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EFFECT OF OXYGEN TENSION ON THE GENERATION OF F_2-ISOPROSTANES AND MALONDIALDEHYDE IN PEROXIDIZING RAT LIVER MICROSOMES

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Abstract—Although numerous methods have been developed for the detection of lipid peroxidation, it is generally recognized that most of these lack specificity and/or sensitivity, particularly when applied to *in vivo* situations. We have reported recently that a series of prostaglandin F_2-like compounds, termed F_2-isoprostanes, are formed *in vivo* from the free radical catalyzed peroxidation of arachidonic acid and appear to be a useful marker of oxidant stress. Because the formation of other products of lipid peroxidation, such as alkanes and malondialdehyde (MDA), are affected by oxygen tension, which may influence their usefulness as markers of oxidant stress, we carried out a systematic study of the generation of F_2-isoprostanes at various oxygen concentrations and compared these changes with the generation of MDA. The disappearance of the F_2-isoprostane precursor, arachidonic acid, was used as a reference measure. Rat liver microsomes were peroxidized using an iron-ascorbate system. The incubations were carried out in sealed flasks at 37° under N_2 and various concentrations of O_2 up to 100%. F_2-isoprostanes were quantified by mass spectrometry and MDA by the thiobarbituric acid reaction. Microsomal fatty acids were measured by gas chromatography. Both MDA and F_2-isoprostane formation increased in a time-dependent manner up to 15 min. Their formation correlated with a loss of polyunsaturated fatty acid and with an increase in O_2 tension up to 21% O_2. At oxygen tensions above 21%, MDA generation continued to increase, while F_2-isoprostane generation and arachidonic acid loss did not. Levels of MDA and F_2-isoprostanes increased a maximum of 65 and 9.4 times baseline values, respectively. These studies, therefore, define factors that influence the formation of F_2-isoprostanes in an *in vitro* model of lipid peroxidation. Further, they demonstrate that higher O_2 tensions do not block formation of F_2-isoprostanes and validate their usefulness for assessing lipid peroxidation under high, as well as low, oxygen tension.

Key words: peroxidation; lipid; eicosanoid; prostaglandin; isoprostane

Free radical catalyzed lipid peroxidation has been implicated in the pathogenesis of a wide variety of human disorders [1–4]. Nonetheless, much remains to be understood about the mechanisms of oxidant injury *in vivo*. It has been reported previously that auto-oxidation of fatty acids *in vitro* results in the formation of PG‖-like compounds [5–7]. Recently, we reported that a series of PGF_2-like compounds, termed F_2-isoprostanes, are produced *in vivo* in humans as products of free radical catalyzed peroxidation of arachidonic acid independent of the cyclooxygenase enzyme [8]. Formation of F_2-isoprostanes proceeds through intermediates comprised of four positional peroxyl radical isomers of arachidonic acid which undergo endocyclization to yield PGG_2-like bicyclic endoperoxides. The endoperoxides are then reduced to F-ring isoprostanes. F_2-isoprostanes are primarily formed *in situ* from arachidonic acid esterified in phospholipids and subsequently released preformed into the circulation, presumably by a phospholipase(s) [9]. Levels of esterified F_2-isoprostanes in tissues such as liver increase dramatically in animal models of free radical injury, and quantification of the F_2-isoprostanes has proved to be an important advance in our ability to assess oxidant status *in vivo* [10]. Nonetheless, factors that modulate the generation of F_2-isoprostanes have not been explored carefully. Because other methods used to measure lipid peroxidation, such as malondialdehyde and alkane generation, are affected by oxygen tension [11], we carried out a systematic study of the generation of F_2-isoprostanes in an *in vitro* model system of peroxidizing rat liver microsomes at various O_2 concentrations and compared these changes with the production of MDA. The disappearance of the F_2-isoprostane precursor, arachidonic acid, was used as a reference measurement.

MATERIALS AND METHODS

$FeCl_3$ and CCl_4 were purchased from the Fisher Scientific Allied Corp. (Pittsburgh, PA). ADP and the fatty acid reference standard were purchased from the Sigma Chemical Co. (St. Louis, MO). [$^2H_4$]Prostaglandin $F_{2\alpha}$ standard for quantification of

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‖ Abbreviations: PG, prostaglandin; and MDA, malondialdehyde.

the F_2-isoprostanes was purchased from the Cayman Chemical Co. (Ann Arbor, MI).

Hepatic microsomes were isolated by differential centrifugation from male Sprague–Dawley rats (200–250 g) purchased from Harlan Sprague–Dawley, Inc. (Indianapolis, IN) [12]. They were given food and water *ad lib.* and housed in alternating 12-hr light and dark cycles. The rats were fasted overnight prior to microsome isolation. A buffer containing 50 mM Tris–HCl and 150 mM KCl (adjusted to pH 7.4) was used for the isolation and incubation medium. Incubations were performed in a shaking water bath at 37° in 25-mL sealed flasks; the total volume was 5 mL and the protein concentration of the solution was 0.55 ± 0.16 mg/mL (mean ± 1 SD, N = 11). The flask atmospheres with less than 21% oxygen were adjusted by flushing the flask with nitrogen for 15 min and injecting graded amounts of oxygen. All reagents used were purged with nitrogen.

Lipid peroxidation was initiated after a 5-min preincubation of microsomes at 37° by the addition of 5 μM iron, 2 mM ADP, and 1 mM ascorbic acid. This peroxidation system was employed so that data obtained regarding F_2-isoprostane formation could be compared with previous studies in which alkane generation was examined [11]. Depending on the experiment, incubation times varied from 2.5 to 90 min. The reactions were terminated by withdrawing mixture aliquots and immediately processing them for MDA, F_2-isoprostanes, or fatty acid composition as described below.

MDA was quantified by measuring thiobarbituric acid reactive material employing a colorimetric assay as described [13]. Esterified F_2-isoprostanes in microsomal phospholipids were quantified as free F_2-isoprostanes after base hydrolysis of lipids, purification and derivatization [9]. Analysis was performed using gas chromatography/mass spectrometry, employing stable isotope dilution techniques with $[^2H_4]PGF_{2\alpha}$ as an internal standard as described [14]. Microsomal phospholipid fatty acids were quantified as described, using pentadecanoic acid as a standard [15].

Experiments were also carried out to compare the generation of F_2-isoprostanes with that of MDA *in vivo* in rats administered CCl_4 (1 mL/kg) intragastrically [16]. One hour after administration, the animals were killed, the livers were harvested, and F_2-isoprostanes and MDA were quantified as described [9, 17]. Statistical evaluation of data was performed using Student's *t*-test.

RESULTS

Initially, we examined the time course of formation of F_2-isoprostanes in peroxidizing rat liver microsomes exposed to 21% O_2 and compared this with the formation of MDA. The results are shown in Fig. 1. As is evident, after initiation of peroxidation, the generation of MDA increased rapidly and dramatically (Fig. 1A) with much of the generation occurring in the first 5–10 min and reaching a plateau within 10–15 min. In an analogous manner, F_2-isoprostane formation increased rapidly over a similar period of time, reaching maximum levels within 10 min. Thereafter, however, levels

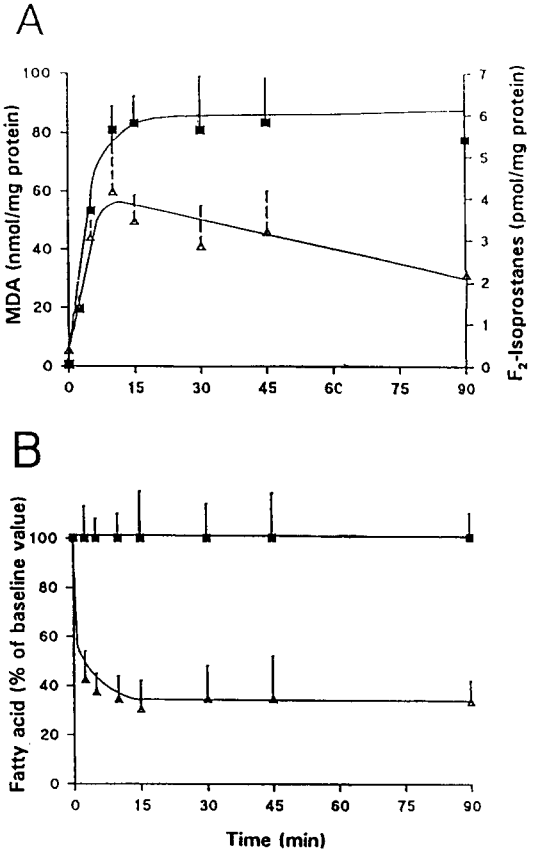

Fig. 1. (A) Time course of F_2-isoprostane (△) and MDA (■) formation in rat liver microsomes after addition of iron, ADP, and ascorbate at an O_2 tension of 21%. (B) Per cent disappearance of palmitic (■) and arachidonic (△) acids in the same microsomal incubations. Baseline values for palmitic and arachidonic acid were 331 ± 30 and 462 ± 34 nmol/mg protein, respectively. Values are means ± SD; N = 5 experiments.

decreased and by 90 min were approximately 45% lower than at 10 min. The reason for the decline in F_2-isoprostanes over time may be explained by prior observations in which we have shown that F_2-isoprostanes, once formed, can be removed from tissue phospholipids *in vivo*, presumably by phospholipase A_2 [9]. Microsomal preparations contain phospholipase A_2, and thus it is possible that after formation, F_2-isoprostanes are hydrolyzed from microsomal phospholipids [18]. Since the method employed to measure esterified F_2-isoprostanes in these studies involves extraction of lipids using a modified Folch procedure [9], free F_2-isoprostanes are excluded because they do not extract into the organic phase at a neutral pH. Thus, to test the hypothesis that free F_2-isoprostanes increase in microsomal incubations over time, free isoprostanes were quantified in addition to esterified F_2-isoprostanes in microsomal incubations at baseline, after 10 min, and after 90 min of peroxidation [14]. Levels of free compounds were undetectable at baseline, were 5 ± 5% of esterified

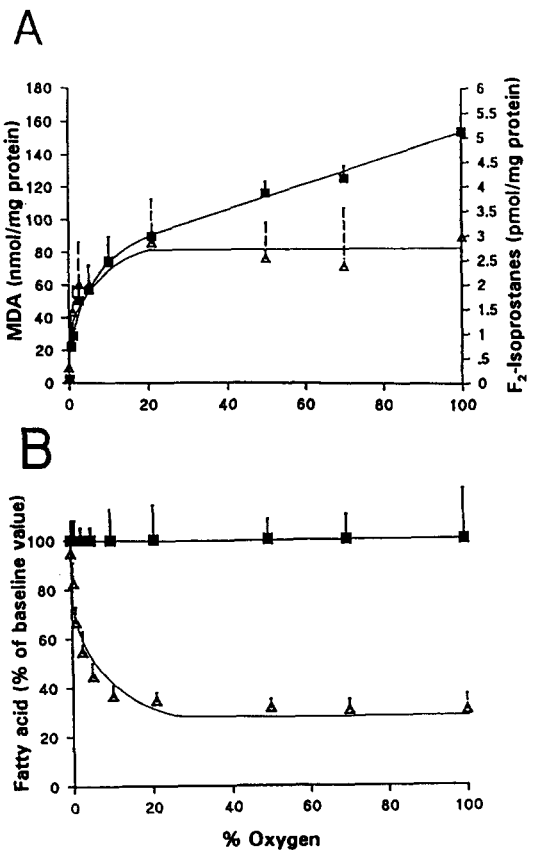

Fig. 2. (A) Oxygen dependence of F_2-isoprostane (△) and MDA (■) increases in rat liver microsomes after addition of iron, ADP, and ascorbate for 20 min. (B) Per cent disappearance of palmitic (■) and arachidonic (△) acid at different O_2 tensions. Baseline values for palmitic and arachidonic acid were 347 ± 41 and 494 ± 34 nmol/mg protein, respectively. Values are means ± SD; N = 5 experiments.

levels at 10 min, and by 90 min were 40 ± 12% of esterified levels (N = 3). Thus, these data support the concept that hydrolysis of F_2-isoprostanes from microsomal phospholipids accounts for the decline in esterified levels of compounds over time.

As shown in Fig. 1B, arachidonic acid was depleted rapidly in peroxidizing microsomes with most of the loss occurring in the first 10 min. The loss paralleled F_2-isoprostane formation. In these studies, disappearance of arachidonic acid was compared with that of palmitic acid, which as a saturated fatty acid is not subject to peroxidative damage, and, therefore, was used to correct for differences in extraction efficiency.

The relationship between F_2-isoprostane and MDA generation at various oxygen concentrations was then examined. The results are shown in Fig. 2. In Fig. 2A, a comparison of the generation of F_2-isoprostanes and of MDA is shown. At concentrations of O_2 up to 21%, the generation of both lipid peroxidation products paralleled each other. At O_2 concentrations above 21%, however, F_2-isoprostane formation plateaued, while MDA concentrations continued to increase, albeit at a slower rate than at lower O_2 tensions. Maximum fold increases of MDA and F_2-isoprostanes were 65 and 9.4 times baseline values, respectively. As shown in Fig. 2B, concentrations of arachidonic acid decreased dramatically as oxygen tension increased, and the loss correlated with F_2-isoprostane generation. Half-maximal loss of arachidonic acid occurred at 5% O_2. Again, losses of arachidonic acid are expressed in relation to concentrations of palmitic acid.

In Table 1, increases in F_2-isoprostane formation under different O_2 tensions compared with the generation of MDA and loss of arachidonic acid are compiled. When the amount of F_2-isoprostanes generated over a range of O_2 concentrations was compared with the quantity of arachidonic acid consumed, there was little variation, with approximately 1 mol of F_2-isoprostanes formed for each 130,000–170,000 mol of arachidonate consumed. The number of moles of MDA generated per mole of arachidonate lost, however, varied more widely; at O_2 tensions below 21%, approximately 1 mol of MDA was generated for each 5–6 mol of arachidonic acid consumed, while the ratio was 1:2 at higher O_2 concentrations. In addition, as is apparent, the total amount of MDA generated far exceeded the quantity of F_2-isoprostane formed.

As a final set of experiments, the relationship between MDA and F_2-isoprostane generation was compared in an *in vivo* model of lipid peroxidation involving the administration of CCl_4 to rats. CCl_4 induces marked lipid peroxidation in the livers of animals due to its metabolism by the cytochrome P450 enzyme system to the trichloromethyl radical, which oxidizes unsaturated fatty acids present in tissue phospholipids. For these studies, liver tissue levels of F_2-isoprostanes and MDA in rats treated with CCl_4 for 1 hr were compared. The results are shown in Table 2. Increases in esterified F_2-isoprostanes were approximately 80 times baseline, whereas they were only 2.7 times baseline for MDA.

DISCUSSION

The present studies were undertaken to examine the relationship of the formation of F_2-isoprostanes, which are novel products of free radical catalyzed lipid peroxidation, and the generation of a commonly used measure of lipid peroxidation, MDA, in peroxidizing rat liver microsomes. The loss of the F_2-isoprostane precursor, arachidonic acid, was used as a reference measurement.

Several important observations emerged from the studies reported herein. First, the generation of F_2-isoprostanes was influenced to a large degree by the oxygen tension present in the reaction medium. Small amounts of F_2-isoprostanes were generated at very low O_2 tensions, but their formation increased markedly as O_2 concentrations increased up to 21% and above this concentration their production plateaued. Further, at concentrations of O_2 less that 21%, F_2-isoprostane generation correlated with MDA generation but above 21% O_2, MDA concentration continued to increase. At all oxygen concentrations, the formation of F_2-isoprostanes

Table 1. *In vitro* changes of oxygen-dependent lipid peroxidation parameters in rat liver microsomes incubated for 20 min with iron, ADP and ascorbate*

Oxygen tension (%)	AA (nmol loss)	MDA (nmol gain)	MDA/AA ratio	F_2-isoprostanes (pmol gain)	F_2-isoprostanes/AA ratio
0.5	94 ± 34	20 ± 12	1/5	0.55 ± 0.56	1/170,000
1	157 ± 39	27 ± 7	1/6	1.13 ± 0.56	1/140,000
5	276 ± 24	55 ± 14	1/5	1.71 ± 0.42	1/160,000
21	326 ± 20	87 ± 3	1/4	2.54 ± 0.92	1/130,000
100	341 ± 34	152 ± 12	1/2	2.66 ± 0.98	1/130,000

* All values for arachidonic acid (AA), MDA, and F_2-isoprostanes are per mg microsomal protein. Baseline values at 0% oxygen were: for arachidonic acid, 494 ± 34 nmol/mg protein; MDA, 2.3 ± 1.3 nnmol/mg protein; and for F_2-isoprostanes, 0.28 ± 0.22 pmol/mg protein.

Table 2. Increase in F_2-isoprostanes and MDA in rat liver 1 hr after intragastric CCl_4

	MDA (nmol/g liver)	F_2-isoprostanes (pmol/g liver)
Control rats	200 ± 132	13 ± 2
Rats administered CCl_4 (1 mL/kg)	527 ± 132	1040 ± 160
Fold-increase	2.7*	80.2†

Rat liver tissue used in these studies contained 12.4 μmol arachidonic acid/tissue. Values for MDA and F_2-isoprostanes are means ± SD, N = 5.

* $P < 0.005$ compared with baseline.

† $P < 0.001$ compared with baseline.

correlated with the loss of its precursor, arachidonic acid.

The fact that F_2-isoprostane production correlates with precursor unsaturated fatty acid loss is important since it implies that F_2-isoprostanes may be a useful measure of lipid peroxidation at high O_2 tensions. This is in contradistinction to alkane formation, another tool used to assess lipid peroxidation. At low O_2 tensions, pentane or ethane formation correlates with fatty acid oxidation, whereas the generation of pentane decreases at higher O_2 tensions despite increasing loss of polyunsaturated fatty acids [11]. The reason for this is unknown but may be due to the preferential formation of lipid peroxidation products other than alkanes at higher O_2 tension.

The cause for the dissociation between MDA formation, F_2-isoprostane generation and arachidonate loss at higher O_2 concentrations is unclear but may be explained by the fact that whereas F_2-isoprostanes derive only from arachidonic acid, MDA is generated by other fatty acids which display different rates of peroxidation at different O_2 tensions based on the number of double bonds contained in the molecule. It has been shown previously that compounds possessing more double bonds are peroxidized to a greater extent at lower O_2 tensions [11]. Thus, it is possible that arachidonic acid, which is highly unsaturated, is the primary source for MDA in peroxidizing microsomes at lower O_2 tensions and thus the generation of MDA and loss of arachidonate correlate. At higher O_2 tensions, however, other fatty acids containing fewer double bonds, such as linoleic acid, may contribute a relatively greater fraction to the generation of MDA, thus altering the relationship between MDA formation and arachidonic acid consumption.

In addition to the *in vitro* studies reported, we sought to compare the generation of F_2-isoprostanes with that of MDA in an *in vivo* model of lipid peroxidation, employing the administration of CCl_4 to rats. It is of interest to note that unlike the generation of MDA and isoprostanes *in vitro*, the increases in F_2-isoprostanes that were detected in the liver tissue of animals treated with CCl_4 were far greater than for MDA (Table 2). This finding may be explained in several ways. First, it is possible that CCl_4 administration to rats results in less MDA formation relative to isoprostanes than does the *in vitro* iron-ascorbate system studied. Indeed, the generation of MDA in rat liver microsomes incubated with CCl_4 is less than in microsomes incubated with iron-ascorbate probably because the formation of the trichloromethyl radical requires the P450 enzyme system, which may be damaged during microsomal purification or may be inactivated by reaction with CCl_4 adducts [11, 12]. On the other hand, in preliminary experiments, we observed that the yield of F_2-isoprostanes in microsomes incubated with CCl_4 also was proportionally less compared with incubations using iron-ascorbate (data not shown), making this explanation unlikely. More likely, however, is the fact that MDA is rapidly metabolized *in vivo* and may be unstable in an oxidizing environment. Thus, the detection of increases in MDA *in vivo* may be more difficult than for F_2-isoprostanes, which are relatively stable [9, 10, 19]. Third, for these studies, MDA was quantified using a colorimetric assay to measure thiobarbituric acid reactive material. In addition to MDA generated from lipid peroxidation, it is well known that other substances such as sugars, proteins and pigments will cross-react in the assay to yield thiobarbituric acid reactive material [19]. Although numerous methods such as HPLC purification have been developed to circumvent this problem, it is clear that no method is entirely satisfactory [20]. This is

particularly true when the assay is utilized to measure complex biological fluids and tissue extracts. Thus, while frequently used as an index of lipid peroxidation, MDA values obtained from biological sources must be interpreted with caution. Therefore, it is possible, for example, that in the studies reported herein, the fold-increases in MDA in the livers of CCl_4-treated rats were artificially low due to interfering substances elevating baseline measurements. Nonetheless, while MDA and F_2-isoprostane increases are similar in peroxidized rat microsomes *in vitro*, it appears as though the latter measure may provide a better index of lipid peroxidation *in vivo*.

In summary, these studies have provided data regarding factors controlling the generation of F_2-isoprostanes in an *in vitro* model of lipid peroxidation and validate their usefulness for measuring lipid peroxidation under a variety of O_2 concentrations. Further, these studies may provide insight into conditions modulating the formation of F_2-isoprostanes *in vivo*.

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