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EVIDENCE THAT FREE FATTY ACID-IRON COMPLEXES DIRECTLY INITIATE LIPID PEROXIDATION IN VITRO AND IN VIVO: A NEW MECHANISM OF OXIDATIVE STRESS

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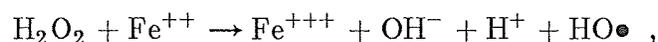
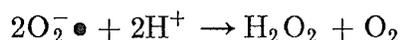
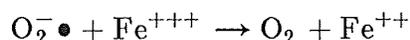
Abstract -- Through a series of biochemical and histochemical experiments we explored the novel hypothesis that iron and free fatty acids, liberated after tissue injury, combine to form liposoluble complexes that directly initiate lipid peroxidation. The addition of 100 μ M ferric iron to 30 mM linoleate suspensions at pH 7.4 produced time dependent lipid peroxidation, measured as conjugated diene formation. Complexes of 100 μ M ferric iron and 600 μ M pentanoate also initiated formation of conjugated dienes in linoleate suspensions and formation of malondialdehyde-like materials in rat liver slices. A histochemical stain for free fatty acids revealed positive reactions within cell membranes in traumatized regions of rat liver tissue that underwent compression injury followed by thirty minutes of blood perfusion, but not in non-traumatized control regions. The diaminobenzidine- H_2O_2 histochemical reaction for iron, revealed increased levels of redox cyclable iron in the membranes and the cytoplasm of traumatized hepatocytes. We propose that traumatic injury initiates cascades leading to liberation of iron from storage proteins and free fatty acids from membranes, which combine, distribute to the lipid domains of cell membranes, and directly initiate lipid peroxidation.

Keywords--Conjugated dienes, Free fatty acid-iron complexes, Initiation, Lipid hydroperoxide, Liposomes, Lipid peroxidation, Oxidative Stress, Perferryl Fe-oxo complexes, Perferryl iron, Oxo-iron complexes, Trauma

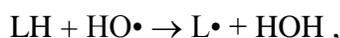
INTRODUCTION

Oxidative stress leading to lipid peroxidation, as well as oxidative modification of proteins, low density lipoproteins, and nucleic acids, has been linked to the pathophysiology of many disease states, including rheumatoid arthritis, [1] ischemia/reperfusion injury, [2-5] atherosclerosis, [6, 7] paraquat poisoning, [8] and traumatic injury to the central nervous system. [9, 10] Although oxidative modification of non-lipid species has received some attention, [11-15] lipids are generally considered to be the most important targets of oxidative stress in biological systems. [10, 16-24] Lipid oxidation has been suggested to disrupt the architecture of cell membranes by the introduction of polar (-OOH and -OH) groups into the lipophilic central regions of phospholipid bilayers, [23] by subsequent degradation of lipid peroxides into smaller molecules, and by toxic effects of lipid oxidation products, including 4-hydroxynonenal, which is both a direct cytotoxin and a potent chemoattractant for leukocytes. [25-28] Activated leukocytes, in turn, are prodigious sources of biological oxidants that may easily perpetuate the cycle of oxidative stress. [29, 30]

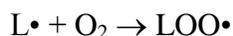
The most quoted chemical mechanism for initiation of lipid peroxidation has been the abstraction of hydrogen atoms from polyunsaturated lipids by hydroxyl radicals (HO•) generated by the superoxide driven Fenton reaction. [2, 4, 12] Superoxide (O_2^-), the primary oxygen radical in biological systems, may be produced by oxidoreductases, [31, 32] endothelial cells, [30, 33] and activated leukocytes. [29, 34, 35] According to the prevailing view, [36] the redox cycling of suitably chelated iron in the presence of superoxide and hydrogen peroxide may lead to the generation of the hydroxyl radical,



followed by the initiation of lipid peroxidation,



where LH is a polyunsaturated lipid, and subsequent chain propagation,



The hydroxyl radical (HO•) is one of the most effective oxidants in nature, which is ordinarily created under extreme physical conditions, such as those prevailing in fields of high intensity gamma irradiation, [37, 38] high temperature flames, [39-41] or intergalactic dust clouds. [42] The intellectual appeal of the superoxide driven Fenton reaction as a pathophysiologic mechanism is that it provides a route by which such a powerful oxidant may be readily generated in aqueous solutions under physiologic conditions of temperature, pH, and ionic strength. Further, there is

rather abundant evidence that interventions such as administration of superoxide dismutase (SOD) or strong iron chelators (like deferoxamine), which would be expected to disrupt Fenton chemistry, attenuate oxidative stress caused by ischemia/reperfusion in the rabbit heart, [43] rabbit kidney, [22, 44] cat intestine, [45] and dog heart, [46-48] as well as oxidative stress associated with CNS trauma, [49, 50] cardiac arrest and resuscitation, [51-53] and hemorrhagic shock. [54-58]

Although the roles of superoxide, iron, and lipid peroxidation in the forgoing scheme are rather well established, several lines of evidence cast doubt upon the importance of HO• as a direct initiator of lipid peroxidation and related pathophysiologic alterations in vivo. A major physical limitation for initiation of lipid peroxidation by HO• is the requirement that the highly reactive HO• radical, initially generated in the aqueous phase, penetrate the hydrophobic central region of the membrane. Hydroxyl radicals are generally considered to be such indiscriminate oxidants that they react within one or two molecular diameters with aliphatic or aromatic carbon compounds [59-61], including amines, amino acids, lipids, and nucleic acids that are likely to be present in the immediate environment or on the membrane surface. Aust and coworkers [62] noted HO• scavengers and catalase prevented lipid peroxidation in detergent solubilized lipids but not microsomal lipids in micellar form. Although HO• can initiate lipid peroxidation in solubilized, homogenous reaction systems, [62, 63] lipid peroxidation in liposomes or microsomes initiated by adding iron-ATP, ferrous-ferric complexes, or ferric ascorbate is not usually inhibited by catalase or scavengers of HO•. [63] Rush and Koppenol, in an uncomplicated chemical system, found kinetic evidence against direct formation of HO• from chelated ferrous iron and hydrogen peroxide at pH 6. [64] Steiner [65], using dimethyl sulfoxide as a molecular probe for HO•, found marginal evidence for a burst of hydroxyl radicals during ischemia/reoxygenation of rat kidney slices, even though lipid peroxidation, preventable by iron chelators and antioxidants, readily occurs in this model. [66, 67] Similarly, sensitive cytochemical [58] and biochemical [59] probes for intracellular bursts of hydrogen peroxide in postischemic liver have failed to reveal evidence of heightened Fenton chemistry; although lipid peroxidation in postischemic liver is readily demonstrated in the laboratory. [66-70]

Review of this prior work and some intriguing preliminary studies in our own laboratory led us to propose a new hypothesis regarding the initiation of lipid peroxidation in biological membranes. The alternative initiation mechanism involves the direct oxidation of polyunsaturated fatty acids by free fatty acid-ferric iron complexes, without the necessary involvement of HO• or H₂O₂. In brief, this alternative mechanism begins with the liberation of low molecular weight iron species, the concurrent liberation of free fatty acids, the formation of free fatty acid-iron complexes and their uptake into phospholipid bilayers, and direct, non-enzymatic initiation of lipid peroxidation by such complexes (Figure 1). The liberation of "free" or low molecular weight forms of iron from high molecular weight forms, notably ferritin, may occur either via the action of calcium activated proteases following tissue trauma or ischemia [71, 72] or via the release of low molecular weight iron from ferritin by superoxide [73] generated by xanthine oxidase, [2, 5] mitochondrial metabolism, [74] and activated leukocytes. [29, 30, 34, 75]

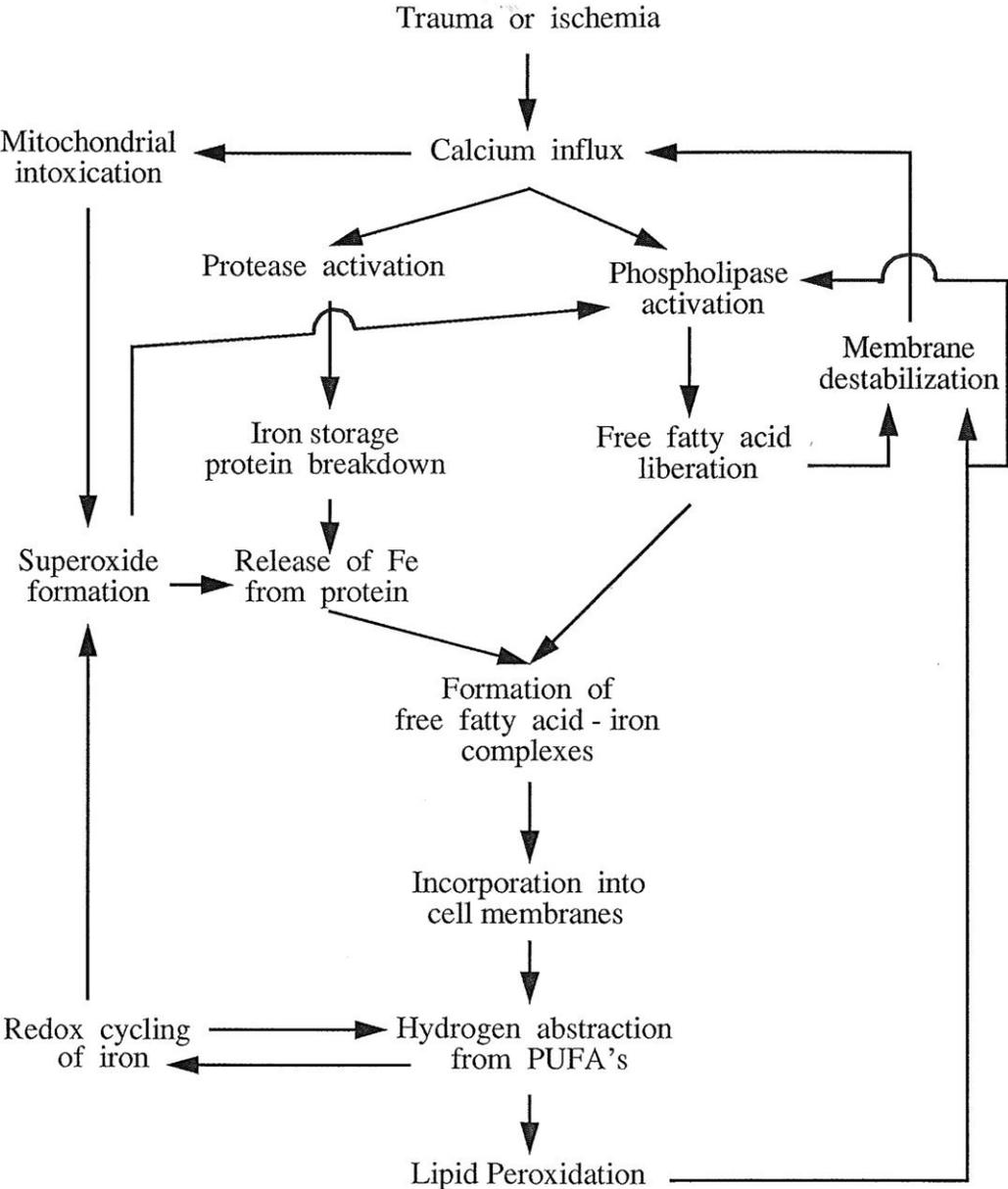


Fig. 1. Proposed pathophysiological cascade of cellular events leading to lipid peroxidation and cell death. Note positive feedback loops leading to accelerated release of iron and free fatty acids. The scheme shows proposed routes for sustained lipid peroxidation after trauma or ischemia.

Thus, lipid peroxidation and attendant tissue damage *in vivo* may not necessarily be dependent upon superoxide and may [76, 77] or may not [78, 79] be inhibited by superoxide dismutase. The concurrent liberation of free fatty acids, most probably by calcium activated phospholipases following tissue ischemia, trauma, thermal injury, etc. [80, 81] may provide lipid soluble chelators of iron, capable of carrying the iron into the hydrophobic interior regions of phospholipid bilayers within cell membranes. [82] Here, in the immediate proximity of the oxidizable double bonds of membrane lipids, we propose that the ferric iron-free fatty acid complexes may directly initiate lipid peroxidation, without the involvement of HO• or H₂O₂.

We further propose that lipid oxidation is accompanied by reduction of the iron to the ferrous form, followed by autoxidation or reoxidation to the ferric form and further redox cycling of the iron, thus creating a self perpetuating mechanism that leads to continued lipid peroxidation, calcium intoxication, and membrane destabilization that hastens membrane disruption and cell death (Figure 1).

The present paper provides evidence from a diverse series of experiments suggesting that such complexes form both *in vitro* and *in vivo* and are capable of initiating lipid peroxidation. In particular, biochemical studies were performed to determine if free fatty acid-iron complexes could initiate lipid peroxidation *in vitro*. Then companion histochemical studies were performed to search for evidence for the proposed free fatty acid-iron complexes *in vivo*.

MATERIALS AND METHODS

Reagents and pharmaceuticals

Ferric-EDTA, linoleic acid, oleic acid, palmitic acid, pentanoic acid, quinacrine (6-chloro-9-[(4-diethylamine)-1-methyl-butyl]amino-2-methoxyacridine), rubeanic acid (dithiooxamide), spectrophotometric grade cyclohexane, 2-thiobarbituric acid and Tris (hydroxymethyl-aminomethane) buffer were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Copper sulfate, hydrogen peroxide, methanol, and trichloroacetic acid were obtained from Fisher Scientific Company, Fair Lawn, New Jersey, USA. Ferric chloride, ferrous sulfate, glacial acetic acid, potassium ferrocyanide and spectrophotometric grade chloroform were obtained from Mallinckrodt, Inc., Paris, Kentucky, USA. 3,3'-diaminobenzidine-4HCL was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin, USA. The Tris-Ringer buffer solution consisted of 4.0 mM KCl, 140 mM NaCl, 2.0 mM CaCl₂, 4.0 mM Tris buffer, and 200 mg/dL d-glucose, pH 7.4. In some experiments, lactate buffer was used, which consisted of 4.0 mM KCl, 100 mM NaCl, 2.0 mM CaCl₂, 40.0 mM lactate, and 200 mg/dL d-glucose, pH 7.4.

Conjugated diene (CD) production in micellar suspensions of polyunsaturated fatty acids

Unlike other indicators of lipid oxidation such as malondialdehyde and ethane formation, conjugated dienes provide a more quantitative yield of reaction products that are proximal to the actual initiation of lipid peroxidation. [36, 83, 84] Also, using conjugated dienes as the end-point permitted studies of the possible anoxic oxidation of lipids by ferric iron, thereby providing

evidence excluding oxygen based initiators. Such work is not possible with malondialdehyde-like materials (MDA-LM), since the formation of MDA-LM from conjugated dienes requires molecular oxygen. [36, 83]

To investigate the hypothesis that iron can directly initiate lipid peroxidation in micellar suspensions of polyunsaturated fatty acids (PUFAs), conjugated diene formation in vitro was studied. To test tubes containing 1.0 ml of 30 mM free fatty acid salt (linoleate, oleate, or palmitate) in suspension at pH 7.4, 0.1 ml of 1.0 mM FeSO₄, FeCl₃, ZnCl₂ or 0.9% NaCl was added while vortexing. The final concentration of iron or zinc was 100 μM. This concentration of low molecular weight chelate iron ("free iron") was chosen to be similar to that previously measured in postischemic dog brain and heart. [85, 86] The test tubes were then incubated in room air for 30 minutes at 37 °C in a shaking water bath. To study the hypothesis that ferric iron and not ferrous iron, was the initiator of conjugated diene (CD) formation in PUFAs, linoleate samples with either FeSO₄ or FeCl₃ additions were also performed in an argon saturated (anoxic) test tube, in which autoxidation of ferrous iron to the ferric form would not occur. In these experiments, anoxia was maintained by continuous bubbling of the fatty acid suspensions with argon gas.

To investigate the time course of iron initiated CD formation in linoleate, 3.5 ml of 30 mM linoleate were mixed with 0.35 ml of 1000 μM ferric chloride by vortexing. The mixture was then placed in a shaking water bath in room air at 37 °C. A time 0 sample was taken from fresh linoleate. (In preliminary experiments, similar results were obtained if the time 0 sample was taken either just before or just after iron addition, indicating that no iron dependent lipid peroxidation occurred during the conjugated diene assay itself). Subsequent 0.5 ml samples 5, 10, 15, 30, 45, and 60 minutes after iron addition were taken from the reaction mixture and assayed for conjugated dienes, as subsequently described. To test the hypothesis that lipid soluble free fatty acid-iron complexes could initiate CD formation, 0.1 ml of 1 mM FeCl₃ / 6 mM pentanoate was added to 1.0 ml of 30 mM linoleate (pH 7.4) vortexed and incubated in room air for 30 minutes at 37 °C. Pentanoic acid only and pentanoic acid / ferric chloride only (no linoleate) controls were also studied.

Measurement of conjugated dienes

Conjugated dienes were measured by a modification of the spectrophotometric method described by Recknagel and Glende. [84] A 0.5 ml aliquot of sample was taken and added to 3.0 ml of chloroform methanol 2:1. The mixture was then vortexed for 2 minutes and then centrifuged for 5 minutes at 1700g. A 0.5 ml aliquot of the bottom chloroform layer was removed, added to a clean test tube and taken to dryness under a stream of argon gas. Then, 2.0 ml of spectrophotometric grade cyclohexane were added, and the test tube was gently vortexed. Conjugated dienes were then assayed by scanning the sample against a cyclohexane blank and reading the absorbance from 300 to 200 nm. The conjugated dienes exhibited an absorbance peak at 232 nm, as expected.

The absorbances at 220, 225, 232, 240 and 245 nm were obtained and conjugated diene concentrations were calculated using a computer program for peak extraction implementing the double derivative method of O'Haver [87, 88] and based on reported UV spectra [89] and an extinction coefficient [89] of 27,000 M⁻¹ cm⁻¹.

Lipid peroxidation in fresh rat hepatic tissue by free fatty acid / iron complexes in vitro.

To investigate the hypothesis that iron / free fatty acid complexes initiate lipid peroxidation in organized tissue, we used an in vitro liver slice model developed in our laboratory and described previously. [66, 67, 90] Tissues were taken from male Wistar rats which had been anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Rat liver was selected for study because it readily undergoes lipid peroxidation in similar models of tissue trauma and oxidative stress, [67, 90] and so provides a convenient basis for the study of free fatty acid and iron effects. Upon reaching the stage of surgical anesthesia, each rat was given a 0.5 ml bolus of 1000U/ml heparin via the dorsal penile vein. After a 2 min circulation time, the liver was surgically exposed and the portal vein was cannulated. The inferior vena cava was then cut and the liver was perfused in situ with cold lactate buffer to remove blood elements. The liver was then excised, and tissue samples were processed according to protocols described subsequently. The tissue slices were placed into 50 ml Erlenmeyer flasks containing air equilibrated lactate buffer solution.

Tissue manipulations

Previous research in our laboratory [90] had indicated that tissue trauma is a very effective inducer of lipid peroxidation in the short term organ culture model. Trauma is several times more effective at inducing lipid peroxidation in this model, measured as MDA-LM formation, as anoxia / reoxygenation without trauma. Accordingly, we chose trauma as our model of oxidative stress in rat hepatic tissue. Minimally traumatized control tissue samples were prepared as follows: a large lobe of rat liver was excised, moistened with buffer, and gently cut into thin (approx. 1 mm) strips with a #11 scalpel blade. Then, the tissue strips were gently cut again, perpendicular to the planes of the first series of cuts. This technique produced small tissue blocks about 1 mm x 1 mm x 2-3 mm. These tissues were then washed three times in cold lactate buffer, divided into samples of approximately 0.4 grams, then added to the buffer-containing 50 ml Erlenmeyer flasks. The cutting was done prior to any tissue incubations. The tissues were incubated with shaking in room-air equilibrated buffer at 37 °C for 30 min. In addition to the "minimally traumatized" tissues described above, samples of "maximally traumatized" tissue, which were vigorously minced without washing into similar sized pieces using iris scissors, were also studied for comparison with minimally traumatized tissue samples.

The fresh hepatic tissue slices were incubated in a 37 °C shaking water bath for 30 minutes in room air. In some experiments, the tissues were exposed to lipid-soluble iron in the form of 100 μM Fe^{+++} complexed with 600 μM pentanoate. In other experiments, tissues were exposed to 100 μM ferric-EDTA (1:1), a water soluble form of iron. Pentanoate only and tissue only controls were included. For comparison, samples of minimally and maximally traumatized liver tissue were prepared, and incubated as above for 30 minutes. In a time course study with the pentanoate / ferric iron complex, 6 separate flasks were incubated for 5, 10, 15, 30, 45 or 60 minutes. A time 0 sample was taken directly from fresh liver tissue. The extent of lipid peroxidation was determined by measuring malondialdehyde-like materials (MDA-LM). Flask contents, including tissue and buffer, were homogenized in a Teflon / glass homogenizer and assayed for MDA-LM by the spectrophotometric method of Buege and Aust, [91] as modified by Salaris and Babbs. [66]

Traumatic injury to rat hepatic tissue in vivo and in situ

To study free fatty acid liberation and iron liberation in traumatized rat liver tissue a model of tissue trauma in vivo was developed. Rats were anesthetized with sodium pentobarbital (60 mg/kg). The liver was then surgically exposed and a supporting steel plate was placed under the liver. Trauma was induced by dropping a 5.5 g steel bolt (1.0 cm diameter hex-head-side down) through a 1.5 cm inside diameter plastic guide tube five times on the liver from a height of 13 cm. Trauma was evident as a circular, bruised region on the surface of the liver. The steel plate was then removed and the incision loosely closed with towel clips. A saline moistened gauze was placed over the incision to prevent tissue drying and the rat was placed under a warm lamp for thirty minutes to allow tissue responses to traumatic injury to develop. Rectal temperature was monitored and maintained at 37-38 °C. Then one of several histochemical methods was used to detect the presence of either free fatty acids or iron in and around the traumatized region.

Histochemical detection of free fatty acid liberation during traumatic injury to hepatic tissue

To detect free fatty acids in situ, a modification of Holczinger's histochemical technique for free fatty acids [92, 93] was employed. This method utilizes the formation of insoluble complexes of free fatty acids and copper, which then reacts with rubeanic acid to form a visible reaction product for light microscopy. Thirty minutes after trauma, the portal vein in the rat was cannulated with an 18 gauge cannula. The inferior vena cava was cut and the liver was perfused with 60 ml of Tris buffer solution to remove blood constituents. The liver was then perfused with 50 ml of an isotonic solution of 75 mM copper sulfate in 75 mM NaCl, followed by 80 ml of Tris buffer solution to remove the excess copper sulfate. Then, the liver was perfused with 50 ml of 0.1% w/v rubeanic acid in 70% v/v ethanol. Finally, the liver was then perfused with 40 ml of isotonic saline to remove excess rubeanic acid, followed by 50 ml of Trumps fixative solution (1% gluteraldehyde, 4% formaldehyde in neutral phosphate buffer).

The liver was then excised and trimmed into sections representing traumatized and non-traumatized regions of the liver, which were submitted for routine tissue processing for light microscopy. Seven micron thick sections were stained with hematoxylin and eosin. Two control groups of rats were studied. In one group, just prior to traumatizing the tissue, the rats were treated with 10 mg/kg of the phospholipase inhibitor, quinacrine, [94, 95] dissolved in saline, given via the dorsal penile vein. In a second control group, an organic solvent cocktail consisting of 50 ml ethanol, 50 ml methanol and 1 ml glacial acetic acid was perfused through the liver prior to the copper / rubeanic staining sequence. The acidic alcohol perfusion was intended to produce conditions in which the free fatty acids would be extracted from the cell membranes [93] and thus not be detected.

Tissue processing for iron staining

In another series of weight drop experiments utilizing intact, blood perfused livers, the portal vein of each rat was cannulated with an 18 gauge cannula after 30 minutes of post-traumatic blood perfusion. The inferior vena cava was cut and the liver was perfused with 60 ml Tris buffer solution to remove blood constituents, followed by 50 ml of Trumps fixative solution. A

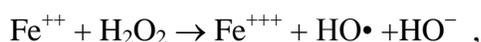
traumatized block and a non-traumatized control block of the same liver were then removed, stored in fixative and processed for light or electron microscopy. In other experiments, the liver was traumatized (as described above) just prior to the Tris buffer flush without post-traumatic blood perfusion. This provided a control in which the presence or absence of redox cyclable iron could be observed immediately after trauma. Unstained, paraffin embedded sections were dewaxed and rehydrated by standard methods. To detect iron histochemically, the slides were then incubated for 60 minutes either in Perl's reagent or in a hydrogen peroxide and diaminobenzidine solution as described subsequently. At the end of the incubation period, the tissues were rinsed, counterstained with eosin (Perl's reagent treated) or methyl green (DAB reagent treated), and permanently mounted.

Conventional histochemical stain for iron

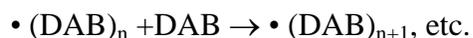
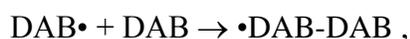
Initial attempts to detect iron in cell membranes of traumatized hepatic cells employed a modification of Perl's method for iron, [96] in which ferric iron in the tissue combines with added ferrocyanide to form ferric ferrocyanide precipitate (Prussian blue). Seven micron thick sections of dewaxed and rehydrated tissues were placed into a mixture of 3% HCl w/v and 7% w/v potassium ferrocyanide solution which had been previously saturated with Prussian blue by the addition of a small amount of ferric chloride solution and subsequent low speed centrifugation. After 1 h, the tissues were washed and counterstained with eosin and examined under the light microscope. Some light blue staining around the cell membranes was visible in the cells near the traumatized region, but it was concluded that a more sensitive method of staining and detecting iron was desirable.

In situ histochemical detection of redox cyclable iron using hydrogen peroxide and diaminobenzidine (DAB)

To detect redox cyclable iron at neutral pH in situ, with greater sensitivity than conventional iron stains, we modified the method of Kuo and Fridovich [97] for staining iron-containing proteins in electrophoretic gels. This method exploits the catalytic activity of iron on the diaminobenzidine polymerization reaction as a histochemical procedure, presumably based upon the following coupled reactions:



followed by chain propagation in the usual manner,



until chain termination occurs



The result is a brown DAB polymer, which persists through further tissue processing and is readily visible by light microscopy. To implement this approach, the dewaxed and rehydrated slides were placed in 4 mM Tris Ringer buffer (pH 7.4) containing 40 mM H₂O₂ and 2.5 mM DAB. Incubation time at room temperature was 60 minutes.

Quantitative morphometry

Cellular profiles of H&E counterstained slides were examined by light microscopy at 200X magnification by an observer who was unaware of the treatment or control group designation of the specimen. In the free fatty acid studies, the copper-rubeanic acid reaction product appeared as amorphous, amber-brown deposits in and around cell membranes, which were scored positively if reaction product was clearly visible. In the free redox cyclable iron studies, the DAB reaction product appeared as amber-brown deposits in and around cell membranes, which were scored positively if reaction product was clearly visible. Counts of profiles of positively stained hepatocyte membranes were made within a 400 x 400 μ^2 test area, delimited by a micrometer eyepiece on the light microscope. The tissue on a 1 in. x 3 in. glass slide was scanned in vertical strips at successive 2.5 mm horizontal increments from left to right.

Defining trauma

In order to independently define traumatized areas of the tissues microscopically, the following five criteria were established. At least three of the five criteria had to be present for the area to be defined as traumatized. These criteria were independent of any histochemical staining that may have been observed and are as follows: (1) irregular tears in the normal cellular matrix not attributable to sectioning artifact, (2) "naked nuclei" or those without surrounding cytoplasmic components, (3) cytoplasmic fragments without nuclei, (4) lighter staining, coagulated cytoplasm in cells near traumatized area, and (5) hemorrhage. These characteristics were not observed in non-traumatized control tissues.

Stereology

To quantify the surface area, S_v , of stained cell membranes per unit volume of tissue in units of mm^2/mm^3 , we employed the stereological techniques of Weibel [98] and Elias [99], in which S_v was computed from the observed number of intersections of positively stained membranes with measured test lines superimposed on the light microscopic field by a micrometer eyepiece. Microscopic sections of non-traumatized tissues were randomly labeled and studied by an observer who was unaware of the treatment protocol. The tissue was scanned in vertical strips, each 2.5 mm apart. The ten horizontal lines of the micrometer eyepiece grid served as the test line for the stereology counts (total length = 10 x 400 μm = 4000 μm) in non-overlapping fields. Each time a positively stained membrane crossed a horizontal test line, a count was recorded. The traumatized tissues were similarly labeled and studied. For these tissues, the sections were scanned vertically until a region of tissue trauma was encountered and then an extensive sampling of the area was made to maximize the information obtained in the traumatized areas. The numbers of counts and total test line length were then added and the S_v calculated as

$$S_v = 2 \frac{\sum I}{\sum L},$$

where $\sum I$ is the sum of the positive membrane intersections observed for all the grids counted and $\sum L$ is the sum of the test line lengths for all of the grids counted. [99]

Electron microscopy

To detect iron in cell membranes at the ultrastructural level, we employed the DAB/hydrogen peroxide reaction used in light microscopic studies. Liver tissue was traumatized and blood perfused in vivo and in situ and then fixed with Trump's solution as described previously. Representative trimmed regions of traumatized and non-traumatized liver tissues were then cut into small (< 0.25 mm thick) sections using a Smith and Farquhar TC-2 tissue sectioner (Sorvall). These small sections were then placed in the DAB and H₂O₂ reaction mixture for 1 hour. The liver slices were then rinsed in phosphate buffered 1% glutaraldehyde--4% formaldehyde and postfixed with 1% osmium tetroxide in phosphate buffer for 1 hour. Samples were then dehydrated through a graded 30% to 100% ethanol series, rinsed twice in propylene oxide and infiltrated with graded propylene oxide-epoxy resin mixture (Poly/Bed 812, Polysciences, Warrington, Pennsylvania, USA). Tissue blocks were all sectioned at 70 nm and photographed at the same intensity on the JEOL, JEM-1000X electron microscope. Negatives were printed using an automatic processor and exposed for identical times and intensities to minimize photographic variations in the apparent density of the reaction product.

Data analysis

An analysis of variance (ANOVA) was run to test each of the null hypotheses that 1) iron alone did not initiate formation of conjugated dienes from poly-unsaturated free fatty acids, 2) ferric and ferrous were not different as initiators of conjugated diene formation, 3) conjugated diene formation did not change as a function of time after iron addition, 4) complexes of ferric iron and free fatty acids did not cause conjugated diene formation in PUFAs, 5) complexes of ferric iron and free fatty acids did not induce lipid peroxidation in fresh rat hepatic tissue, 6) free fatty acids did not increase in traumatized hepatic cells, and 7) the amounts of free, redox cyclable iron detected in traumatized hepatic cells were not different from those in control cells. The ANOVA's were preceded by Bartlett's chi-square tests for homogeneity of variance. [100] If the variances of compared data sets were not similar, a logarithmic or square root transformation of the data was performed [101] and the ANOVA repeated on the transformed data. Specific comparisons were made using Scheffe's multiple range test. A p-value of 0.05 was considered significant.

RESULTS

Oxidation of purified fatty acids

Figure 2 illustrates the formation of conjugated dienes following the simple addition of iron to linoleate suspensions at pH 7.4. Conjugated dienes (CDs) are formed by the spontaneous rearrangement of the polyunsaturated lipid alkyl radical immediately after hydrogen atom abstraction. [83, 102] Hence, they are the earliest measurable products of lipid oxidation. There was a significant effect of iron on conjugated diene formation ($F = 38.6$, $p < 0.01$). Compared to Fe^{++} and Fe^{+++} ions, salts of the non-transition metal ion Zn^{++} did not induce CD formation. In the argon sparged (anoxic) samples, CD formation by ferrous iron was significantly less than in the air exposed samples ($F = 75.6$, $p < 0.01$), while CD formation in response to ferric iron was unaffected by anoxia. The quantitative yield of CDs in linoleate suspensions was nearly equal to the molar amount of ferric iron added ($100 \mu\text{M}$). No CD formation was detected upon iron addition to either saturated (oleic) or monounsaturated (palmitic) fatty acids, by the methods described above (data not shown). CD formation required Fe^{+++} iron.

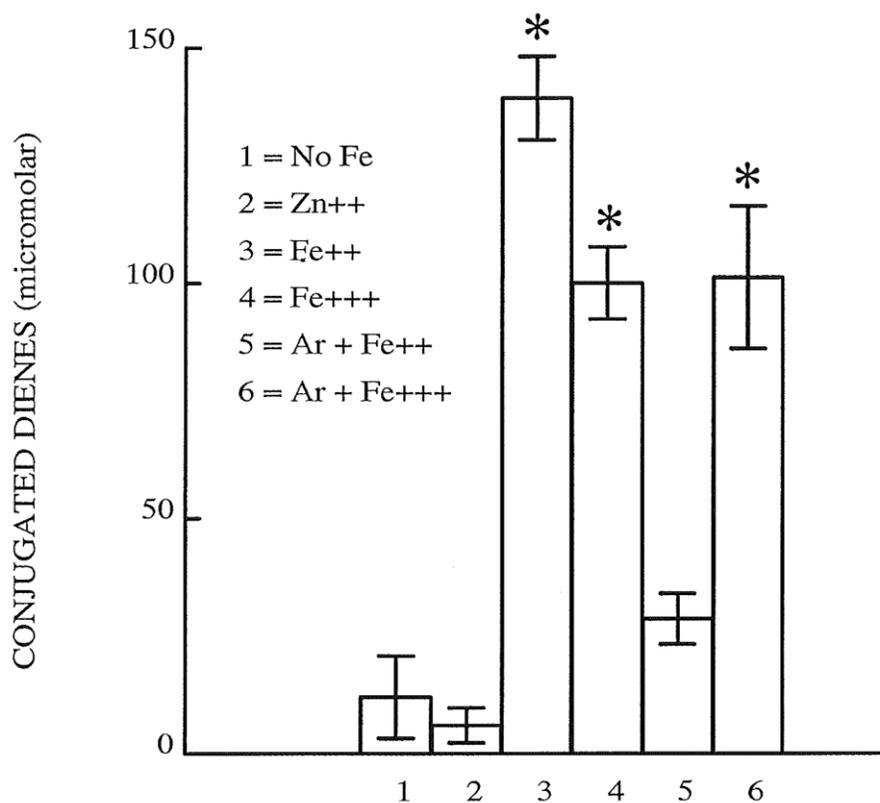


Fig. 2. Formation of conjugated dienes following the addition of $100 \mu\text{M}$ iron or zinc to 30 mM suspensions of purified linoleate at pH 7.4. Bars represent mean \pm SE for $n = 6$ experiments. Legend: 1 = linoleate only; 2 = linoleate plus Zn^{++} ; 3 = linoleate plus ferrous iron; 4 = linoleate plus ferric iron; 5 = anoxic linoleate plus ferrous iron; 6 = anoxic linoleate plus ferric iron. * = significant from linoleate only control at $p < 0.05$.

Figure 3 illustrates the time course of conjugated diene formation from linoleate at pH 7.4 over a 60 minute period after iron addition. The increase in CDs with respect to time was significant ($F = 10.1, p < 0.05$). There was an initial phase of rapid CD formation during the first 5 minutes, followed by a phase of much slower CD formation after that time. Quantitatively, the initial rise matched the initial concentration of Fe^{+++} iron ($100 \mu M$). Thereafter, the continued slow rise in CD concentration is consistent with slow autoxidation of Fe^{++} back to Fe^{+++} in the presence of molecular oxygen.

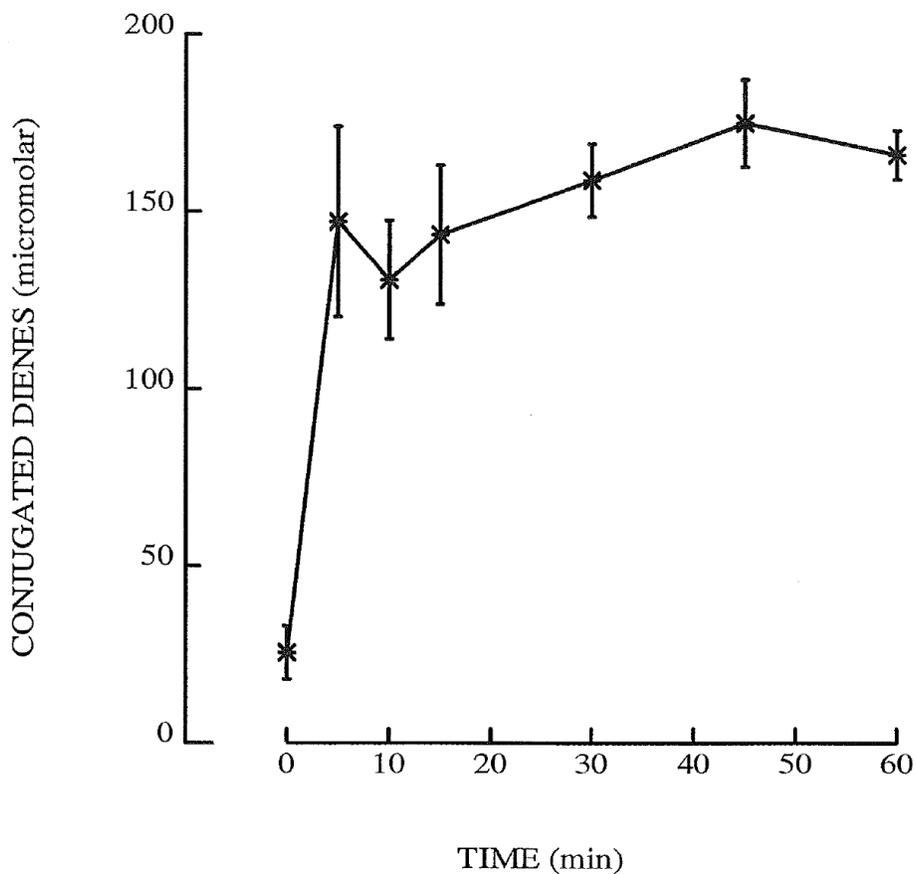


Fig. 3. Time course of conjugated diene formation in 30 mM suspensions of purified linoleate incubated with $100 \mu M$ ferric iron at pH 7.4. Graph shows means \pm SE for $n = 4$ experiments.

Figure 4 illustrates the formation of conjugated dienes in linoleate suspensions following the addition of iron complexed with pentanoate at pH 7.4. There was a significant effect of the iron-pentanoate complex on conjugated diene formation ($F = 69.2$, $p < 0.01$). The two controls of iron-pentanoate only and pentanoate without iron generated minimal CDs.

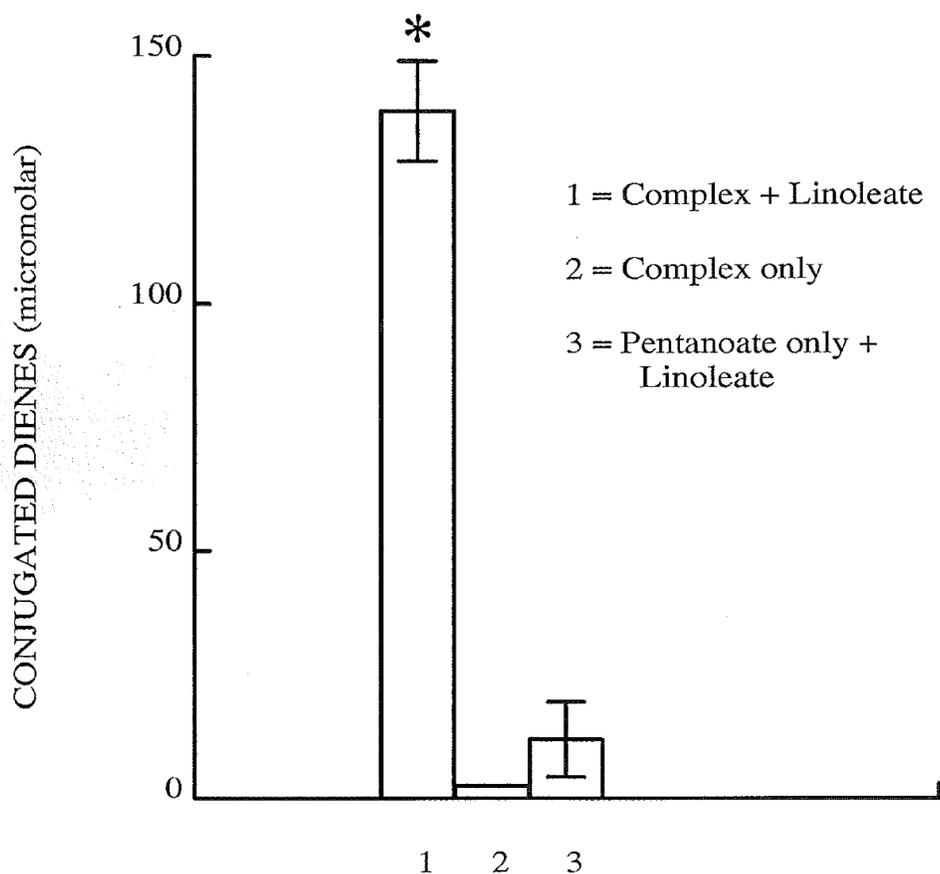


Fig. 4. Formation of conjugated dienes following the addition of 100 μM ferric iron complexed with 600 μM pentanoate to 30 mM suspensions of purified linoleate at pH 7.4. Bar #1 represents mean \pm SE for $n = 6$ experiments; bars 2 & 3 represent means \pm SE for $n = 3$ experiments. Legend: 1 = complex plus linoleate; 2 = complex only; 3 = pentanoate only plus linoleate. * = significant from pentanoate only plus linoleate control at $p < 0.05$.

Oxidation of rat liver slices

Figure 5 demonstrates the formation of MDA-LM as indicators of lipid peroxidation in rat hepatic tissues following the addition of either lipid soluble or water soluble iron salts. The addition of pentanoate only to non-traumatized tissues had no effect on MDA-LM production when compared to untreated, non-traumatized tissue ($F = 1.5$, $p > 0.05$). Water soluble iron in the form of ferric-EDTA slightly and significantly increased MDA-LM production ($F = 9.5$, $p < 0.05$). However, the addition of ferric iron complexed with pentanoate, caused a large increase in the amount of measured MDA-LM compared to all the other treatments of non-traumatized tissue ($F = 252.4$, $p < 0.01$). The effect of the more lipid soluble ferric-pentanoate complex was similar to that produced by maximal tissue trauma.

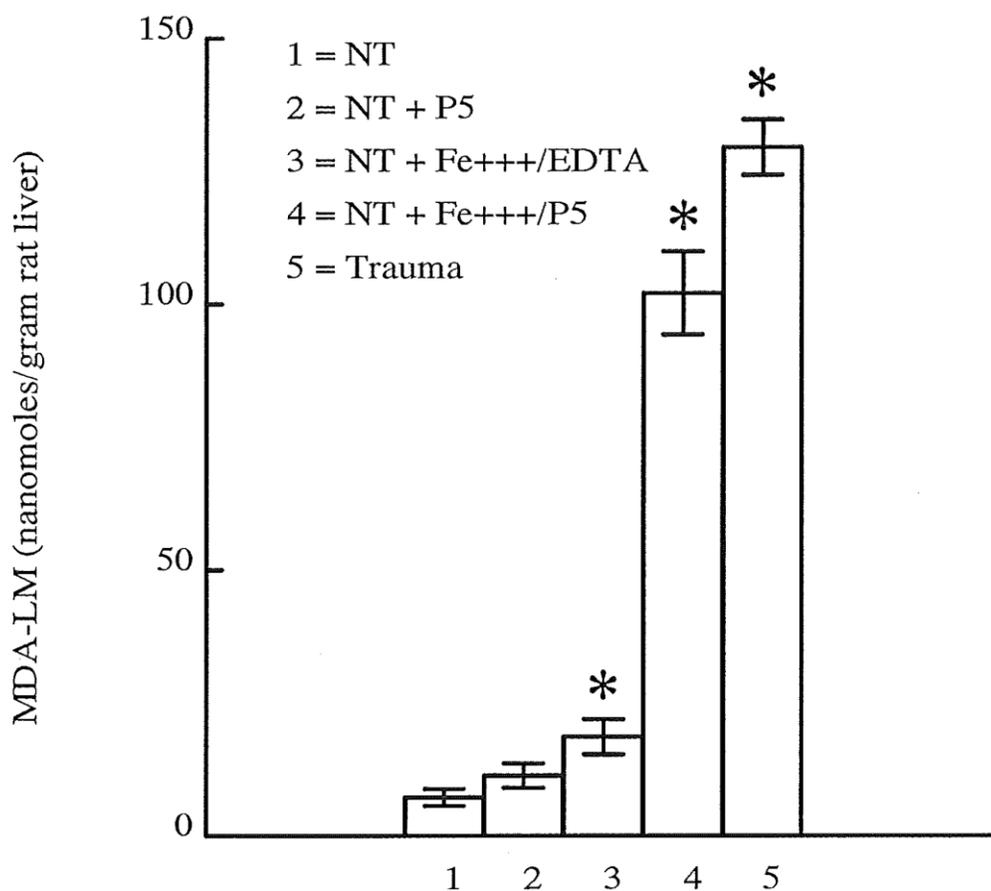


Fig. 5. Formation of MDA-LM in rat hepatic tissue slices following the addition of either lipid soluble or water soluble iron salts. Bars represent mean \pm SE for $n = 6$ experiments. Legend: 1 = non-traumatized tissues (NT); 2 = non-traumatized tissues plus 600 μ M pentanoate (P5); 3 = non-traumatized tissues plus 100 μ M ferric-EDTA (1:1); 4 = non-traumatized tissues plus 100 μ M ferric iron complexed with 600 μ M pentanoate; 5 = traumatized (vigorously minced) tissues without complex added. * = significant from non-traumatized tissues at $p < 0.05$.

Figure 6 illustrates the time course of MDA-LM formation in non-traumatized rat liver tissue incubated with the ferric/pentanoate complex. The increase in MDA-LM with time was significant ($F = 85.8$, $p < 0.01$). As with the formation of CDs in linoleate, there was an early rapid phase of MDA-LM generation, perhaps followed by a later, slower phase. Unlike the linoleate experiment, however, there was a brief initial lag phase in the formation of MDA-LM, possibly attributable to antioxidants such as vitamin E in the cell membranes.

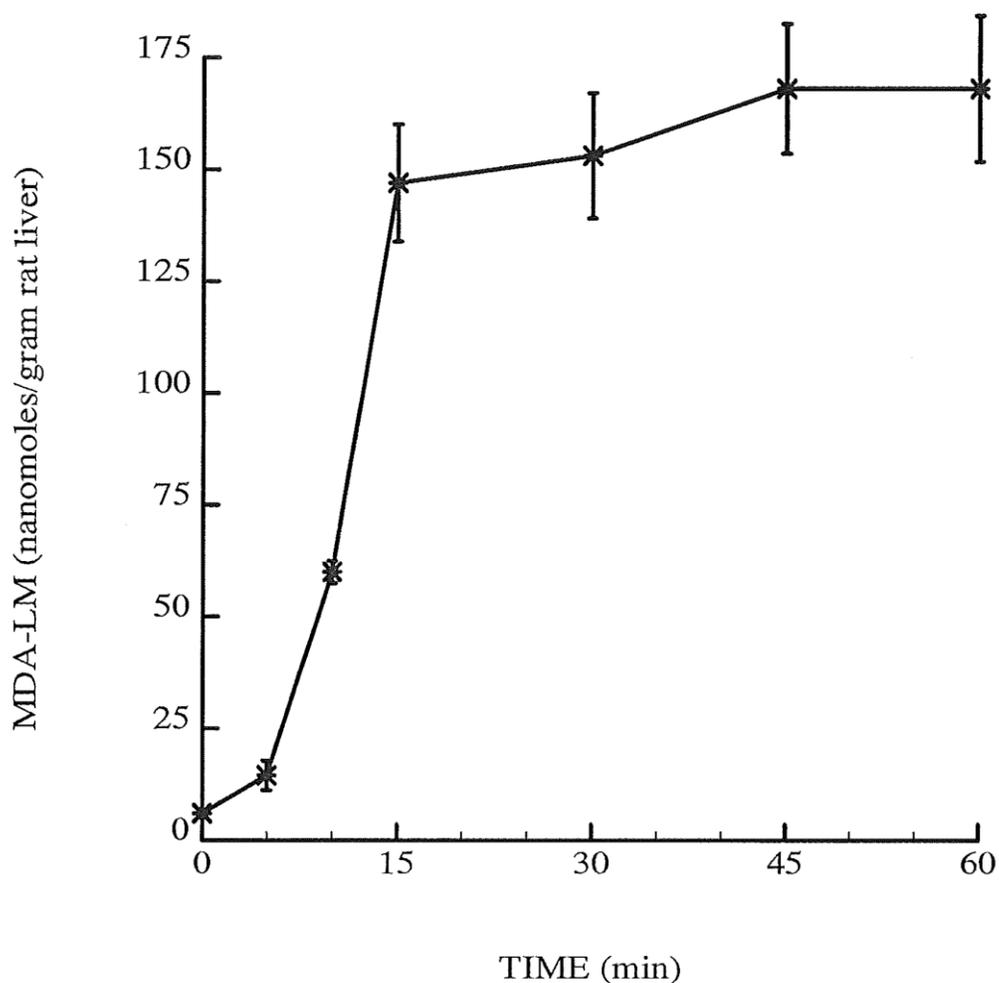


Fig. 6. Time course of MDA-LM formation in non-traumatized rat liver slices incubated with $100 \mu\text{M}$ ferric iron complexed with $600 \mu\text{M}$ pentanoate. Graph shows means \pm SE for $n = 4$ experiments.

Free fatty acid accumulation in membranes

Figure 7 illustrates normal, non-traumatized tissue that has undergone the staining procedure for the detection of free fatty acids. Note the normal microanatomy of the liver lobule with central vein.

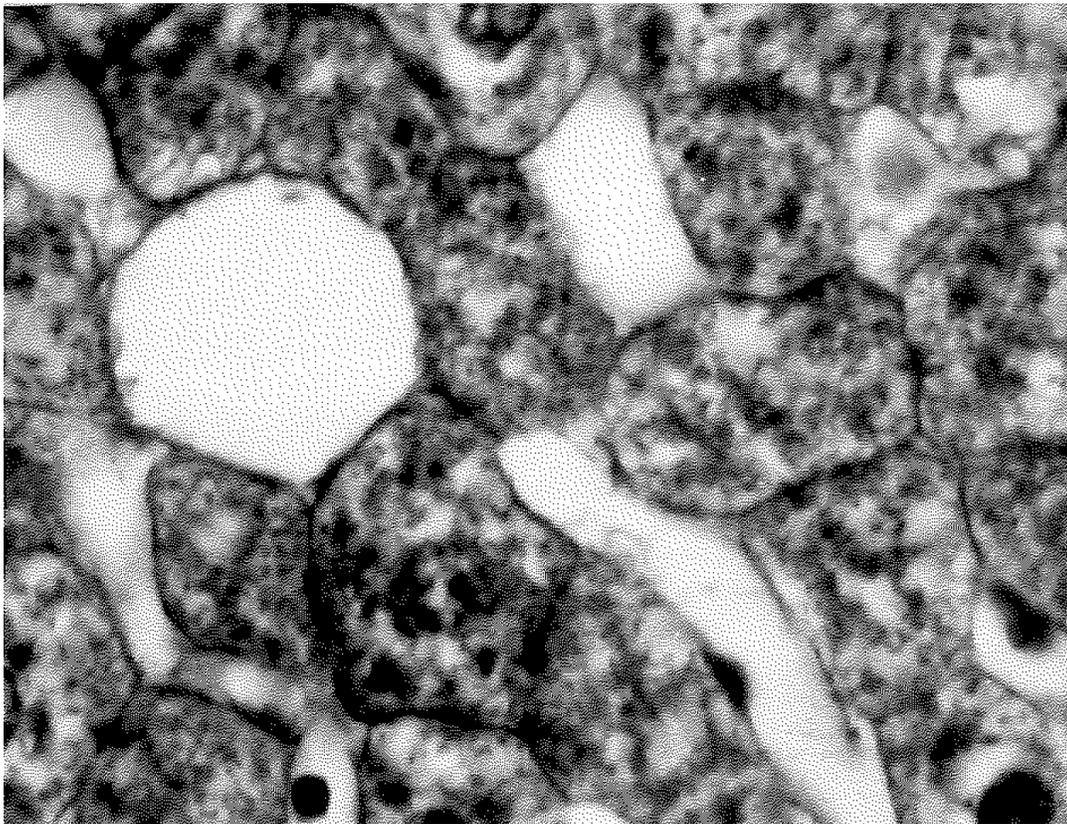


Fig. 7. Photomicrograph of normal non-traumatized liver tissue counterstained with methyl green that has undergone the staining procedure for the detection of free fatty acids. 400X.

In contrast, Figure 8 illustrates traumatized hepatic tissue stained for free fatty acids. This photomicrograph demonstrates the criteria used to identify traumatized regions as well as positive histochemical staining. Hemorrhage, cytoplasmic coagulation, naked nuclei, and cytoplasmic fragments are evident in this section. This field also clearly demonstrates the dark histochemical stain in the cell membranes (arrow) indicating the presence of free fatty acids.

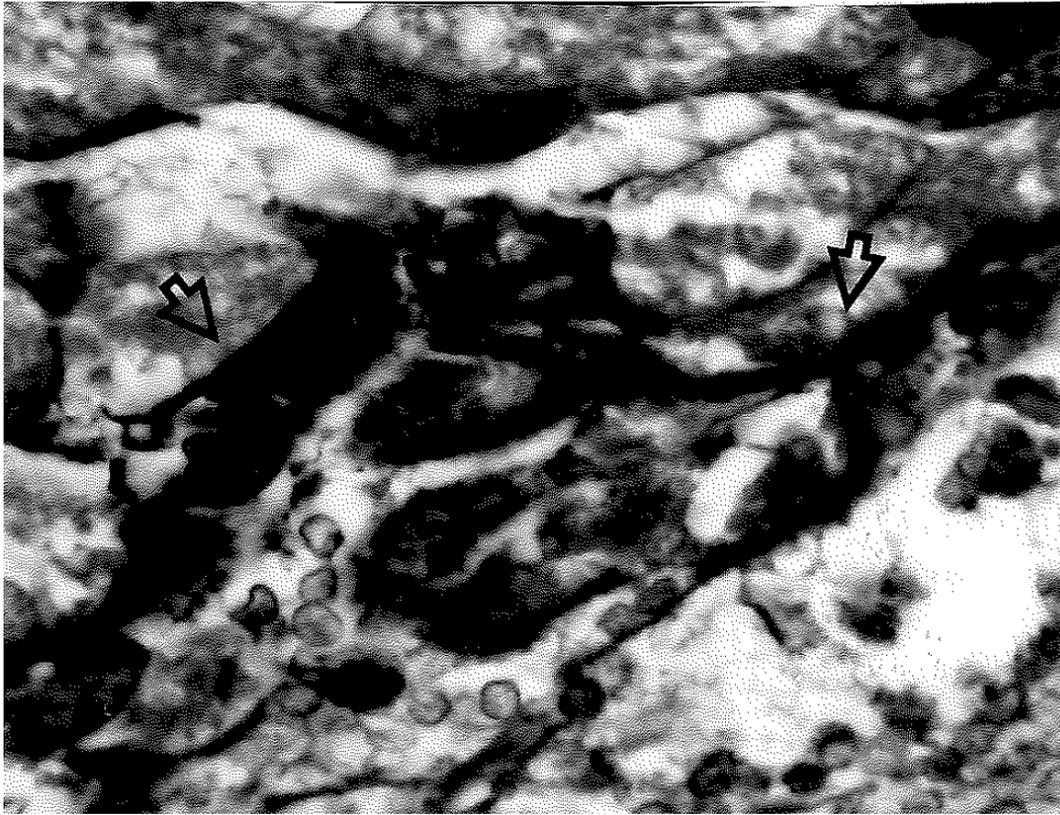


Fig. 8. Photomicrograph of traumatized liver tissue counterstained with methyl green that has undergone the staining procedure for the detection of free fatty acids. Note the presence of hemorrhage and cytoplasmic disruption. Also note the dark histochemical stain present in the cell membranes (arrows) indicating the presence of free fatty acids. 400X.

As a control, Figure 9 illustrates traumatized rat hepatic tissue treated with the organic solvent extraction (to remove fatty acids) prior to staining for free fatty acids. This field illustrates the lack of dark staining within the membranes that was present in the untreated experimental tissues. Figure 10 illustrates traumatized rat hepatic tissue treated with 10 mg/kg of the phospholipase inhibitor, quinacrine. Although the area of trauma is clearly evident, there is minimal dark membrane staining, which was typical. These control experiments support the interpretation that positive histochemical staining represents free fatty acids, which are produced in traumatized tissue by phospholipases.

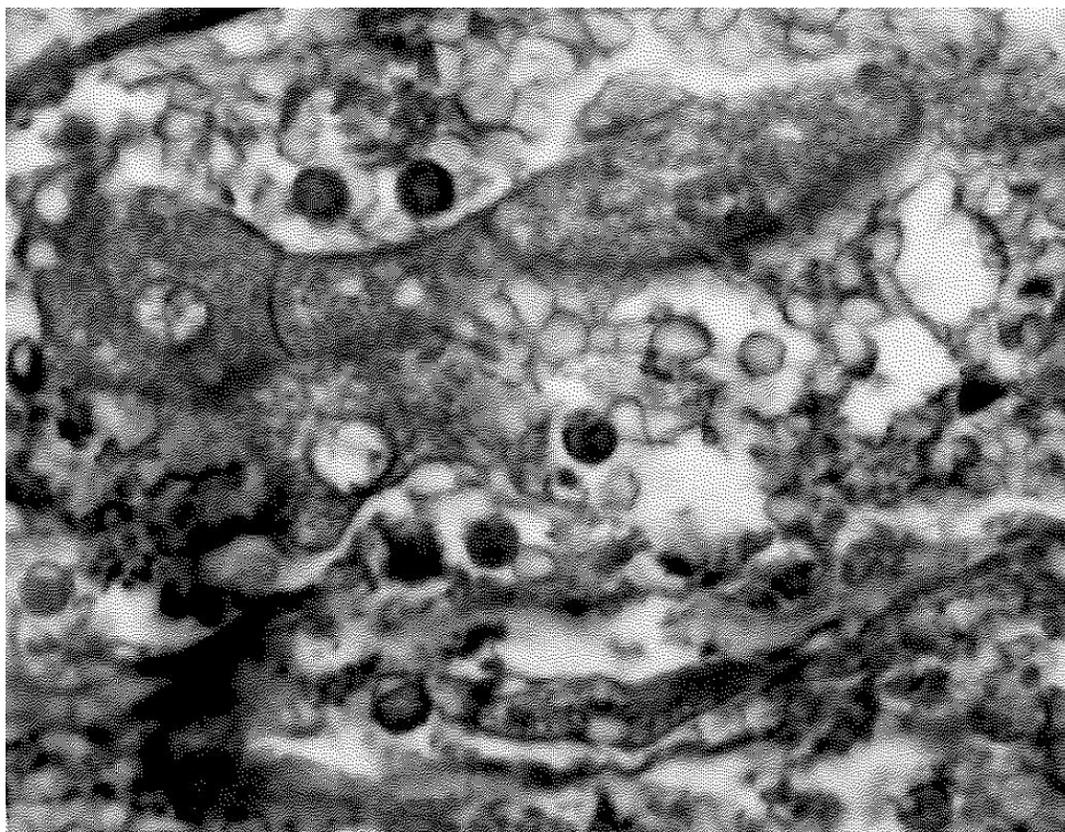


Fig. 9. Photomicrograph of traumatized liver tissue counterstained with methyl green and treated with the organic extraction prior to the staining procedure for the detection of free fatty acids. Note the presence of hemorrhage and cytoplasmic fragments indicative of trauma. Note the lack of dark histochemical stain in the cell membranes. 400X.

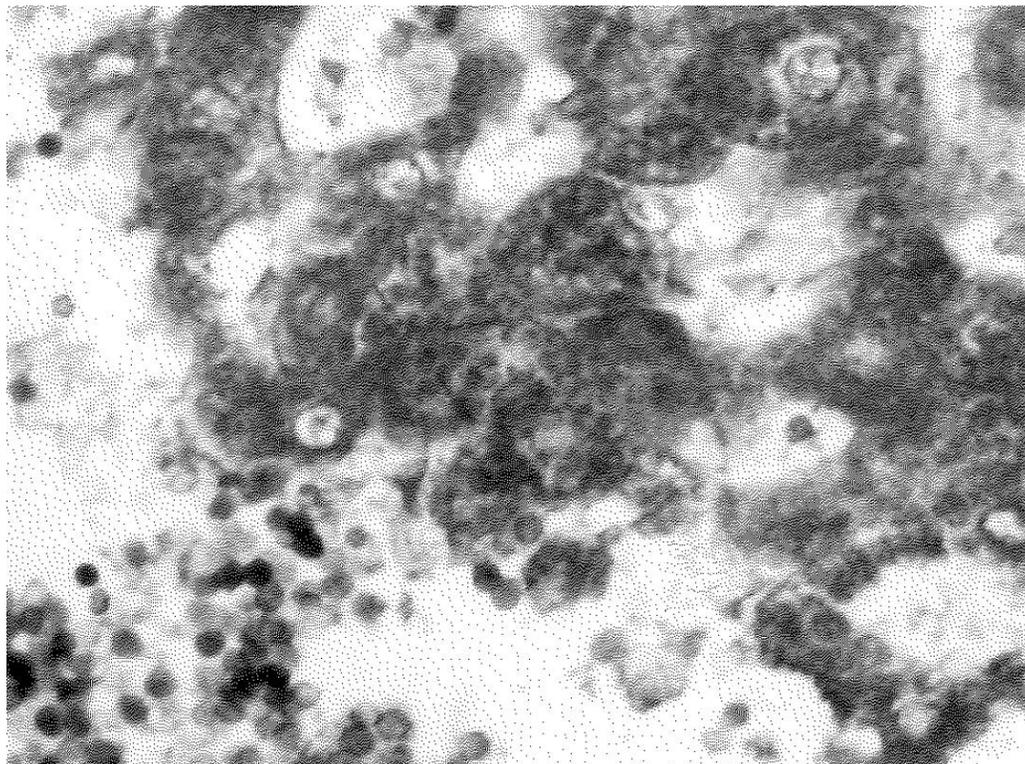


Fig. 10. Photomicrograph of traumatized liver tissue counterstained with methyl green and treated with 10 mg/kg of the phospholipase inhibitor, quinacrine, just prior to traumatic insult that has undergone the staining procedure for the detection of free fatty acids. Note the lack of dark histochemical stain in the cell membranes that was present in the non-quinacrine treated experimental livers. 400X.

Figure 11 summarizes the results of quantitative morphometry of sections stained for free fatty acids in non-traumatized and traumatized rat hepatic tissue. The results of stereological calculations are expressed in square millimeters of positively stained membrane per cubic millimeter of tissue (mm^{-3}). In non-traumatized tissues, membrane staining for free fatty acids was minimal and there was no significant effect of treatments expected to remove free fatty acids or suppress their formation ($F = 1.89$, $p > 0.05$). The area of membranes stained for free fatty acids was significantly greater in untreated traumatized tissues than in untreated non-traumatized tissues ($t = 11.72$, $p < 0.05$). However, comparable positive staining did not occur in the organic extracted tissues ($t = 1.81$, $p > 0.05$) or the quinacrine treated tissues ($t = 2.73$, $p > 0.05$). A group-wise comparison of the traumatized tissues indicated that both the organic extraction treatment and the quinacrine treatment significantly reduced membrane staining ($F = 37.5$, $p < 0.01$) for free fatty acids in traumatized tissues.

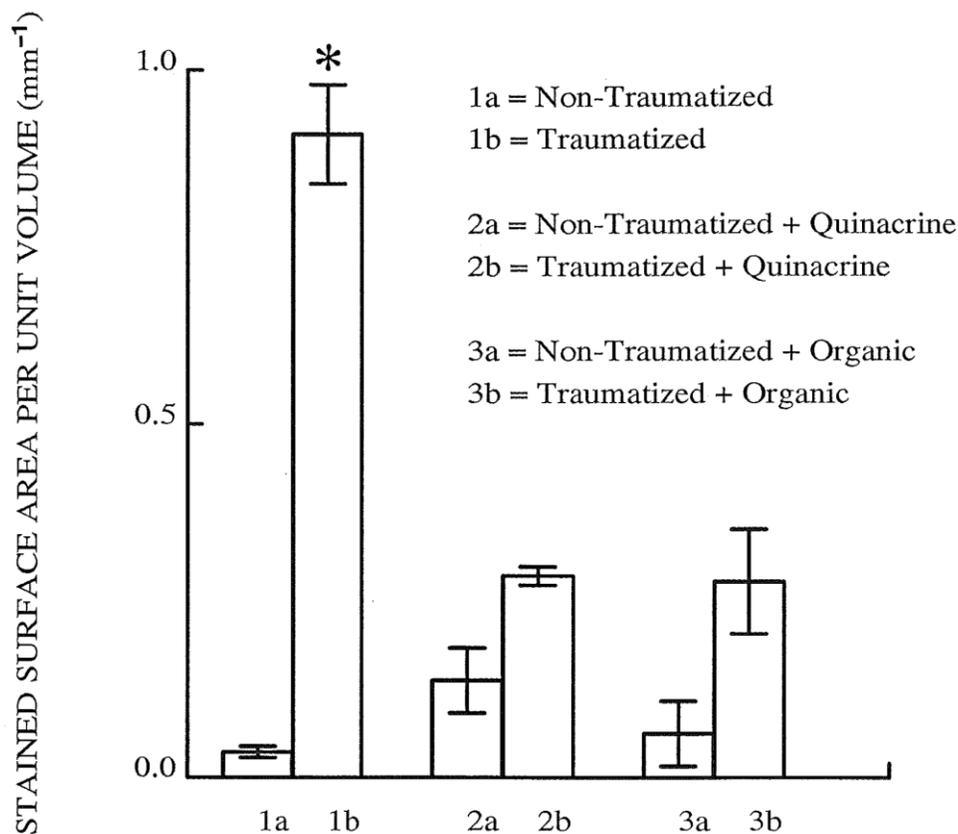


Fig. 11 Quantitative morphometry of sections stained for free fatty acids in non-traumatized and traumatized rat hepatic tissue. Bars represent means \pm SE for $n = 3$ experiments. Legend: 1a = non-traumatized; 1b = traumatized; 2a = non-traumatized plus quinacrine; 2b = traumatized plus quinacrine; 3a = non-traumatized plus organic extraction; 3b = traumatized plus organic extraction. * = significant from non-traumatized control at $p < 0.05$.

Iron accumulation in membranes

Figure 12 illustrates the DAB-H₂O₂ reaction for iron in traumatized hepatic tissue after 30 minutes of in situ blood perfusion. These sections revealed probable dark membrane staining (arrow) accompanied by various degrees of amorphous brown cytoplasmic staining in traumatized cells at the light microscopic level. Importantly, the spatial pattern of piecemeal staining of traumatized cells for redox cyclable iron was similar to the staining pattern that was observed with the Cu⁺⁺-rubeanic acid technique for free fatty acids.

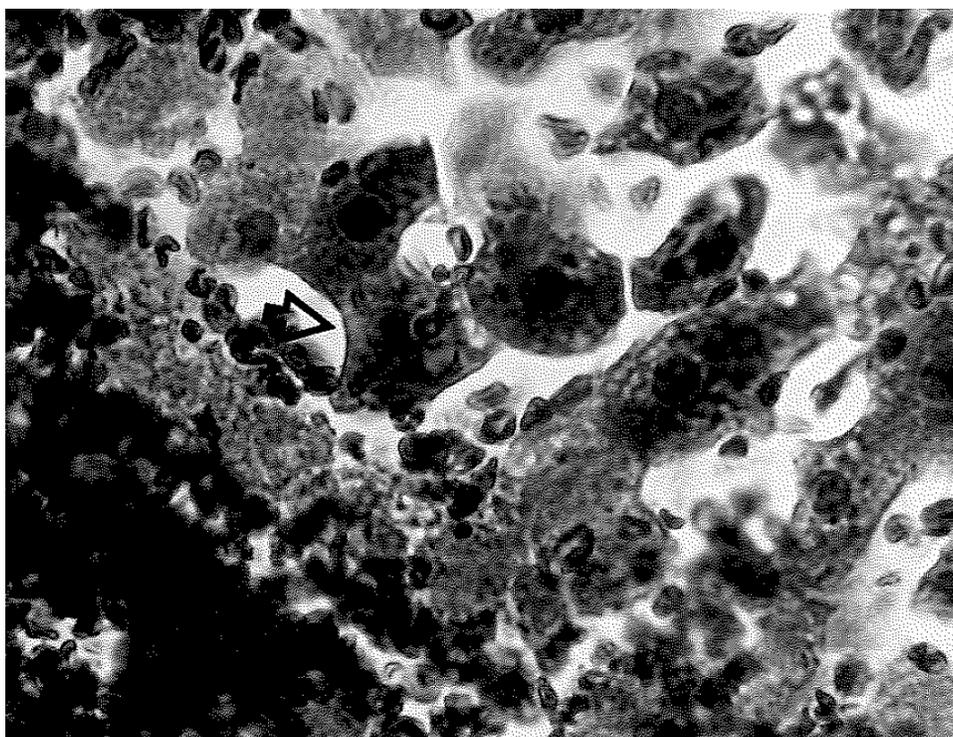


Fig. 12. DAB-H₂O₂ reaction for iron in traumatized hepatic tissue after 30 minutes of in situ blood perfusion. Sections were counterstained with methyl green. These sections illustrate dark brown membrane staining (arrow) accompanied by various degrees of cytoplasmic staining in cells in the traumatized region. 400X.

The observed brown cytoplasmic staining of hepatocytes varied in intensity in different areas but was only associated with the traumatized regions of the tissue, perhaps indicating the presence of free redox cyclable iron in the cytoplasm of the cell as well as in the intracellular membrane matrices of the endoplasmic reticulum and mitochondria. Staining due to peroxidase activity in hepatocytes was unlikely since non-traumatized cells in the same paraffin embedded sections did not stain with DAB-H₂O₂. Red blood cells in the specimen consistently showed a positive (internal control) reaction for iron.

Figures 13 and 14 illustrate the results of negative control experiments. Figure 13 illustrates control, non-traumatized rat hepatic tissue stained with the DAB-H₂O₂ reaction and counterstained with methyl green. Note normal hepatic microstructure with intact liver lobe and central vein. Figure 14 illustrates freshly traumatized hepatic tissue without the 30 minute blood perfusion time. Traumatized area is defined by the presence of the indicators described previously. These slides revealed little membrane staining but a tendency towards an amorphous, brown staining of the cytoplasmic regions of the cells in the traumatized region (arrow). This cytoplasmic staining was absent in the non-traumatized tissues and only vaguely present in the traumatized tissues without blood perfusion. Also note the darkly stained red blood cells (*) which serve as a positive control, since heme iron does redox cycle [103] to cause DAB polymer formation.

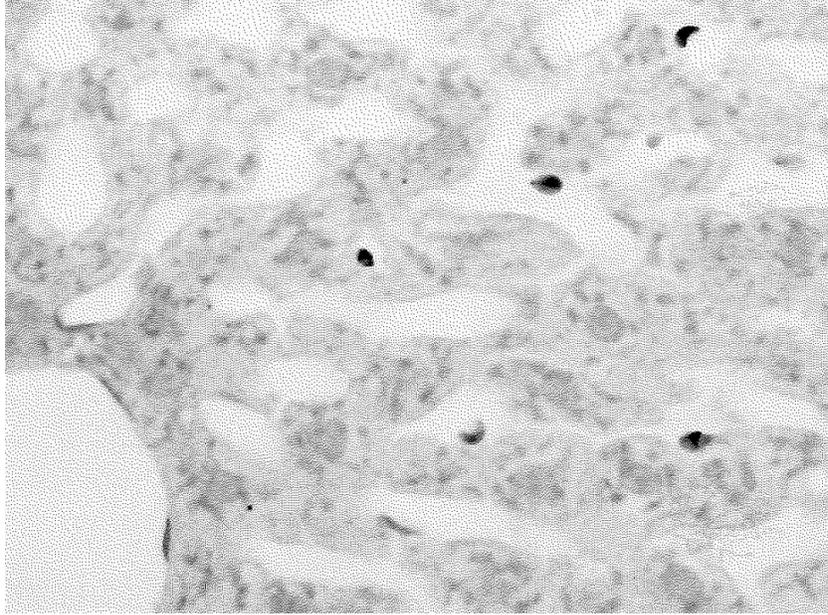


Fig. 13. Negative control DAB-H₂O₂ reaction for iron in non-traumatized hepatic tissue. Sections were counterstained with methyl green. These sections illustrate the lack of dark brown membrane and cytoplasmic staining. Note the darkly stained red blood cells which served as a positive internal controls for redox cyclable iron. 400X.

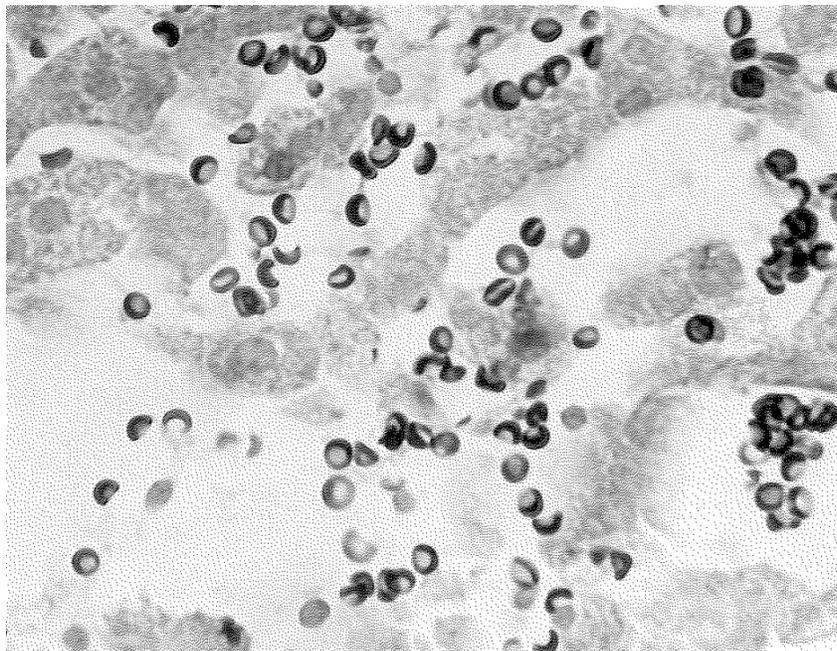


Fig. 14. Negative control DAB-H₂O₂ reaction for iron in traumatized hepatic tissue without the 30 minutes of in situ blood perfusion. Sections were counterstained with methyl green. Hepatocytes show little dark brown membrane staining with only a small degree cytoplasmic staining in cells in the traumatized region. Many positive red cells are present. 400X.

Figure 15 summarizes the results of stereologic analysis of membrane staining for iron by the DAB-H₂O₂ reaction. There was a highly significant effect of tissue trauma ($F = 73.4$, $p < 0.01$). Membrane staining was slightly and significantly increased after trauma without blood perfusion ($F = 20.7$, $p < 0.01$), compared to non-traumatized controls, and membrane staining was greatly increased after trauma followed by 30 min in situ blood perfusion ($F = 116.4$, $p < 0.01$). The density of membrane surface positively stained for low molecular weight chelate iron was on the same order of magnitude as the density of membrane surface positively stained for free fatty acids.

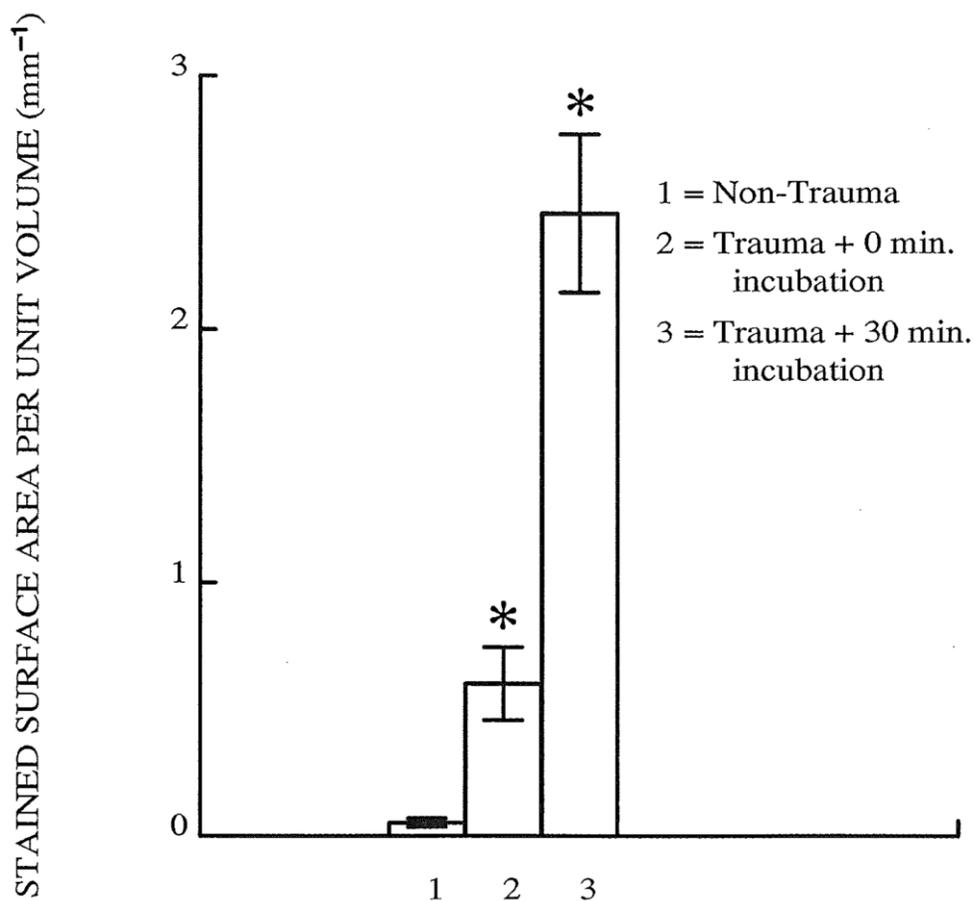


Fig. 15. Stereological analysis of membrane staining for iron by the DAB-H₂O₂ reaction. Bars represent means \pm SE for $n = 4$ experiments ($n = 3$ for non-traumatized tissues). Legend: 1 = non-traumatized tissues; 2 = traumatized tissues plus 0 min blood perfusion; 3 = traumatized tissues plus 30 min blood perfusion. * = significant from non-traumatized controls at $p < 0.05$.

Electron microscopy

Because the heavy cytoplasmic component of the DAB-H₂O₂ reaction product tended to mask membrane localization at the light microscopic level; we performed electron microscopy. Figure 16 illustrates the ultrastructural appearance of DAB-H₂O₂ reaction product in traumatized hepatic tissues. In both non-traumatized and traumatized tissues, peroxisome staining (P) was observed. This result was expected, since previous work in our laboratory had utilized the DAB-H₂O₂ reaction to stain for peroxidase activity in peroxisomes. [68] In the application of the DAB-H₂O₂ reaction employed here, the catalytic activity of low molecular weight chelate iron is visualized as a pseudoperoxidase. In traumatized tissues, individual cells revealed either of two cytoplasmic staining patterns: light or heavy. Some cells in the traumatized area showed signs of cytoplasmic disruption, vacuolization, and severe swelling of the mitochondria.

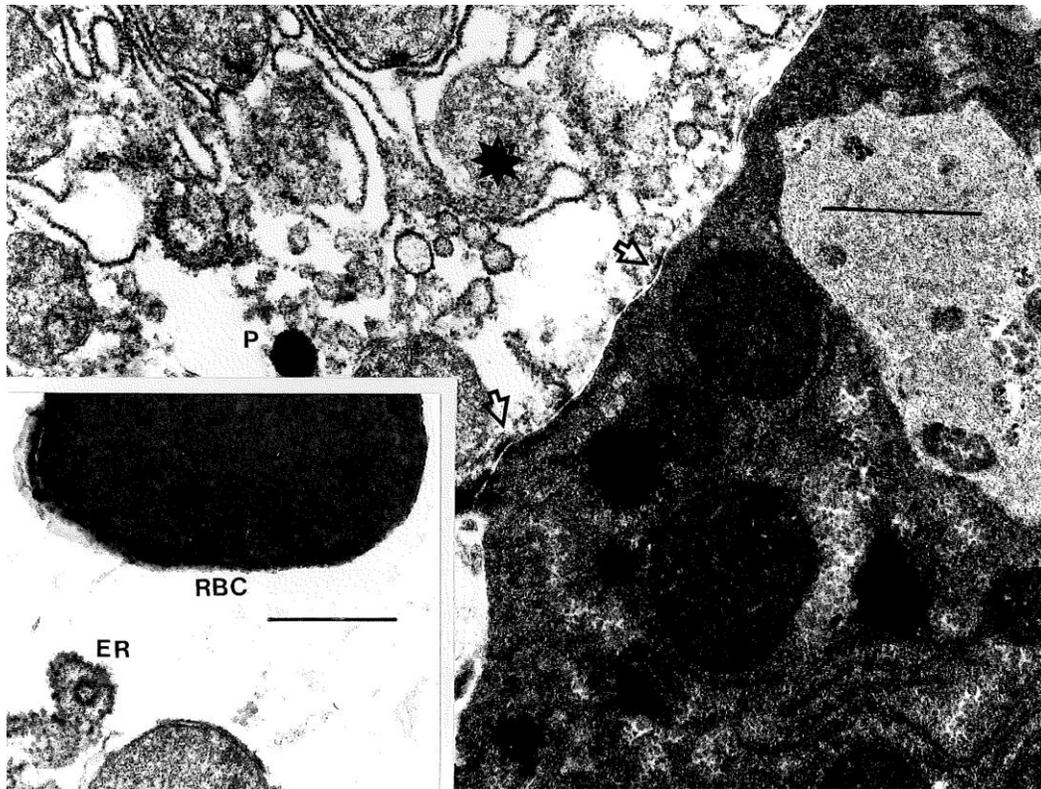


Fig. 16. Electron micrograph of traumatized hepatic tissue stained for iron using the DAB-H₂O₂ reaction. Note individual cells exhibiting evidence of mitochondrial swelling and cytoplasmic disruption (*). Also note densities in cell membranes (arrows) suggesting that reaction product is present in traumatized, but not in non-traumatized tissues. Note similar density of the peroxisome (P) indicating residual peroxidase activity. Bar = 1 micron. Inset shows red blood cell (RBC) and nearby endoplasmic reticulum (ER) at 40,000X to illustrate the concentration of the reaction product in and around membrane structures. Inset bar = 0.5 micron.

Additionally, in these same cells, there was electron dense reaction product associated with internal and external cell membranes. The piecemeal distribution of reaction product observed by electron microscopy correlated well with what was observed by light microscopy (see Figure 12). Intracellularly, the electron microscope revealed reaction product in mitochondrial membranes and in rough endoplasmic reticulum (RER). The dense reaction product appeared to be "layered" out on the RER and was readily observed at 40,000X as well as on the plasma membrane of the nearby red blood cell (insert).

As a control, Figure 17 illustrates traumatized hepatic tissues that did not undergo the DAB-H₂O₂ reaction for redox cyclable iron. In these tissues, the ultrastructural evidence for trauma, such as a mitochondrial disruption, etc., was observed, but the electron dense reaction product that was tightly bound to the lipid bilayers of the rough endoplasmic reticulum, mitochondria, and plasmalemma of injured hepatocytes was not observed.

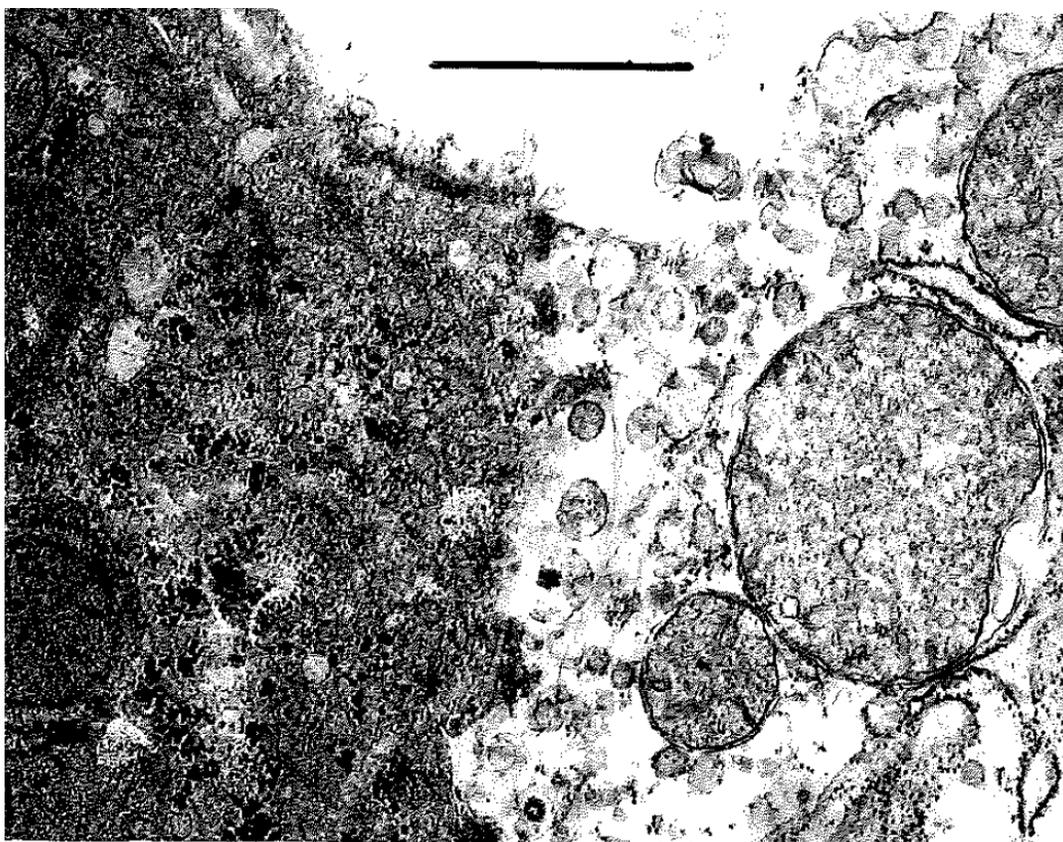


Fig. 17. Control traumatized hepatic tissues that did not undergo the DAB-H₂O₂ reaction for redox cyclable iron. The electron dense reaction product in the lipid bilayers of the rough endoplasmic reticulum, mitochondria, and plasmalemma was not observed. Bar = 1 micron.

As an additional control, non-traumatized tissues under electron microscopy did not reveal the staining patterns mentioned above but did reveal relatively normal liver cell structure (Figure 18).

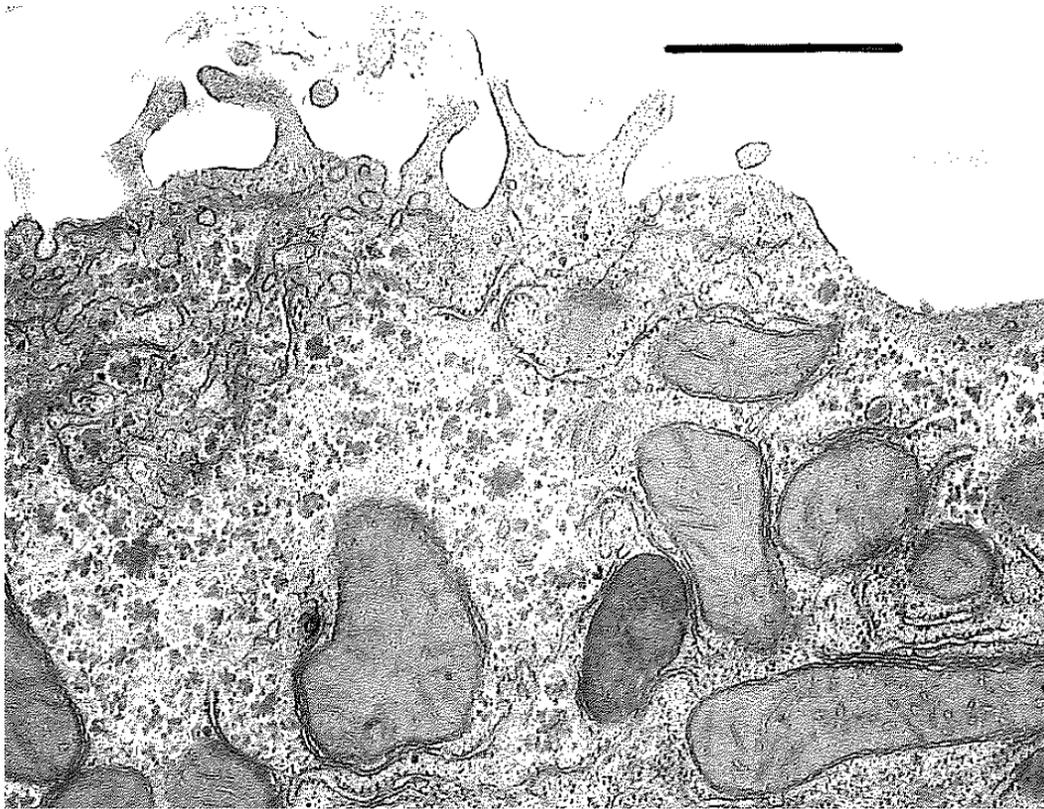


Fig. 18. Electron micrograph of control, non-traumatized hepatic tissue stained for iron using the DAB-H₂O₂ reaction. Note normal tissue structure and membrane staining. The peroxisomes show increased density expected on the basis of residual peroxidase activity. Bar = 1 micron

DISCUSSION

The forgoing results demonstrate that products of lipid peroxidation, including conjugated dienes and MDA-LM can be formed directly by the interaction of free fatty acid-iron chelates with purified polyunsaturated fatty acids or with tissue slices. The companion results demonstrate that both free fatty acids and redox cyclable iron can be detected in membranes of traumatized hepatic tissues known to undergo extensive lipid peroxidation. Taken together, these results provide rather straightforward evidence for a novel mechanism of tissue injury by free fatty acid-iron complexes.

The original inspiration for this work came from previous studies in our laboratory demonstrating the importance of trauma as a stimulus for oxidative stress in hepatic tissue [90] and the absence of detectable increases in hydrogen peroxide [68] or expected lethal bursts of hydroxyl radicals [65] in models of oxidative stress induced by anoxia and reoxygenation. In turn, we developed the working hypothesis that oxidative damage following trauma or transient ischemia might be due to

the direct effects of iron rather than the direct effects of reactive oxygen species. To test this hypothesis, we employed the conjugated diene assay to investigate the ability of pathobiologically realistic amounts low molecular weight chelate iron [85, 86] to directly oxidize micellar polyunsaturated fatty acids. Importantly, ferric iron in the absence of oxygen was capable of directly oxidizing linoleate to form conjugated dienes. Linoleate suspensions treated with ferrous iron in the absence of oxygen did not form CDs. However, if ferrous iron is added in the presence of oxygen (about 200 μM O_2 in air equilibrated water [104] vs. 100 μM iron) CD formation returned. This latter result is explained by the autoxidation of ferrous ions chelated to free fatty acids



a phenomenon which is readily observable as a color change (colorless \rightarrow yellow-orange) in 1 mM free fatty acid-iron suspensions.

Upon further reflection, it became evident that the iron would probably be most capable of initiating lipid oxidation *in vivo* in a lipid soluble form. Hence, we utilized the complex of ferric iron and pentanoate. This short chain (5 carbon) free fatty acid salt was chosen because the free fatty acid itself was soluble in water as well as in a lipid environment and because it formed stable yellow suspensions with ferric iron that did not adhere to laboratory glassware. Our idea was that these more hydrophobic short chain free fatty acid-iron complexes could be formed in an aqueous environment and then migrate into the lipid micelles or lipid bilayers.

In the conjugated diene studies, these free fatty acid-iron complexes could in fact induce formation of conjugated dienes in micellar linoleate suspensions at pH 7.4. Based on these results, it was concluded that the initial results with ferric or ferrous iron only were probably due to the spontaneous formation of lipid soluble free fatty acid-iron salts, which became incorporated into micellar structures to initiate conjugated diene formation by a non-Fenton mechanism.* According to this hypothesis, Fenton's reaction may still occur *in vivo*, but it is important as a source of ferric iron rather than as a source of $\text{HO}\cdot$.

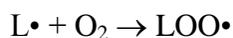
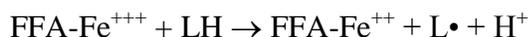
The next step was to see if these free fatty acid-iron complexes could cause lipid peroxidation in fresh tissue. For these experiments, we employed our previously published model of short term organ culture [66-68] and exposed fresh liver tissue to the free fatty acid-iron complexes in the form of iron-pentanoate. In this model system, the Fe-FFA complex was able to significantly increase MDA-LM production in liver slices in a time dependent manner quite similar to that observed when iron was added to air-equilibrated suspensions of polyunsaturated fatty acids. A slight lag period in MDA-LM production was observed within the first five minutes of

* Regarding non-Fenton mechanisms of lipid peroxidation, Gutteridge [105] argued that in practice, liposomes and microsomes always some pre-existing lipid hydroperoxides, so that chain initiation can occur without $\text{HO}\cdot$ in the presence of ferrous iron, $\text{LOOH} + \text{Fe}^{++} \rightarrow \text{LO}\cdot + \text{OH}^- + \text{Fe}^{+++}$, a process that decomposes lipid hydroperoxides into chain propagating alcoxyl radicals. However, this possibility is excluded as a major factor in the experiments we report here with purified lipids, since in these experiments ferrous iron added to the lipids anaerobically caused little CD formation. Only when oxygen was added, did CDs appear, as was the case when ferric iron was added anaerobically.

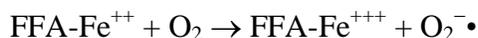
incubation, and may be explained either by the time required for the complexes to distribute to the lipid bilayer of the cell membranes or by local antioxidants such as Vitamin E. The results also clearly demonstrated that the more lipophilic free fatty acid-iron complex was highly effective in producing MDA-LM during the incubation period, compared to the water soluble ferric-EDTA chelate. The results with ferric-EDTA were not unexpected since, in contrast to iron pentanoate complexes, water soluble ferric-EDTA, as previously shown by Borg and Schaich, [106] does not distribute well to the lipid phase of free fatty acid suspensions.

Finally, in support of the hypothesis that such direct initiation mechanisms may occur *in vivo*, we found histochemical evidence of increased free fatty acids in the cell membranes near areas of trauma in rat liver. Companion histochemical stains for iron revealed a very similar microscopic distribution of redox cyclable iron in traumatized cells in local concentrations comparable to that found within normal red blood cells (about 17 mM). In electron micrographs redox cyclable iron stain was associated with membrane structures.

Accordingly, we suggest that low molecular weight chelate iron and both long chain and short chain free fatty acids spontaneously form water insoluble complexes that are analogous to soap scum that is formed from complexes of free fatty acid salts and heavy metals in hard water areas. Just as do household soap scums, such complexes tend to deposit on surfaces, which *in vivo* are comprised of phospholipid membranes. (Indeed, we humorously refer to the mechanism herein proposed as "the soap scum hypothesis".) To the extent that such complexes enter the lipid domains of the cell membrane, we suggest that free fatty acid-ferric iron complexes are able to abstract hydrogen atoms from polyunsaturated fatty acids and directly initiate the lipid peroxidation cascade:

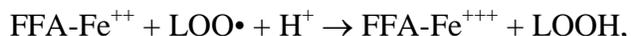


We also speculate that the initial reaction of ferric-FFA complexes with the methylene C-H bond of a polyunsaturated fatty acid may be aided by the 5-membered ring structure of the intermediate complexes, favoring a concerted reaction that simultaneously protonates the carboxy acid, reduces the iron, and liberates the free fatty acid radicals. Subsequent redox cycling of the iron, in the presence of molecular oxygen,



returns the ferrous iron to the ferric state and the complex is able to oxidize another lipid.

Alternatively, complexed ferrous iron may reduce the peroxy radicals themselves [107, 108]



to continue the cycle of lipid oxidation. This later reaction may explain previous observations that

mixtures of Fe^{++} and Fe^{+++} iron induce greater initial rates of lipid peroxidation than the same total iron in purely ferrous or ferric forms. [109]

If the forgoing novel mechanism is correct, the question then arises, how do these complexes arise during naturally occurring instances of oxidative stress? The larger pathophysiologic cascade that we propose is initiated by non-selective entry of calcium into cells that are stressed by either ischemia or trauma. [17] It has long been known that rapid influx of calcium occurs in ischemia due to failure of ATP dependent ion pumps. [110] We further suggest that trauma may physically disrupt cell membranes making them leaky (at least temporarily) to calcium, therefore allowing intracellular calcium intoxication.

Elevated levels of cytosolic calcium will result in mitochondrial intoxication, as a result of calcium uptake, followed by uncoupling of the electron transport chain and subsequent depletion of cellular energy stores. [111-113] Concurrently, elevated cytosolic calcium may activate membrane phospholipases [114, 115], which begin liberating free fatty acids in the vicinity of the cell membrane. In the case of ischemic damage, several reports have demonstrated that free fatty acid concentrations increase in ischemic dog myocardium. [81, 116] Also, Otani et al showed that the phospholipase inhibitor, mepacrine, caused significant improvement in high energy phosphates, coronary blood flow and ventricular function of postischemic pig myocardium. [117]

We found similar results histochemically in traumatized liver tissue, as would be expected as a result of phospholipase activation. Further, pretreatment with the phospholipase inhibitor, quinacrine, sharply attenuated histochemical staining for free fatty acids. Theoretically, the detergent-like action of the free fatty acids may well destabilize the membrane making it more leaky to ions like calcium or by acting like ionophores and so continuing the cycle of calcium intoxication. This phenomenon provides an opportunity for positive feedback (Figure 1): calcium intoxication begets free fatty acid release, which begets more calcium intoxication. In support of this concept, Philipson and Ward [118] have shown that free fatty acids increased the permeability of sarcolemmal vesicles to ionized calcium by as much as 150%.

The present studies also suggest that in addition to their direct physical effects on the membrane, free fatty acids may act chemically as chelators of low molecular weight chelate iron liberated from stores within the injured hepatocytes. Although the catalytic iron concentration in vivo is in the low micromolar range, around 5 micromoles/kg except in iron-overload diseases, on a volume averaged basis, the partitioning of liposoluble iron complexes within membranes, detected in the present work histochemically, could raise the local iron concentration in the immediate vicinity of oxidizable fatty acids perhaps 40 fold [119] to 200 micromoles/kg. In traumatized tissues, where increased iron liberation may occur, the intra-membranal value may be even greater. This value is clearly sufficient to support lipid peroxidation, as shown both by previous investigations [63] and by the companion in vitro studies reported here. Iron may be liberated from ferritin by the action of calcium activated proteases [72] degrading the macromolecular structure of ferritin and thus liberating low molecular weight chelate iron. [120] The iron may also be released from ferritin by the increased amounts of superoxide that are generated by calcium intoxicated mitochondria. [73] Hydrogen peroxide can degrade hemoglobin (which is abundant in traumatized tissue) to release low molecular weight chelate iron. [63]

Thus, intracellular calcium intoxication and oxidant stress from excess superoxide and hydrogen peroxide can create the raw materials required for free fatty acid-iron complex formation and subsequent lipid peroxidation. In turn, oxidants and products of lipid peroxidation themselves may further reinforce the free original fatty acid-calcium cycle. Au and coworkers, [121] for example, showed calcium dependent activation of membrane phospholipase A2 activity in isolated brain capillaries by free radicals generated by xanthine oxidase, hypoxanthine, and iron. EGTA (ethylene glycol bis(β -aminoethyl ether) N, N, N', N' tetraacetic acid) abolished the action of oxygen radicals on phospholipid breakdown and fatty acid release, suggesting a role for extracellular calcium ions in phospholipase activation by free radicals. Sevanian and Kim [122] also demonstrated that phospholipase A2 can be activated in the absence of calcium by peroxidized fatty acids in the phospholipids. Further, Braughler [17] has reported that lipid hydroperoxides themselves, in concentrations as low as 1 micromolar increase the permeability of synaptosome preparations to calcium. Hence, yet another route for positive feedback is provided. Importantly, in Braughler's system the strong iron chelator, deferoxamine, completely inhibited both evidence of lipid peroxidation and radiolabelled calcium uptake into synaptosomes. [17]

In conclusion, we present several lines of evidence that iron and free fatty acid complexes form in the traumatized rat hepatic tissue model and that these complexes are capable of directly initiating lipid peroxidation without the intermediate formation of hydroxyl radicals. We further propose that a calcium dependent initiation mechanism of iron liberation and phospholipase activation furnishes the necessary low molecular weight chelate iron and free fatty acids. Once started, this cascade leading to lipid oxidation and membrane damage is sustained and amplified by several potential positive feedback loops embedded in the "soap scum" mechanism of lipid peroxidation (Figure 1), making it a biochemically explosive process more likely than not to be pathologically important. An intellectually satisfying virtue of the free fatty acid iron hypothesis proposed herein is that it unifies the roles of lipid peroxidation and intracellular calcium intoxication in the pathophysiology of cell death.

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ABBREVIATIONS

CD--conjugated diene DAB--diaminobenzidine

EDTA--ethylenediaminetetraacetic acid FFA--free fatty acid

H&E--hematoxylin and eosin

MDA-LM--malondialdehyde-like material· PUFA--polyunsaturated fatty acid

SOD--superoxide dismutase

Tris--Tris-hydroxymethylaminomethane

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