Iron loading of cultured hepatocytes

Effect of iron on 5-aminolaevulinate synthase is independent of lipid peroxidation

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Cultured chick embryo hepatocytes were iron-loaded with ferric nitrilotriacetate. Iron-loading was confirmed by both quantitative cellular iron determinations and ultrastructural studies. With iron-loading, lipid peroxidation, as detected by malonaldehyde released into the medium, occurred at a linear rate for 12 h, after which time the rate of malonaldehyde production decreased. No cell toxicity, as detected by lactate dehydrogenase release, was noted. The amount of malonaldehyde recovered in the medium after 18 h of exposure to iron represented 24–33% of the total malonaldehyde that could be produced by incubating lysed cells with iron and ascorbate. Cellular glutathione was not affected by iron-stimulated lipid peroxidation, but was increased by allylisopropylacetamide. Although iron-loading by itself had no effect on activity of 5-aminolaevulinate synthase, the first and rate-limiting step in haem synthesis, iron-loading in the presence of the porphyrogenic drug allylisopropylacetamide increased levels of 5-aminolaevulinate synthase 6-fold over levels induced by the drug alone. The antioxidant, butylated hydroxytoluene, totally inhibited iron-stimulated lipid peroxidation, but did not interfere with the effect of iron-loading to potentiate an increase in 5-aminolaevulinate synthase. After 18 h of exposure to iron, followed by a change to fresh medium, the iron remaining within the cells did not stimulate further lipid peroxidation over the following 18 h, but did potentiate an increase in 5-aminolaevulinate synthase on exposure to allylisopropylacetamide. It therefore appears that lipid peroxidation is not the mechanism by which iron potentiates induction of hepatic 5-aminolaevulinate synthase.

One of the effects of iron excess in experimental animals is the induction of hepatic 5-aminolaevulinate synthase, the first and rate-limiting enzyme for haem synthesis (De Matteis & Sparks, 1973; Ibrahim *et al.*, 1981; Bonkowsky *et al.*, 1981). Iron is also well known to potentiate the induction of hepatic 5-aminolaevulinate synthase by porphyrogenic drugs such as allylisopropylacetamide (De Matteis & Sparks, 1973; Bonkowsky *et al.*, 1981; Stein *et al.*, 1970). The mechanism by which iron promotes these effects on 5-aminolaevulinate synthase remains unclear, but may involve depletion of a regulatory haem pool due to either decreased synthesis of haem or increased breakdown of haem (Bonkowsky *et al.*, 1979a).

Lipid peroxidation, catalysed by iron, could be involved in the effect of iron on hepatic 5-amino-

laevulinate synthase (Bonkowsky *et al.*, 1979a). When added to mitochondrial and microsomal suspensions, iron rapidly catalyses peroxidation of unsaturated lipids with extensive membrane damage (Hunter *et al.*, 1963; Wills, 1969; Pederson & Aust, 1975; Kornbrust & Mavis, 1979). Such damage to membranes or membrane-bound enzymes could lead to depletion of a regulatory haem pool by decreasing activity of certain enzymes in the haem synthetic pathway. Alternatively, depletion of regulatory haem might be secondary to increased haem catabolism, a phenomenon that has been associated with iron-stimulated lipid peroxidation in microsomal preparations (Levin *et al.*, 1973). However, since intact cells possess several means of protection against peroxidative damage (Chance *et al.*, 1979; Tappel, 1980; Kappus & Sies, 1981; Flohe, 1982), it is not yet clear whether iron-excess causes pathogenically important lipid peroxidation *in vivo*.

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione.

Although lipid peroxidation has been detected in iron-loaded rats by measuring expired ethane (Dillard & Tappel, 1979; Dougherty *et al.*, 1981), it is not certain that the source of ethane was hepatic. Other workers using isolated rat hepatocytes exposed to various forms of iron have also detected lipid peroxidation by various means (Hogberg *et al.*, 1975; Stacey & Priestley, 1978; Dianzani *et al.*, 1981; de Ruiter *et al.*, 1982; Stacey & Kappus, 1982), but these cells may have lacked normal protection against lipid peroxidation due to limited viability (Tanaka *et al.*, 1978) or selenium deficiency (Newman & Guzelian, 1982).

In order to study the effects of iron-loading and lipid peroxidation on 5-aminolaevulinate synthase, we used primary cultures of chick hepatocytes. These cells survive for several days in culture and maintain inducibility of both 5-aminolaevulinate synthase (Granick *et al.*, 1975) and several species of cytochrome *P*-450 (Althaus *et al.*, 1979). In the present paper, we show that treatment of chick embryo hepatocytes with the chelate ferric nitrilotriacetate leads to substantial iron-uptake and lipid peroxidation without toxicity. We find that iron-loading markedly potentiates the increase in 5-aminolaevulinate synthase activity by the porphyrinogenic drug allylisopropylacetamide. However, our results show that lipid peroxidation is not the underlying mechanism for this effect of iron. Levels of cellular GSH were also measured, since this thiol is important for protection against damage by oxidants (Chance *et al.*, 1979; Tappel, 1980; Kappus & Sies, 1981; Flohe, 1982).

Portions of this work have appeared previously in abstract form (Shedlofsky *et al.*, 1982a,b).

Experimental

Hepatocyte cultures

Primary cell cultures from livers of 16-day White Leghorn chicken embryos were prepared as described previously (Sinclair *et al.*, 1982). After 20 h, the cells were rinsed twice with Williams E medium (Flow Laboratories, McLean, VA, U.S.A.) without insulin and, after a third medium change, chemicals were added. Cultures were maintained in either 3.5 cm- or 6.0 cm-diameter culture dishes (Corning, Corning, NY, U.S.A.).

Chemicals used

Ferric citrate was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Conalbumin-iron complex [23% (w/w) iron/protein], trisodium nitrilotriacetate, butylated hydroxytoluene, butylated hydroxyanisole, thiobarbituric acid, *o*-phthalaldehyde, GSH, Triton X-100 and insulin were all obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Malonaldehyde bis(dimethyl-

acetal) was obtained from Aldrich (Milwaukee, WI, U.S.A.). 2-Allyl-2-isopropylacetamide and 2-propyl-2-isopropylacetamide were gifts from Hoffmann-LaRoche Laboratories (Nutley, NJ, U.S.A.). Iron-dextran was a gift from Fisons (Holmes Chapel, Cheshire, U.K.). Desferrioxamine mesylate was obtained from Ciba-Geigy (Summit, NJ, U.S.A.).

Stock ferric nitrilotriacetate was prepared by the method of White & Jacobs (1978) by dissolving ferric citrate and nitrilotriacetate in water to final concentrations of 5 mM and 10 mM respectively and adjusting the pH of the mixture to 7.4 with solid NaHCO₃. The spectrum of the complex formed had a peak at 469 nm. Nitrilotriacetate was a 10 mM aqueous solution adjusted to pH 7.4. Ferric pyridoxal isonicotinoyl hydrazone was prepared by the method of Ponka *et al.* (1979).

Assays

Quantitative cellular iron determinations were made by using the method of Barry & Sherlock (1973) on cells rinsed twice with saline and scraped into iron-free tubes.

Release of lactate dehydrogenase activity into the medium was used as a measure of cell toxicity. Lactate dehydrogenase was assayed by the method of Wroblewski & Ladue (1955), and activity was calculated from a standard curve obtained with purified enzyme (bovine heart; Worthington, Freehold, NJ, U.S.A.). To determine total cellular lactate dehydrogenase content, cultures were exposed to 0.2% (w/v) Triton X-100 for 1 h, a treatment previously shown to lyse all cells without inhibiting the enzyme (Sinclair *et al.*, 1981). None of the chemicals used interfered with the enzyme assay or decreased the total enzyme present.

Malonaldehyde, assayed by a modification of the method of Buege & Aust (1977), was used as a measure of lipid peroxidation. Either medium or cells (scraped off the plate into saline) were mixed with an equal volume of an aqueous solution containing thiobarbituric acid (0.5%, w/v), trichloroacetic acid (20%, w/v), HCl (0.25 M) and butylated hydroxytoluene (0.02%, w/v, added in ethanol). A blank for each sample was prepared by mixing medium or pellet suspension with the same solution not containing thiobarbituric acid. Samples and blanks were heated at 80°C for 15 min, cooled and centrifuged. Malonaldehyde was measured by scanning the sample versus blank supernatants on an Aminco DW2 spectrophotometer in the split-beam mode and measuring the difference in absorbance between the peak at 532 nm and a flat area of the spectrum at 610 nm. The blank for each sample ensured that the malonaldehyde peak was not affected by changes in phenol red, which absorbs at 506 nm in the acid solution and is present in the Williams E medium. Malonaldehyde, prepared

from malonaldehyde bis(dimethylacetal) by the method of Burger *et al.* (1980), gave a $\Delta A_{\text{mM}, 533\text{nm} - 610\text{nm}}$ of $160\text{mM}^{-1}\cdot\text{cm}^{-1}$. This ΔA was used to calculate malonaldehyde concentrations.

Cellular GSH content was determined by using the fluorimetric method of Hissin & Hilf (1976) and total cellular glutathione (GSH + GSSG) was determined by the enzymic method of Tietze (1969) as modified by Anderson & Meister (1980).

5-Aminolaevulinate synthase activity was assayed, as previously described (Sinclair & Granick, 1977), using intact cells. We have recently found that when this assay is performed on cells previously incubated in medium without insulin, formation of 5-aminolaevulinate is linear only within the first 2 h (P. R. Sinclair, personal communication). We have therefore modified the assay to a 1 or 2 h incubation as indicated. Cytochrome P-450 was measured as previously described (Sinclair *et al.*, 1979) and proteins were assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Microscopy

Cultures were viewed routinely with a Nikon Inversion Microscope with phase-contrast optics. Prussian Blue staining of cells for iron was performed after fixing the cells with 70% ethanol. Cells were prepared for transmission electron microscopy as described by Pettengill *et al.* (1980) and viewed on a Phillips 201 electron microscope.

Results

Accumulation of iron by cultured hepatocytes

Cellular iron content was measured after treatment of the cells with several different iron preparations (Fig. 1). Cultures not exposed to iron complexes contained 22 nmol of Fe/3.5 cm plate. Treatment with $100\mu\text{M}$ -ferric nitrilotriacetate resulted in the highest Fe accumulation (140 nmol of Fe/3.5 cm plate). With $90\mu\text{M}$ -ferric citrate, 55 nmol of Fe accumulated, but with higher concentrations toxicity was noted, as judged by loss of cells adhering to the culture plate and a lower cellular iron content per plate. Ferric pyridoxal isonicotinoyl hydrazone ($200\mu\text{M}$) caused accumulation of 52 nmol of Fe without evidence of toxicity, but higher concentrations could not be tested because of difficulty in keeping the complex soluble in the medium. Neither iron-dextran nor iron-saturated conalbumin (1–5 mg/ml of medium; results not shown) increased cellular iron above control levels. Because $100\mu\text{M}$ -ferric nitrilotriacetate optimally iron-loaded cells, we used this complex for further studies.

Prussian Blue staining of cells iron-loaded with ferric nitrilotriacetate showed abundant iron within the hepatocytes with only minimal staining of

Fig. 1. Iron uptake by cultures of chicken embryo hepatocytes exposed to various iron-complexes

Cultured hepatocytes on 3.5 cm plates were prepared as described in the Experimental section and exposed to increasing concentrations of iron for 18 h. Each plate contained 0.45–0.55 mg of protein. Iron determinations were carried out as described in the Experimental section after removing the medium and rinsing the cells twice with saline. ●, Ferric nitrilotriacetate; ○, ferric citrate; ■, ferric pyridoxal isonicotinoyl hydrazone; □, iron-dextran. Each point is the mean of two plates, whose values varied by less than 10%.

fibroblasts. Transmission electron microscopy revealed that iron-exposed cells, but not control cells, contained numerous electron-dense bodies, 6–7 nm in diameter, characteristic of ferritin cores. These structures were distributed throughout the cytoplasm, frequently aggregated in large vesicles, and were absent from mitochondria and nuclei.

Lipid peroxidation stimulated by iron

After exposure of cultures to $100\mu\text{M}$ -ferric nitrilotriacetate, malonaldehyde, a product of lipid peroxidation (Hunter *et al.*, 1963; Wills, 1969; Pederson & Aust, 1975; Kornburst & Mavis, 1979), was readily detected in the medium (Fig. 2). When assayed at 18 h, the amount of malonaldehyde present in the medium was 10 times greater than that recovered in the cells. Therefore, malonaldehyde in the medium was used to measure lipid peroxidation. Medium malonaldehyde increased linearly with time up to 12 h, then began to reach a plateau. Ascorbic acid ($100\mu\text{M}$) initially enhanced the rate of malonaldehyde production by iron, but by 18 h caused no increase in malonaldehyde compared with iron alone. Addition of iron to medium removed from 24 h-old control cultures did not cause generation of malonaldehyde. Chemically

Fig. 2. Time course of iron-stimulated malonaldehyde production in hepatocyte cultures treated with and without ascorbic acid

Cultures on 3.5 cm plates were exposed to chemicals for the times indicated, and malonaldehyde was determined in the medium as described in the Experimental section. ●, Ferric nitrilotriacetate (100 μM); ○, 100 μM-ferric nitrilotriacetate + 100 μM-ascorbic acid. Each point shows total malonaldehyde/plate (mean ± S.D.) of three plates; if no error bar is shown, then the S.D. fell within the symbol. □, 100 μM-Ascorbic acid alone in a separate culture experiment (mean of duplicate plates).

synthesized malonaldehyde remained stable in the medium when added to control cultures (results not shown). Production of malonaldehyde by intact cells required incubation in medium since cultures incubated in buffered saline with iron (with or without ascorbic acid) for 18h produced only 9–15% as much malonaldehyde as when incubated in complete medium.

When cells exposed to 100 μM-iron for 18h were rinsed, scraped into 2 ml of water and the cell lysate incubated with an additional 0.2 μmol of iron and 0.4 μmol of ascorbic acid for 3h, two to three times more malonaldehyde was produced (16.3 nmol) than was already formed by the intact iron-exposed cells and recovered in the medium. Although this experiment did not determine whether the malonaldehyde produced from the cell lysate represented breakdown of all the peroxidizable lipid, it did confirm that the intact cells exposed to iron still contained lipids that could be a source of malonaldehyde.

Fig. 3 shows that in the first 18h of iron exposure, the amount of malonaldehyde produced was dependent on the concentration of iron. However, if the same iron-treated cultures were then rinsed and incubated in fresh medium with 100 μM-iron for a second 18h period (Fig. 3), much less malonaldehyde was generated, the amount being inversely

Fig. 3. Malonaldehyde production in hepatocyte cultures exposed to increasing concentrations of ferric nitrilotriacetate (FeNTA) on the first day of iron exposure (●) and subsequent malonaldehyde production in the same cultures exposed to iron the next day (○)

Hepatocyte cultures on 3.5 cm plates were exposed to increasing concentrations of iron for 18h and malonaldehyde was determined on portions of medium as described in the Experimental section. Cells were then rinsed twice, fresh medium added and then all cultures were exposed to 100 μM iron for a second 18h period, after which time malonaldehyde was again determined. If no iron was added on the second day to a culture iron-loaded on the first day, malonaldehyde production was only slightly above control values (≤ 0.4 nmol). Each point shows total malonaldehyde/plate (mean ± S.D. of three plates; if no error bar is shown, then S.D. fell within symbol).

related to the amount of malonaldehyde produced during the first 18h of iron exposure. Cells iron-loaded during the first 18h period (with 100 μM-ferric nitrilotriacetate) and not exposed to any further iron during the second 18h period produced almost no further malonaldehyde (0.7 ± 0.1 , compared with a control value of 0.4 ± 0.2 nmol/plate) even though the cells still contained 140 nmol of Fe/plate (Fig. 1).

The antioxidants butylated hydroxytoluene and butylated hydroxyanisole both inhibited iron-stimulated lipid peroxidation (Table 1). Complete inhibition of malonaldehyde generation occurred with either 227 μM butylated hydroxytoluene or 67 μM butylated hydroxyanisole.

We also studied iron-stimulated lipid peroxidation in the presence of the porphyrinogenic drug allylisopropylacetamide, since one of our main interests was to determine whether lipid peroxidation was involved in the ability of iron to potentiate induction of 5-aminolaevulinic synthase by this

Table 1. *Effect of antioxidants and allylisopropylacetamide (AIA) on iron-stimulated lipid peroxidation*

Cells on 3.5 cm plates (0.4–0.5 mg of cell protein) were exposed to various chemicals for 18 h and malonaldehyde measured in the medium as described in the Experimental section. The concentration of ferric nitrilotriacetate (FeNTA) was 100 μ M with respect to Fe and 200 μ M with respect to nitrilotriacetate; and nitrilotriacetate alone (NTA) was 200 μ M. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were added in 5 μ l of ethanol. This amount of ethanol alone had no effect on malonaldehyde accumulation. Values are means $\pm$ S.D. of three plates or duplicate values.

Treatment	Malonaldehyde accumulation (nmol/plate)
NTA	0
FeNTA	4.7 $\pm$ 0.1
FeNTA + BHT (156 μ M)	0.7 $\pm$ 0.5
FeNTA + BHT (227 μ M)	0
FeNTA + BHA (34 μ M)	0.7, 0.8
FeNTA + BHA (67 μ M)	0
NTA + AIA (0.14 or 1.77 mM)	0
FeNTA + AIA (0.14 mM)	4.1 $\pm$ 0.3
FeNTA + AIA (1.77 mM)	4.2 $\pm$ 0.1
FeNTA + AIA (1.77 mM) + BHT (227 μ M)	0

drug. As shown in Table 1, iron, in the presence of either a low or a high concentration of allylisopropylacetamide, caused malonaldehyde generation, but both concentrations of this drug decreased iron-stimulated malonaldehyde by 10%. Butylated hydroxytoluene inhibited iron-stimulated malonaldehyde production in the presence of allylisopropylacetamide.

Lipid peroxidation in intact cells exposed to iron was not associated with obvious toxic changes in cell morphology as indicated by phase microscopy. Transmission electron microscopy showed no alterations in membrane structure of either mitochondria or endoplasmic reticulum. However, there was an increase in cytoplasmic vacuolation, a finding that has been noted previously in iron-loaded rat liver (Bonkowsky *et al.*, 1979b).

To quantify cellular damage, we measured release into the medium of the intracellular enzyme, lactate dehydrogenase. As shown in Fig. 4, iron exposure did not cause enzyme release even with the addition of ascorbic acid to enhance lipid peroxidation (see Fig. 2). Addition of the antioxidant butylated hydroxytoluene, however, was associated with some cell toxicity. Greater cell toxicity was noted when butylated hydroxytoluene was administered in combination with 0.14 mM-allylisopropylacetamide or 100 μ M iron. Allylisopropylacetamide, at the higher concentration of 1.77 mM, however, consistently prevented the toxicity caused by butylated hydroxy-

Fig. 4. *Release of lactate dehydrogenase (LDH) caused by exposure of hepatocyte cultures to ferric nitrilotriacetate (FeNTA), nitrilotriacetate (NTA), allylisopropylacetamide (AIA), antioxidants and ascorbic acid* Lactate dehydrogenase released into medium was determined as described in the Experimental section from cultured cells on 3.5 cm plates exposed to chemicals for 18 h. Antioxidants used were butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). Total lactate dehydrogenase activity determined by using control cultures exposed to 0.2% Triton X-100 for 1 h was 2.05 units. The means of duplicate plates are presented and all duplicates were within 5% of the mean. *In other experiments, butylated hydroxytoluene alone caused up to 62% release of total lactate dehydrogenase.

toluene, without affecting the antioxidant capacity of this agent.

Potentiation by iron of drug-mediated induction of 5-aminolaevulinate synthase

As shown in Table 2, under the assay condition used, 5-aminolaevulinate synthase activity was below the level of detection in control cells or in cells exposed to 100 μ M-ferric nitrilotriacetate alone. When the cultures were exposed to either propylisopropylacetamide or allylisopropylacetamide, both of which induced cytochrome *P*-450 in the culture (Table 2) (Althaus *et al.*, 1979; Sinclair *et al.*, 1979), activity of 5-aminolaevulinate synthase rose to detectable levels. The effect of iron-loading was to increase induction of 5-aminolaevulinate synthase activity 6–13-fold when the cultures were simultaneously exposed to iron and

Table 2. Effect of iron on 5-aminolaevulinate synthase activity and levels of cytochrome P-450 in cultures exposed to allylisopropylacetamide (AIA) and propylisopropylacetamide (PIA)

Activity of 5-aminolaevulinate synthase was determined as described in the Experimental section using cultures on 3.5 cm plates exposed to chemicals for 18 h. In a parallel experiment, concentrations of cytochrome P-450 were measured as described in the Experimental section using cultures on 6 cm plates. Results are means $\pm$ s.d. of three plates. **P* values for the one-tailed *t* test on the cytochrome P-450 concentrations with or without iron are 0.1 to 0.3 and are therefore not considered significant. Concentrations of ferric nitrilotriacetate (FeNTA) and nitrilotriacetate (NTA) were as given in Table 1.

Treatments	5-Aminolaevulinate synthase (nmol/mg of protein per h)	Cytochrome P-450 (pmol/mg of protein)
No AIA		
NTA	<0.3	37 $\pm$ 4
FeNTA	<0.3	38 $\pm$ 3
AIA (0.07 mM) + NTA	0.8 $\pm$ 0.6	278 $\pm$ 28
+ FeNTA	5.2 $\pm$ 0.8	241 $\pm$ 16*
AIA (0.14 mM) + NTA	0.8 $\pm$ 0.1	
+ FeNTA	6.9 $\pm$ 0.2	
AIA (0.36 mM) + NTA	0.7 $\pm$ 0.2	221 $\pm$ 15
+ FeNTA	9.6 $\pm$ 1.6	197 $\pm$ 5*
AIA (1.77 mM) + NTA	2.1 $\pm$ 0.2	137 $\pm$ 6
+ FeNTA	12.3 $\pm$ 0.1	117 $\pm$ 5*
PIA (0.14 mM) + NTA	0.9 $\pm$ 0.1	344 $\pm$ 33
+ FeNTA	11.1 $\pm$ 1.3	295 $\pm$ 30*

either propylisopropylacetamide or allylisopropylacetamide for 18 h. As shown in Fig. 5, the 5-aminolaevulinate synthase induced by the combination of 1.77 mM-allylisopropylacetamide and iron increased linearly for 20 h. In contrast, the increase in 5-aminolaevulinate synthase produced by exposure to 1.77 mM-allylisopropylacetamide alone was less, and reached a plateau by 8 h or earlier.

To test whether lipid peroxidation was involved in the mechanism by which iron potentiated the induction of 5-aminolaevulinate synthase by allylisopropylacetamide, we added the antioxidant butylated hydroxytoluene (227 μ M) to cultures exposed to 1.77 mM-allylisopropylacetamide and 100 μ M-iron. As shown in Fig. 5, the antioxidant had no effect on the potentiation of 5-aminolaevulinate synthase activity mediated by iron, even though lipid peroxidation was completely prevented (Table 1).

To determine whether 5-aminolaevulinate synthase induction by allylisopropylacetamide could be potentiated by iron 'sequestered' after 18 h of iron-loading, cells were exposed to 100 μ M-iron for 18 h, rinsed with fresh medium and then exposed to allylisopropylacetamide for a second 18 h period. As shown in Table 3, 'sequestered' iron was even better than freshly added iron in potentiating induction of 5-aminolaevulinate synthase activity (7.8 compared with 5.5 nmol of 5-aminolaevulinate/mg of protein per h). Lipid peroxidation was not detected during the induction of 5-aminolaevulinate synthase with 'sequestered' iron.

Effect of iron on cytochrome P-450 levels

As shown in Table 2, both propylisopropyl-

Fig. 5. Lack of effect of butylated hydroxytoluene on synergism by iron of drug-mediated induction of 5-aminolaevulinate synthase

Time course of 5-aminolaevulinate synthase activity in cultures on 3.5 cm plates exposed to 1.77 mM-allylisopropylacetamide and either nitrilotriacetate (open symbols), or 100 μ M-ferric nitrilotriacetate (filled symbols). ○ and ●, No antioxidant; □ and ■, 227 μ M-butylated hydroxytoluene. 5-Aminolaevulinate production in 2 h was determined as described in the Experimental section. Each point is the mean $\pm$ s.d. of three plates. (If no error bar is shown, s.d. was within the symbol.) After 20 h exposure to iron and allylisopropylacetamide, 4.2 nmol of malonaldehyde accumulated; butylated hydroxytoluene prevented malonaldehyde production.

Table 3. 5-Aminolaevulinate synthase activity in cultured cells iron-loaded either before or simultaneously with allylisopropylacetamide (AIA) exposure

5-Aminolaevulinate synthase activity was determined as described in Table 2 after exposure to 100 μ M-ferric nitrilotriacetate (FeNTA) and 0.14 mM-allylisopropylacetamide (AIA) for the times indicated. After the first 18 h period, cultures were rinsed twice and fresh medium added before chemical exposure for the second 18 h period. (Approx. 3–4 h elapsed before the second addition of chemicals.) Each value is the mean $\pm$ S.D. for three plates.

Chemical exposure during:		5-Aminolaevulinate synthase (nmol/mg of protein per h) after:	
First 18 h	Second 18 h	First 18 h	Second 18 h
None	FeNTA and AIA	<0.4	5.5 $\pm$ 0.5
FeNTA	AIA	<0.4	7.8 $\pm$ 0.5
FeNTA and AIA	None	4.3 $\pm$ 0.5	2.0 $\pm$ 0.1

acetamide and allylisopropylacetamide increased levels of cytochrome P-450 after 18 h of exposure. Iron caused a slight decrease in levels of cytochrome P-450 induced by propylisopropylacetamide or allylisopropylacetamide, but the decreases did not reach statistical significance.

Effects of ferric nitrilotriacetate on GSH

Intracellular GSH, important for protection against damage by oxidants (Chance *et al.*, 1979; Tappel, 1980; Kappus & Sies, 1981; Flohe, 1982), was measured to see whether iron-loading and iron-catalysed lipid peroxidation affected the concentration of this thiol. As shown in Table 4, at 18 h, iron alone had little effect on GSH levels despite stimulation of lipid peroxidation (Fig. 1). At no time during iron exposure did GSH levels fall (measured at 0.5, 1.5, 3, 6 and 12 h after addition of iron; results not shown). Allylisopropylacetamide, at all concentrations tested, approximately doubled the GSH content of the cells by 18 h and time courses revealed no initial fall. In the presence of both iron and allylisopropylacetamide, GSH levels were further increased by 2.6–3.2-fold over levels of untreated controls (Table 4).

In several experiments, the antioxidants butylated hydroxytoluene and butylated hydroxyanisole were added to see if the increase in GSH produced by allylisopropylacetamide or the further increase caused by iron with allylisopropylacetamide would be abolished by inhibiting lipid peroxidation. Both antioxidants alone, however, caused increases in cellular GSH of 1.4–1.6-fold (results not shown), and it was therefore difficult to interpret any effect of inhibiting lipid peroxidation. If the antioxidants were given in combination with allylisopropylacetamide and/or iron, increases of 1.9–3.6-fold over those in untreated cells were seen.

The GSH levels given above were measured by using the fluorimetric method (Hissin & Hilf, 1976). To corroborate this method, in some experiments the enzymic assay for total glutathione was also performed (Tietze, 1969). The pattern and extent of

Table 4. Effect of allylisopropylacetamide (AIA) and iron on cellular GSH

GSH was determined as described in the Experimental section using the fluorimetric assay. Cells on 3.5 cm plates were exposed to chemicals for 18 h. The values (mean $\pm$ S.D.) are results from ten separate experiments. Data are expressed as percentages of control values because there was variation among experiments in the absolute concentration of cellular GSH. The cellular GSH concentration in controls for the ten experiments was 15.3 $\pm$ 5.3 (mean $\pm$ S.D.). The concentrations of ferric nitrilotriacetate and nitrilotriacetate were as given in Table 1.

	Cellular GSH (% of control)	
	Nitrilotriacetate	Ferric nitrilotriacetate
AIA	100 $\pm$ 12	113 $\pm$ 20
AIA (0.14 mM)	196 $\pm$ 36	258 $\pm$ 55
AIA (0.71 mM)	205 $\pm$ 31	320 $\pm$ 93
AIA (1.77 mM)	212 $\pm$ 4	297 $\pm$ 15

change in total glutathione seen with the enzymic assay was similar to that found with the fluorimetric assay. There was no increase in total GSH with iron, a 2.4-fold increase with allylisopropylacetamide (0.14 mM) and a 3.2-fold with the drug and iron.

Discussion

Iron-loading of cultured cells

Chick embryo hepatocytes in culture were readily iron-loaded by using the nitrilotriacetate chelate of ferric iron (Fig. 1). This chelate is known to increase the iron and ferritin content of cultured Chang cells and to do so to levels greater than those obtained with saturated transferrin (White & Jacobs, 1978). In the chick cultures other iron complexes, including saturated conalbumin, were less effective than ferric nitrilotriacetate in iron-loading. Both Prussian Blue staining, showing hepatocyte-associated iron, and transmission electron microscopy, showing an

abundance of ferritin with formation of siderosomes, confirmed iron uptake.

Iron-stimulated lipid peroxidation in cultured cells

Lipid peroxidation, as measured by malonaldehyde generation, was readily detected in cultured hepatocytes exposed to iron (Figs. 2 and 3). Although the assay for malonaldehyde has been criticized for not accurately reflecting lipid peroxidation in intact animals (Plaa & Witschi, 1976), a number of workers have recently found that malonaldehyde production by isolated rat hepatocytes correlated well with other assays for lipid peroxidation, including alkane evolution (Gee & Tappel, 1981; Smith *et al.*, 1982; Stacey & Kappus, 1982), fluorescence (Koster *et al.*, 1981; Smith *et al.*, 1982) and chemiluminescence (Smith *et al.*, 1982). In our studies using chick embryo hepatocytes, malonaldehyde in the medium provided a simple and sensitive test for lipid peroxidation. Because the chick embryo hepatocytes remained viable and intact for several days, we were able to follow the course of iron-stimulated lipid peroxidation over longer periods than has been done previously, and thereby correlate lipid peroxidation with other effects of iron.

As shown in Fig. 4, iron-stimulated lipid peroxidation, even when enhanced with ascorbic acid, was not associated with cell toxicity as assayed by either lactate dehydrogenase release or morphological changes seen on microscopy. Several groups, using isolated rat hepatocytes exposed to ADP-Fe complexes (Stacey & Priestly, 1978; Ekstrom & Hogberg, 1980; Gee & Tappel, 1981) or agents that deplete cellular GSH (Stacey & Klaasen, 1981, 1982), have also found poor correlations between lipid peroxidation and cellular toxicity. Therefore, it should not be assumed that cellular lipid peroxidation necessarily leads to cell toxicity.

With the intact chick embryo hepatocytes, the amount of malonaldehyde produced with iron exposure was much greater when the cells were incubated in complete medium rather than in saline, suggesting that cellular metabolism was required for malonaldehyde production, possibly for iron transport into the cell. The amount of malonaldehyde produced by the intact cell, however, was only 24–33% of the total malonaldehyde produced by incubation of lysed cells with iron and ascorbic acid. Malonaldehyde formation has been found to correlate with a 60–80% loss of arachidonate and docosahexenoate from microsomal membrane phospholipid (Jordan & Schenkman, 1982). If we assume that malonaldehyde derives from the same sources in the cultured cells used in the present study, our results suggest that 20–25% of arachidonate and docosahexenoate can be lost by peroxidation from membrane phospholipids without measurable damage to the cells.

As shown in Fig. 2, the rate of malonaldehyde production declined after 12 h of exposure to iron, either with or without ascorbic acid. If cells were then re-exposed to iron, little additional malonaldehyde was generated (Fig. 3), even though two to three times as much peroxidizable lipid was still present as determined by the cell lysate studies. A number of factors may be responsible for these findings. Iron-loading may have increased or activated some type of inhibitor of lipid peroxidation, although the production of such an inhibitor does not appear to require elevated GSH levels (see below). It is also possible that the rate of iron uptake by the cells may have decreased as the cells become iron-loaded. In addition, with time, iron may have become 'sequestered' by being incorporated into ferritin or other intracellular components and rendered unable to stimulate lipid peroxidation. In support of this latter possibility, cells that had been iron-loaded, washed and then incubated in fresh medium generated almost no malonaldehyde, even though approx. 140 nmol of iron/plate was still present within the cells. This finding would seem to suggest that only newly acquired iron is involved in lipid peroxidation. The concept of a short-lived 'metabolically active' iron pool has been considered previously (Bonkowsky *et al.*, 1981). However, as discussed below, the iron sequestered after 18 h of iron exposure still potentiated induction of 5-aminolaevulinate synthase (Table 3), even though it no longer stimulated lipid peroxidation. Therefore, iron may be transformed through several metabolic pools, each of which exerts various effects on such processes as lipid peroxidation and haem synthesis.

The antioxidants butylated hydroxytoluene and butylated hydroxyanisole were found to inhibit malonaldehyde production when added to the culture with iron (Table 1) and could, therefore, be used to assess the involvement of lipid peroxidation in the effect of iron on 5-aminolaevulinate synthase activity (see below). In some experiments, butylated hydroxytoluene proved toxic at concentrations required to completely prevent lipid peroxidation (Fig. 4). However, the presence of the higher concentration of allylisopropylacetamide (1.77 mM) by an unknown mechanism prevented toxicity with 227 μ M-butylated hydroxytoluene without affecting this agent's antioxidant capacity.

Effects of iron-loading and allylisopropylacetamide on GSH levels

Although some workers using the ADP-Fe complex in isolated rat hepatocytes found lower levels of GSH with iron-stimulated lipid peroxidation (Hogberg *et al.*, 1975), others have not confirmed these findings (Stacey & Priestly, 1978) and we did not find lower levels of cellular GSH in iron-loaded chick hepatocyte cultures (Table 4).

Since GSH is important for protection against damage by oxidants, it is important to distinguish between (1) experiments in which a decrease in cellular GSH as the 'primary' event leads to lipid peroxidation (Power *et al.*, 1977; Stacey & Priestly, 1978; Ekstrom & Hogberg, 1980; Stacey & Klaasen, 1981, 1982) and (2) experiments such as ours in which lipid peroxidation is the 'primary' event. In the latter case, our findings show that iron-stimulated lipid peroxidation itself does not lower cellular GSH levels.

Allylisopropylacetamide was found to increase GSH levels in the absence of detectable lipid peroxidation. The increase in GSH was linear without any initial decrease (results not shown). Although allylisopropylacetamide has been reported to decrease hepatic GSH slightly when administered to rats (Edwards *et al.*, 1978; Maines, 1980), perhaps due to the formation of an adduct between the drug and GSH, our results in the cultured hepatocytes differ for reasons we do not understand. Iron-loading plus exposure to allylisopropylacetamide was associated with a further increase in GSH levels over levels increased by the drug alone. It could not be determined, however, whether this effect of iron was related to lipid peroxidation, because the antioxidants themselves affected GSH levels.

The effects of iron on hepatic 5-aminolaevulinate synthase induction

It has been known for some time that iron-loading *in vivo* potentiates the induction of 5-aminolaevulinate synthase in animals exposed to porphyrogenic drugs (Stein *et al.*, 1970; De Matteis & Sparks, 1973; Bonkowsky *et al.*, 1981). In the present studies, we demonstrate that iron-loading with ferric nitrilotriacetate of cultured hepatocytes exposed to the drugs propylisopropylacetamide and allylisopropylacetamide also markedly potentiated induction of 5-aminolaevulinate synthase (Table 2). These findings clearly show that this potentiation is an effect of iron directly on the liver.

The regulation of hepatic 5-aminolaevulinate synthase activity has been postulated to involve a regulatory haem pool for which there is strong circumstantial evidence (Bonkowsky *et al.*, 1979a, 1981). A fall in the amount of haem in this regulatory pool would lead to induction of 5-aminolaevulinate synthase. Both propylisopropylacetamide and allylisopropylacetamide increased levels of cytochrome P-450 in the cultured hepatocytes (Table 2), and presumably caused some depletion of haem in the regulatory pool, as synthesis of new cytochrome P-450 utilized available haem. Iron-loading may have potentiated the induction of 5-aminolaevulinate synthase by causing a further depletion of haem in the regulatory

pool. One possible way iron could do this is by stimulating lipid peroxidation, which could theoretically block haem synthesis at some point, or cause an increase in haem catabolism (Levin *et al.*, 1973; Bonkowsky *et al.*, 1979a, 1980). Our data, however, clearly show that iron-stimulated lipid peroxidation in the intact cells bears no relationship to the effect of iron to potentiate the induction of 5-aminolaevulinate synthase. Butylated hydroxytoluene, which totally inhibited lipid peroxidation in the culture, did not alter the effect of iron on 5-aminolaevulinate synthase (Fig. 5). Furthermore, when iron was 'sequestered' within the cell, and failed to cause detectable lipid peroxidation, iron still potentiated 5-aminolaevulinate synthase induction by allylisopropylacetamide, and did so even better than when the drug and iron were administered simultaneously (Table 3).

Although lipid peroxidation does not explain the effect of iron to potentiate 5-aminolaevulinate synthase induction, it is still likely that iron causes depletion of haem in the regulatory pool by either blocking haem synthesis, possibly at the uroporphyrinogen decarboxylase step (Kushner *et al.*, 1975), or by increasing haem catabolism, probably via increased haem oxygenase (Maines & Kappas, 1976; Ibrahim *et al.*, 1981). Previous data from our group (Bonkowsky *et al.*, 1980) using rats treated with ferric citrate, and preliminary data using the iron-loaded chick embryo hepatocyte culture (Shedlofsky *et al.*, 1982b), suggest that iron in the presence of allylisopropylacetamide causes an increase in haem breakdown without a block of haem synthesis.

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