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Effect of Oxygen Concentration on the Formation of Malondialdehyde-Like Material in a Model of Tissue Ischemia and Reoxygenation

Steven C. Salaris

Charles F. Babbs
Purdue University, babbs@purdue.edu

EFFECT OF OXYGEN CONCENTRATION ON THE FORMATION OF MALONDIALDEHYDE-LIKE MATERIAL IN A MODEL OF TISSUE ISCHEMIA AND REOXYGENATION

STEVEN C. SALARIS and CHARLES F. BABBS

Biomedical Engineering Center, and School of Veterinary Medicine, Purdue University, West Lafayette, Indiana, USA.

ABSTRACT

This study was conducted to explore the functional relationship between oxygen concentration during tissue reoxygenation after ischemia and the extent of postischemic lipid peroxidation, an indicator of reoxygenation injury. Excised rat liver or kidney tissue was rendered ischemic for 1 h at 37°C, minced into 1 mm^3 fragments, and then reoxygenated for 1 h in flasks of buffered salt solution containing various amounts of oxygen. Production of malondialdehyde-like material (MDA) was measured to indicate lipid peroxidation. MDA production was minimal at oxygen tensions less than 10 mmHg, increased sharply from 10 to 50 mmHg, and plateaued at approximately 100 mmHg. A similar functional relationship was produced by a simple mathematical model of free radical mediated lipid peroxidation in biological membranes, suggesting that MDA production is indeed caused by free radical oxidation of membrane phospholipids and that the oxygen effect is governed by simple competition between chain propagation and chain termination reactions within the membrane. These experimental and analytical results confirm that relatively low concentrations of oxygen arc sufficient to produce oxidative damage in post-ischemic tissues.

Key words: Deferoxamine, Free radicals, Hydroxyl radical, Lipid peroxidation, Methylene blue, Reoxygenation injury, Reperfusion injury, Superoxide ion, Xanthine oxidase

INTRODUCTION

The concept of reperfusion or reoxygenation injury implies that an important component of tissue damage observed after a period of ischemia and reperfusion is caused by events during the reperfusion phase, which necessarily involve the return of molecular oxygen. By most accounts, the injury associated with reoxygenation is initiated by the formation of active oxygen species such as the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2). These can compound primary ischemic injury by a variety of possible mechanisms, including the generation of highly reactive hydroxyl radicals (HO·) via the iron catalyzed Haber-Weiss reaction and subsequent initiation of lipid peroxidation in biological membranes, which then proceeds by radical chain mechanisms [1-5].

Many studies of reperfusion and/or reoxygenation injury, including our own [6, 7] and especially those utilizing isolated, perfused hearts [8-11], have been done using 95 to 100% oxygen for reoxygenation after ischemia or hypoxia. Ranges of oxygen concentration are generally not tested. If toxic free radicals were formed only at high oxygen tensions, for example PO_2 >100 mmHg, as might occur in vascular endothelial cells, then one simple strategy to minimize reoxygenation injury in clinical practice would be to resuscitate with room air rather than oxygen.

To date, the oxygen requirements for free radical formation and/or lipid peroxidation in postischemic tissues have not been rigorously studied. Demopolous [12], for example, addressed this issue only in terms of idealized curves. The large rate constant for the addition of oxygen to lipid alkyl radicals (L· + $O_2 \rightarrow$ ·LOO ; k = 9 x 10^6 $M^{-1}sec^{-1}$) [13], would seem to indicate that only low PO_2's are necessary for lipid peroxidation. However, there are many competing reactions and complexities of free radical chemistry in vivo that could allow for the formation of other products.

If the toxic effects of oxygen after ischemia were to occur only at relatively high oxygen concentrations, while the beneficial effects of reoxygenation were achieved at lower concentrations, then a therapeutic window for postischemic oxygen therapy would exist that would permit restoration of aerobic metabolism with minimum reoxygenation injury. In support of this possibility are Negovskii's intriguing studies of the 1960s, in which dogs resuscitated from hemorrhagic shock with 100% oxygen developed microhemorrhages in the brain [14], while those similarly resuscitated with room air did not. On the other hand, if the toxic effects of oxygen in postischemic tissues appear at concentrations similar to those necessary to restore mitochondrial function, then the potential for reoxygenation injury is an inevitable accompaniment to oxygen therapy and must be suppressed by other strategies.

To investigate oxygen effects after ischemia we used tissue slices incubated in short term organ culture, as originally described for the study of carbon tetrachloride toxicity by Tappel and coworkers [15, 16] and subsequently modified in our laboratory for the study of reoxygenation injury [17]. Tissue slices offer a useful and convenient compromise between biochemical and physiological model systems. The microscopic organization of tissues is preserved, while the

chemical composition, including, in particular, the PO_2 of the incubation media, can be easily and precisely controlled to mimic the extracellular milieu in bulk tissues. When the thickness of the tissue slices is approximately 1 mm, the oxygen gradients that develop with distance from the surface are similar to those that develop in vivo with distance from perfused capillaries [18]. Moreover, tissues may be conveniently exposed to a cycle of ischemia and reoxygenation by pre-incubating freshly excised tissue in humidified argon gas, prior to mincing and subsequent incubation in oxygen containing buffer.

The production of tbiobarbituric acid reactive substances or malondialdehyde equivalents, for simplicity abbreviated here as MDA equivalents, provides a sensitive indicator of free radical dependent reoxygenation injury. Our previous studies with tissue slices [17] had shown that MDA equivalent production by postischemic rat liver and kidney is abolished by treatment with the iron chelator deferoxamine, the liposoluble, chain breaking antioxidant, BHT, and by methylene blue, which probably acts to inhibit production of superoxide by xanthine oxidase [19]. This simple system would thus appear to exhibit the classical features of reoxygenation injury as proposed by Saugstad [20] and McCord [1]--dependence upon enzymatically generated superoxide, upon the iron catalyzed Haber Weiss reaction, and upon free radical chain oxidations. Accordingly, we conducted the following study to determine the functional relationship between oxygen concentration during the post-ischemic period and one well-known indicator of reoxygenation injury.

METHODS

Materials

TRIS buffer and 2-thiobarbituric acid were obtained from Sigma Chemical Company (St. Louis, MO 63178), trichloroacetic acid from Fisher Scientific Company (Fair Lawn, NJ 07410), and methylene blue, from Aldrich Chemical Company (Milwaukee, WI 53233). Deferoxamine, Lot #30315, was kindly provided by CIBAGEIGY Pharmaceuticals Division (Suffern, NY 10901). The TRIS-Ringer buffer solution consisted of 4.0 mM KC1, 100 mM NaCl, 2.0 mM $CaCl_2$, 40 mM TR1S buffer, and 200 mg/dL glucose, pH 7.4. TRIS buffer was chosen rather than phosphate because its solubility in the presence of calcium permitted a greater buffering capacity to neutralize lactic acid produced by tissues during ischemia than the usual phosphate buffers.

Tissues

Tissues were taken from 30 male Wistar rats which were anesthetized with sodium pentobarbitol (60 mg/ kg, i.p.). Rat liver and kidney were selected for study because they produce abundant MDA equivalents in this model of ischemia/reoxygenation [17], and so provide a good basis for the study of oxygen effects. The liver and kidneys were excised, and tissue samples of approximately 0.3-0.4 grams were then placed into stoppered 50 ml Erlenmeyer flasks containing argon saturated (anoxic) TRIS-Ringer solution. Liver and kidney tissues were separately divided among 9 flasks, making a total of 18 flasks, which included ischemic but not reoxygenated controls and eight flasks for each tissue that were resupplied with various amounts of oxygen at the end of ischemia.

Ischemia/Reoxygenation

For the ischemic phase, the 50 ml Erlenmeyer flasks, with tissue samples and 3.9 ml of argonated TRIS-Ringer buffer were flushed with argon gas, re-stoppered, and kept in a warmed, 37°C, non-shaking water bath for 50 min to create ischemia. The tissues were not minced or shaken at this time to minimize the diffusion of metabolites from the tissue into the media, which does not occur in vivo during ischemia.

Tissue mincing and drug treatment

After the 50 min of ischemia, the tissues were minced with iris scissors to a size of approximately 1 mm on a side. Mincing was done to provide greater surface area for subsequent oxygen diffusion during the reoxygenation phase and also to promote greater drug distribution into the tissue slices that were treated with drugs. In some experiments, deferoxamine or methylene blue was added prior to reoxygenation. These drugs were dissolved in 0.1 ml of TRIS Ringer buffer and added to the flasks to achieve a final concentration of deferoxamine 100 mg/L (150 µM), or methylene blue 10 mg/L (27 µM). Non-drug treated flasks received 0.1 ml buffer. After the addition of the treatment to each flask, the head spaces of the flasks were quickly flushed with argon gas. Because the density of argon is greater than that of oxygen, there was little contamination of the buffer and tissues with oxygen during the mincing and treatment phase. The flasks were then recapped, swirled gently, and returned to the 37°C water bath for 10 min to allow drug diffusion into the tissue prior to reoxygenation.

Reoxygenation

After the 60 min of ischemic anoxia, a volume (V_1) of argon was removed from each flask and a volume (V_2) of oxygen gas was added so as to achieve a specified oxygen concentration ranging from 0 to 100%, and corresponding to calculated partial pressures of 0, 5, 10, 20, 40, 100, 250, 500, and 700 mmHg. Argon was removed and oxygen was added via a stopcock and 18 gauge needle, inserted through the rubber stopper of each flask, to which a syringe could be attached. The correct volumes, V_1 and V_2, of gases to withdraw and add back were calculated using the ideal gas law ($PV = nRT$).

After introduction of oxygen, all flasks were returned to a 37°C shaking water bath for 60 min of reoxygenation. The PO_2's within the flasks prepared by this procedure were checked by injecting the buffers into a Corning Model 165 pH Blood Gas Analyzer. Four samples were tested at each specified PO_2. The relation between measured PO_2 and calculated (target) PO_2 is presented in Table 1. From the linear regression function for these data, the prevailing PO_2 was calculated for all similarly prepared flasks. These measured values were used as the independent variable, PO_2, in preparing plots. Metabolic oxygen demand of the tissues (about 0.2 ml O_2/h) was negligible with respect to the amount of oxygen available in the flask, for all oxygen concentrations greater than 1%, hence we assumed that oxygen concentration in the flasks did not change as a result of tissue metabolism during the 1 h reoxygenation period.

Table 1. Target and Measured Oxygen Tensions in Experimental Flasks

Specified target PO_2	Measured PO_2
0	6 ± 1
5	17 ± 5
10	23 ± 2
20	28 ± 5
40	49 ± 5
100	104 ± 12
250	235 ± 11
500	453 ± 31
700	699 ± 11

Thiobarbituric acid assay for malondialdehyde

Malondialdehyde was measured by the spectrophotometric method of Buege and Aust [21]. Flask contents, including tissue and buffer were homogenized in a Teflon/glass homogenizer. The total homogenate was combined with 4.0 ml of TCA-TBA reagent [21] in a test tube and mixed thoroughly. Each tube was covered with a glass marble and heated for 15 min in a temperature controlled heating block (Dri-bath) at 90-95°C. After cooling, the flocculent precipitate was removed by centrifugation at 1500 g for 5 min. The absorbance of the supernatant was determined spectrophotometrically at 500, 530, and 560 nm against a blank containing 4 ml of TCA-TBA reagent, 3.9 ml buffer and 0.1 ml of the corresponding drug or buffer. Standard curves were prepared using authentic malonaldehyde (bis-dimethylacetal) [1,1,3,3-tetramethoxypropane], available from Aldrich Chemical Company, Inc. (Milwaukee, WI 53233, Cat # 10,838-3).

A simple double derivative technique [17, 22] was used to calculate the concentration of MDA equivalents in the spectrophotometer cuvet as

$$c = \frac{2A_{530} - A_{500} - A_{560}}{(2 - b_{500} - b_{560})\varepsilon d},$$

where subscripts represent wavelengths in nanometers, A is absorbance, the constants $b_{500} = A_{500}/A_{530}$ for authentic MDA standard, and $b_{560} = A_{560}/A_{530}$ for authentic MDA standard, ε is the molar extinction coefficient for authentic MDA (1.56×10^5 $M^{-1}cm^{-1}$), and d is the light path length through the cuvet. This relationship, derived previously [17], provides a measure of concentration based upon the 530 nm peak of genuine MDA, which is insensitive to background interference that does not exhibit a peak at 530 nm.

Data analysis

For each tissue type an analysis of variance (ANOVA) was run to test the null hypotheses that (1) MDA equivalent production was the same at all of the PO_2's and (2) MDA equivalent production was the same for all drug treatments. The ANOVA was preceded by a Bartlett's chi-square test for homogeneity of variance [23]. If the variances of compared data sets were not similar, a logarithmic transformation of the data was performed [24] and the ANOVA repeated on the transformed data. Specific comparisons were made using Duncan's multiple range test. A p-value of 0.05 was considered significant.

RESULTS

Figures 1 and 2 illustrate the effects of various PO_2's on the postischemic production of MDA equivalents in rat liver and kidney respectively. The data are plotted as mean ± standard error for n = 10 organs in each group. In both liver and kidney of the rat, production of MDA equivalents increases sharply as PO_2 increases from 0 to 50 mmHg, and then begins to plateau. MDA equivalent production was quenched in the methylene blue and deferoxamine treated tissues, compared to the untreated organs. In the untreated liver and kidney, all differences between reoxygenated and non-reoxygenated organs were statistically significant. Analysis of drug treatments showed that production of MDA equivalents in methylene blue and deferoxamine treated organs was significantly less than in untreated organs.
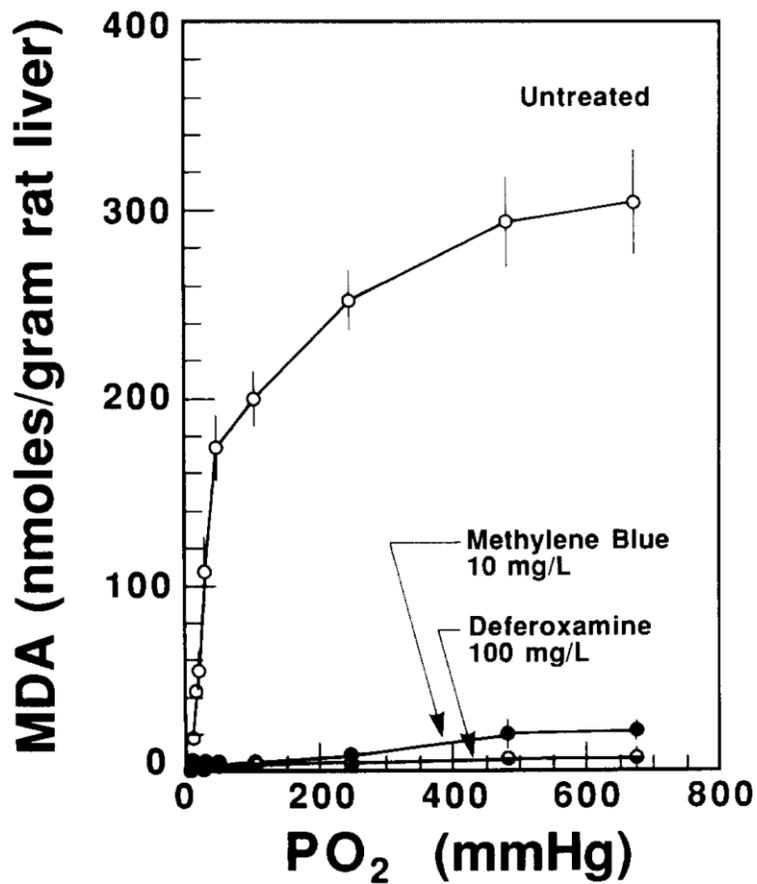
Fig. 1. Production of malondialdehyde equivalents (MDA) in rat liver slices during 1 h reoxygenation after 1 h warm ischemia at 37°C. Error bars represent ± 1 SEM. PO_2, values plotted are measured levels determined during preliminary experiments (Table 1) for each oxygen/argon mixture. Untreated liver generates MDA equivalents during reoxygenation in an oxygen dependent manner. Both methylene blue and deferoxamine strongly suppress MDA production.
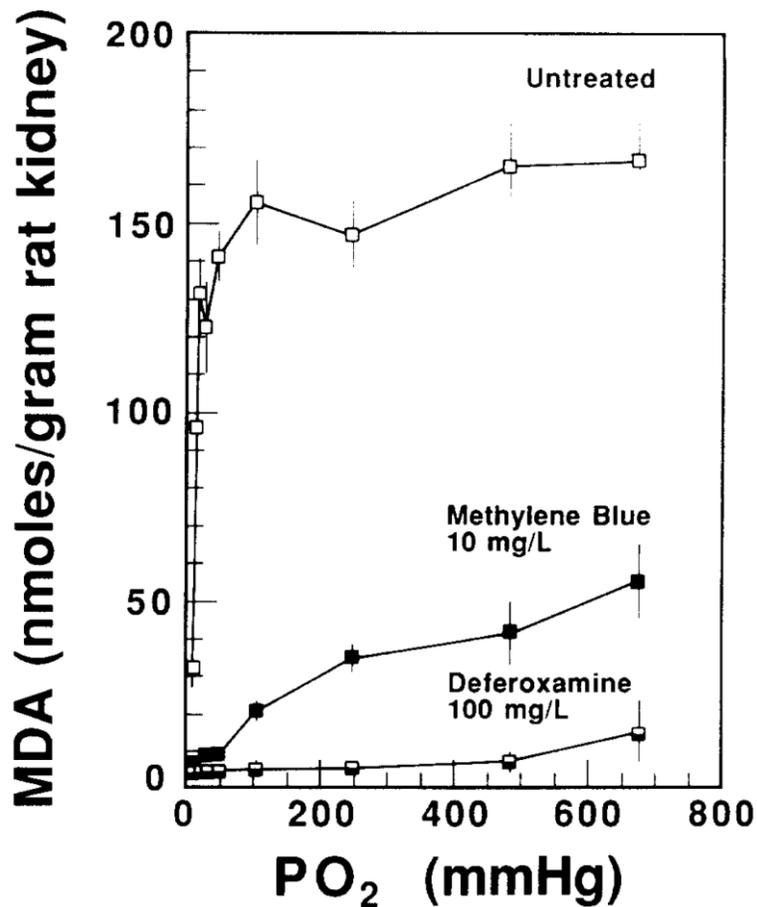
Fig. 2. Production of malondialdehyde equivalents (MDA) in rat kidney slices during 1h reoxygenation after 1 h warm ischemia at 37°C. Error bars represent ± 1 SEM. PO$_2$ values plotted arc measured levels determined during preliminary experiments (Table 1) for each oxygen/argon mixture. Untreated kidney generates MDA equivalents during reoxygenation in an oxygen dependent manner. Methylene blue suppresses and deferoxamine abolishes MDA production.

DISCUSSION

The present study illustrates the strong dependence of the formation of postischemic oxidation products, measured as MDA equivalents, upon the prevailing oxygen concentration. Preliminary computer modeling of this phenomenon by an approach previously described [25] suggested that in essence the oxygen dependence of membrane lipid peroxidation can be explained by a much simpler series of competing reactions in phospholipid membranes themselves. These reactions are as follows.

Initiation:

$$R\cdot\ +\ LH \longrightarrow L\cdot \qquad \text{(initiation rate, } R_i) \qquad (1)$$

Chain propagation:

$$L\cdot\ +\ O_2 \longrightarrow LOO\cdot$$
$$k_2\ =\ 9\ \times\ 10^6\ M^{-1}\ S^{-1(13)} \qquad (2)$$

$$LOO\cdot\ +\ LH \longrightarrow LOOH\ +\ L\cdot$$
$$k_3\ =\ 11\ M^{-1}\ S^{-1(13,26-28)} \qquad (3)$$

Chain termination:

$$L\cdot\ +\ L\cdot \longrightarrow L-L$$
$$k_4\ =\ 1\ \times\ 10^9\ M^{-1}\ S^{-1(13,29,30)} \qquad (4)$$

$$L\cdot\ +\ LOO\cdot \longrightarrow LOOL$$
$$k_5\ =\ 5\ \times\ 10^7\ M^{-1}\ S^{-1(13)} \qquad (5)$$

$$LOO\cdot\ +\ LOO\cdot \longrightarrow LOOL\ +\ O_2$$
$$k_6\ =\ 6\ \times\ 10^5\ M^{-1}\ S^{-1(13,26,31)} \qquad (6)$$
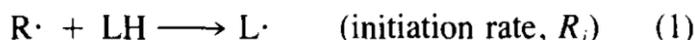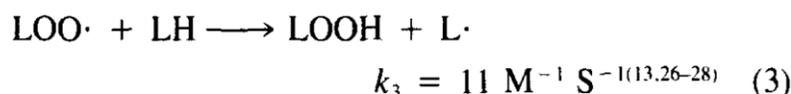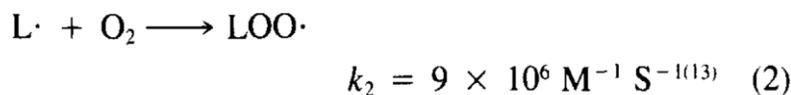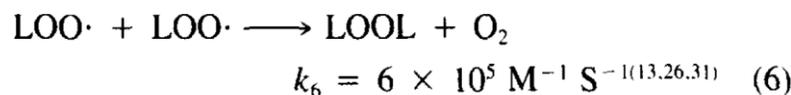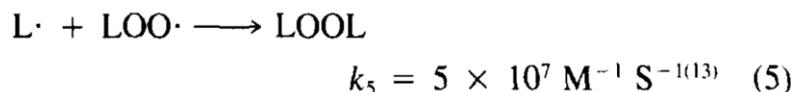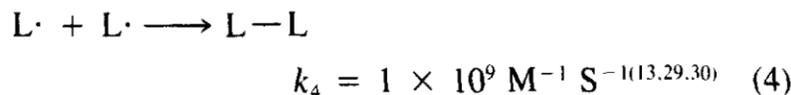
where R_i is the rate of initiation (M/S), k values indicate rate constants for the bimolecular reactions ($M^{-1}sec^{-1}$), and LH indicates an oxidizable membrane lipid.

Swern [32] has given a general expression for the kinetics of such oxidations in which the oxidation rate is given by the expression

$$\frac{-d[LH]}{dt} = \sqrt{R_i}\, k'\, [LH]\, \frac{[O_2]}{k''\, [LH]\, +\, [O_2]}$$

in which hybrid constants k' and k" are defined as

$$k' = k_3/\sqrt{(k_6)} \text{ and } k'' = (k_3/k_2)\sqrt{(k_4/k_6)}.$$

From this expression the shape of the function relating the rate of lipid oxidation to oxygen concentration, which is of immediate interest, can be expressed as a percentage of the maximal oxidation rate by the ratio

$$r = 100\, \frac{[O_2]}{k''\, [LH]\, +\, [O_2]}\, ,$$

in which the concentration of unsaturated lipids in membrane material *per se* can be estimated to be about 1 M from typical densities and molecular weights of membrane phospholipids and allowing about 30% of the interior membrane volume for proteins and non-oxidizable components. Then, taking the values for k_2 through k_6 just presented to obtain k" and plotting the results in relation to experimental data from the present study, one finds the relationships presented in Figure 3. On the whole, the shapes of the oxygen dependence curves that we obtained for liver and kidney experimentally are explained by the six-reaction model (dashed curve) for membrane lipid peroxidation within experimental error.
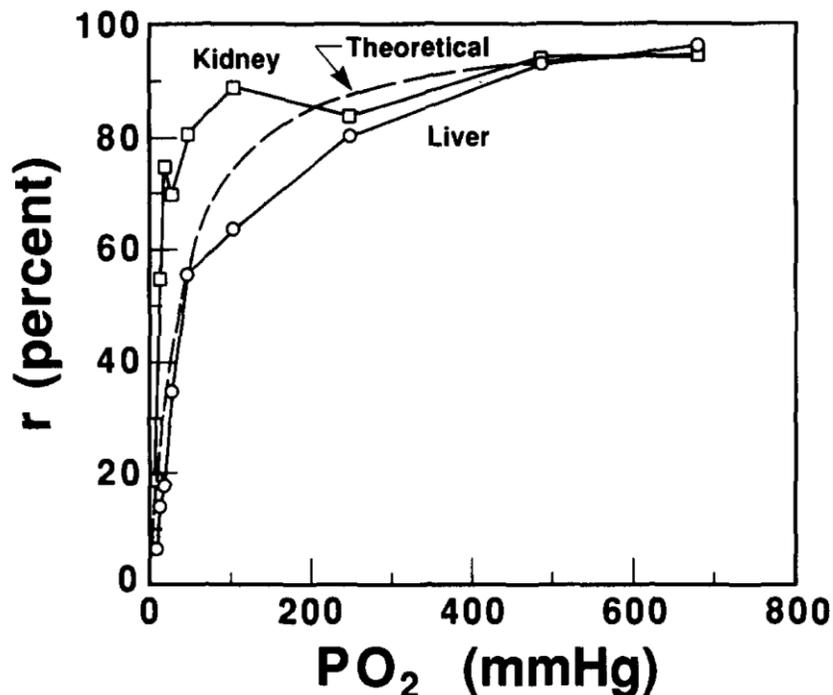
Fig. 3. Relative MDA equivalent (MDA) production as a function of oxygen tension obtained experimentally (solid curves) and theoretically (dashed curve), expressed as a percent of maximal response, assuming approximately 95% maximal response at 700 mmHg. A conversion of 1 μM oxygen = 0.7 mmHg partial pressure of oxygen was assumed in plotting the dashed curve, which was obtained from the expression $r = 100 [O_2]/(k'' [LH] + [O_2])$, using a membrane compartment lipid concentration, [LH], of 1 M and a dimensionless hybrid constant, k'' of 50×10^{-6}, as defined in the text. The oxygen effect is reasonably well predicted by the theoretical expression, which describes the balance of chain propagation and chain termination reactions in the membrane compartment.

Our interpretation of the observed oxygen effect in the tissue slice model is that it reflects the competition between chain propagation and chain termination reactions for lipid peroxidation within biological membranes. A related implication of this interpretation is that membrane lipids are indeed the major source of MDA-equivalents in this system, although it is well known that MDA-like materials can also be produced from the oxidation of nucleic acids, amino acids and certain complex carbohydrates [33-36]. The ability of the highly lipid soluble antioxidant, butylated hydroxytoluene (BHT), to abolish production of MDA equivalents under conditions of ischemia and reoxygenation identical to those of the present study [17] also suggests to us that most of the measured MDA-equivalents are coming from the lipid compartment.

The pathophysiologic implication of the observed oxygen effect is that the critical extracellular PO_2 necessary for reperfusion injury is relatively low. It we equate the oxygen concentration in our experimental incubation flasks with that in arterial blood, recognizing that diffusion into tissue must occur in each case, then about half the maximal effect is observed with a prevailing PO_2 of only 40 mmHg. Since the normal arterial PO_2 of a subject breathing room air is approximately 100 mmHg, this finding suggests the inevitability of some degree of membrane oxidation during reperfusion. We thus found no evidence for the existence of a therapeutic window of oxygen concentrations sufficient to restore aerobic metabolism after ischemia with minimal risk of reoxygenation injury.

Indeed, our results are quite consistent with the proposition of Downey and coworkers [37] that reperfusion injury can occur without reperfusion in small myocardial infarcts supplied by tenuous collateral circulation. That is, even though a coronary artery occlusion is never re-opened, oxygen radical injury may occur in the subepicardial regions and border zones of the resulting infarct, owing to residual oxygen delivery provided by collateral vessels. This proposition is supported by the clinical observation that plasma levels of MDA-like materials in patients with acute myocardial infarction, in the absence of thrombolytic therapy, are twice those of healthy subjects without infarction [38].

Inhibition of MDA equivalent production in our tissue slice model, regardless of oxygen tension, by both deferoxamine and methylene blue is also interesting. This finding is consistent with a Haber-Weiss-Fenton mechanism for initiation of lipid peroxidation [39] as well as with the mechanism involving a ferrous-dioxygenferric chelate complex, proposed by Aust and coworkers [40-42], in which ferric iron must be partially reduced to the ferrous form, perhaps by superoxide.

We studied methylene blue because it is well absorbed into tissue slices [17] and because its mechanism of xanthine oxidase inhibition probably suppresses superoxide production by the enzyme without blocking oxidation of hypoxanthine or xanthine or formation of urate [19, 43, 44]. The proposed chemistry is as follows. Methylene blue competes with molecular oxygen to accept electrons from xanthine oxidase, forming leukomethylene blue. The leukomethylene blue promptly autoxidizes back to the blue form with production of hydrogen peroxide directly, bypassing the formation of superoxide, as has been shown in vitro in the presence of cytochrome-c and oxygen [45]. Thus the net reaction in the presence of both xanthine oxidase and methylene blue is

$$\text{xanthine} + O_2 + 2\ H^+ \rightarrow \text{uric acid} + H_2O_2.$$
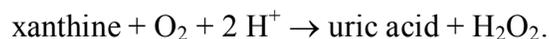
Assuming this mechanism, one can interpret our results with methylene blue as further circumstantial evidence that both superoxide and xanthine oxidase are involved in postischemic lipid peroxidation. Further, the importance of superoxide as a reductant of chelated ferric iron [46] is indirectly confirmed, since the presumed excess H_2O_2, alone is inadequate to induce formation of MDA-like material.

CONCLUSION

This report characterizes experimentally the dependence of postischemic lipid peroxidation, indicated by formation of malondialdehyde-like materials in liver and kidney tissue, upon oxygen concentration, and suggests that the observed effect is explained fundamentally by the balance of chain propagation and chain termination reactions for free radical mediated lipid peroxidation within biological membranes. The process is clearly dependent upon the availability of oxygen and iron, and may also require superoxide production by xanthine oxidase or a related enzyme in rat liver and kidney.

REFERENCES

1. McCord, J. M. Oxygen-derived free radicals in postischemic tissue injury. N. Engl. J. Med. 312:159-163; 1985.

2. Babbs, C. F. Role of iron ions in the genesis of reperfusion injury following successful cardiopulmonary resuscitation: preliminary data and a biochemical hypothesis. Ann. Emerg. Med. 14:777-783; 1985.

3. Aust, S. D.; Morehouse, L A.; Thomas. C. E. Hypothesis paper--role of metals in oxygen radical reactions. J. Free Radical Biol Med. 1:3-25; 1985.

4. Bulkley, G. B. The role of oxygen free radicals in human disease processes. Surgery 94:407-411; 1983.

5. Nayler, W. G.; Elz, J. S. Reperfusion injury: laboratory artifact or clinical dilemma? Circulation 74:215-221; 1986.

6. Badylak, S. F.; Simmons, A; Turek, J; Babbs, C. F. Protection from reperfusion injury in the isolated rat heart by postischemic deferoxamine and oxypurinol administration. Cardiovasc. Res. 21:500-506; 1987.

7. Badylak, S. F.; Babbs, C. F. The effect of carbon dioxide, lidoflazine, and deferoxamine upon long term survival following cardiorespiratory arrest in rats. Resuscitation 13:165-173; 1986.

8. Hearse, D. J.; Humphrey, S. M.; Chain, E. B. Abrupt reoxygenation of the anoxic potassium-arrested perfused rat heart: a study of myocardial enzyme release. J. Molec. Cell Cardiol. 5:395-407; 1973.

9. Gauduel, Y.; Duvelleroy, M. A. Role of oxygen radicals in cardiac injury due to reoxygenation. J. Molec. Cell Cardiol. 16:395-407; 1984.

10. Guarnieri, C.; Flamigni, F.; Caldarera, C. M. Role of oxygen in the cellular damage induced by re-oxygenation of hypoxic heart. J. Molec. Cell Cardiol 12:797-808; 1985.

11. Myers, C. L.; Weiss, S. J.; Kirsh, M. M.; Shlafer, M. Involvement of hydrogen peroxide and hydroxyl radical in the oxygen paradox: reduction of creatinine kinase release by catalase, allopurinol, or deferoxamine, but not by superoxide dismutase. J. Molec. Cell Cardiol. 17:675-684; 1985.

12. Demopolous, H. B.; Flamm, E. S.; Pietronigro, D. D.; Seligman, M. L. The free radical pathology and the microcirculation in the major central nervous system disorders. Acta Physiologica Scand. Suppl. 492:91-119; 1980.

13. Uri, N. Physio-chemical aspects of autoxidation. In: Lundberg, W. O., ed. Autoxidation and antioxidants. New York: Wiley-Interscience; 1961:55-106.

14. Negovskii, V. A. Resuscitation and artificial hypothermia. New York: Consultants Bureau; 1962 (translated from the Russian by Basil Haigh).

15. Sano, M.; Motchnik, P. A.; Tappel, A. L. Halogenated hydrocarbons and hydroperoxide-induced lipid peroxidation in rat tissue slices. J. Free Radical Biol. Med. 2:41-48; 1986.

16. Fraga, C. G.; Leibovitz, B. E.; Tappel, A. L. Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices: characterization and comparison with homogenates and microsomes. Free Radical Biol. Med. 4:55-161; 1988.

17. Salaris, S. C.; Babbs, C. F. A rapid, widely applicable screen for drugs that suppress free radical formation in ischemia/reperfusion. J. Pharmacological Methods 20:335-345; 1988.

18. Trowell, O. A. The culture of lymph nodes in vitro. Exp. Cell Res. 3:79-107; 1952.

19. Fridovich, I. Personal communication, September, 1988.

20. Saugstad, O. D.; Hallman, M.; Abraham, J. L.; Epstein, B.; Cochrane, C.; Gluck, L. Hypoxanthine and oxygen induced lung injury: a possible basic mechanism of tissue damage? Pediat. Res. 18:501-504; 1984.

21. Buege, J. A.; Aust, S. D. Microsomal lipid peroxidation. Methods in enzymology 51:302-310; 1978.

22. O'Haver, T. C. Potential clinical applications of derivative and wavelength modulation spectrometry. Clin. Chem. 25:1548-1553; 1979.

23. Cooper, B. E. Statistics for experimentalists. Oxford: Pergamon Press; 1969.

24. Anderson, V. L.; McLean, R. A. Design of experiments: a realistic approach. New York: Marcel Dekker; 1974.

25. Babbs, C. F.; Griffin, D. W. Scatchard analysis of methane sulfinic acid production from dimethyl sulfoxide: a method to quantify hydroxyl radical formation in physiologic systems. Free Radical Biol. Med. 6:493-503; 1989.

26. Semenov, N. N. Some problems of chemical kinetics and reactivity. Vol. 2. New York: Pergamon Press; 1959.

27. Denisov, E. T.; Mitskevich, N. 1.; Agabekov, V. E. Liquid phase oxidation of oxygen containing compounds. New York: Consultants Bureau; 1969.

28. Reich, L.; Stivala, S. S. Autoxidation of hydrocarbons and polylegins--kinetics and mechanisms. New York: Marcel Dekker; 1969.

29. Stevens, G. C.; Clarke, R. M.; Hart, E. J. Radiolysis of aqueous methane solutions. J. Phys. Chem. 76:3863-3867; 1972.

30. Hickel, B. Absorption spectra and kinetics of methyl and ethyl radicals in water. J. Phys. Chem. 79:1054-1059; 1975.

31. Frimer, A. A. Organic reactions involving the superoxide anion. In: Patai, S., ed. The Chemistry of functional groups peroxides. New York: John Wiley & Sons; 1983.

32. Swern, D. Primary products of olefinic autoxidations. In: Lundberg, W. O., ed. Autoxidation and antioxidants. Vol. I. New York: Wiley-lnterscience; 1961.

33. Slater, T. F. Free radical mechanisms in tissue injury. London: Pion Limited; 1972.

34. Slater, T. F. Overview of methods used for detecting lipid peroxidation. Methods in enzymology 105:283-299; 1984.

35. Bird, R. P.; Draper, H. H. Comparative studies on different methods of malonaldehyde determination. Methods in Enzymology 105:299-305; 1984.

36. Gutteridge, J. M. C.; Toeg, D. Iron-dependent free radical damage to DNA and deoxyribose. Separation of TBA-reactive intermediates. Int. J. Biochem. 14:891-893; 1982.

37. Akizuki, S.; Yoshida, S.; Chambers. D. E.; Eddy, L. J. Parmley, L. F.: Yellon, D. M.; Downey, J. M. Infarct size limitation by the xanthine oxidase inhibitor, allopurinol, in closed-chest dogs with small infarcts. Cardiovasc. Res. 19:686-692; 1985.

38. Dousset, J. C.; Trouilh, M.; Foglietti, M. J. Plasma malonaldehyde levels during myocardial infarction. Clinica Chimica Acta 129:319-322; 1983.

39. Gutteridge, J. M. C.; Richmond, R.; Halliwell, B. Inhibition of the iron-catalzyed formation of hydroxyl radicals from superoxide and lipid peroxidation by desferrioxamine. Biochem. J. 184:469-472; 1979.

40. Tien, M.; Svingen, B. A.; Aust, S. D. Initiation of lipid peroxidation by perferryl complexes. In: Oxygen and oxyradicals in chemistry and biology. New York: Academic Press; 1981:147- 152.

41. Bucher, J. R.; Tien, M.; Aust, S. D. The requirement for ferric in the initiation of lipid peroxidation by chelated ferrous iron. Biochem. Biophys. Res. Comm. 111:777-784; 1983.

42. Minotti, G.; Aust, S. D. The requirement for Iron(III) in the initiation of lipid peroxidation by Iron(II) and hydrogen peroxide. J. Biol. Chem. 262:1098-1104; 1987.

43. Kelner, M. J.; Alexander, N. M. Methylene blue directly oxidizes glutathione without the intermediate formation of hydrogen peroxide. J. Biol. Chem. 260:15168-15171; 1985.

44. Kelner, M. J., Bagnell, R.; Alexander, N. M. Competitive inhibition of paraquat induced superoxide production in flavin enzymes by methylene blue. Proceedings 4th International Congress on Oxygen Radicals, 1987:100-101.

45. McCord, J. M.; Fridovich, 1. The utility of superoxide dismutase in studying free radical reactions II. The mechanism of the mediation of cytochrome-c reduction by a variety of electron carriers. J. Biol. Chem. 245:1374-1377; 1970.

46. Thomas, C. E.; Morehouse. L. A.; Aust, S. D. Ferritin and superoxide-dependent lipid peroxidation. J. Biol. Chem. 260: 3275-3280; 1985.