Iron Chelation as a Possible Mechanism for Aspirin-induced Malondialdehyde Production by Mouse Liver Microsomes and Mitochondria

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Abstract

To investigate the possibility that lipid peroxidation is the mechanism responsible for aspirin-induced liver damage, pure neutralized acetylsalicylic acid (ASA), 0.6–90.9 mM, was added to calcium-aggregated mouse liver microsomes followed by incubation in NADPH buffer at 37°C for 60 min and subsequent measurement of malondialdehyde (MDA). MDA production at ASA concentrations from 1.2 to 4.6 mM was greater than control ($P < 0.004$). Peak MDA values were observed with 4.6 mM ASA, 39.58 ± 6.73 nmol MDA/mg protein vs. 16.16 ± 2.85 ($P < 0.004$). Higher concentrations of ASA were inhibitory compared with the value at 4.6 mM ($P < 0.001$). Aspirin had similar effects on MDA production by mouse liver mitochondria. MDA production with either ASA or buffer was completely suppressed by the potent iron-chelating agents desferrioxamine and alpha,alpha' dipyridyl when these were added to the microsomal preparations. Since MDA production in this system is known to be affected by iron-chelating agents (enhanced at low concentration, inhibited at higher concentration), the iron-chelating properties of ASA were investigated. Conductivity titration curves of Fe(OH)$_3$ added to water or ASA suggested that the ASA was complexing with iron. The presence of an iron-ASA complex was established by high pressure liquid chromatographic analysis of the solution from this study. We conclude that aspirin enhances MDA production by hepatic microsomes and mitochondria via an aspirin–iron chelate and that this represents at least one mechanism by which aspirin may produce liver damage.

Introduction

Although the hepatotoxic potential of aspirin was first described in 1971 (and over 300 cases of aspirin-related hepatic injury have been reported), the precise mechanism of injury remains unknown (4). However, some evidence has accumulated that salicylates have an injurious effect on hepatocyte plasma and organelle membranes, resulting in the loss of important metabolites from the cell. For example, Kaplowitz et al. (5) have observed decreased hepatic glutathione in rats given aspirin or sodium salicylate by the intraperitoneal route. These workers demonstrated that incubation of liver slices in the presence of sodium salicylate (100 mg/dl) was associated with leakage of glutathione from liver into the supernate. Aspirin has no effect on the enzymes regulating the synthesis and catabolism of glutathione. Similarly, Tolman et al. (6) reported that administration of graded doses of sodium salicylate to rat liver cells cultured in monolayers was associated with loss of lactic dehydrogenase into the culture media. Finally, Martens and Lee (7) demonstrated that incubation of isolated hepatic mitochondria with salicylate plus calcium induced loss of 90% of the intramitochondrial NAD$^+$ and NADP$^+$ into the surrounding medium.

Peroxidation of membrane long chain polyunsaturated fatty acids is a common mechanism whereby membrane damage can occur (8). Since aspirin can stimulate lipid peroxidation in erythrocytes incubated in the presence of an oxidant stress (H$_2$O$_2$) (9), the possibility that aspirin might also stimulate lipid peroxidation in hepatic microsomes and mitochondria was investigated.

Hunter et al. (10) have demonstrated that hepatic mitochondria form lipid peroxides when exposed to even minute amounts of iron. The central role of iron in stimulating NADPH-dependent lipid peroxidation in hepatic microsomes was first described in 1964 by Hochstein et al. (11). Subsequently Wills (12) observed that no other metal ion could adequately replace inorganic iron in this system. Utilizing a model system made from extracted microsomal lipids as substrates for peroxidation, Tien et al. (13) noted that addition of the iron-chelating agent EDTA to this system enhanced lipid peroxidation. Thus, in the present study, after we had described the ability of aspirin to enhance hepatic mitochondrial and microsomal lipid peroxidation, the possibility that aspirin could chelate iron was investigated.

Methods

Microsomal malondialdehyde (MDA)[1] production

Livers were obtained from male, Balb C mice nourished with mouse chow (Ralston-Purina Co., St. Louis, MO) and given ad libitum water. Average mouse weight was 18.4±0.2 g. Food and water were withdrawn just before killing by cervical dislocation and rapid hepatectomy was performed. Calcium aggregated microsomes were obtained by a modification of the method of Roders et al. (14) as follows: a 10% wt/vol whole liver homogenate in 0.154 M NaCl was centrifuged at 2,700 g for 10 min (Accuspin FR centrifuge with a TH-4 horizontal rotor with buckets; Beckman Instruments, Inc., Fullerton, CA). (At this high ionic strength, the mitochondria are completely sedimented.[2]

1. *Abbreviations used in this paper:* ASA, acetylsalicylic acid; MDA, malondialdehyde.
2. Recknagel, R. O., personal communication.

The postmitochondrial supernatant fraction was diluted 1:2 (vol/vol) with 0.154 M NaCl, and 1.0 M $CaCl_2$ was added for a final concentration of 10 mM Ca^{++}. After thorough mixing, the suspension was centrifuged at 20,000 g for 15 min (J2-21 centrifuge with JA-17 rotor; Beckman Instruments, Inc.). The resulting pellet was resuspended by sonication and diluted (10% wt/vol) with phosphate-buffered saline (0.51 mM KH_2PO_4, 3.44 mM K_2HPO_4, and 150 mM NaCl, pH 6.6). This yielded a suspension containing 100 mgeq calcium aggregated microsomes per milliliter. (A milligram equivalent of microsomes is that amount of microsomes derived from 1 mg wet weight of whole liver.) All work involved with isolation of the microsomes was carried out at 4°C.

Microsomes were incubated aerobically in a water bath with agitation in a system containing 0.3 mM NADPH, phosphate-buffered saline (as above), and 25 mgeq calcium aggregated microsomes per milliliter, at 37°C (values refer to final concentration). Just before incubation, neutralized (pH 7.0) acetylsalicylic acid (ASA) (2-acetoxybenzoic acid) (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline or an equal volume of buffer alone was added to the microsomal incubation media. According to the manufacturer, ASA was of the highest (> 99%) purity available. MDA was measured after 60 min of incubation by the thiobarbituric acid method (15). Results were expressed on the basis of microsomal protein analyzed (16) before the addition of aspirin to the incubate. The standard curve for MDA was measured by thiobarbituric acid reacting with an acidic ethanolic solution of tetraethoxypropane. To determine if aspirin affected either this reaction or the absorbance value of the product, the MDA standard curve was conducted in the presence of aspirin in concentrations of 0.16, 0.32, 1.3, 2.5, and 17.6 mM. Aspirin in these concentrations had no effect on the MDA standard curve. To test the possibility that obtaining the microsomes via calcium aggregation affected the results, similar experiments were performed with microsomes obtained by the conventional method of sequential differential centrifugation (17). MDA production in microsomes obtained in this fashion and incubated with 2.3 mM ASA was 44.6±1.7 nmol MDA (n = 6) vs. 15.9±1.1 (n = 6) for controls (P < 0.001) (similar to values described in the Results section for calcium-aggregated microsomes).

In some experiments with calcium-aggregated microsomes, an NADPH-generating system (18) was used in place of NADPH, utilizing 5 mM $MgCl_2$, 3 mM D,L-isocitrate, 0.07 U isocitrate dehydrogenase/ml, 100 mM NADP, and 2.5 mM nicotinamide (quantities expressed as final concentrations) in the incubation mixture. When the generating system was used with calcium-aggregated microsomes, results were similar to those obtained with NADPH: 39.03±0.96 nmol MDA/mg protein in microsomes incubated with 2.3 mM ASA (n = 6) vs. 16.37±0.43 for controls (n = 6) P < 0.001.

Mitochondrial MDA production

Livers were obtained from male Balb C mice as described for the microsomal studies. Mitochondria were isolated according to a modification of the method by Hogeboom (19). A 10% wt/vol whole liver suspension was homogenized in 0.25 M sucrose, pH 7.4, using a loose-fitting pestle in a 15-ml Wheaton glass tissue grinder. The homogenate was layered carefully over an equal volume of 0.34 M sucrose and centrifuged (model J2-2 centrifuge with a JA 17 rotor; Beckman Instruments, Inc.) at 700 g (1,800 rpm) for 10 min. The supernatant fraction was centrifuged at 5,000 g (6,100 rpm) for 10 min. The mitochondrial pellet was resuspended (5% wt/vol) in 0.25 M sucrose and centrifuged twice at 24,000 g (13,250 rpm) for 10 min, utilizing fresh 0.25 M sucrose between centrifugations. The supernatant fraction was discarded and the resulting pellet was resuspended and diluted (5% wt/vol) with phosphate-buffered saline as described for the microsomes. From then on, the procedure was identical to that described for the latter organelles, with the exception that the concentration of NADPH was 0.6 mM. The incubate contained ~ 2-3 mg mitochondrial protein per milliliter. The iron content of all solutions was determined by the ferrozine method (20, 21)[2] (see below). Any solutions containing measurable iron were passed over an iron-chelating resin

(Chelex 100; Bio-Rad Laboratories, Richmond, CA) and the absence of iron verified by the ferrozine method (20, 21).[2] All work involved with isolation of the mitochondria was carried out at 4°C.

Ferrozine procedure for determination of iron

The stock solution of aspirin was analyzed for the presence of ferric (Fe III) and ferrous (Fe II) iron by the method of Stookey (20) and Carter (21) as modified by Recknagel.[2] An Fe III standard was used to verify the efficiency of the reducing agent (hydroxylamine) and an Fe II standard was used to assure that the ferrous iron complex with ferrozine would be detected spectrophotometrically. (Ferrozine is the salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine).

Conductimetric titration

Titration was performed by the dropwise addition of a $Fe(OH)_3$ solution to deionized water or to a solution of 3% ASA. Conductivity was measured with a conductivity meter (4503; Markson Science Inc., Phoenix, AZ).

Synthesis of iron–aspirin chelate

The method of Williams et al. (22) was modified with substitution of ferrous sulfate for copper sulfate pentahydrate. ASA (3.60 g, 0.02 mol) was allowed to dissolve in a solution of potassium bicarbonate (2.02 g, 0.02 mol) in 30 ml water. To this solution was added $FeSO_4 \cdot 7 H_2O$ (2.78 g, 0.01 mol) in 20 ml water, slowly and with constant stirring. The aquamarine precipitate that formed was filtered under an N_2 atmosphere and vacuum dried overnight at room temperature in a dessicator containing calcium chloride. Subsequently, the dried precipitate was dissolved in ethanol (pH 4.0), and insoluble particulate matter was removed using a Sep-pak C_{18} unit (Waters) by elution with $H_2O/MeOH/CH_3COOH$ 50:48:1 (vol/vol/vol).

High pressure liquid chromatographic identification of aspirin and aspirin–iron chelate (23)

Pure neutralized ASA was dried under N_2, dissolved in methanol, and injected directly onto column. The solution (of $Fe(OH)_3$ and ASA) from the conductimetric titration study was prepared for HPLC by passage through a Sep-pak C_{18} unit (see above) to remove particulate matter. HPLC was carried out using a pump (model 501) attached to an ultraviolet–Vis LC spectrophotometer (model 481) and data module (model 740; Millipore, Waters Chromatography Division, Milford, MA). Samples of ASA alone, the conductimetric titration solution, and the iron-ASA chelate, were injected onto a Nova-pac C_{18} column via an injector (model UK6; Millipore, Waters Chromatography Div.) and eluted with $H_2O/MeOH/CH_3COOH$ 52:48:1 (vol/vol/vol) at 1 ml/min.

Results were calculated as $k' = (V_s - V_0)/V_0$, where V_s is volume of peak in question and V_0, void column.

Statistics

Data are given as mean±SE. Nonpaired two-tailed student's t test was used to compare treatment vs. control. One-way analysis of variance was used to compare results using various concentrations of ASA. Significance level was P < 0.05.

Results

The results of addition of varying concentrations of ASA on MDA synthesis by microsomes are shown in Fig. 1. The data demonstrate an increase in MDA production as medium ASA concentration was increased up to 4.6 mM. Higher concentrations of ASA were inhibitory compared with the value at 4.6 mM (P < 0.001). Time course data in Fig. 2 demonstrate that the effect of ASA on MDA production was already apparent by 15 min. When microsomes were incubated with salicylic acid (a major metabolite of aspirin) (24) in place of aspirin, increased MDA production was observed. 24.51±5.53 nmol
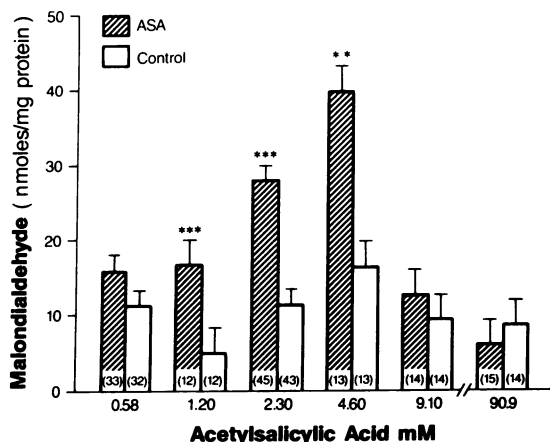
Figure 1. The effect of various concentrations of pure neutralized ASA on MDA production was studied in calcium-aggregated mouse liver microsomes (14) incubated in the presence of 0.3 mM NADPH at 37°C for 60 min. The concentrations of aspirin (expressed in millimolar above) would correspond to the following values: 10, 22, 42, 83, 164, and 1,636 mg/dl. Symbols for statistical significance utilized in Figs. 1, 3, and 4 are as follows: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ for treated vs. control. n, Nos. in parentheses.

MDA/mg protein for 2.3 mM salicylic acid ($n = 9$) compared with 9.66 ± 1.12 for controls ($P < 0.02$) ($n = 9$). However, when a minor metabolite of aspirin, gentisic acid, (24) was used in place of aspirin, there was inhibition of MDA formation. Results were 7.28 ± 1.28 nmol MDA/mg protein for microsomes incubated with 2.3 mM gentisic acid ($n = 14$) compared with 12.30 ± 1.01 ($P < 0.004$) ($n = 15$) for controls.

Since iron contamination of buffers in microsomal preparations is common (25), and would promote lipid peroxidation, the possibility that the stock solution of ASA was contaminated with iron was investigated. The stock solution of aspirin contained 0.2 μg iron/ml. Thus, the final iron concentration in the microsomal incubation medium was only 0.1 μM when an ASA stock solution was used to achieve a final incubate ASA concentration of 2.3 mM. When microsomes were incubated with this amount of ASA, results were 27.16 ± 1.82 nmol MDA/mg protein vs. 3.36 ± 0.99 with 0.1 μM FeSO$_4$ ($P < 0.001$). To ascertain that the trace iron contaminant in the ASA solution was not acting synergistically with ASA to enhance MDA production, all iron was removed from ASA stock solution (as well as all buffers and reagents used) by passage of these solutions over Chelex 100. Absence of iron in the resin treated solutions was verified with the
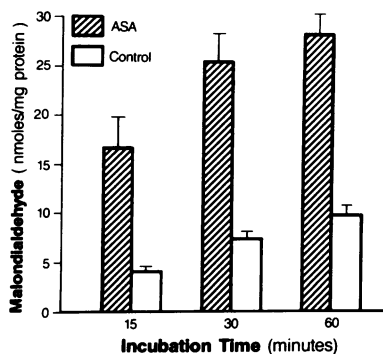
ferrozine method (20, 21). When the treated solutions were used, results for 2.3 mM ASA were 22.05 ± 0.61 nmol MDA/ mg protein for ASA ($n = 6$) vs. 8.41 ± 0.49 for controls ($n = 6$) ($P < 0.001$).

The effect of desferrioxamine (a potent iron-chelating agent and superoxide scavenger) (26) on MDA production by microsomes was studied. As shown in Fig. 3, when microsomes were incubated with buffer and desferrioxamine or aspirin plus desferrioxamine, MDA production was almost completely abolished. Similar results were obtained when alpha',alpha dipyridyl (an iron chelator without superoxide scavenging activity) was used in place of desferrioxamine.

Data in Fig. 4 indicate that aspirin also affected MDA production by mouse liver mitochondria studied over a similar range of aspirin concentrations as those employed in the microsomal studies. In the mitochondria as well as in microsomes, aspirin at low concentration stimulated MDA production while it inhibited the process at the higher concentration.

To study the possibility that ASA may be forming a complex with iron to bring about the observed increase in MDA production, the effect of ASA on the electrical conductance of Fe(OH)$_3$ was analyzed. In Fig. 5, it can be seen that addition of ferric hydroxide to deionized water resulted in a linear increase in conductance directly proportional to the concentration of Fe(OH)$_3$. When the same conductimetric titration was performed with Fe(OH)$_3$ added to a 3% (0.166 M) ASA solution, there was no increase in conductance until the molarity of the Fe(OH)$_3$ exceeded that of the ASA solution.

When an aliquot from the conductimetric titration study described above was injected onto a reverse phase HPLC column (see Fig. 6), there appeared two species from the Fe(OH)$_3$–aspirin solution, the first peak with $k' = 1.10$, and a second with $k' = 2.00$. When the stock solution of aspirin was studied in similar fashion by HPLC, a single peak with $k' = 1.08$ was observed. Fig. 6 also illustrates an HPLC tracing from an experiment in which the method of Williams et al. (22) was utilized to produce an iron chelate complex as a precipitate. The precipitate was subsequently resuspended in
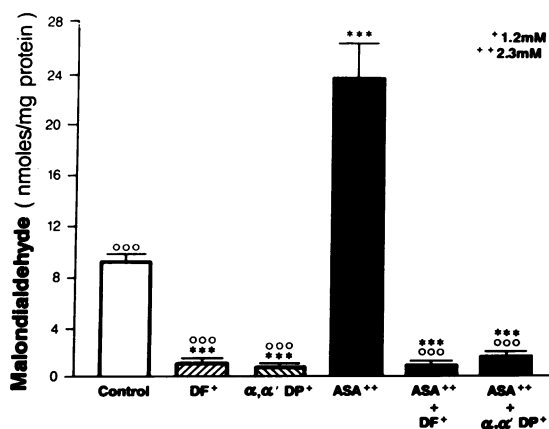

Figure 2. A time course study of MDA production by calcium-aggregated hepatic microsomes (see Fig. 1) treated with 2.3 mM ASA or buffer.

Figure 3. The effect of the iron-chelating agents desferrioximine (DF) and alpha,alpha' dipyridyl (α,α' DP) on MDA production by calcium-aggregated mouse liver microsomes (14) was studied in the absence (control) or presence of 2.3 mM ASA added to the microsomal suspension just before incubation in the presence of 0.3 mM NADPH at 37°C for 60 min. Open circles compare results to ASA alone and asterisks are used for comparison with control.
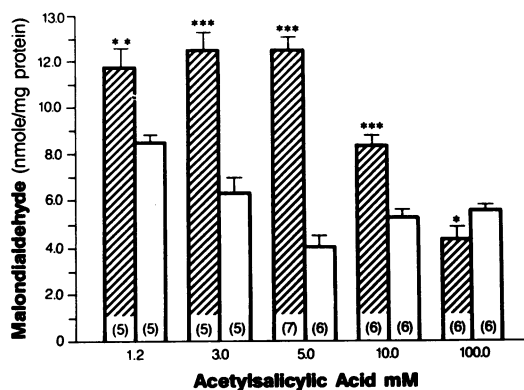
Figure 4. The effect of various concentrations of pure neutralized ASA on MDA production was studied in mouse liver mitochondria incubated in the presence of 0.6 mM NADPH at 37°C for 60 min.

Figure 6. HPLC chromatograms of the 1.4 mM pure neutralized ASA analyzed by the method of Ali (23), the conductimetric titration solution shown in Fig. 5 containing 0.6 M $Fe(OH)_3$ in 0.17 M ASA, and the putative iron–aspirin chelate synthesized by the method of Williams et al. (22). Samples were eluted with H_2O/ MeOH/CH_3COOH, 52:48:1 (vol/vol/vol) at 1 ml/min.

eluting solvent and injected onto the same column. There again appeared two species with k' value of the first very similar to that of aspirin alone; k' of the second peak suggested the presence of a complex different than that obtained from the conductimetric titration experiment.

Discussion

The results of our studies demonstrate that addition of pure neutralized ASA to mouse liver microsomes or mitochondria incubated in the presence of NADPH is associated with a roughly dose-dependent increase in MDA production over the range of concentrations from 1.2 to 4.6 mM (equivalent to 22–83 mg/dl). These results are consistent with observations by Stockman et al. (9) of increased MDA production by erythrocytes incubated in the presence of aspirin and hydrogen peroxide. Regillo et al. (27) also postulated a possible role for lipid peroxidation in aspirin-induced gastric injury.

Our studies have provided three separate lines of evidence that aspirin can complex with iron: the conductimetric titration study, the HPLC analyses, and the effect of iron chelators on ASA-induced hepatic microsomal MDA production. Martell et al. (28) have commented on the utility of electrical conductance measurements for studying the properties of chelates; our conductimetric titration study clearly demonstrates that aspirin can complex with iron. The HPLC studies of both the titration solution and the synthetic iron–aspirin chelate provide further evidence of such a complex. The results of our
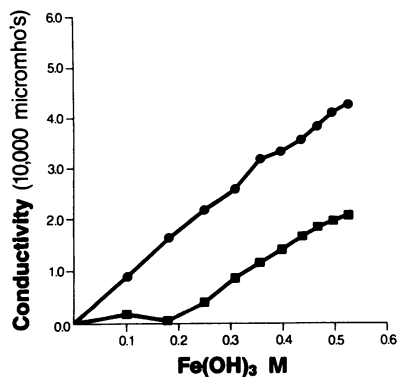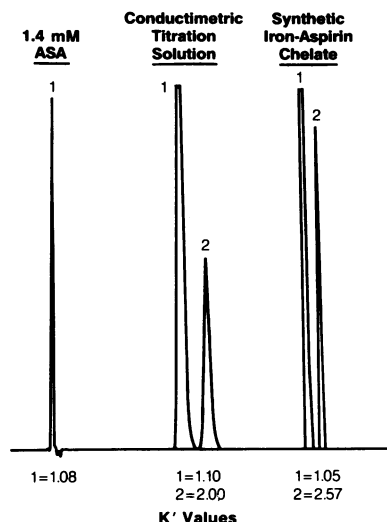

Figure 5. Effect of ASA on conductivity of a solution of $Fe(OH)_3$. Conductimetric titration was performed with a conductivity meter (Markson Science Inc.) with dropwise addition of a solution of $Fe(OH)_3$ to deionized H_2O (— ● —) or to a solution of 3% ASA (— ■ —).

studies with desferrioxamine and alpha,alpha' dipyridyl also suggest that aspirin-induced lipid peroxidation is mediated via iron.

Tien et al. (13) have reviewed the various ways in which the chelating agent EDTA can influence lipid peroxidation. When the molar ratio of EDTA-iron was less than one, the agent enhanced lipid peroxidation in phospholipid liposomes. In systems utilizing Fe^{2+}, it was thought that EDTA chelation of Fe^{2+} facilitated the autoxidation of Fe^{2+} leading to lipid peroxidation via reduction of hydrogen peroxide to form the hydroxyl radical that initiated the peroxidation. In systems utilizing the Fe^{3+}, the chelating agent was thought to enhance lipid peroxidation via facilitation of the reduction of Fe^{3+}. When the concentration of EDTA exceeded that of Fe^{3+} in a xanthine oxidase reaction mixture containing ADP-Fe^{3+}, lipid peroxidation was inhibited, probably as a consequence of the EDTA removing the Fe^{3+} from the ADP. Our results of stimulation of MDA production by aspirin in liver microsomes or mitochondria at relatively low ASA concentrations and inhibition of MDA production at high ASA concentrations are similar to the concentration dependent effects of EDTA described above.

Our results of aspirin-induced lipid peroxidation in liver are in agreement with the effect of the drug on stimulating MDA production by gastric mucosa (27) and erythrocytes (9). We are fully aware of the classical studies in which aspirin was shown to inhibit prostaglandin synthetase (29, 30), thereby decreasing arachidonic acid metabolism. Since MDA is a peroxidation product of arachidonic acid, an inhibitor of prostaglandin synthetase, such as aspirin, would be expected to inhibit production of MDA. Some authors assess prostaglandin synthetase activity by assaying for inhibition of MDA formation from arachidonic acid (31). Utilizing this assay, Roth et al. (31) have demonstrated that aspirin decreases prostaglandin synthetase (i.e., MDA formation from arachidonic acid) in sheep seminal vesicle microsomes. The type of effect on MDA production in response to aspirin is probably explained both by the varying iron content of the tissue studied as well as the dose of aspirin utilized (see Figs. 1 and 4). Both liver and erythrocytes contain large amounts of iron. When rats are given parenteral radioiron, the majority ($\sim$ 75%) is taken up by liver plus circulating red blood cells and marrow (32). Fur-

thermore, ASA administered orally to rats increases the hepatic uptake of ^{59}Fe (33). In liver, apparently the effect of iron chelation by aspirin is predominant over the inhibition of prostaglandin synthetase by aspirin.

There are a number of possible sources of endogenous microsomal iron that might be available for chelation. Thomas and Aust (34) have recently shown that much of endogenous iron in microsomes is ferritin (83% of the nonheme microsomal iron). However, note that Bonkowsky et al. (35) have demonstrated that the "microsomal iron content" depends on the technique utilized to isolate the microsomal fraction. In their studies, microsomes isolated by a calcium aggregation method (10 min centrifugation at 1,500 g) contained less iron than those isolated by differential centrifugation (30 min centrifugation at 105,000 g). This difference in iron levels may explain why MDA production in microsomes obtained by differential centrifugation in our studies and then incubated with 2.3 mM aspirin was 44.6±1.7 nmol MDA/mg protein compared with 27.2±1.8 for values obtained using calcium aggregated microsomes incubated in the same concentration of aspirin.

At least in vitro, ferritin iron can be released by O_2^- with subsequent promotion of the peroxidation of microsomal lipids. However, it is not necessary to invoke ferritin as the site of iron release in liver microsomes. Montgomery et al. (36) as well as Thomas and Aust (34) have postulated the presence of a small, nonheme iron pool in microsomes, which is not ferritin, and, which is capable of promoting lipid peroxidation. Maines (37) has suggested that the iron released through the destruction of microsomal heme (mediated in her experiments by allylisopropylacetamide) promotes lipid peroxidation. Bonkowsky et al. (35) demonstrated that the iron content of rat liver mitochondria is approximately half that of microsomes. Our data, demonstrating that equivalent doses of aspirin promote less MDA formation in hepatic mitochondria than in microsomes (see Figs. 1 and 4), suggests that organelle iron content and MDA production correlate directly.

We suggest that our observations of in vitro aspirin-induced hepatic organelle lipid peroxidation may be of relevance to Reye's Syndrome, a hepatic encephalopathy of childhood in which a number of studies have demonstrated a strong epidemiologic association with aspirin intake (38–41). The link between our observations and Reye's Syndrome is all the more intriguing because of reports of products of lipid peroxidation in blood (42) and liver (43) of children with this syndrome. Since Bacon et al. (44) have demonstrated that in vivo iron loading in experimental animals is associated with evidence of lipid peroxidation in hepatic microsomes and mitochondria, our studies also raise the possibility of increased susceptibility to aspirin-induced hepatotoxicity in hemochromatosis. However, the medical literature does not contain any reports of such increased susceptibility.

Whereas caution must be exercised in extrapolating from our in vivo effects, it is of interest that Nakagawa et al. (45) have recently reported increased MDA in whole liver homogenates obtained from rats treated with several doses of oral aspirin. However, even this information does not necessarily establish a direct causal relationship between aspirin administration and tissue lipid peroxidation. As is the case with CCl₄ and other hepatotoxicants, the administration of which is associated with evidence of hepatic lipid peroxidation, there is a gap in our understanding of the precise relationship between

the initial reactant species, membrane lipid peroxidation, secondary mechanisms, such as changes in mitochondrial function and increased cytosolic calcium, and the eventual unfolding of histopathology (8).

In conclusion, our results suggest that ASA may chelate endogenous hepatic iron and thereby (either by direct or indirect mechanisms) promote lipid peroxidation. This hypothesis merits further investigation as a possible mechanism for aspirin-related hepatotoxicity.

Acknowledgments

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