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LIPID PEROXIDATION STIMULATED BY IRON NITRILOTRIACETATE IN RAT LIVER

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Abstract—The complex of ferric iron with nitrilotriacetate (iron-NTA) given i.p. is an unusually potent stimulus for lipid peroxidation (LP) *in vivo*, as monitored by exhaled alkanes. Localization of ^{59}Fe-labeled NTA radioactivity in mouse liver and accumulation of thiobarbituric acid (TBA)-reacting material in liver after i.p. injection suggested that the effect of i.p. iron-NTA could be primarily hepatic. It was found that 100 μM iron-NTA added to a hepatocyte suspension gassed with air stimulated ethane production (3 ± 1 pmoles/10^6 cells/min) versus an undetectable control, and at a sensitivity of 0.083 pmole/10^6 cells/min. Under similar conditions, hepatocytes stimulated by iron-NTA generated low level chemiluminescence (CL) in parallel with formation of TBA-reactants; the generation of CL was concentration related. Liver was homogenized and fractionated by ultracentrifugation: iron-NTA stimulated CL in whole liver homogenate as in intact cells. The greater part of this activity localized to the microsomal and mitochondrial fractions where NADH or NADPH was required. Using rat liver microsomes, it was shown that iron-NTA in the presence of NADPH stimulated two phases of CL with an initial phase maximum in 1–2 min (phase 1) which decreased abruptly to be followed by a prolonged rise (phase 2); NADH could replace NADPH. Ferrous iron (as chloride) caused a burst of CL, whereas ferric iron was inactive. However, complex differences exist between CL stimulated by Fe(II) and by iron-NTA in the presence of reducing equivalents. Under conditions resulting in the production of CL, a microsomal system with iron-NTA and reducing equivalents accumulated TBA-reactants in parallel with the stimulated CL and rapid increase in oxygen consumption. Both desferrioxamine and butylated hydroxyanisole were able to strongly inhibit the CL stimulated by iron-NTA. When iron-NTA and iron-ADP were compared in the microsomal system, similar responses were obtained but major differences characterized the effects of these iron chelates on whole cells with the ADP complex being relatively inactive. We conclude that iron-NTA stimulated free radical reactions in liver by undergoing cyclic oxidation and reduction and that these reactions utilized oxygen, generated CL, and formed TBA-reactants and ethane. At a subcellular level, the reactions of iron-NTA resembled those reported for iron-ADP.

Many papers attest to the ability of iron in various forms to support lipid peroxidation in a variety of tissues [1–4]. This has been investigated most extensively in the case of iron-stimulated lipid peroxidation in liver microsomes [3]. In this system, iron complexed with ADP or EDTA has received the most attention: in each case, reducing equivalents (e.g. as NADPH) must be present before lipid peroxidation will proceed.

We recently described the marked stimulation of lipid peroxidation, measured as an increase in exhaled ethane, by iron(III)-nitrilotriacetate (iron-NTA)† administered parenterally to mice [5]. In these experiments, expired ethane and pentane increased 400 times at a dose of complexed iron of 7.5 mg/kg, given i.p. Previous papers had documented that the same iron complex possesses organ toxicity, repeated administration causing diabetes [6] and kidney tumours in rats [7]. Because the liver was the major site for uptake of radiolabeled iron given as iron-NTA, and because parenteral administration of this iron chelate was associated with accumulation of thiobarbituric acid (TBA)-reacting material in the liver [5], we postulated that this organ is the major site for stimulation of LP by iron-NTA. The effect of the chelate on isolated liver cells and the microsomal fraction of rat liver has therefore been studied. The endpoints used have been the accumulation of TBA-reactants, generation of alkanes (in particular ethane), measurement of low level chemiluminescence (CL), by cells and/or cell fractions, and correlation of these variables with altered oxygen uptake.

METHODS

Animals. Male Wistar rats weighing 200–300 *g* were obtained from Woodlyn Farms, Guelph, Ontario. Rats were boarded in a controlled environment with a 12-hr light and 12-hr dark cycle. Access was permitted *ad lib.* to Purina Rodent Chow Pellets and to water.

Reagents. Reagents were from the Sigma Chemicals Co. (St. Louis, MO) with the exception of butylated hydroxyanisole from the Grand Island Biological Co. (Buffalo, NY) and inorganic reagents which were of reagent grade or better from a variety of suppliers. The preparation of iron-NTA was

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† Abbreviations: CL, chemiluminescence; NTA, nitrilotriacetic acid; and TBA, thiobarbituric acid. Iron complexes are indicated as iron-chelator (e.g. iron-EDTA); where the oxidation state of iron is not shown it is understood to be Fe(III).

modified from that of Awai *et al.* [8]; 0.16 M NTA (prepared from the free acid by dissolution in two equivalents of NaOH), 0.1 M Fe $(NO_3)_3$ and 0.117 M Na HCO_3 were mixed in order, in the ratio 1:1.081:3.324. The resulting 20 mM Fe^{3+} solution had a pH of 7.0, and the molar ratio of chelator to iron was 1.5:1. To prepare Fe(II)-NTA, rigorous precautions were taken to exclude oxygen, and all solutions used were saturated with argon. $FeCl_2$ was dissolved in an appropriate volume of NTA to give a molar ratio of 1:1.5, otherwise using the same methods as for the preparation of Fe(III)-NTA.

Preparation of hepatocytes. This was performed as previously described [9].

Subcellular fractionation. A 20% homogenate of whole liver from fasted rats was prepared in 50 mM Tris–HCl/0.15 M KCl buffer, pH 7.4, and centrifuged at 900 *g* for 15 min. The pellet was discarded and the supernatant fraction was centrifuged at 900 *g* for 20 min. The supernatant fraction, including the loose fluffy layer above the pellet, was removed, leaving behind a "heavy" mitochondrial fraction. This fraction contained <5% of the total cytochrome P-450 [10]. The supernatant fraction was then centrifuged at 20,000 *g* for 20 min, and the sediment was discarded. The supernatant fraction from this step was centrifuged at 100,000 *g* for 1 hr to yield the microsomal fraction. The foregoing details refer to experiments designed to localize iron-NTA-stimulated LP to particular subcellular fractions; the preparation of microsomes for the remaining experiments was similar except that a 25% homogenate of liver was used, the 9000 *g* spin was omitted, and buffer contained 1 mM EDTA. Also, microsomes were washed in Tris buffer without EDTA, recentrifuged at 100,000 *g* for 1 hr, and resuspended in the wash. As far as possible, reagents used for preparing subcellular fractions were freed of oxygen by bubbling argon.

Gas chromatography. Alkanes were measured using a Varian 2700 gas chromatograph equipped with a 5 m × 3 mm outside diameter, stainless steel column packed with Porasil C (120–160 mesh). The flame ionization detector (FID) was operated at 225°, and the injection port was held at the same temperature. A suspension of hepatocytes (5 ml; 5×10^6 cells/ml) was incubated in a 25-ml sealed Erlenmeyer flask, agitated on a rotary mixer at 160 rpm, and maintained at 37°. Cells were suspended in Hanks' balanced salt solution with 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) buffer (25 mM, pH 7.4). One milliliter of headspace gas was sampled using a gas-tight Hamilton syringe and injected directly onto the column of the gas chromatograph. Oven temperature was held at 100° for 2 min followed by a 20°/min increase to 200°. The signal from the FID was processed by a Hewlett–Packard HP3390 recording integrator.

TBA-reactants. The method of Buege and Aust [11] was followed except that 0.01% (w/v) of butylated hydroxyanisole was included in the reaction mixture.

Chemiluminescence. The photon counter was a Thorn-EMI-Gencom instrument comprising a model C10 photon counter, model 3000 R regulated power supply operated at 1500 VDC, and FACT 50 MK III thermoelectrically cooled housing for an EMI 9558A red-sensitive photomultiplier tube which was mounted vertically. This device is equipped with an evacuated window immediately adjacent to the photomultiplier tube. Above the evacuated window a light-tight aluminum chamber was constructed; this was held thermostatted at 37° and could accommodate a 6-place cell culture plate (Linbro Plastics No. FB6). The lid of the culture dish was replaced with a specially designed cover which included miniature stirring motors driving Teflon paddles at 17 rpm to ensure agitation of the contents of each of the six wells. Provision was made for gassing the chamber and the individual wells, a shutter was provided between the photomultiplier tube and the cell culture chamber, and provision was also made for the addition of reagents during the course of experiments. The output of the photon counter was recorded on a strip chart recorder.

Oxygen uptake. To measure oxygen uptake, 1.3 ml of a microsomal suspension was introduced into a closed, stirred chamber, thermostatted at 37° and equipped with a Clarke-type polarographic electrode (Transidyne General Corp., model 730). The polarographic current was measured using a Keithley electrometer (model No. 602), and the output of the electrometer was recorded on a strip chart recorder.

RESULTS

To verify that liver was a potential source of the ethane production resulting from *in vivo* exposure of mice to iron-NTA [5], isolated liver cells were incubated with the complex and ethane production was followed for 60 min. Untreated cells produced ethane at less than the detectable rate (0.08 pmole/10^6 cells/min over a 1-hr incubation) which is in accordance with the data presented in Ref. 12. Hepatocytes exposed to 100 μM iron-NTA, on the other hand, accumulated ethane in the headspace at 6.7 pmoles/10^6 cells/min (Fig. 1A). This experiment was complemented by a study of CL and accumulation of TBA-reacting materials by hepatocytes incubated under similar conditions. Figure 1B shows the similar time course for the increase in CL and accumulation of TBA-reactants following stimulation of hepatocytes by iron-NTA. The decrease in cell viability was minimal (<10%) over 1 hr at the iron-NTA concentrations studied; however, beyond this time a concentration-dependent increase in cell damage (assessed by trypan blue staining) was observed. Other experiments indicated that untreated cell preparations of low viability (<85% viable) produced ethane at a detectable rate; therefore, lipid peroxidation apparent in cells incubated with iron-NTA for periods greater than 1 hr may reflect both the peroxidation of intact and viable cells, as well as of their degradation products.

Examination of the rate of increase of CL during the first 10 min of hepatocyte incubation with iron-NTA (Fig. 1B) reveals an early peak of CL (Phase I) occurring 2–3 min after addition of iron-NTA to cells, followed by a slower, continuous increase (Phase II). Such an early maximum of activity was less evident, but nevertheless present, in the rate of accumulation of TBA reactants. This lack of reso-

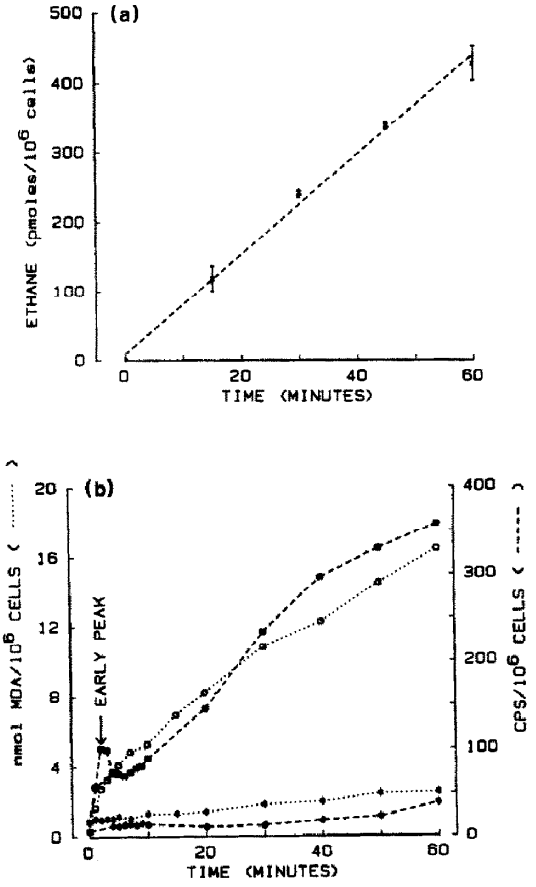

Fig. 1. Time course of lipid peroxidation in hepatocytes exposed to iron-NTA. (a) Hepatocytes were incubated in sealed Erlenmeyer flasks agitated on a rotary mixer. Iron-NTA (100 μM) was added to the medium at zero time, and ethane was determined in the gas phase by gas chromatography at the times shown. (b) Hepatocytes were incubated under similar conditions to those specified for the determination of alkane production. The concentration of iron (as iron-NTA) was 100 μM and of NTA (control) 150 μM. At the times shown, an aliquot of suspension containing 6×10^6 cells was transferred to a well in the photon counting equipment, and 0.2 ml of suspension was used to measure accumulated TBA-reactants.

lution of Phase I was taken to be consistent with the integrating nature of the assay for TBA-reacting material accumulating in the suspension.

Having established the similar responses of hepatocytes to 100 μM iron-NTA using the three endpoints of LP documented in Fig. 1, we studied the dependence of CL alone on different concentrations of the iron complex. Figure 2 shows the concentration-dependent CL response of hepatocytes to iron-NTA in the range of 5–50 μM. The biphasic nature of the CL response was consistently observed only at the higher concentrations of iron-NTA (<50 μM). When the rate of increase of CL during Phase II was plotted as a function of the iron-NTA concentration, the relationship was linear up to 50 μM iron-NTA.

We attempted to localize the subcellular site of lipid peroxidation stimulated by iron-NTA using CL as the endpoint for these studies. Rat liver was homogenized: the CL produced in response to 50 μM iron-NTA was similar to that observed using an equivalent quantity of intact hepatocytes but, if a source of reducing equivalents was supplied (240 μM NADPH), the CL response in homogenate (but not in cells) was enhanced markedly. The whole liver homogenate was therefore fractionated as indicated in Methods. A comparison was made between the stimulation by iron-NTA of CL in the heavy mitochondrial fraction, which was largely free of contaminating microsomal material, and the microsomal fraction. As expected, in the absence of reducing equivalents (NADPH or NADH), stimulated CL was not observed when iron-NTA was added to the microsomal fraction, and the stimulated CL output in the case of the heavy mitochondrial fraction was less than 5% of that obtained with NADPH. In the presence of 240 μM NADH or NADPH, iron-NTA (50 μM) stimulated CL in both microsomal and mitochondrial suspensions. Similar patterns and rates of CL were obtained when either NADH or NADPH was incubated with iron-NTA and the mitochondrial suspension (1 mg protein/ml). On the other hand, NADH and NADPH differentially supported microsomal iron-NTA-dependent CL. NADH yielded rates which were similar to those obtained with the mitochondrial suspensions, whereas the use of NADPH resulted in 50–100% greater CL after 6 min of incubation as well as a more distinct Phase I peak preceding the steady increase associated with Phase II CL.

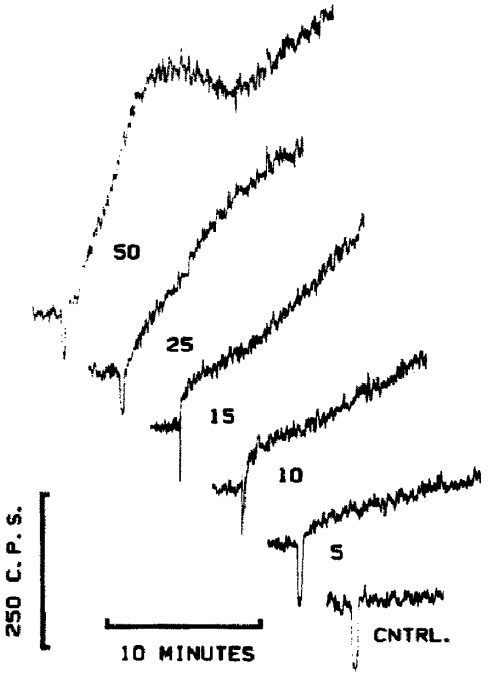

Fig. 2. Hepatocyte chemiluminescence as a function of iron-NTA concentration. Two milliliters of a suspension of hepatocytes (3×10^6 cells/ml) was placed in a well of the photon counting equipment, and background CL was recorded. Iron-NTA was then added to a final concentration ranging from 5 to 50 μM, and CL was recorded for 10–15 min.

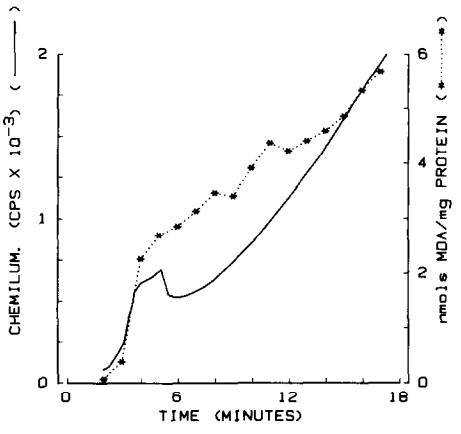

Fig. 3. Measurement of TBA-reactants compared with chemiluminescence as indices of microsomal lipid peroxidation. A suspension of liver microsomes (2 mg protein/ml) containing the NADPH-generating system described under Methods was incubated in either a well of the photon counting equipment (2 ml) or an Erlenmeyer flask agitated in a Dubnoff incubator at 37° (0.2 ml). At the time indicated, iron (as iron-NTA) was added to a final concentration of 100 μM. Photon counts were read from the digital counter.

Figure 3 shows the effect of iron-NTA on CL and the accumulation of TBA-reactants in microsomes incubated in the presence of NADPH and documents results similar to those obtained using intact cells (Fig. 1). The time course, however, was different, with the experiment run over a shorter period of time (18 min). Microsomes were incubated under conditions which permitted good aeration and were sampled at intervals of 1–2 min for determination of TBA-reactants. The same microsomal preparation was observed continuously for 18 min with the photon counting equipment. Traces copied from the analogue recorder again show two phases of CL; after a decrease in CL at the end of Phase I, the Phase II light output increased approximately linearly for the duration of the experiment. TBA-reactants also showed a biphasic rate of increase although the temporal resolution of our sampling technique was lower and, in any event, a drop in accumulated TBA-reactants would not have been expected.

Figure 4 shows the effect of 100 μM iron-NTA on the rate of oxygen consumption by microsomes in the presence of NADPH. The system used in this instance differed from that used for measuring CL in that it was closed while the CL apparatus was designed to allow constant aeration. Keeping this difference in mind, it is seen that the increase in oxygen consumption was immediate, rising to a maximum approximately 2 min after iron-NTA addition. By 3 min, very little oxygen remained to support LP.

An important question concerns the comparison of stimulation of LP by iron-NTA and by unchelated iron salts. This comparison has proven to be somewhat complicated and will be reported in more detail in a later paper. However, using CL as an indicator that LP is occurring, certain generalizations appear to be valid. Ferric iron, added as $Fe(NO_3)_3$ was without effect on both hepatocytes and microsomes, whether or not NADPH was present. Ferrous iron, added as chloride, always caused a transient increase in CL when added to microsomes; in the presence of NADPH a slow increase in CL followed this transient increase. The magnitude of the slow response to ferrous iron in the presence of NADPH is much less than that due to an equimolar amount of ferric iron as iron-NTA. Also, we have been unable to demonstrate that the abrupt increase in CL that follows addition of Fe(II) to microsomes is associated with the marked increase in oxygen consumption which was easily demonstrated when Fe-NTA was added (Fig. 4). The effect of Fe(II) (as chloride) on hepatocytes has been variable: a rapid and variable increase in CL occurred but the question remains whether this is due to interaction with intact cells, or products derived from damaged cells present in the system.

Although mitochondrial CL was stimulated by the NADPH/iron-NTA combination, further experiments were restricted to study of the microsomal system. The experiment depicted in Fig. 3 is analogous to that of Fig. 1B and demonstrates that the monitored CL output from microsomes closely parallels the accumulation of TBA-reactants. Again, it should be noted that the phase I response of CL was matched by a rapid increase in TBA-reactants which was then followed by a slower, approximately linear accumulation similar to phase II of the CL. Further confirmation that the CL arising from the microsomal system is associated with lipid peroxidation was sought. Figure 5 shows the progressive inhibition of iron-NTA-stimulated CL by increasing concentrations of both the antioxidant butylated hydroxyanisole (Fig. 5A) and the chelating agent desferrioxamine (as the mesylate) (Fig. 5B).

Since the discovery of Hochstein *et al.* [2] that iron-ADP complex is a potent stimulator of lipid peroxidation in liver microsomes, this phenomenon has been studied intensively, and a comparison of

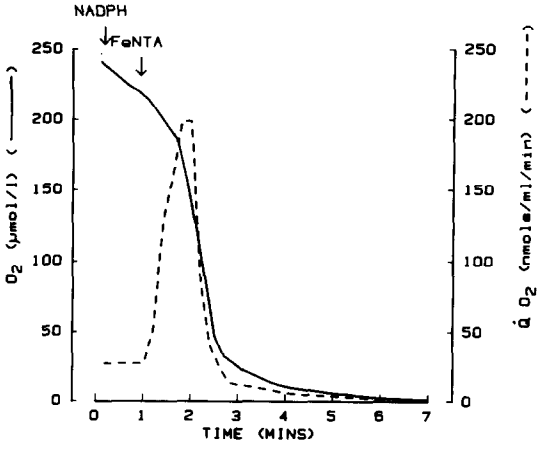

Fig. 4. Stimulation of microsomal oxygen consumption by NADPH/iron-NTA. A 1.3-ml suspension of liver microsomes identical to that used for the experiments shown in Fig. 3 was introduced into a closed, thermostatted (37°), stirred chamber, and oxygen consumption was monitored with a Clarke electrode. NADPH (100 μM) and iron-NTA (100 μM) were introduced at the times shown. The broken line shows the rate of oxygen consumption based upon the slope of the polarographic trace.

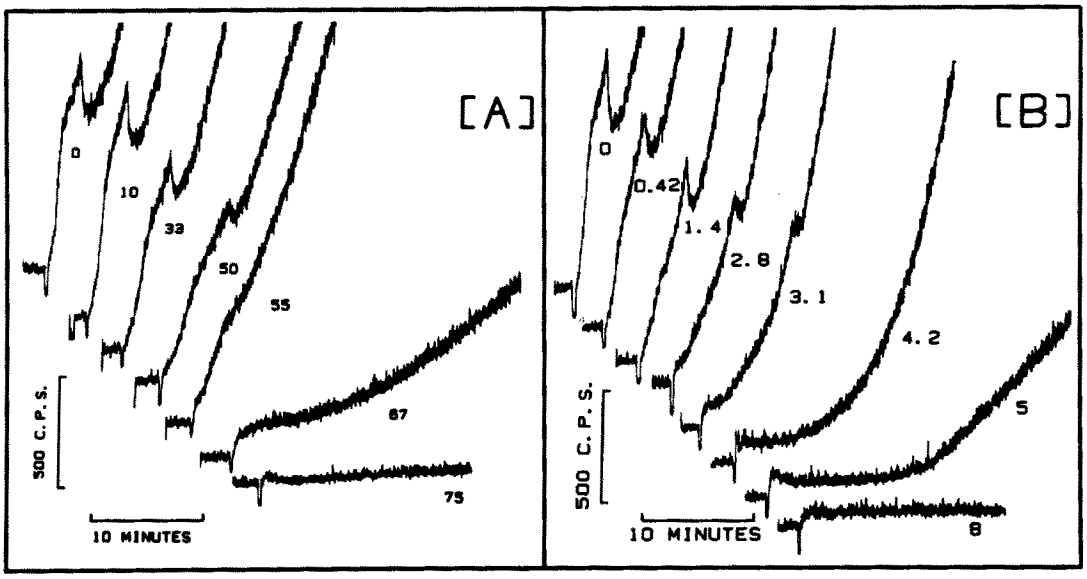

Fig. 5. Inhibition of NADPH/iron-NTA-mediated chemiluminescence in microsomes. CL of a microsomal suspension was determined as described for Fig. 3. except that 50 μM iron-NTA was added after the background photon count had been recorded. Inhibition of CL was produced by adding desferrioxamine (A) or butylated hydroxyanisole (B) at the micromolar concentrations specified, before recording background CL.

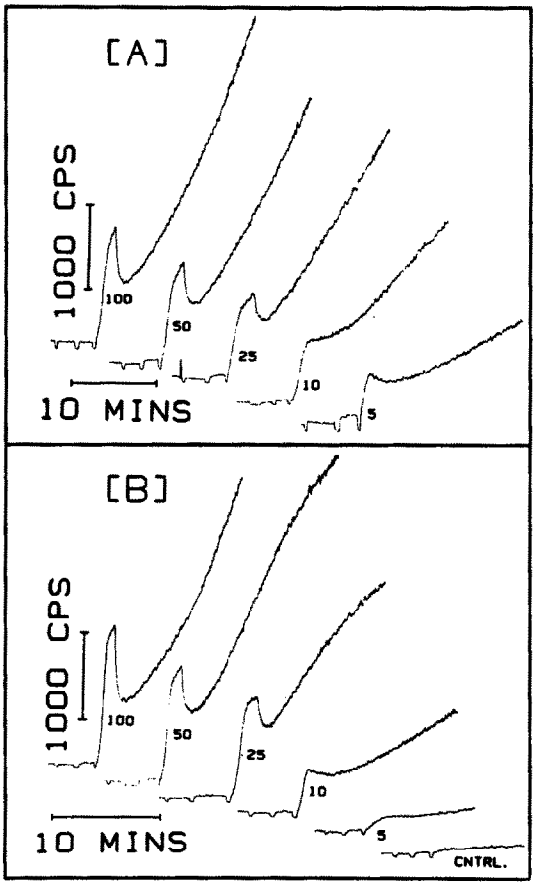

Fig. 6. Comparison of iron-NTA with iron-ADP as stimulants of microsomal chemiluminescence. Conditions were as for Fig. 5 (note the increased attenuation). Iron-NTA (A) or iron-ADP (B) was added at the indicated concentrations (μM) after recording the background CL.

iron-NTA with iron-ADP was of interest. Figure 6, A and B, contrasts the stimulation of CL in a microsomal suspension by these two agents. Except for minor differences at concentrations of iron-complex below 25 μM, the responses were identical for the first 10 min. However, this was not the case in intact cells. Figure 7 shows the stimulation of CL in hepatocytes by iron-ADP and should be compared with Fig. 2 in which CL stimulated by iron-NTA in the same hepatocyte preparation is shown. On addition of iron-ADP, a rapid concentration-related emission of light occurred which leveled out to a slow rise or plateau within 1 min. At no iron concentration tested (up to 100 μM) did the ADP complex elicit a response characterized by an early peak followed by a prolonged rising count rate as seen with iron-NTA.

DISCUSSION

We have demonstrated that, in suspensions of rat hepatocytes, homogenate from rat liver, rat liver mitochondria and rat liver microsomes, iron-NTA stimulated CL and the accumulation of TBA-reactants. In suspensions of hepatocytes we have demonstrated a parallel increase in the production of ethane, and in the microsomal system we have demonstrated that the addition of iron-NTA in the presence of an NADPH-generating system was accompanied by an abrupt increase in oxygen consumption. The majority of methods used to monitor lipid peroxidation are indirect but the relationship between those reactions which lead through hydrogen abstraction and/or production of activated oxygen species to lipid hydroperoxides, and the endpoints we have measured and equated with lipid peroxidation, are generally accepted. We have shown previously [5] that iron-NTA is toxic and stimulates alkane production in mice *in vivo*. In

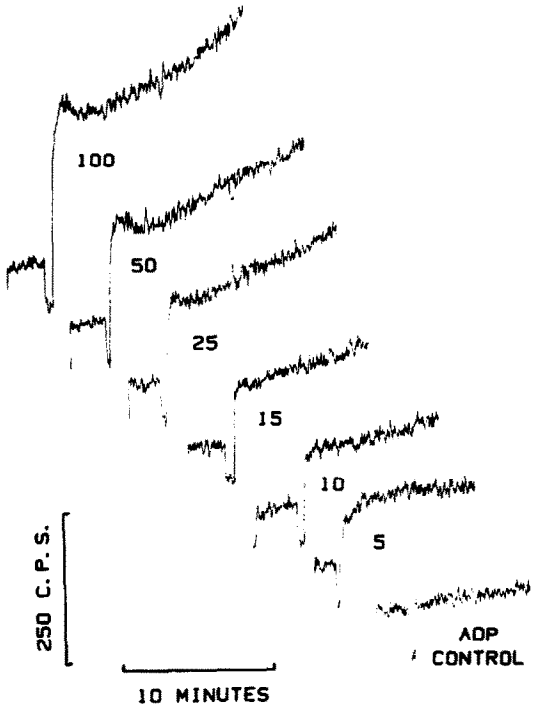

Fig. 7. Dose–response study of iron-ADP-stimulated chemiluminescence from hepatocytes. Conditions were as for Fig. 2 but iron-ADP complex (see Methods) was used in place of iron-NTA.

unpublished work we have demonstrated that the same holds true in rats although the LD_{50} for this species was 2- to 3-fold higher than in mice. Similarly, some of the experiments reported above have been repeated with mouse liver microsomes, and we have no reason to believe that fundamental differences exist between the responses of these two species. Having demonstrated that a considerable portion of a dose of ^{59}Fe-labeled NTA localizes in mouse liver following i.p. injection [5], the experiments reported here further emphasize the potentially important role that stimulated hepatic LP plays in intact animals administered iron-NTA parenterally.

As previously emphasized [13, 14], CL, or low level photon emission, has proven to be a most convenient endpoint. The experiments shown in Figs. 1B and 3, in particular, demonstrate the close correlation between this endpoint and the assessment of LP, in both cell and microsome suspensions, by measuring the accumulation of TBA-reacting material. The photon counting equipment is operated at −30° in order to obtain a low background count-rate from the red-sensitive photocathode of the EMI photomultiplier tube used. Experiments have been reported in which a limited number of either high- or low-pass gelatin filters have been used to characterize the wavelength of emission contributing to the CL signal. We have performed similar experiments and have shown that the major fraction of emission is indeed at wavelengths longer than 605 nm. It has been suggested that this arises from the transition of singlet to triplet state oxygen and that the singlet oxygen is formed when two lipid peroxyradicals interact [13]. However, details of the chemistry involved remain uncertain and more direct measurements of postulated intermediates are required.

We have made some comparison between the much studied stimulation of LP by the Fe(III) complex with ADP (in the presence of a reducing system) and that due to iron-NTA under similar circumstances. We found that the ADP complex was relatively ineffective in intact cells; however, the abilities of the two complexes to stimulate microsomal LP were similar over the periods of time (<20 min) studied. The low activity of iron-ADP relative to iron-NTA in the hepatocyte system may be attributed to: (1) differences in iron uptake by the cells, (2) lower intracellular stability of iron-ADP compared with iron-NTA, or (3) metabolism of the nucleotide.

Attention was drawn previously [15] to a biphasic CL response occurring when liver microsomes are reacted with *tert*-butyl hydroperoxide. The biphasic responses we have documented (Figs. 2 and 6) are different in that they occurred in the absence of added hydroperoxide and involved consumption of dissolved oxygen. This accounts in part for the biphasic responses seen (see Fig. 4), but we do not believe that dependence of subsequent CL production (Phase II) upon oxygen diffusing back into our stirred system completely explains the events observed. Rather, it is likely that iron complex, oxygen and membrane lipids interact to form hydroperoxides, an increase in the concentration of which supports the CL of Phase II. This hypothesis is supported by the increasing concentration of TBA-reactants documented in Fig. 3. Of course, at low iron concentrations (Fig. 6) the events referred to as Phase I will not exhaust available oxygen and the temporary decrease in CL between Phases I and II will not be seen.

Much emphasis has been placed upon microsomal lipid peroxidation, and the role in this of the flavoprotein, NADPH cytochrome P-450 (cytochrome *c*) reductase (EC 1.6.2.4) [16, 17]. Thus, the similar CL generated by a mitochondrial fraction minimally contaminated with smooth endoplasmic reticulum was unexpected. Also unexpected was the support of CL by both NADPH and NADH, in the presence of iron-NTA. However, further study of the dependence of LP on reduced pyridine nucleotide concentration may show larger differences than the data presently available.

Iron-NTA has been prepared in both ferrous and ferric forms, and comparisons have been made between these two forms and also between these NTA complexes and iron as either ferric or ferrous salts. In the absence of a supply of reducing equivalents, and using a microsomal system, Fe(II)-NTA behaved in a fashion similar to, but less potent than, $FeCl_2$. However, ferric iron was inactive under all circumstances tested unless given in the presence of a chelator such as NTA or ADP.

The results obtained suggest that the role played by the iron-NTA complex may not differ substantially from that of iron-ADP except for the stability within the cell of the complexing agent and the ability of the complex to introduce iron into the hepatocyte. In both cases, a continuous supply of

reducing equivalents in the form of NADPH or NADH was required, suggesting that cyclic reduction and oxidation (redox cycling) of the iron complex was occurring. The differences between supply of electrons from NADPH and NADH appeared to be relatively minor, the major constraint being the availability of the reducing agent which, at the cytosolic faces of the membranes at risk, would be NADPH.

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