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KINETIC EVALUATION OF FREE MALONDIALDEHYDE AND ENZYME LEAKAGE AS INDICES OF IRON DAMAGE IN RAT HEPATOCYTE CULTURES

INVOLVEMENT OF FREE RADICALS

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Abstract—The present study relates to the effect of ferric iron supplementation on lipid peroxidation of adult rat hepatocyte pure cultures. Lipid peroxidation was evaluated by free malondialdehyde (MDA) using size exclusion chromatography (HPLC) as a specific and sensitive method. The ferric iron used under its complexed form with nitrilotriacetic acid (NTA) exhibited a prooxidant activity corresponding to an increase of free MDA recovery in the cells and in the culture medium. This enhancement of lipid peroxidation in the hepatocyte cultures supplemented with ferric iron was correlated with an intracellular enzyme leakage (lactate dehydrogenase and transaminase), suggesting that lipid peroxidation and enzyme release represented good parameters for cytotoxicity evaluation. The toxic effect of Fe-NTA on hepatocyte cultures was a function of the incubation time (from 0 to 48 hr) and of the concentration of ferric iron loading (i.e. 5, 20 and 100 μM). The mechanism by which Fe-NTA induced cellular damage involved free radical production, as increasing amounts of free radical scavengers corresponded to diminishing rates of both total free MDA and enzyme release. However, this reducing capacity varied from one scavenger to another, where they exhibited preferentially a decrease in lipid peroxidation or in enzyme leakage. This suggested a dissociation between the two parameters of cytotoxicity considered. Lipid peroxidation corresponding to alterations of both inner membranes and the plasma membrane, whereas enzyme release mainly corresponded to the damage of plasma membrane. Subsequently, some scavengers (superoxide dismutase, mannitol, α tocopherol, β carotene) presented an intracellular activity, as they reduced mostly lipid peroxidation. Other ones (catalase, dimethylpyrroline *N*-oxide, thiourea) seemed essentially efficient in protecting the external plasma membrane, as shown by an important decrease in enzyme leakage.

Intraperitoneal injection of ferric iron from ferric nitrilotriacetate (Fe-NTA) induced significant iron deposition in rat hepatic cells [1, 2]. Excess of iron deposition in the liver characterizes the hepatic disease hemochromatosis [3]; moreover, fibrosis and cirrhosis occur after many years of iron overload. Despite clinical evidence for the liver toxicity of excess hepatocellular iron, the role of iron in the pathogenesis of liver injury has not yet been fully established experimentally [2, 4, 5].

Although iron is well known to induce lipid peroxidation [6], the relationship between lipid peroxidation and iron damage was not clearly elucidated. Is lipid peroxidation one of the contributing factors of iron injury or a secondary phenomenon?

The mechanism whereby iron stimulates lipid peroxidation seemed to involve the intermediate formation of oxygen free radical species such as $OH^{\cdot}$ or $O_2^{\cdot-}$ produced either by the Fenton reaction, or by the Haber–Weiss reaction or both [7]:

$$O_2^{\cdot-} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^{\cdot} + OH^- \text{(Fenton)}$$

$$H_2O_2 + O_2^{\cdot-} \xrightarrow{Fe^{3+}/Fe^{2+}} O_2 + OH^{\cdot} + OH^- \text{(Haber–Weiss)}.$$

Fe^{3+}/Fe^{2+} ratio is an important factor for the initiation of lipid peroxidation and the interconversion $Fe^{2+} \longleftrightarrow Fe^{3+}$ is necessary. When iron is used under its ferric form like Fe(III)-NTA, lipid peroxidation initiation is possible since small amounts of Fe^{2+} are always present in the ferric solution [8].

During the Haber–Weiss reaction, ferric iron is converted into ferrous iron by superoxide anion which in turn reacts with hydrogen peroxide to produce hydroxyl radicals.

Hydroxyl radicals could initiate lipid peroxidation and thus causes membrane cell injury [9] by two different manners [10]: (i) directly, by causing structural modifications in membrane like changes in membrane lipid organization and fluidity [11]; and (ii) indirectly, by producing toxic products from lipid hydroperoxide decomposition especially oxygen free

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radicals and aldehydes such as malondialdehyde (MDA) and 4-hydroxyalkenals, in particular 4-hydroxynonenal [12]. All these compounds could combine with cellular targets such as DNA, proteins and lipids.

The aims of this work were: (i) to demonstrate that Fe-NTA is able to induce lipid peroxidation in adult rat hepatocyte pure cultures; (ii) to observe if lipid peroxidation can be directly correlated to an hepatocyte damage considering the effect of Fe-NTA on lactate dehydrogenase and transaminase leakage; and (iii) to clarify the mechanism of the Fe-NTA induction of lipid peroxidation on hepatocyte cultures and the role of free radical scavengers.

Lipid peroxidation was estimated by measurement of MDA, a by-product of lipid hydroperoxide decomposition. MDA was usually evaluated by the thiobarbituric (TBA) method which recovered the dosage of "TBA reactive substances," since other aldehydes reacted with TBA. More recently, some workers have developed a new method using HPLC to measure only free MDA which is a sensitive index for lipid peroxidation evaluation [13]. We have applied this technique to follow lipid peroxidation in rat hepatocyte cultures loaded with ferric iron (Fe-NTA). The kinetic of free MDA formation was determined in the cells as well as in the culture medium after various supplementations of Fe-NTA.

MATERIAL AND METHODS

Reagents

1,1,3,3-Tetramethoxypropane, superoxide dismutase (SOD) from bovine erythrocytes, catalase from bovine liver, β carotene, 5,5-dimethylpyrroline *N*-oxide (DMPO) and cytochalasin B were obtained from the Sigma Chemical Co. (St Louis, MO). α Tocopherol was provided by Hoffmann-Laroche (Neuilly-sur-Seine, France). Mannitol, thiourea and methylamine were purchased from Prolabo (Paris, France).

The ferric nitrilotriacetate solution (Fe-NTA) was prepared according to the method of White and Jacobs [14]. Briefly, 470 mg nitrilotriacetate acid disodium salt (Sigma) were dissolved in 100 mL of sterile water with a consequent drop in pH of about 2. Then 200 mg ferric ammonium citrate (Merck, Darmstadt, F.R.G.) were added and after complete solubilization of the ferric iron nitrilotriacetate complex the pH was adjusted to 7 with sodium bicarbonate. The final concentration of ferric iron was 10 mM and the molar ratio nitrilotriacetic acid-ferric iron was 2/1.

In order to study the kinetics of iron incorporation into the cells, 10 μL ^{59}Fe ferric chloride (13 μCi/mg Fe; Radiochemical Centre, Amersham, U.K.) were added to 10 mL of ferric nitrilotriacetate solution. Addition of 200 μL of ^{59}Fe Fe-NTA solution to 100 mL of culture medium gave a final iron concentration of 20 μM. The ^{59}Fe Fe-NTA solution was sterilized before use by passage on a 0.22 μm filter.

Cell isolation and culture

Adult rat hepatocytes were isolated from 2-month-old Sprague–Dawley rats by cannulating the portal vein and perfusing the liver with a collagenase solution, as previously described [15]. The cells were collected in Leibovitz medium containing, per mL: 1 mg bovine serum albumin and 5 μg bovine insulin. Cell suspension was filtered on gauze and allowed to sediment for 20 min in order to eliminate cell debris, blood and sinusoidal cells. The cells were then washed three times by centrifugation at 150 *g*, tested for viability and counted. Then, the hepatocytes were suspended in a mixture of 75% Eagle's minimum essential medium and 25% medium 199 with Hank's salts, supplemented with 10% fetal calf serum and containing, per mL: kanamycin (50 μg), streptomycin (50 μg), penicillin (7.5 I.U.), bovin insulin (5 μg), bovine serum albumin (1 mg) and $NaHCO_3$ (2.2 mg). Usually 2.5×10^6 hepatocytes were plated in 25 cm^2 Nunclon flasks corresponding to *ca.* 200 μg of protein/mL of culture medium. The medium was changed 3–4 hr later, renewed the day after with the same medium as above but deprived of serum and supplemented with 10^{-7} M dexamethasone. For experimental purposes, some cultures were maintained in the presence of nitrilotriacetic acid alone (NTA) or ferric iron nitrilotriacetate (Fe-NTA) in order to obtain final iron concentrations of 5, 20 and 100 μM. More precisely, each sample with Fe-NTA was compared to control cultures without any supplementation and to cultures supplemented with 200 μM NTA which is the higher amount of NTA added to the cultures in the experimental procedure.

Free MDA evaluation

HPLC procedure. MDA quantification was performed according to a method described previously [16]. The HPLC system (LDC Milton Roy) was equipped with a Spherogel-TSK G1000 PW size exclusion column 7.5 mm i.d. × 30 cm (Cluzeau, France). The eluant was composed of 0.1 M disodium phosphate buffer, pH 8 at a flow rate of 1 mL/min, at ambient temperature. The absorbance was monitored at 267 nm and the sensitivity was set at 0.05 AUFS (absorbance units full scale). The injections were performed by an automatic injector (LDC Promis) set at a volume of 250 μL. The data were recorded and integrated by a CI 3000 LDC integrator.

Preparation of free MDA standard. Five mL of 1,1,3,3-tetramethoxypropane were hydrolysed in 5 mL of 0.1 N HCl during 5 min in boiling water. This solution was then diluted 1000 times in 0.01 M Na_2HPO_4 buffer pH 7.45, corresponding to a 6 μM MDA solution. The concentration of MDA in samples was calculated using a standard curve of free MDA.

Preparation of the samples for HPLC analysis. Free MDA was quantified from culture medium and from hepatocytes.

Culture media were collected and hepatocytes were washed twice with 0.01 M phosphate buffer, pH 7.45. They were resuspended in 1 mL of the same buffer. The cells were lysed using an ultrasonic homogenizer. An aliquot was stored at −17° until the protein content was estimated.

The samples (culture media or cell homogenates) were filtered through 500 daltons membrane ultrafilter (Millipore, France) in a 10 mL Amicon (U.S.A.) cell pressurized at 4 bars with nitrogen gas. The

filtrate was used for the HPLC procedure. All experiments were performed at least on triplicate cultures. Protein content was determined on defrozen cell homogenates according to the Bradford reaction [17], using the Bio-Rad reagent, bovine serum albumin serving as standard and performed by a Cobas-Bio automatic analyser.

Determination of enzyme leakage

The eventual damage of cultured hepatocytes after different incubation times with various amounts of Fe-NTA was evaluated by measuring lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) both in the cells and in the culture medium. These assays were performed using Roche kit procedures (France) and a Cobas-Bio automatic analyser. The limit of sensitivity of the assays was about 0.4 mI.U./mL.

Free radical scavengers supplementation

Superoxide dismutase (SOD), catalase, mannitol and thiourea were dissolved in Hepes buffer. β Carotene and α tocopherol were solubilized in dimethylsulfoxide (DMSO). Dimethylpyrroline *N*-oxide (DMPO) was prepared according to Floyd *et al.* [18] and was diluted in Hepes buffer. These scavengers were added to 100 μM Fe-NTA supplemented culture medium, at a final concentration of 100, 500, 1000 and 1500 I.U./mL for SOD; 100, 1000 and 10,000 I.U./mL for catalase; 10, 25 and 50 mM for mannitol; 50, 100 and 500 mM for thiourea; 1.8, 4.5 and 9 mM for β carotene; 250, 500 and 1000 μM for α tocopherol; and 3.2, 16 and 32 mM for DMPO.

The cells were cultured for 5 hr in the absence or presence of these various reagents and the samples containing SOD or catalase were compared to control cultures containing the corresponding boiled enzyme.

At the end of incubation, the evaluation of free MDA and of LDH release was performed as described above.

Addition of inhibitors of endocytosis

Cytochalasin B and methylamine were used as inhibitors of endocytosis; they were respectively dissolved in DMSO and Hepes buffer and were provided to the cultures at a final concentration of 25 μg/mL for cytochalasin B and 40 mM for methylamine. Fe-NTA (100 μM) alone or in the presence of SOD (500 I.U./mL) or catalase (10,000 I.U./mL) was added 15 min after the addition of one of the above inhibitors of endocytosis. The total amount of MDA present in the cultures and the magnitude of LDH release into the culture medium were determined following 5 hr exposure.

RESULTS

Iron overload

The ability of iron under its complexed form with NTA to enter the cells is shown by Fig. 1. Labelled iron penetration in the hepatocytes occurred very early during the first hour of treatment. Iron concentration increased progressively in the cells, was more than duplicated in 3 hr and was 10 times higher after 48 hr. These results were in agreement with our previous report [19], where iron quantification was obtained in cell pellets by flameless atomic spectrophotometry and iron overload was confirmed by the staining method of Tirmann-Schmelzer [20] which consisted firstly in the reduction of ferric ions by ammonium sulfur, and secondly by a blue staining of ferrous ions with a mixture of potassium ferricyanide and hydrochloric acid.

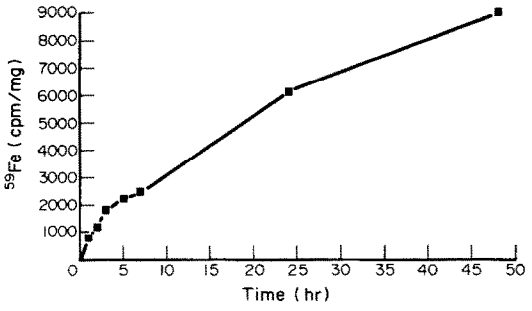

Fig. 1. Kinetics of incorporation of iron into the cells. Hepatocytes in culture were incubated during various times with 20 μM ^{59}Fe Fe-NTA. Results are means ± SD of four experiments.

MDA evaluation

Determination of free MDA in the cells. Upon the addition of iron, free MDA in the cells increased immediately and reached a maximum during the first hours of incubation, then the concentration of MDA in the cells decreased rapidly until a stabilized value (Fig. 2a). This phenomenon was more marked with 100 μM than with 5 and 20 μM Fe-NTA. The increase of intracellular free MDA was positively correlated with the amount of iron supplemented while control cells, with or without NTA, only presented a weak level of MDA.

Determination of free MDA in culture medium. The concentration of MDA increased rapidly in the culture medium and reached a maximum after 12 hr of incubation with 5, 20 and 100 μM Fe-NTA (Fig. 2b). After this time the MDA value slightly decreased until 24 hr of treatment and remained at a stable level. As noted in the cells, the extracellular free MDA increased with the concentration of Fe-NTA.

Determination of total free MDA produced by hepatocytes. Total amount of free MDA corresponding to MDA in the cells and MDA in the culture media depended on time and on the concentration of iron added to the cultures (Fig. 2c). For example, the increase of total MDA corresponding to 5, 20 and 100 μM Fe-NTA represented, respectively, 5, 20 and 55 times the amount found in controls at 5 hr incubation time and 4, 12 and 45 times after 12 hr of incubation.

Approximately 95% of total MDA were found in the extracellular medium (Fig. 2b and c).

Enzyme leakage determination

As lipid peroxidation is often associated to cellular damage, enzyme leakage was determined simultaneously with MDA in the cultures maintained in the presence of 5 and 100 μM Fe-NTA. Hepatocytes

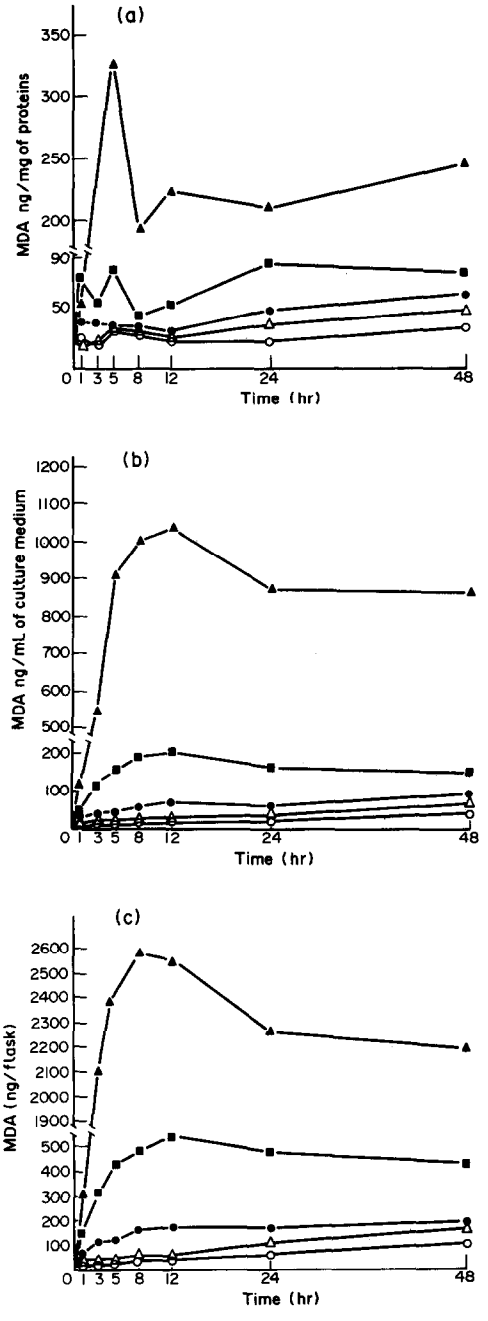

Fig. 2. Free MDA in the cells (a), in the culture medium (b) and total free MDA in the flask (c) after different times of incubation with or without various amounts of Fe-NTA: (○) control: hepatocyte culture without any supplementation; (△) hepatocyte culture supplemented with 200 μM NTA; (●) hepatocyte culture supplemented with 5 μM Fe-NTA; (■) hepatocyte culture supplemented with 20 μM Fe-NTA; (▲) hepatocyte culture supplemented with 100 μM Fe-NTA.

treated with 5 and 100 μM Fe-NTA showed a higher LDH, AST and ALT release than in the controls supplemented or not with NTA (Fig. 3a–c). More precisely, enzyme leakage after 8 hr of, respectively, 5 and 100 μM Fe-NTA supplementation, increased 3 and 15 times for LDH, 4 and 18 times for AST and 4 and 18 times for ALT, as compared to control cultures, and after 24 hr of iron supplementation, these amounts increased, respectively, 6 and 20 times for LDH, 2.5 and 6 times for AST and 5 and 13 times for ALT. Moreover, this extracellular leakage was well correlated with the intracellular enzyme decrease, especially for LDH and ALT (data not shown).

Determination of the mechanism whereby Fe-NTA induced lipid peroxidation on hepatocyte cultures

Endocytosis of SOD and catalase. Inhibitors of endocytosis were used to show whether the efficiency of SOD and catalase dissolved in culture medium depended on their internalization by the cells. The percentage of total MDA and of LDH release (Table 1) were expressed in comparison to cultures only supplemented with 100 μM Fe-NTA. Cultures supplemented with iron and SOD or catalase showed a decrease of both total MDA and LDH leakage, corresponding to an increase of cell viability. This protective effect of these scavengers was reduced when the cells were previously treated with inhibitors of endocytosis like methylamine or cytochalasin B (Table 1). However, there was a discrepancy in the reductive activity of these scavengers; it appeared that SOD efficiency was more affected by inhibitors of endocytosis than catalase since the low levels of total MDA (29%) and LDH leakage (66%) observed with SOD were greatly re-enhanced with a simultaneous addition of inhibitors of endocytosis (95% for MDA and about 90% for LDH), whereas the protective effect of catalase was effective even in the presence of these inhibitors of endocytosis (no great modification for MDA and re-augmentation from 4 to about 20% for LDH). This suggested that catalase did not need an intracellular localization in order to be active.

Free radical scavengers and lipid peroxidation. Fe-NTA enhancement of lipid peroxidation in hepatocyte cultures was supposed to involve reactive oxygen species such as probably hydroxyl radicals $OH^{\cdot}$, superoxide anion $O_2^{\cdot-}$, hydrogen peroxide H_2O_2, singlet oxygen $^1O_2$ for the induction and $^1O_2$, peroxy radicals $ROO^{\cdot}$ for the propagation of the reaction [21]. In order to determine the respective influence of each oxygenated intermediate on Fe-NTA induced lipid peroxidation, free radical scavengers were tested at various concentrations on hepatocyte cultures supplemented with 100 μM Fe-NTA. The results were expressed in per cent of MDA level compared to cultures only supplemented with iron which represented 100%. The possible interference of the scavenger on HPLC assay for MDA evaluation was investigated using standard cultures only supplemented with the scavenger or with the boiled SOD or catalase. The results demonstrated that there was no difference in these controls as compared to MDA levels in cell cultures without any supplementation (data not shown). In the presence of Fe-NTA, total MDA was reduced by all the free radical scavengers employed (Table 2), suggesting that all the corresponding oxygen species could be implicated in the stimulation of lipid peroxidation by iron. This ability of reducing the total MDA level

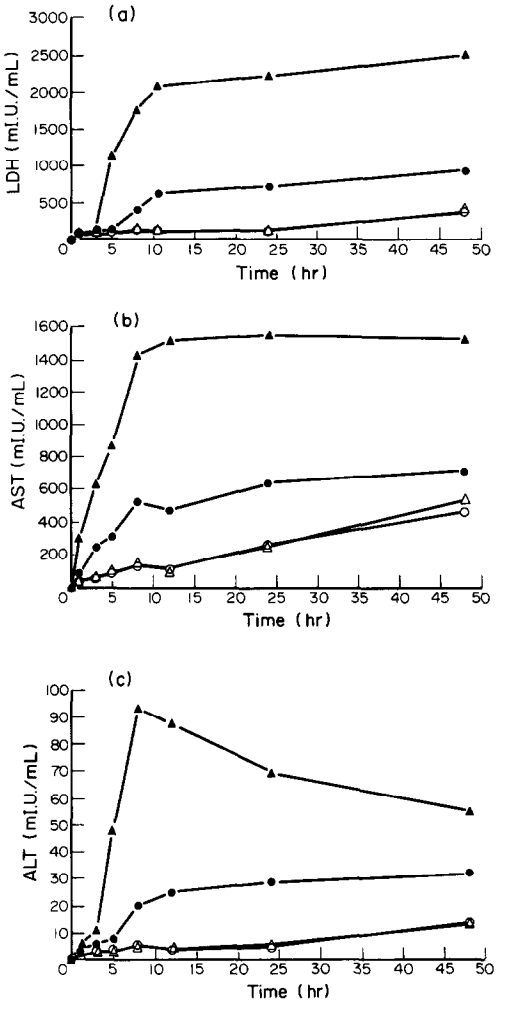

Fig. 3. Cumulative enzyme release in the culture medium (a: LDH, b: AST, c: ALT) following different incubation times in the presence or in the absence of Fe-NTA: (○) control: hepatocyte culture without any supplementation; (△) hepatocyte culture supplemented with 200 μM NTA; (●) hepatocyte culture supplemented with 5 μM Fe-NTA; (▲) hepatocyte culture supplemented with 100 μM.

depended on the scavenger used but not on its concentration, excepted for the liposoluble scavengers (α tocopherol, β carotene) where the increase of their concentration induced a decrease in MDA level, to reach quite a complete inhibiting effect with the higher dose of α tocopherol. SOD and the liposoluble scavengers appeared to be the most effective agents studied for reducing total MDA in the hepatocyte cultures. Since 95% of total MDA corresponded to extracellular MDA, the evolution of MDA level in the culture media in the presence of scavengers was similar to total MDA modifications (Table 2). In contrast to these inhibitions observed for total MDA and for extracellular MDA, the influence of the scavengers on intracellular MDA level was more various. β Carotene and α tocopherol were the only scavengers used able to reduce intracellular lipid peroxidation, whereas the other tested scav-

Table 1. Inhibition of endocytosis of SOD (500 I.U./mL) and catalase (10,000 I.U./mL) by methylamine (40 mM) and cytochalasin B (25 μg/mL)

	Fe-NTA			Fe-NTA + SOD			Fe-NTA + catalase		
	—	Methylamine	Cytochalasin B	—	Methylamine	Cytochalasin B	—	Methylamine	Cytochalasin B
% Total MDA	100 ± 2	96 ± 2	99 ± 1	29 ± 2	96 ± 1	92 ± 2	41 ± 3	39 ± 2	46 ± 4
% LDH	100 ± 3	79 ± 3.5	88 ± 5	66 ± 2.5	110 ± 8	81 ± 4.5	4 ± 4.5	22 ± 1	16 ± 1.8

After 15 min of preincubation of the cells by these inhibitors of endocytosis or by their solvent, Fe-NTA (100 μM) ± SOD or catalase was added directly to the cultures without changing the media; the percentage of total MDA and LDH release were evaluated after 5 hr of incubation. The results were expressed in per cent of cultures only supplemented with iron representing 100% except in presence of cytochalasin B where they were compared to cultures supplemented with iron and DMSO. Results are expressed as means ± SD of triplicate determinations.

Table 2. Effects of free radical scavengers

	Fe-NTA (100 μM)										
	SOD (I.U./mL)					Catalase (I.U./mL)			Mannitol (mM)		
	0	250	500	1000	1500	100	1000	10,000	10	25	50
% Total MDA	100 ± 2	31 ± 1	29 ± 2	33 ± 2	32 ± 3	36 ± 2	42 ± 6	41 ± 3	73 ± 5	43 ± 3	31 ± 2
% Intracellular MDA	100 ± 3	201 ± 7	256 ± 5	364 ± 4	378 ± 5	217 ± 4.5	441 ± 9	468 ± 13	533 ± 14	320 ± 13	280 ± 8
% Extracellular MDA	100 ± 2	19 ± 1	12 ± 1	11 ± 0.7	11 ± 2	23 ± 1.8	13 ± 5	11 ± 1.2	41 ± 4	24 ± 3	14.6 ± 0.4
% LDH release	100 ± 1	82 ± 2	66 ± 2.5	62 ± 3	60 ± 0.6	—	14 + 3	4.1 ± 0.5	—	90 ± 3.5	79 ± 3

Various concentrations of free radical scavengers were added simultaneously to 100 μM Fe-NTA and the cells were incubated with iron (100 μM) or with iron and DMSO and corresponded to 100%. Results are expressed as means ± SD of triplicate

engers showed a contradictory property of increasing intracellular MDA level. Moreover, the very high values of intracellular MDA obtained in the presence of thiourea could probably be explained by the appearance of a toxic event resulting from a pro-oxidant effect occurring when high concentrations of thiourea were used in the presence of iron.

Free radical scavengers and LDH leakage. Complementary to the study of the effect of free radical scavengers on iron induced-lipid peroxidation, the influence of these scavengers on LDH leakage was investigated. The possible interference of these scavengers on the activity of LDH was investigated and did not reveal any undesired interaction (data not shown). As reported in Table 2, LDH release was reduced in a function of the amount and of the type of free radical scavenger used. When this reduction was compared to the decrease observed for total MDA level, it appeared that some scavengers seemed to be more efficient than other ones; for example, thiourea and DMPO were more protective than SOD and mannitol when considering LDH release, whereas SOD seemed to be more effective in reducing the production of total MDA (29% of MDA produced) than in decreasing LDH release (66% of LDH released). These variations in the reductive activity of these scavengers exhibited a discrepancy between the evolution of total MDA and of LDH release, showing that these two parameters for the evaluation of cytotoxicity did not correlate well. These results suggested that lipid peroxidation and enzyme leakage were two distinct phenomena. Subsequently, the free radical scavengers employed could be separated into two classes; those which were more effective in reducing lipid peroxidation than in decreasing LDH leakage (SOD, mannitol, α tocopherol and β carotene) and those which had more influence on enzyme release than on MDA production (catalase, thiourea and DMPO).

DISCUSSION

Hepatocytes in culture are a good system for studying liver functions because they maintain many liver-specific morphological features and biochemical functions for at least several days after plating [22].

This study shows that iron accumulation was effective and induced lipid peroxidation in adult rat hepatocyte cultures in dose and time related manner and that this enhancement of lipid peroxidation can be correlated with intracellular enzyme leakage corresponding to cell injury. Free radical scavengers were protective on cell cultures supplemented with iron; they however exhibited a discrepancy in their ability to decrease total MDA level and enzyme release, suggesting that the mechanism whereby they reduced iron cytotoxicity was not always the same and depended on the kind of free radical eliminated, on the lipophilicity of the scavenger and then on its intracellular localization.

Lipid peroxidation in iron overloaded hepatocyte cultures

Ferric nitrilotriacetate was chosen for overloading hepatocytes since the uptake of ferric iron from NTA complex was about 30 times higher than from fully saturated transferrin or from ferric citrate [23]. Moreover, Shedlofsky [24], testing different iron complexes on cultured chick embryo hepatocytes, showed that optimal iron loaded cells, with lowest toxicity, was obtained by Fe-NTA. Furthermore, only minimal metabolism of NTA occurred in the liver *in vitro* [25, 26]. It should also be noted that Fe-NTA was probably closed to the so-called non-transferrin bound iron, which has been advocated as being of potential great importance in terms of iron overload and iron toxicity in hemochromatosis [27, 28]. The use of Fe-NTA in our culture model provided a significant and dose-dependent iron accumulation, as we recently described [19] and the increase of iron content in the liver cells correlated with enhancement of lipid peroxidation [29].

In our study, free MDA production was a function of the amount of Fe-NTA loading and of the incubation time and free MDA was recovered both inside the cells and in the culture medium. The presence of MDA in the culture medium could be due to a leakage of MDA from dead cells, to a release by viable cells as a mechanism of cellular detoxification, or to an extracellular production by the external plasma membrane. We tried to elucidate these hypotheses using free radical scavengers as discussed later. Under kinetic aspects, we have found an increase of free MDA both in intracellular and extracellular media during the 5 hr after Fe-NTA loading, after which time the MDA level greatly decreased in the cells whereas a slight reduction occurred in the culture medium, to reach a stabilized value. This discrepancy between intracellular and extracellular

on MDA production and on LDH release

Fe-NTA (100 μM)											
Thiourea (mM)			DMPO (mM)			β Carotene (mM)			α Tocopherol (μM)		
50	100	500	3.2	16	32	1.8	4.5	9	250	500	1000
84 ± 4	80 ± 3	191 ± 36	55 ± 2	46 ± 3	45 ± 4	82 ± 4	30 ± 3	17 ± 1	44 ± 1	43 ± 2	1.7 ± 0.4
476 ± 12	609 ± 18	2870 ± 582	449 ± 12	587 ± 14	631 ± 17	79 ± 2.9	74 ± 5.5	69 ± 0.7	3.2 ± 0.9	2.6 ± 0.4	ND
56 ± 1.4	43 ± 0.7	2 ± 0.4	34 ± 1.7	10.4 ± 0.7	3.5 ± 0.5	82 ± 8.5	27 ± 1.5	14 ± 1	47 ± 1	46 ± 2.5	1.9 ± 0.4
42 ± 2.2	—	13.3 ± 0.4	—	3.5 ± 0.2	2.7 ± 0.6	85 ± 1	82 ± 2.6	60 ± 0.7	66 ± 1.6	62 ± 1.3	49 ± 0.7

5 hr before free MDA and LDH leakage evaluations. The results were expressed in per cent of cultures only supplemented determinations.

MDA evolutions could be surprising since if a decrease of intracellular MDA was consecutive to a leakage into the culture medium, extracellular the MDA level should increase. This might however be explained by other phenomena occurring simultaneously to this leakage and reducing the MDA level, like a degradation of MDA, consequence of its weak stability or by a reduction of MDA production consecutive to iron storage or corresponding to a fall in the amount of polyunsaturated fatty acids in the membranes [30]. Our results confirmed the rapid uptake of iron from the Fe-NTA complex [31] and were in agreement with Yamanoi *et al.* [32] who found that intraperitoneal injection of Fe-NTA produced a peak of MDA in the liver at 6 hr and gradually fell after. This author explained this reduction of MDA by the incorporation of iron from Fe-NTA into ferritin protein which corresponded to the iron storage molecule. When considering total MDA, we noticed that the production was biphasic: during the first hours of incubation with iron, total MDA increased regularly and rapidly and then, after 24 hr of stimulation by iron, it reached a constant value which might result, as we previously noticed for extracellular MDA, from the occurrence of an equilibrium between MDA production and MDA degradation and from a reduction of MDA production.

Evolutions of enzyme leakage and lipid peroxidation as indices of cellular damage

In our model of rat hepatocyte pure culture, the introduction of Fe-NTA was responsible for a leakage of the intracellular enzyme with a dose-dependent response. In the presence of iron alone, we have also found a good relationship between kinetics of total MDA production by hepatocytes and kinetics of enzyme leakage into the media, especially for ALT. However, in cultures supplemented with a combination of iron and free radical scavengers, there was a wide divergence between the reduction of MDA production and the decrease of LDH release, suggesting that the mechanism of the influence of iron on these two parameters was different.

Involvement of free radicals in iron cytotoxicity and mechanism of protection by free radical scavengers

Iron added to hepatocytes in culture induced a toxic process which could be reduced by the use of free radical scavengers. It has been shown on liver microsomes that iron ions were presumably involved in the initiation step of lipid peroxidation [33] via the intermediate formation of oxygen free radical species [34]. Since, in our study, Fe-NTA could induce lipid peroxidation and since this lipid peroxidizing ability was inhibited by antioxidants, hepatocyte injury induced by Fe-NTA was believed to depend on an oxidative mechanism. Although all the free radical scavengers employed were effective in reducing iron toxicity by reducing both lipid peroxidation and enzyme release, indicating that all the kinds of free radicals were implicated in this process, this activity was more pronounced on one of these two parameters of cytotoxicity. When considering that enzyme release was known to represent essentially plasma membrane damage whereas lipid peroxidation corresponded to alterations of total cellular membranes, i.e. plasma membrane and inner membranes, which represented 98% of the total membranes of the hepatocyte [35], it could be suggested that lipid peroxidation exhibited the intracellular toxicity of iron, while enzyme leakage described only the external action of iron on plasma membrane. Subsequently, the scavengers which preferentially reduced lipid peroxidation (like SOD, mannitol, α tocopherol and β carotene) exhibited an intracellular activity and the oxygenated intermediates eliminated by these scavengers ($O_2^{\cdot-}$, $OH^{\cdot}$, $ROO^{\cdot}$, $^1O_2$) appeared to be generated inside the cells. On the contrary, the scavengers which principally reduced enzyme release (catalase, DMPO, thiourea) seemed to present a peripheral activity, showing that the corresponding oxygenated species (H_2O_2, $OH^{\cdot}$) were produced at the plasma membrane. Moreover, the study of the uptake of SOD and catalase by the cells has revealed that these scavengers added to culture medium could be taken up by fluid phase endocytosis into the cells [36]. The important reducing effect of inhibitors of endocytosis on SOD activity allowed us to ascertain an intracellular localization, while the fact that catalase was not greatly influenced by these inhibitors of endocytosis confirmed our previous hypothesis suggesting only a pericellular action on plasma membrane.

Plasma membrane fluidity was however recognized to be an important factor in the expression of the Fe-NTA induced cytotoxicity of Erlich ascite tumor cells [37] and it has been reported that lipid peroxidation increased with liposomal membrane permeability [38]. The special arrangement of the

membrane constituents provided it a structural antioxidant capability [39] which can be preserved by free radical scavengers. This restoration of normal permeability of plasma membrane could explain the increase of intracellular free MDA level observed with some of the free radical scavengers. The intact plasma membrane prevented the diffusion of free MDA produced inside the cells into the extracellular medium and then it accumulated in the hepatocyte. This lack of intracellular reduction of these scavengers could also be explained by different ways: first, the corresponding free radical was not generated inside the cell, which could probably not be the case; the second possibility was that the scavenger might not be able to reach the sites of production of free radicals, maybe because of a rapid degradation or of an aqueous solubility which conditioned its intracellular localization and then its diffusion into lipid phases. α Tocopherol and β carotene were the only tested scavengers which were liposoluble and then, their high capability of diffusion into membranes could probably explain their ability to reduce the free MDA level both inside the cells and in the culture medium. However, another explanation for their particular efficiency in reducing lipid peroxidation could be their properties of scavenging peroxy radical ROO· and singlet oxygen $^1O_2$, involved, not in the initiation, but in the propagation of lipid peroxidation, suggesting that these scavengers were also effective by acting on a different step of lipid peroxidation.

This rat hepatocyte culture model seemed to be a good tool for investigating the Fe-NTA damaging effect as shown by an enhancement of free MDA level and enzyme release. Our model could also be useful for further understanding of the influence on Fe-NTA stimulated lipid peroxidation of iron chelators such as desferrioxamine or other xenobiotics or medicinal forms such as liposomal-encapsulated SOD [40] supposed to have scavenging properties.

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