Analysis of Cardiac Membrane Phospholipid Peroxidation Kinetics as Malondialdehyde: Nonspecificity of Thiobarbituric Acid-reactivity[1]

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When exposed to xanthine oxidase (superoxide)-dependent, iron-promoted Fenton chemistry, purified cardiac membranes evidenced, by the thiobarbituric acid (TBA) test, a virtually instantaneous peroxidative response with a maximal linear rate of 5.8 nmol malondialdehyde (MDA)-equivalents/mEquivalents lipid ester reacted/min. Yet when the lipids purified from these same membranes and reconstituted into liposomes were peroxidized under identical reaction conditions, the TBA test indicated that a pronounced (~20-min) lag period preceded a maximal peroxidation rate of only 2.1 nmol MDA-equivalents/mEquivalents lipid ester reacted/min. After 120 min of peroxidation, the cardiac membranes yielded some 300 nmol TBA-reactive MDA-equivalents/mEquivalent ester, whereas the isolated membrane lipids evidenced ~40% less TBA-reactivity. To verify that these quantitative and kinetic differences in membrane (phospho)-lipid peroxidation occurred with removal of the lipids from their membrane milieu, the MDA produced during both cardiac membrane peroxidation and the peroxidation of the lipids derived therefrom was isolated as its free anion by ion-pair high-pressure liquid chromatography. As quantified spectrophotometrically, true MDA production during myocardial membrane peroxidation was identical in kinetics and in amount to the production of TBA-reactive substance from the peroxidized isolated membrane lipids. These results demonstrate that significant non-MDA, TBA-reactive species are generated during the peroxidation of cardiac membranes, especially before the maximal rates of bona fide MDA production. As a direct consequence, artifactual levels and kinetics of membrane lipid peroxidation do result.
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Free radical-mediated membrane lipid peroxidation through Fenton-type, metal-promoted oxygen chemistry is increasingly being appreciated as a causative pathogenic factor, especially in myocardial injury (2). Consequently, importance rests with the reliable detection and quantitative assessment of the extent and dynamics of membrane lipid peroxidation. The most extensively employed method for the detection and analysis of peroxidation is the thiobarbituric acid (TBA) test, predicated upon the reactivity of a colorless aldehyde end-product of lipid peroxidation, malondialdehyde (MDA), with TBA to produce a red adduct (3). The convenience, ease and and rapidity of the TBA test have tended, in practice, to mitigate against such considerations as the nonspecificity of TBAs chemical reactivity (4), the low efficiency of fatty-acid hydroperoxide breakdown to MDA (5), and the effects that procedural modifications have upon color development (6). The TBA test has been employed repeatedly in a variety of cardiac oxidative injury models to measure myocardial membrane lipid peroxidation (e.g., 7,8).

This report details quantitative and kinetic investigations of the relationship between TBA-reactivity and bona fide MDA production during the peroxidation of cardiac membranes and their lipids. A defined, physiologically relevant oxidative injury system based upon xanthine oxidase (XOD)-mediated, superoxide ($O_2^{\cdot-}$)-dependent, iron-promoted Fenton chemistry (2) is employed. The results demonstrate that only when isolated cardiac membrane lipids are peroxidized does TBA-reactivity reflect MDA production and, hence, lipid peroxidation. The fact that no qualitative or quantitative reliability could be demonstrated to support the use of the TBA test as an analytical tool with which to monitor the peroxidation of the cardiac membranes themselves would urge that caution be exercised when applying the TBA test to membrane/membraneous organelle systems.

EXPERIMENTAL PROCEDURES

Materials. Allopurinol, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), dithiothreitol, tris(hydroxymethyl)aminomethane (Tris), hydroxylamine hydrochloride, ADP, TBA, and tetramethoxypropane were from Sigma Chemical Co. (St. Louis, MO). XOD (analytical preparation from bovine milk; 1.0 unit/mg) and superoxide dismutase (SOD; analytical preparation from bovine erythrocytes; 5000 units/mg) were purchased from Boehringer-Mannheim (Indianapolis, IN). Desferrioxamine B (Desferal) was a gift from Ciba A.G. (Basle, Switzerland). α-Tocopherol was synthesized by Hoffmann-La Roche. All solvents were of analytical grade (Burdick and Jackson, Muskegon, MI).

Isolation of rat myocardial membranes. The procedure was carried out in a dehumidified cold-room (3 C). Conscious male Sprague-Dawley rats (~275 g; Charles River, Boston, MA) maintained on a normal rodent diet were decapitated. Each heart was rapidly excised and perfused via the aorta with 20.0 ml ice-cold 10 mM HEPES buffer, pH 7.4. The trimmed ventricular muscle masses were pooled, minced, and homogenized (100 mg tissue/ml ice-cold HEPES) for 3 × 5 sec with a Tissumizer (Tekmar, Cincinnati, OH) at maximal setting. The homogenate was filtered through four-ply cheesecloth, and the myocardial membranes were isolated from the filtrate by utilizing KCl extraction to remove contractile protein and differential centrifugation in 10.0 mM Tris-HCl, pH 7.4, containing 2.0 mM dithiothreitol (9). The membranes were used immediately after having been washed twice with 25.0 ml 10 mM HEPES-0.145 M KCl, pH 7.4.

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Abbreviations: HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; MDA, malondialdehyde; $O_2^{\cdot-}$, superoxide anion radical; SOD, superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1); TBA, thiobarbituric acid; Tris, tris(hydroxymethyl)-aminomethane; XOD, xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2); HPLC, high pressure liquid chromatography.

Lipid extraction and quantification. Lipids were extracted and purified from the myocardial membranes by a modified Bligh-Dyer (10) procedure (11). The final chloroform phase containing the purified lipid represented quantitative recovery of myocardial membrane lipid: >98% of the radioactivity associated with the organic fraction obtained by direct saponification (12) of membrane metabolically labeled in vivo from [^{14}C]acetate, sodium salt, (54.0 mCi/mmol, sp. act. [New England Nuclear, Boston, MA]) was recovered in the saponified fraction from the lipid extracted out of an identical amount of membrane. The myocardial membrane total lipid extract was resolved into its constituent phospholipid and neutral lipid fractions by Sep-Pak silica column chromatography (Waters, Milford, MA) (13). All lipids were stored in chloroform under argon at −20 C (14).

Lipid phosphate was determined microchemically on perchloric acid digests (15). The hydroxamate reaction was used to measure lipid ester (16); L-α-phosphatidylcholine dipalmitate (Avanti Polar Lipids, Birmingham, AL) was used as standard.

Preparation of cardiac liposomes. A known amount of myocardial membrane lipid in chloroform was placed into a rotating glass flask and taken to dryness under nitrogen at room temperature. The lipid was taken up in 10 mM HEPES-0.145 M KCl, pH 7.4, and was resuspended by indirect anaerobic sonication for 15 min at room temperature. The liposomes were used immediately.

Peroxidation reaction system. Cardiac liposomes or membranes (at equal lipid concentrations) were subjected to $O_2^{\cdot-}$-dependent, iron-promoted peroxidation in glass tubes. Ninety minutes before the start of the reaction, a chelate was formed in 10.0 mM HEPES-0.145 M KCl buffer, pH 7.4, between Fe^{3+} (1.0 mM $FeCl_3$, final conc.) and ADP (10.0 mM, final conc.) with continuous stirring at room temperature. The peroxidation reaction, in a final volume of 1.0 ml, contained the following components at their specified final concentrations: 10 mM HEPES-0.145 M KCl, pH 7.4; 1.0 mM hypoxanthine; 0.1 mM Fe^{3+}-1.0 mM ADP chelate; 125 μg lipid (as membrane or liposomal suspension); and 10 mUnits XOD. The reaction was started by adding the XOD, mixing, and incubating at 37 C in a shaking water bath. Membrane/liposome samples also were incubated in parallel to the same final lipid concentration but without free radical generator (i.e., without hypoxanthine, Fe-ADP, and XOD). The peroxidation incubation was ended on ice by lipid extraction, acidification, or SOD addition, depending upon the subsequent analysis (below). Solutions of α-tocopherol were prepared under yellow lighting in ethanol such that the final solvent concentration in the peroxidation reaction was 1,000-fold below that which affected peroxidation. Desferrioxamine and allopurinol were solubilized in 10 mM HEPES-0.145 M KCl, pH 7.4. Dose-response curves to define, quantitatively, the effects of these agents were generated with the programming assistance of RS/1 software (BBN Corp., Cambridge, MA) on an IBM PC-AT (IBM, Boca Raton, FL).

Determination of the production of conjugated dienes and TBA-reactive substance during peroxidation. For conjugated diene analysis, lipids were extracted (11) out of 2.0 ml of peroxidation reaction mixture and the parallel, nonperoxidized membrane or lipid samples. The purified lipids were dissolved in 1.0 ml spectro-grade cyclohexane. Spectra of the lipids were taken against cyclohexane from 190 nm–400 nm in a DU-7 kinetic spectrophotometer (Beckman, Palo Alto, CA). Spectra of the lipids of nonperoxidized liposomes and membranes and of the peroxidation reaction mixture itself were the blank spectra. Raw spectra were corrected for Rayleigh scattering (17), and the nonperoxidized lipid and reagent blank spectra were subtracted from the appropriate experimental, peroxidized lipid spectra to obtain difference spectra. Calculation of net conjugated diene formation was made from the difference spectra using the molar absorptivities given (18).

The TBA test was conducted by the following modification of published methods (5,6,19). The peroxidation incubation was terminated on ice by acidification with 0.15 ml ice-cold 76% TCA-2.3 N HCl per 1.0 ml sample (pH = 2.2), and 0.35 ml of a freshly-prepared mixture of H_2O/7.14 M butylated hydroxytoluene in ethanol/1.51% TBA in 0.2 M Tris, pH 7.0, (1:1:5, v/v/v) was added. After thorough mixing, the samples (final pH = 2.4) were incubated in an 80 C shaking water bath for 30 min. After this time, the tubes were plunged into an ice-water bath, and the TBA test was immediately stopped with 0.5 ml ice-cold 91% TCA followed by 2.0 ml $CHCl_3$. After centrifugation for 30 min at 2000 × g, 4 C, the absorbance of the upper phase at 532 nm was read. Various amounts (9.8–40.0 nmol) of MDA standard, freshly prepared by acidification of 1,1,3,3-tetramethoxypropane with 76% TCA-2.3 N HCl (0.15 ml acid mixture with 1.0 ml suitably diluted tetramethoxypropane), were subjected to the identical TBA test procedure as the basis for constructing a standard curve of TBA-reactivity as MDA-equivalents. Computer-assisted regression analysis of the standard curve was used to quantify the molar amounts of MDA-equivalents in the experimental samples. Net peroxidative production of MDA-equivalents was taken as the difference in TBA-reactivity between peroxidized samples that had been incubated in the complete peroxidation reaction system and non-peroxidized samples that had been incubated in parallel without free radical generator. The TBA reaction per se was not affected by the components of the radical generator, α-tocopherol, desferrioxamine, allopurinol or SOD.

Chromatographic MDA isolation and quantification. When the peroxidation reaction was to be analyzed by high pressure liquid chromatography (HPLC) for MDA content, the peroxidation incubation was stopped by adding SOD to a final concentration of 10^{-7} M and freezing the mixture in dry ice-acetone. MDA was then isolated from the sample by an ion-pair HPLC technique (20) based on the work of Bull and Marnett (21). The HPLC system (Beckman Instruments) was equipped with a μBondapak C_{18} stainless-steel 3.9 mm × 30 cm analytical column preceded by a radially packed C_{18} guard column (Millipore/Waters, Milford, MA). MDA, as the enolate anion, was resolved out of the peroxidation reaction by isocratic elution at 3.0 ml/min with a mobile phase of acetonitrile/50.0 mM myristyltrimethylammonium bromide in 0.9 mM sodium phosphate, pH 6.7 (15:85, v/v). Detection was based on UV absorbance at 267 nm, and quantitation was computerized by peak integration with reference to MDA standard. Net peroxidative production of MDA was taken as the difference in MDA content between peroxidized samples that had been incubated in

the complete peroxidation reaction system and parallel, nonperoxidized samples that had been incubated without free radical generator. MDA for the calibration chromatograms was prepared by hydrolysis of malonaldehyde-bis-diethylacetal (Merck, Darmstadt, Germany) with H_2SO_4, as described (22). Absolute MDA concentration in the solution was checked by UV spectrophotometry at 267 nm with the extinction coefficient of 34,000 M^{-1} cm^{-1} (22).

Assessment of "bound" MDA. To liberate any bound MDA before HPLC analysis, membranes/liposomes were hydrolyzed in base (NaOH) at pH 12.0, 60 C, for 30 min (23). The hydrolysis reaction mixtures then were subjected to rapid ultrafiltration through a YM2 membrane (Amicon, Danvers, MA) to exclude high-molecular-weight (>1000) molecules and the cardiac membranes or liposomes themselves. The clarified filtrates were then neutralized (pH ~7.4) with HCl before HPLC analysis for MDA. Bound MDA was considered the difference in MDA content between parallel samples that had or had not been hydrolyzed. Recovery of standard MDA in this procedure was >96%.

Protein determination. Protein was quantified with a dye-binding microassay (24).

RESULTS

Properties of the isolated myocardial membranes. The biochemical characteristics of the isolated rat-heart membranes are summarized in Table 1. The efficiency of myocardial contractile protein extraction by salt was readily apparent from the dramatically lower protein content of the membranes with respect to starting homogenate. In contrast, the membranes represented over 70% of the heart-muscle homogenate lipid. The high recovery of cardiac phospholipid, localized virtually exclusively in membrane (25), and the three-fold increase in lipid:protein ratio of the membranes over homogenate indicate that the isolated membranes were representative of the myocardial membranes in situ. Additional support for this conclusion rests with the finding that, upon sucrose density-gradient subfractionation of the myocardial membranes (26), ~90% of the total membrane phospholipid was found associated with mitochondria, ~7% with sarcoplasmic reticulum and ~3% with sarcolemma; this distribution is reminiscent of the relative membrane areas of the three organelles in the intact heart muscle cell (27).

Conjugated diene production and TBA-reactivity during myocardial membrane and membrane lipid peroxidation. With exposure of isolated cardiac membranes and the total membrane lipid or phospholipid but not neutral lipid to $O_2^{\cdot-}$-dependent, iron-promoted oxy-radical chemistry, net formation of lipid conjugated dienes was observed (Fig. 1). Because in all cases diene formation was normalized to the actual amount of lipid reacted, the parallelisms in both the pattern and level of lipid conjugated diene production from the intact cardiac membranes and from the purified membrane total lipid and phospholipid would indicate that the phospholipid was the membrane target for oxidative damage. By 120 min of reaction, the levels of diene intermediates decreased when, presumably, the propagation phase of peroxidation ended as conjugated

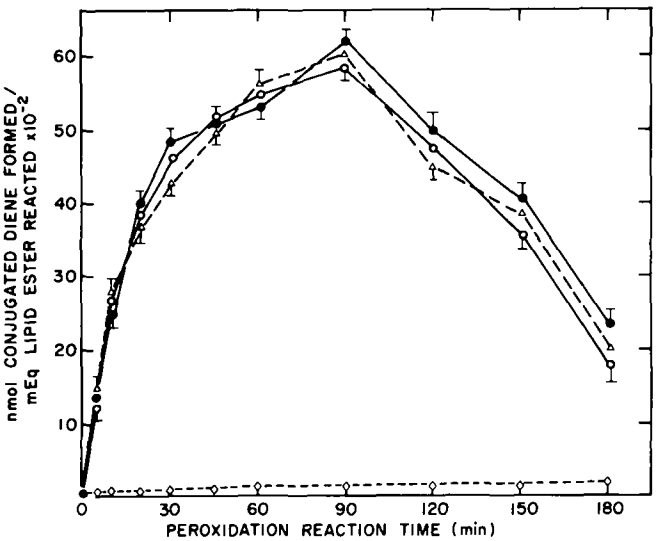

FIG. 1. Conjugated diene production during myocardial membrane and membrane lipid peroxidation. Isolated myocardial membrane (●—●) and the membrane total lipid (○—○), phospholipid (△—△) and neutral lipid (◇—◇) purified therefrom were subjected to peroxidative injury by exposure to XOD ($O_2^{\cdot-}$)-dependent, iron-promoted Fenton chemistry. Samples were taken over 180 min of reaction, and the lipids were extracted and analyzed spectrophotometrically for conjugated diene content. The mean difference in conjugated diene content between the peroxidized samples and non-peroxidized membrane/lipid controls was calculated as the conjugated diene formed during peroxidation. These values, normalized to the amount of lipid reacted, are graphed ± S.D. (n ≥ 6).

TABLE 1

Biochemical Properties of Rat Myocardial Membranes[a]

	Protein		Lipid				
			Ester		Phosphate		
Fraction	(%)	(mg/heart)	(%)	(mEquivalents/heart)	(%)	(μg/heart)	Lipid:Protein
Heart muscle homogenate	100	49.8 ± 2.9	100	6.9 ± 0.3	100	312.0 ± 18.5	0.15 ± 0.01
Myocardial membranes	24.1 ± 0.5	12.0 ± 1.0	72.5 ± 2.6	5.0 ± 0.3[b]	73.2 ± 1.9	228.5 ± 18.4	0.45 ± 0.02

[a]Myocardial membranes were isolated from rat-heart ventricular muscle essentially as described (9). Results are means ± S.D. (n = 6).
[b]90.5 ± 2.6% of the lipid ester is found in membrane phospholipid; the remainder is associated with neutral lipid.

intermediates increasingly converted to products such as lipid peroxides and MDA (5,6).

Exposure of myocardial membranes or liposomes comprised of either the total membrane lipid or the membrane phospholipid to free radical generator resulted in the production of TBA-reactive substance (Fig. 2). As was the case with conjugated diene production (Fig. 1), myocardial membrane neutral lipids evidenced a negligible peroxidative response. However, each of the other myocardial substrates for oxy-radical attack, in contrast to their similar patterns of diene production, showed markedly distinctive kinetics of TBA-reactivity. The intact myocardial membranes evidenced a linear propagation phase through the first 30 min of reaction with an apparent maximal peroxidation rate of 5.8 ± 0.3 nmol MDA-equivalents produced/mEquivalents lipid ester reacted/min (mean ± S.D.; n = 6) and an accumulation of some 300 nmol MDA-equivalents by 180 min. The total myocardial membrane lipid complement, however, displayed a conspicuous lag-period of about 20 min, whereupon an apparent maximal peroxidation rate of 2.1 ± 0.1 nmol MDA-equivalents produced/mEquivalents lipid ester reacted/min led to a net accumulation of about 150 nmol MDA-equivalents by 180 min. Production of TBA-reactive substance from the isolated myocardial membrane phospholipid began virtually instantaneously upon exposure to oxy-radicals, with a linear propagation rate and a net accumulation of TBA-reactive substance that approximated those of the total myocardial membrane lipid.

The lag-period in the production of TBA-reactivity from myocardial membrane total lipid is an effect of the high

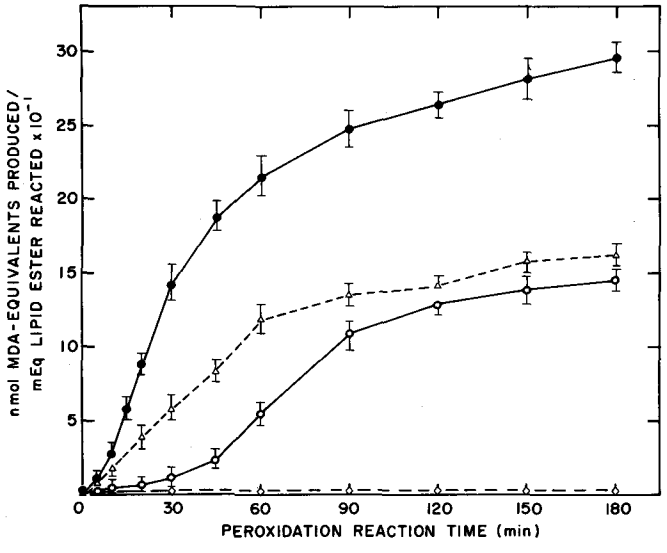

FIG. 2. Generation of TBA-reactive substance during myocardial membrane and membrane lipid peroxidation. Isolated myocardial membranes (●—●) and the membrane total lipid (○—○), phospholipid (△--△) and neutral lipid (◇--◇) purified therefrom were peroxidized by incubation with XOD + hypoxanthine + iron. Membranes/lipids also were incubated in parallel without free radical generator. Samples taken over 180 min of peroxidation reaction were subjected to the TBA test, and the content of TBA-reactive substance in each sample was expressed as MDA-equivalents and normalized to the amount of lipid reacted. The mean difference in MDA-equivalents between peroxidized and non-peroxidized samples was calculated as the MDA-equivalents produced during peroxidation and graphed ± S.D. (n ≥ 6).

myocardial membrane α-tocopherol content (28); the peroxidized isolated membrane phospholipids demonstrated an instantaneous TBA response (Fig. 2). Nonetheless, upon exposure to oxy-radical generator, the membranes, containing both the phospholipids and the α-tocopherol, always generated TBA-reactive substance more rapidly and to a greater extent than did the membrane phospholipids.

HPLC analysis of myocardial membrane peroxidation as MDA. The comparative TBA-reactivity data (Fig. 2) appeared to indicate that profound quantitative and kinetic changes in myocardial membrane phospholipid peroxidation resulted from removal of the lipid out of its membrane milieu. To attempt to verify this conclusion, TBA-reactivity was obviated by isolating MDA throughout the course of myocardial membrane and membrane lipid peroxidation. To avoid the conditions (acid, heat) of the TBA reaction and to ensure complete independence from TBA-reactivity, isolation of, for example, a TBA-MDA adduct was not carried out.

We first defined an effective means of stopping the peroxidation reaction other than by the acidification protocol required for the TBA test, so that precise end-points could be established without jeopardizing subsequent analyses. As shown in Table 2, the XOD ($O_2^{\cdot-}$)-dependent, iron-promoted Fenton chemistry supporting oxidative injury in this system could be blocked at several critical points. Inhibiting XOD with a substrate analog (allopurinol [29]), chelating the iron in the system with desferrioxamine (30), intercepting lipid radicals that would otherwise propagate peroxidation with α-tocopherol (31), and dismutating $O_2^{\cdot-}$ with SOD (32) all were effective means of blocking the generation of TBA-reactive substance from isolated cardiac membrane lipids exposed to XOD + hypoxanthine + iron. The system was most sensitive to SOD: 10.0 nM SOD completely inhibited the generation of TBA-reactive material (as well as the formation of conjugated dienes) from cardiac lipids. Consequently, SOD was added to a final concentration of 100.0 nM to the peroxidation reaction system to dismutate the $O_2^{\cdot-}$ therein and thereby halt the progression of oxidative damage before HPLC analysis for MDA.

To isolate the MDA anion directly out of the peroxidation reaction system without preparatory sample manipulation or clean-up, a variety of amino-phase (33), reverse-phase (34), size-exclusion (35) and ion-pairing (21) HPLC techniques were tested. Ion-pairing chromatography appeared to have the most potential, but the exact conditions of Bull and Marnett (21) offered incomplete resolution of MDA standard from the reactants in the Fenton peroxidation system. With modification of the chromatographic conditions as detailed under Experimental Procedures, MDA could be separated rapidly from the constitutents of the Fenton reaction (Fig. 3) and eluted as a well-resolved peak, 7.0 pmol being the lower-limit of MDA quantitation. Only the material eluting from the column at ~4.9 min evidence TBA-reactivity, had the UV spectrum characteristic of MDA (Fig. 3, inset), and co-eluted with standard MDA. Recovery of known amounts of MDA standard subjected to HPLC isolation was >98%.

With these data, the HPLC technique was adopted to determine directly the MDA produced during oxidative injury to myocardial membranes and their purified lipids

TABLE 2

Inhibition of the Generation of TBA-reactive Substance from Myocardial Membrane Lipid[a]

Agent	Action	Concentration of agent that inhibits TBA-reactivity by: 50%	100%
Allopurinol	XOD inhibition	5.0×10^{-4} M	5.0×10^{-2} M
Desferrioxamine	Iron chelation	—[b]	1.0×10^{-4} M
α-Tocopherol	Chain-breaking antioxidant	8.5×10^{-7} M	1.0×10^{-5} M
SOD	$O_2^{\cdot-}$ dismutation	5.0×10^{-9} M	1.0×10^{-8} M

[a]Liposomes comprised of total myocardial membrane lipid were exposed to XOD + hypoxanthine + iron as radical generator for 60 min either in the absence (control) or presence of varying concentrations of each listed agent. After one hr, the samples were run in the TBA test, and the level of TBA-reactive substance in each was expressed relative to the control samples as inhibition of TBA-reactivity.

[b]Because of the stoichiometric nature of iron chelation by desferrioxamine (30), only complete inhibition was noted at a desferrioxamine concentration equal to the iron concentration in the system.

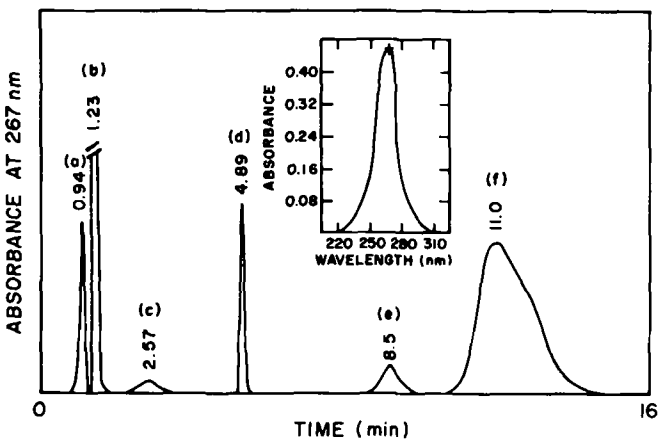

FIG. 3. HPLC isolation of MDA produced during myocardial membrane lipid peroxidation. Myocardial membrane total lipid (125 μg lipid) was reacted with XOD + hypoxanthine + iron in the Fenton peroxidation system described (Experimental Procedures). At one hr of reaction, SOD was added (100 nM, final concentration), and 250 μl of the 1.0 ml sample was subjected to ion-pair chromatography with spectrophotometric detection at 267 nm. Retention times are given above each peak in minutes. The identities of the peaks are (a) hypoxanthine, (b) xanthine, (c) uric acid, (d) MDA, (e) Fe-ADP, (f) ADP. Inset shows the on-line spectrum of the MDA in peak (d) as taken during its elution from the HPLC column; the absorption maximum is indicated by the cross at 267 nm.

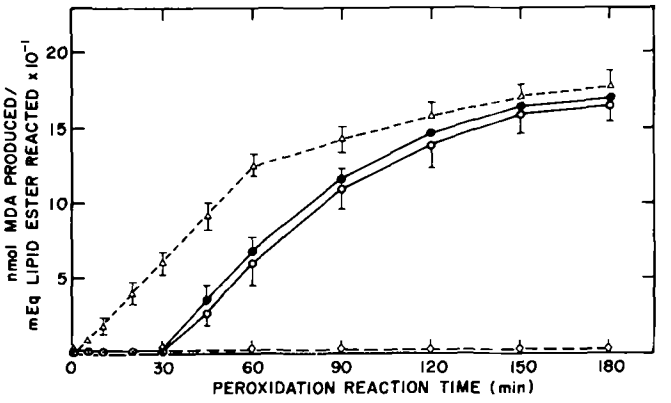

FIG. 4. Production of MDA during myocardial membrane and membrane lipid peroxidation. Isolated myocardial membranes (●—●) and the membrane total lipid (○—○), phospholipid (△—△) and neutral lipid (◇—◇) purified therefrom were peroxidized by exposure to XOD ($O_2^{\cdot-}$)-dependent, iron-promoted Fenton chemistry with XOD + hypoxanthine + iron as oxy-radical generator. Membranes/lipids also were incubated in parallel without free radical generator. Samples were taken over 180 min of peroxidation and were analyzed for their content of MDA (as its enolate anion) by ion-pairing HPLC (Fig. 3). The mean difference in MDA content between peroxidized and non-peroxidized samples was calculated as the MDA produced during peroxidation. MDA production was normalized to the amount of lipid reacted and graphed ± S.D. ($n \geq 6$).

(Fig. 4). Production of MDA during the peroxidation of purified myocardial membrane total lipid, phospholipid or neutral lipid was equivalent in kinetics and magnitude to the accumulation of TBA-reactive substance (expressed as MDA-equivalents; Fig. 2). However, MDA production during peroxidation of isolated myocardial membranes paralleled the MDA production from the purified cardiac membrane lipids (Fig. 4) and did not resemble the levels or pattern of membrane TBA-reactivity (Fig. 2). Because both the TBA-reactivity and MDA production were normalized to the amount of lipid reacted, these results would indicate that non-lipid related TBA-reactive substance generated during myocardial membrane peroxidation was responsible for the apparent attenuation in cardiac membrane lipid peroxidation when the lipids were extracted out of the membranes.

The non-lipid related TBA-reactivity was generated with its own kinetics, as could be demonstrated from the difference curve between TBA-reactivity (as MDA-equivalents) and MDA production upon exposure of myocardial membranes to oxy-radical generator (Fig. 5). It is evident that non-lipid related TBA-reactive substance was generated most rapidly early in the exposure of membranes to the radicals (before ~20 min of reaction) and

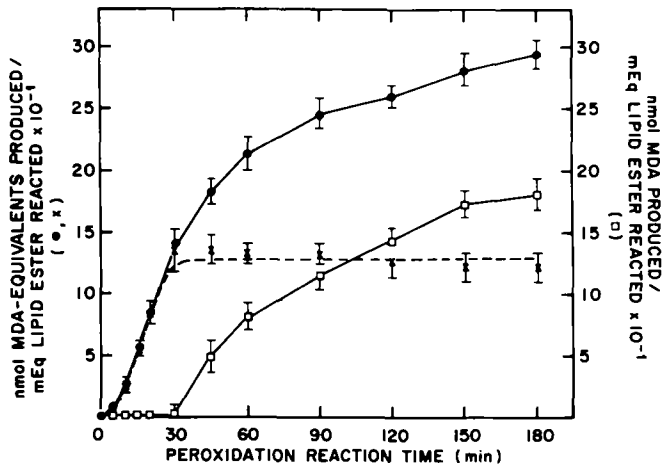

FIG. 5. Production of non-lipid associated TBA-reactive substance during myocardial membrane peroxidation. The generation of TBA-reactive substance (● — ●) and the production of MDA (□—□) during myocardial membrane peroxidation by Fenton-type oxy-radical chemistry are re-plotted from Figs. 2 and 4, respectively. The mean difference (± range) between the amount of TBA-reactive substance and the actual amount of MDA at each sampled time during the 180 min of reaction was calculated and plotted (×——×) as non-lipid related TBA-reactive substance.

ultimately accounted for some 40% of the total TBA-reactivity of the system.

Membrane source of non-lipid related TBA-reactive substance. Several attempts were made to define chemically the source of non-lipid associated TBA-reactivity when myocardial membranes are peroxidized. The acidic conditions and heat of the TBA test will liberate MDA bound to membrane constituents (especially peptide lysine residues) by hydrolyzing Schiff-base linkages (36), whereas the linkages are stable under the mild conditions (no heat) of both the peroxidation reaction (pH = 7.4) and the HPLC run (pH = 6.8). Consequently, additional studies were undertaken in which samples of peroxidized and non-peroxidized membranes were hydrolyzed in base (pH = 12.0) with heating before HPLC isolation of MDA from the filtered, neutralized hydrolysates. This procedure would convert any bound MDA to free MDA that then could be detected as HPLC analyte (23). From comparisons between hydrolyzed membrane samples and non-hydrolyzed samples run in parallel in the peroxidation reaction, some 7% (i.e., 12.2 ± 0.6 nmol MDA/mEq lipid ester reacted; mean ± S.D., n = 6) of the total MDA produced during cardiac membrane peroxidation was in the bound form. This level of bound MDA was too low to account for the 40% difference between membrane TBA-reactivity (as MDA-equivalents) and bona fide MDA generated during membrane peroxidative injury. The bound MDA level remained at ~12 nmol/mEq lipid ester during the course of the peroxidation reaction, indicating that the capacity of the isolated membranes to take up free MDA is limited relative to the MDA yields from peroxidation. This conclusion is substantiated by the finding that cardiac membranes (or their isolated lipids) incubated at 37 C with known amounts of free MDA standard in HEPES-KCl buffer, pH 7.4, yield levels of MDA-equivalents (by the TBA test) or free MDA (by HPLC) that were always >90% of those expected from the standard (data not shown).

Because free acylneuraminic acids are known to react with TBA (37) and cardiac membranes contain acylneuraminic acids (38), it was investigated whether membrane acylneuraminic acids could have contributed to the observed TBA-reactivity of peroxidized rat heart-muscle membranes. To this intent, membranes that had been treated with neuraminidase (acylneuraminyl hydrolase from *Clostridium*; EC 3.2.1.18) (39) or that had undergone controlled acid hydrolysis (40), conditions known to liberate sialic acid from membrane glycoconjugates (41), were peroxidized. The treated cardiac membranes evidenced no statistically significant decrease in the levels and rates of TBA-reactivity development with respect to non-treated membranes (data not shown). Peroxidation of the myocardial membrane protein recovered during the extraction of the membrane lipid did not yield appreciable TBA-reactive substance (data not shown).

DISCUSSION

Growing realization that oxidative damage to membrane lipids is critical in the pathogenesis of a variety of diseases (5,7,8) has placed increased importance upon the application and evaluation of methodology with which membrane lipid peroxidation dynamics are assessed. This study explored in quantitative and kinetic terms the relationships between TBA-reactivity and MDA production during myocardial membrane peroxidation through $O_2^{\cdot-}$ (XOD)-dependent, iron-promoted oxy-radical chemistry of the type believed to occur in myocardial ischemia (2). The data demonstrate that while there is a direct and quantitative relationship between the production of TBA-reactive substance and the generation of free MDA during peroxidation of isolated cardiac membrane (phospho)lipids, no such relationship exists when the myocardial membranes are peroxidized. Generation of non-lipid related, non-MDA TBA-reactive substance with its own kinetics during cardiac membrane peroxidation, if unrecognized, would have led to overestimations of the development and extent of lipid peroxidation and an erroneous peroxidative kinetic profile.

Gutteridge (42) has concluded that the close agreement between the production of TBA-reactive substance and the generation of fluorescent MDA-phospholipid complexes during the peroxidation of isolated ox-brain phospholipid validates the TBA test as an indicator of lipid peroxidation. Although brain membranes were not peroxidized by Gutteridge, and MDA was not isolated, his conclusion is supported by the present data on the reliability of the TBA test in assessing the peroxidation of purified myocardial membrane phospholipids.

Warso and Lands (5,43) have documented extensively that human plasma TBA-reactivity bears no relationship to plasma hydroperoxide level. Specifically, 80% of the TBA-positive material in human plasma was found not to have originated from lipid hydroperoxide conversion to MDA. Quantitatively, these high plasma levels of non-lipid related TBA-reactivity are reminiscent of our finding that ~40% of the TBA-reactive substance produced during the peroxidation of myocardial membrane is not bona fide MDA and does not arise directly from membrane phospholipid peroxidation.

The chemistry and precise source(s) of the non-lipid related TBA-reactive substance generated during cardiac

membrane peroxidation remain to be defined. It is possible that molecular interactions/conformational changes at the membrane level induced during free-radical attack are required to incite formation of TBA-reactive substance beyond that which would be formed from the MDA product of membrane lipid peroxidation (44). Such reasoning has been invoked (45) to explain why the conversion of hydroxyl groups to aldehydes at the termini of cell-surface carbohydrate chains enhances the peroxidation of erythrocyte ghosts by periodate.

The direct quantitative comparison between the production of TBA-reactive substance and the generation of MDA during cardiac membrane and membrane phospholipid peroxidation made herein, along with the work of others in nonmembrane systems (5,42,43), would argue that the TBA test lacks the requisite chemical specificity to be used as a reliable measure, or even as an indicator, of lipid peroxidation, unless purified lipid is the sole oxidative substrate in the system. Independent verification of even the qualitative, comparative significance of a positive TBA test would appear to be necessary to use the TBA reaction as anything other than an empirical indicator of the occurrence of (per)oxidation in biological membrane samples.

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REFERENCES

1. Janero, D.R., and Burghardt, B. (1987) *J. Am. Oil Chem. Soc. 44,* 667-668.
2. Jackson, C.V., Mickelson, J.K., Pope, T.K., Rao, P.S., and Lucchesi, B.R. (1986) *Am. J. Physiol. 251,* H1225-H1231.
3. Nair, V., and Turner, G.A. (1984) *Lipids 19,* 804-805.
4. Gutteridge, J.M.C. (1981) *FEBS Lett. 128,* 343-346.
5. Warso, M.A., and Lands, W.E.M. (1984) *Clin. Physiol. Biochem. 2,* 70-77.
6. Asakawa, T., and Matsushita, S. (1979) *Lipids 14,* 401-406.
7. Kramer, J.H., Mak, I.T., and Weglicki, W.B. (1984) *Circ. Res. 55,* 120-124.
8. Herbaczynska-Cedro, K., and Gordon-Majszak, W. (1986) *Pharmacol. Res. Commun. 18,* 321-332.
9. St. Louis, P.J., and Sulakhe, P.V. (1976) *Int. J. Biochem. 1,* 547-558.
10. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
11. Janero, D.R., and Barrnett, R. (1981) *Anal. Biochem. 111,* 283-290.
12. Brockerhoff, H. (1963) *J. Lipid Res. 4,* 96-99.
13. Juaneda, P., and Rocquelin, G. (1985) *Lipids 20,* 40-41.
14. Janero, D.R., and Barrnett, R. (1981) *J. Chromatogr. 216,* 417-422.
15. Letters, R. (1964) *Biochem. J. 93,* 313-316.
16. Skidmore, W.D., and Entenman, C. (1962) *J. Lipid Res. 3,* 356-363.
17. Watson, B.D., Busto, R., Goldberg, W.J., Santiso, M., Yoshida, S., and Ginsberg, M.D. (1984) *J. Neurochem. 42,* 268-274.
18. Chan, H.W.-S., and Levett, G. (1976) *Lipids 12,* 99-104.
19. Bernheim, F., Bernheim, M.L.C., and Wilbur, K.M. (1948) *J. Biol. Chem. 74,* 257-264.
20. Janero, D.R., and Burghardt, B. (1986) *J. Am. Oil Chem. Soc. 63,* 476-477.
21. Bull, A.W., and Marnett, L.J. (1985) *Anal. Biochem. 149,* 284-290.
22. Esterbauer, H., Lang, J., Zadravec, S., and Slater, T.F. (1984) *Meth. Enzymol. 105,* 319-328.
23. Lee, H.-S., and Csallany, A.S. (1987) *Lipids 22,* 104-107.
24. Pierce, J., and Suelter, C.H. (1977) *Anal. Biochem. 81,* 478-480.
25. Wheeldon, L.W., Schumert, Z., and Turner, D.A. (1965) *J. Lipid Res. 6,* 481-489.
26. Jaqua-Stewart, M.J., Read, W.O., and Steffen, R.P. (1979) *Anal. Biochem. 96,* 293-297.
27. Page, E., McGallister, L.P., and Power, B. (1971) *Proc. Natl. Acad. Sci. USA 68,* 1465-1466.
28. Janero, D.R., and Burghardt, B. (1986) *Fed. Proc. 45,* 1808.
29. Massey, V., Komai, H., Palmer, G., and Elion, G.B. (1970) *J. Biol. Chem. 245,* 2837-2844.
30. Laub, R., Schneider, Y.-J., Octane, J.-N., Touet, A., and Chrichton, R.R. (1985) *Biochem. Pharmac. 34,* 1175-1183.
31. McCay, P.B. (1985) *Ann. Rev. Nutr. 5,* 323-340.
32. McCord, J.M., and Fridovich, I. (1969) *J. Biol. Chem. 244,* 6049-6055.
33. Esterbauer, H., and Slater, T.F. (1981) *IRCS Med. Sci. Libr. Compend. 9,* 749-750.
34. Kakuda, Y., Stanley, D.W., and van de Voort, F.R. (1981) *J. Am. Oil Chem. Soc. 58,* 773-775.
35. Csallany, A.S., Guan, M.D., Manwaring, J.D., and Addis, P.B. (1984) *Anal. Biochem. 142,* 277-283.
36. Gray, J.I. (1978) *J. Am. Oil Chem. Soc. 55,* 539-546.
37. Warren, L. (1959) *J. Biol. Chem. 234,* 1971-1975.
38. Tsuchihashi, H., Sasaki, M., and Nargatomo, T. (1986) *Biochem. Med. Metab. Biol. 36,* 36-44.
39. Schauer, R. (1978) *Meth. Enzymol. 50,* 64-89.
40. Skoza, L., and Mohos, S. (1976) *Biochem. J. 159,* 457-462.
41. Kobata, A. (1979) *Anal. Biochem. 100,* 1-14.
42. Gutteridge, J.M.C. (1977) *Anal. Biochem. 82,* 76-82.
43. Warso, M.A., and Lands, W.E.M. (1985) *J. Clin. Invest. 75,* 667-671.
44. Jain, S.K. (1984) *J. Biol. Chem. 259,* 3391-3394.
45. Beppu, M., and Kikugawa, K. (1987) *Lipids 22,* 312-317.

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