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PRELIMINARY COMMUNICATIONS

FERRIC NITRILOTRIACETATE: A POTENT STIMULANT OF IN VIVO LIPID PEROXIDATION IN MICE

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Iron in excess of requirements is toxic to biological systems but the mechanism of toxicity is uncertain. It has been demonstrated, however, that both free and chelated iron are capable of stimulating lipid peroxidation in vitro (1,2). In vivo this process is presently best detected by measuring expired low molecular weight alkanes (3,4) which are liberated following decomposition of lipid hydroperoxides. In their studies of alkane production following i.p. injections of $FeCl_2$, Dougherty et al. (5) found significant increases in ethane production only in animals sensitized by dietary deficiencies of selenium and vitamin E.

We have shown that iron plays a synergistic role in the hepatotoxicity of 2,3,7,8-tetrachlorodibenzo(p)dioxin in laboratory animals (6). Seeking experimental models to elucidate the mechanism of iron-dependent liver damage we sought a means of delivering iron rapidly to hepatic parenchymal cells. Awai et al. (7) have previously shown that rats, treated daily for five months with ferric nitrilotriacetate (Fe^{3+}-NTA), developed extensive iron deposits in parenchymal cells of the liver while non-parenchymal cells were largely unaffected. This group recently provided evidence for accelerated lipid peroxidation in vitro using liver homogenates obtained from rats given a single injection of Fe^{3+}-NTA (8).

In this paper we demonstrate that Fe^{3+}-NTA was more toxic than free iron and was a more potent stimulus of alkane production than either ferrous chloride or carbon tetrachloride. In addition, ^{59}Fe-labeled Fe^{3+}-NTA was shown to be taken up extensively by hepatic parenchymal cells, and thiobarbituric acid (TBA) reacting material was found in the liver following Fe^{3+}-NTA treatment.

MATERIALS AND METHODS

Male C57Bl/6J mice aged 6 weeks (Jackson Laboratories, Bar Harbor, ME) were housed five per cage with a 12 hr day/night cycle. Free access was allowed to water and chow (Ralston Purina, St. Louis, MO) until use. The mice were used at age 10–12 weeks (22–26 g) and experiments initiated between 1600 and 1800 hr allowing time for calibration of the chromatograph. Expired gases were collected using a system modified from that of Dillard et al. (3). Briefly, mice were placed in a chamber which was swept with hydrocarbon-free air at 105 ml/min. Water and CO_2 were removed from the expired gas and, over a 9-min period, residual gases were adsorbed on a sample loop partially filled with activated alumina and cooled in an ethanol-propanol-liquid nitrogen mixture. To initiate an analysis of adsorbed gases the sample loop was rapidly heated to 90°, and carrier gas was diverted through it to a Varian 2740 gas chromatograph (Varian Instruments, Palo Alto, CA), equipped with a

5 m x 3 mm (O.D.) s.s. column packed with 100-150 mesh Porasil C (Chromatographic Specialties, Brockville, Ontario) and a flame ionization detector. The column was run isothermally at 25° for 3.33 min at which time the oven temperature was programmed to rise at 20°/min to 200°. The output from the gas chromatograph was processed by an HP 3390A recording integrator (Hewlett-Packard, Mississauga, Ontario). Calibration was with a mixture of alkanes ("Scotty" gas mixture: Mandel Scientific, Rockwood, Ontario). The minimum detection level for each alkane was 1.5 pmoles or less and the coefficient of variation for measurement of ethane was better than 5%.

TBA reacting substances were determined by the method of Uchiyama and Mihara (9). TBA (Sigma Chemical Co., St. Louis, MO) was reacted with malonaldehyde bis(dimethyl acetal) (Aldrich Chemical Co., Milwaukee, WI) to form the TBA-malonaldehyde (TBA-MA) complex (10) which, for calibration purposes, was added to liver homogenate from untreated mice and extracted with 1-butanol. Spectrophotometric analyses were performed on a Perkin Elmer model 356 dual wavelength spectrophotometer with the sample wavelength set at 533 nm and the reference wavelength at 510 nm.

NTA, as the free acid, was obtained from the Sigma Chemical Co.; Fe^{3+}-NTA (1:1.5, molar ratio) was prepared according to Awai et al. (11); $[^{59}Fe]FeCl_3$ was from New England Nuclear. Radioactivity was determined in a well-type scintillation counter with similar geometry for all specimens counted. Prior to counting, tissue samples were digested in nitric acid. To prepare liver cells, the organ was digested by perfusion with collagenase using methods similar to those described elsewhere (12). Non-parenchymal cells were prepared separately from parenchymal cells according to the methods of Zahlten et al. (13).

RESULTS AND DISCUSSION

A dose of 7.5 mg Fe^{3+}/kg i.p. as Fe^{3+}-NTA was given to a group of five mice and proved fatal. Death occurred between 2 and 11 hr after injection. Thirty minutes after treatment the quantities of ethane and pentane collected from these mice over a 9-min period increased 427- and 452-fold, respectively, relative to the pretreatment values. These results suggested a rapid and very marked stimulation of lipid peroxidation at this dose, far in excess of that reported for free iron in rats (5). The dose response of mice to Fe^{3+}-NTA and the tissue distribution of $[^{59}Fe]Fe^{3+}$-NTA were therefore studied.

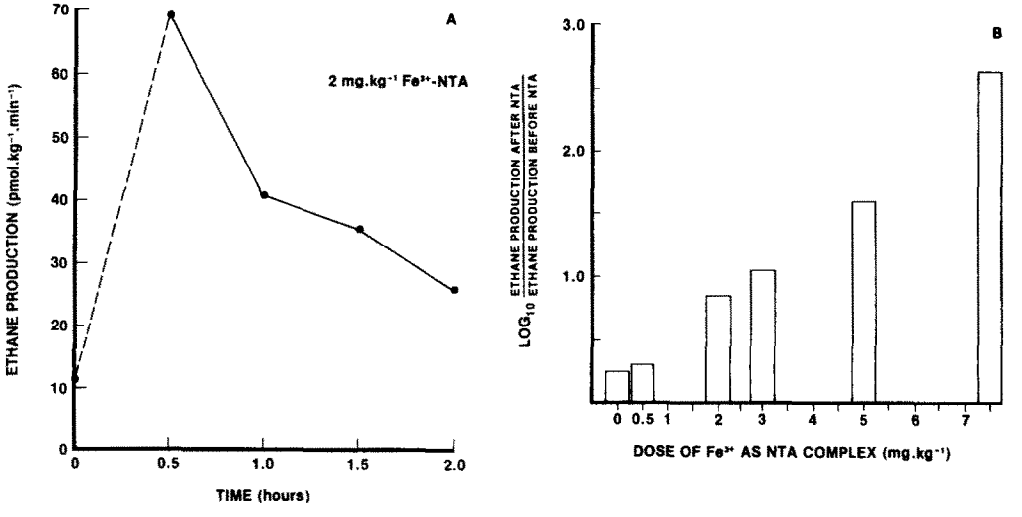

Fig.1. Groups of five C57Bl/6J mice received i.p. injections of NTA or Fe^{3+}-NTA. Ethane production was measured following 9-min collection periods. Panel A shows the time course of ethane production after 2 mg Fe^{3+}/kg as NTA complex; panel B shows the logarithmic relationship between the increase in ethane production and the i.p. dose of iron. Measurements were made 30 min after injection of the complex.

Fe^{3+}-NTA, at a dose level of 2 mg Fe^{3+}/kg, caused a 6.5-fold increase in the production of ethane over the pretreatment values; this declined fairly rapidly, although at 2 hr post-injection the level of expired ethane was still increased 2.4 times (Fig. 1A). A similar rapid rise and fall in alkane excretion was observed at all doses of Fe^{3+}-NTA tested. The logarithm of the increase in ethane production measured at 30 min relative to production before treatment was proportional to the dose of Fe^{3+}-NTA (Fig. 1B). A control injection of NTA alone was without significant effect.

Table 1. Distribution of ^{59}Fe 30 min after a single injection of $[^{59}Fe]Fe^{3+}$-NTA*

Tissue	Liver	Blood†	Kidneys	Spleen	Lungs	Heart
Percent of injected radio-iron dose ± S.D.	36.5 ±3.6	4.5 ±0.7	2.9 ±0.09	0.293 ±0.04	0.277 ±0.04	0.165 ±0.007

*$[^{59}Fe]Fe^{3+}$-NTA (3 mg Fe^{3+}/kg; 2.05 x 10^6 cpm/mg Fe^{3+}) was administered to 3 mice by i.p. injection.

†Total blood content was calculated using 80 g of blood/kg body wt.

Table 1 shows that, in mice, the major site of iron deposition 30 min after injection of Fe^{3+}-NTA is the liver. It is known that iron, given as iron-dextran complex, is initially taken up by Kupffer cells in the liver (14) with no apparent increase in ethane production from rats injected with 500 mg Fe^{3+}/kg in this form (5). We, therefore, were interested in determining the cellular localization of the radio-iron injected as $[^{59}Fe]Fe^{3+}$-NTA. Liver cells were prepared from a mouse injected 30 min previously with $[^{59}Fe]Fe^{3+}$-NTA (3 mg Fe^{3+}/kg, 4.42 x 10^6 cpm/mg). Non-parenchymal cells were prepared from a portion of this suspension, and radioactivity was determined in the fractions obtained. The count rate was 711 cpm/10^6 unseparated cells compared with 9 cpm/10^6 separated, nonparenchymal cells indicating preferential uptake of Fe^{3+}-NTA by hepatocytes. Using the data of Greengard *et al.* (15), based upon morphometric studies of rat liver, the parenchymal cell fraction of liver is 0.57. Assuming that mouse livers are similar, this indicates that more than 99% of the iron delivered to the liver by Fe^{3+}-NTA is taken up by the parenchymal cells.

The TBA test was performed on liver homogenates to determine the presence of TBA reacting substances and expressed in terms of nmoles of malonaldehyde/g of liver. Mice treated 1 hr previously with Fe^{3+}-NTA (7.5 mg Fe^{3+}/kg, N = 3) were found to have 405.7 ± 98.5 nmoles of MDA equivalents/g of liver whereas the NTA-treated controls (N = 3) had 152.5 ± 46.8 nmoles/g of liver. These values are significantly different at $P < 0.01$ by Student's t-test. The indicated errors are ± S.D.

These data lead us to the conclusion that the toxic complex, Fe^{3+}-NTA, markedly stimulates lipid peroxidation in mice. The time course of enhanced alkane production in Fe^{3+}-NTA poisoning is similar to that due to CCl_4, a known promoter of lipid peroxidation, indicating that this phenomenon is not secondary to cell death. Comparison of our data with that of Sagai and Tappel (16) using CCl_4-treated rats, however, suggests that Fe^{3+}-NTA may be 30 or more times more potent as a stimulant of alkane production. The selective uptake of radio-iron by hepatocytes and the detection of TBA reactive materials in liver tissue implicate hepatic parenchymal cells as the major site of Fe^{3+}-NTA-stimulated lipid peroxidation. This, however, does not dismiss the possibility of other target organs nor that the toxicity of peroxigenic compounds may be mediated by release of toxic products of lipid peroxidation. It is evident from these findings that the potential for stimulation of lipid peroxidation *in vivo* by iron compounds is highly dependent on the form of the administered iron. Further research should clarify the relationship between iron-stimulated lipid

peroxidation and the toxicity of this metal.

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