig. 8d). Incubation of hepatocytes from this preparation with 120 μM 4-hydroxynonenal led to an almost total loss of glutathione concomitant with an increase of the conjugate (Fig. 8e).

The stoichiometry of conjugate formation was examined by chromatography with radiolabelled GSH *in vitro*. As Table 2 shows, the loss of radioactivity of GSH corresponded to 7.8 nmol and was accompanied by an increase in radioactivity of glutathione in the conjugate by an amount corresponding to 8.0 nmol. Further, the absorbance of the derivatized *S*-conjugate increased by an amount corresponding to 8.3 nmol when the loss of GSH corresponded to 8.7 nmol (Table 2), and the amount of 4-hydroxynonenal decreased correspondingly, as measured by absorbance at 224 nm (not shown). Thus the stoichiometry is 1:1 as would be the case if glutathione were added to the 3,4 double bond of 4-hydroxynonenal.

Table 1. *Effect of 4-hydroxynonenal on low-level chemiluminescence, alkane, malonaldehyde and diene-conjugate formation by isolated hepatocytes*

Assay conditions were as in Fig. 1. Values of the different parameters are standardized for 10^8 cells and given at 0 min and after 40 min of incubation with 2 mM 4-hydroxynonenal, and are representative of three different hepatocyte preparations.

Assay	Time (min)	Control	4-Hydroxynonenal	4-Hydroxynonenal
				Control
Low-level chemiluminescence [counts $\cdot$ s $^{-1}$ $\cdot$ (10 8 cells) $^{-1}$]	0	1870	1870	
	40	3750	25900	
	Increase (40–0)	1880	24000	12.7
Ethane production [pmol $\cdot$ (10 8 cells) $^{-1}$]	0	0	0	
	40	5	60	
	Increase (40–0)	5	60	12.0
Malonaldehyde formation [nmol $\cdot$ (10 8 cells) $^{-1}$]	0	78	175	
	40	156	261	
	Increase (40–0)	79	86	1.1
Diene-conjugate formation [nmol $\cdot$ (10 8 cells) $^{-1}$]	0	162	295	
	40	434	614	
	Increase (40–0)	272	319	1.2

Table 2. *Incorporation of ^3H from [glycine- ^3H]GSH into *S*-conjugate with 4-hydroxynonenal in vitro*

Radioactivity and relative units of integrator response from h.p.l.c. separation for the retention times of glutathione conjugate (8.6 min) and glutathione (17.0 min) are compared. Values in parentheses correspond to the amount of glutathione or glutathione *S*-conjugate in nmol. Values show the results of one experiment typical of three.

Retention time (min) ...	Radioactivity (c.p.m.)		Integrator response (relative units)	
	8.6	17.0	8.6	17.0
[^3H]GSH (12.5 nmol)	None	26 100 (12.5)	None	18.0 (12.5)
[^3H]GSH + 4-hydroxynonenal (12.5 nmol + 50 nmol) (1 min incubation)	16 700 (8.0)	9900 (4.7)	12.0 (8.3)	5.5 (3.8)

Conclusions

The present study shows that externally added 4-hydroxynonenal can elicit an enhancement of low-level chemiluminescence and alkane production (notably n-pentane) by hepatocytes under constant oxygenation. Both non-invasive methods give an insight into free-radical reactions associated with peroxidation of lipids. However, chemiluminescence can also be observed in systems that do not undergo lipid peroxidation, e.g., during redox cycling of paraquat (Cadenas *et al.*, 1983). The observation that no extra amounts of malonaldehyde or diene-conjugate formation are elicited by 4-hydroxynonenal makes a strict relationship between chemiluminescence and alkane evolution, on the one hand, and lipid peroxidation, on the other, unlikely. It should be mentioned, however, that malonaldehyde and diene conjugate are assayed at the nanomolar level, whereas the alkanes and chemiluminescence reflect compounds at the picomolar level. At present, we are not able to suggest a mechanism that would account for both alkane formation and chemiluminescence but which is not related to the lipid-peroxidation process.

Depletion of glutathione by formation of a glutathione conjugate seems to be unrelated to the production of alkanes or low-level chemiluminescence by hepatocytes. The hypothesis that a rapid loss of glutathione would decrease cellular defences and concomitantly increase oxidative stress (thereby increasing chemiluminescence and alkane production) seems not feasible in view of the following experimental evidence: (a) chemiluminescence and alkane release are enhanced in glutathione-depleted hepatocytes (Figs. 2 and 5); (b) vitamin E pretreatment of rats abolishes chemiluminescence and decreases ethane and n-pentane production by 4-hydroxynonenal significantly (Figs. 3a and 6) and does not affect the decrease of glutathione by the alkenal; (c) whereas in anaerobiosis 4-hydroxynonenal-induced chemiluminescence and alkane production are inhibited, 4-hydroxynonenal-dependent glutathione depletion proceeds as usual.

It is also noteworthy that observing chemiluminescence and ethane formation relies on a high concentration of 4-hydroxynonenal over that of cellular glutathione. In this way only free or non-bound nonenal would be capable of producing the effects that give rise to chemiluminescence and alkane evolution. Other aldehydes, such as acetaldehyde, propionaldehyde or benzaldehyde, also elicited ethane release from perfused liver (Müller & Sies, 1982, 1983). Under normal conditions the cellular glutathione concentration varies between 3 and 10 mM; minute amounts of 4-hydroxynonenal, which, for example, would be formed in a localized peroxidation process, would have a very short half-life by reacting with cellular glutathione. It was

calculated that the half-life of 4-hydroxynonenal in a solution containing 3 mM-glutathione was about 300 s (Esterbauer *et al.*, 1981). Under our experimental conditions, total hepatocyte glutathione in the assay mixture varied between 80 and 150 nmol/ml; concentrations of added nonenal over this value were able to elicit a significant chemiluminescence signal and alkane release. Therefore the higher chemiluminescence and ethane production observed with glutathione-depleted hepatocytes could be interpreted as the effects produced by a larger amount of free 4-hydroxynonenal.

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