Oxidation of myoglobin in isolated adult rat cardiac myocytes by 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid

Frederick P. Walters, Frances G. Kennedy and Dean P. Jones*

Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322, USA

Received 22 August 1983; revised version received 29 September 1983

The oxidation of intracellular myoglobin by 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid was studied in suspensions of isolated adult rat heart cells. Myoglobin was converted to a species identified as ferrylMb by its reaction with Na_2S to form ferrous sulfmyoglobin. This process was time-dependent and concentration-dependent in a manner consistent with direct accessibility of the exogenous peroxide to the cytosolic protein. The results indicate that myoglobin oxidation may be an early sign of oxidative injury and may limit myocardial function by elimination of this short-term O_2 reserve.

Myoglobin *Arachidonate* *Heart* *Prostaglandin* *Lipid peroxide* *Oxidation*

1. INTRODUCTION

15-Hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) is an arachidonate-derived lipid hydroperoxide which is formed by the action of lipoxygenase, an enzyme found in human platelets, leukocytes and other tissues [1–3]. Such peroxides have been suggested to be important in inflammatory processes [4] and are potent inhibitors of enzymes [5,6]. Thus, 15-HPETE and other arachidonate-derived lipid hydroperoxides are potential intermediates in pathological processes. This is of particular interest in the pathogenesis of cardiovascular disease since leukocyte infiltration of the myocardium can be profound in myocarditis [7] and platelet aggregation is common following stress [8] and myocardial infarction [9].

Myoglobin (Mb) is an O_2-binding hemoprotein found in very high concentrations in mammalian heart and thought to function as a short-term O_2 reserve [10] and possibly in facilitation of intracellular O_2 diffusion [11,12]. Loss of Mb has been shown to limit the functional capacity of the heart [13]. Because of the spectral characteristics of Mb, its concentration in isolated heart cells and perfused heart can be directly measured [12,14]. Myoglobin is readily oxidized by a variety of agents, including peroxides [12,14,15]. The purpose of this study was to determine whether the naturally occurring lipid hydroperoxide, 15-HPETE, oxidizes Mb in intact heart cells. The results demonstrate that this oxidation occurs and further suggest that this type of oxidation may reflect on early functional change of oxidative injury to myocytes.

2. MATERIALS AND METHODS

[5,6,8,9,11,12,14,15-^{3}H(N)]Arachidonic acid, spec. act. 60–100 Ci/mol, was obtained from New England Nuclear (Boston) and used to localize the product of the lipoxygenase reaction during isolation. Arachidonic acid, soybean lipoxygenase (type V), enzymes and other chemicals for cell preparation were purchased from Sigma (St. Louis). Silicic acid (325 mesh, chromatographic grade) was obtained from Fisher Scientific (Fair Lawn) and prepared as in [16]. Other chemicals were reagent grade and were purchased locally. Deionized water

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V.

was used throughout. 15-HPETE was biosynthesized from arachidonic acid and isolated by silicic acid chromatography [17] and quantitated by assay with thiobarbituric acid [18].

Cardiac myocytes were isolated from male rats (Kng:(SD)Br, King Animal Laboratories, 250–350 g, fed ad libitum) as in [12]. Cells were quantitated and viability determined in the presence of 0.2% trypan blue [12]. Spectral changes of Mb were typically measured by dual-wavelength scanning spectrophotometry (Aminco DW2a, Travenol Laboratories, Chicago), in the 570–650 nm range with 630 nm as a reference wavelength.

3. RESULTS AND DISCUSSION

Addition of 15 μM 15-HPETE resulted in the loss of the 585 nm absorbance maximum of MbO_2 (fig. 1) in less than 5 min. This loss was not due to deoxygenation since the α-band of cytochrome *a* (605 nm) was not present under these conditions, and addition of O_2-saturated buffer did not cause recovery of the 585 nm band. Spectra of deoxygenated and KCN-treated cells are available for comparison [19]. Because other peroxides oxidize Mb (or MbO_2) to either metMb or ferrylMb, we felt that it was likely that the MbO_2 was being converted to one of these forms. FerrylMb has previously been identified in ethyl hydrogen peroxide-perfused rat heart by addition of Na_2S to produce sulfMb [14,20]. The sulfide adds to the periphery of the porphyrin ring to produce a chlorin structure which has a characteristic intense absorption maximum at 616 nm for the deoxy ferrous form. The maximum shifts to 624 nm in the oxygenated form which is formed at several atmospheres O_2 [21, 22]. Metsulfmyoglobin has an absorption maximum at 595 nm, but has a much lower extinction value [22].

Addition of 1 mM Na_2S to 15-HPETE-treated heart cells produced an absorption maximum at 617 nm as expected for the formation of ferrous sulfMb (fig. 1). This spectrum was the same as that obtained by addition of Na_2S to ethyl hydrogen peroxide-treated cells (not shown) and is therefore indicative of the presence of ferrylMb in the 15-HPETE-treated cells. Addition of Na_2S to untreated oxygenated or deoxygenated cells did not result in the formation of the 617 nm maximum. Furthermore, addition of Na_2S to cells in which

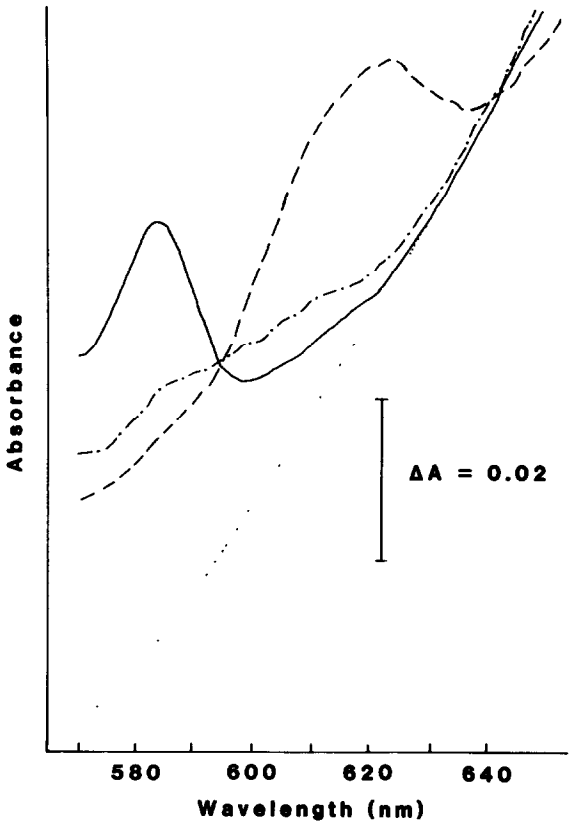

Fig. 1. Oxidation of Mb in isolated cardiac myocytes by 15-HPETE. Spectra were obtained in the dual-wavelength scanning mode of an Aminco DW2a spectrophotometer with 630 nm as the reference wavelength. A spectrum in the 570–650 nm range shows MbO_2 (——) with a maximum absorbance at 585 nm. Addition of 15-HPETE (-.-) produced essentially complete loss of MbO_2 within 5 min. The product of this reaction was predominantly ferrylMb, identified by its conversion to ferrous sulfMb which occurred when 1 mM Na_2S (– – –) was added. Maximum absorbance of ferrous sulfMb occurs at about 617 nm. The light path was 1 cm, sample temperature 37°C, and cell concentration 4×10^5 cells/ml in a Hepes-supplemented modified Krebs-Henseleit buffer (pH 7.4). Data are from one experiment which is representative of studies on 5 cell preparations. The baseline obtained with water under these conditions was characteristically skewed. However, because the turbidity of the cells contributes to the measured ΔA, direct subtraction of this baseline is not valid for correction of the spectra.

Mb was converted to metB by minimal treatment with H_2O_2 [12] did not result in formation of this maximum, but rather caused a minor increase in

absorbance in the 600 nm region. Addition of 1 mM KCN to H_2O_2-treated, anaerobic cells resulted in an increased absorbance in the region of 545 nm, consistent with the formation of cyanometMb. The same addition to 15-HPETE-treated cells had very little effect on the spectrum in this region and was consistent with the identification of the product of the reaction with 15-HPETE as ferrylMb. Using the change in extinction coefficient on forming ferrous sulfMb ($\Delta\epsilon$ = 10.5 $mM^{-1} \cdot cm^{-1}$ for the wavelength pair 617 minus 600 nm calculated from [21]), the amount of ferrous sulfMb formed was 4.9–6.8 nmol/10^6 cells. Since these cells contain about 8 nmol Mb/10^6 cells as isolated, most of the Mb appears to be oxidized by 15-HPETE to the 'ferryl' form.

The initial rate of Mb oxidation by 15-HPETE was linear within the sensitivity of the assay (fig. 2). Free-radical-initiated lipid peroxidation in liver cells typically has a lag phase of several minutes [23]. The absence of a lag phase indicates that Mb is directly oxidized by 15-HPETE. Moreover, the nearly quantitative conversion of Mb to ferrylMb further suggests a direct oxidation since paraquat-initiated injury gives a mixture of oxidation products of Mb in heart cells (unpublished).

Studies of the concentration dependence of Mb oxidation by 15-HPETE showed that an apparent 'threshold' concentration was required for Mb oxidation (fig. 3). This threshold was typically 2–4

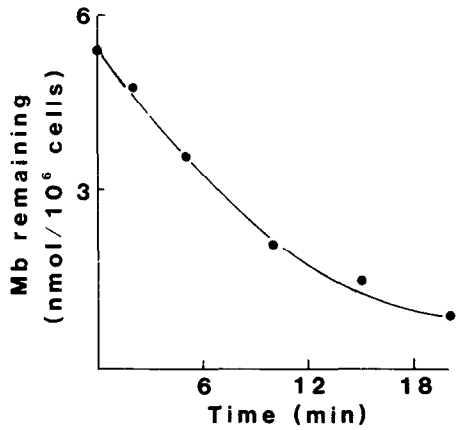

Fig. 2. Time course of Mb oxidation with 15-HPETE. Cells were incubated as described in fig. 1 with 4 μM 15-HPETE and Mb content measured from the $\Delta A_{585-630}$. Data are from one experiment which is representative of 5 cell preparations.

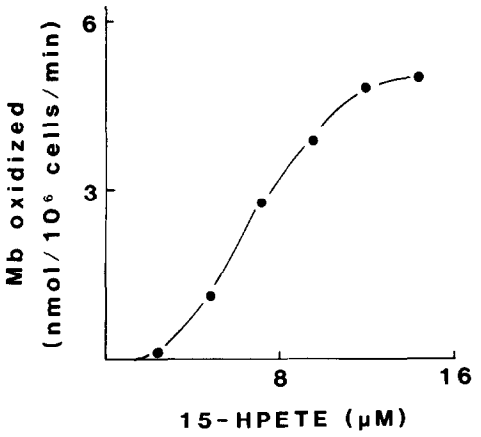

Fig. 3. Concentration dependence of 15-HPETE-dependent oxidation of Mb in isolated cardiac myocytes. Initial rates of loss of MbO_2 were measured with varying concentrations of 15-HPETE. Cells were incubated as described in fig. 1 and Mb loss measured as the $\Delta A_{585-630}$. Data are from one experiment which is representative of 4 cell preparations.

μM 15-HPETE and indicated that either 15-HPETE was not accessible to Mb at lower concentrations or ferrylMb was reduced back to functional Mb at a rate comparable to the rate of oxidation. Reduction of lipid hydroperoxides by glutathione peroxidase has been studied extensively. Since a high glutathione peroxidase activity occurs in heart [24], the reduction of 15-HPETE by this enzyme may be largely responsible for the measured concentration dependence. Further studies on the activity of the glutathione peroxidase system as well as on the reactivity of isolated myoglobin (or oxymyoglobin) and 15-HPETE will be necessary to explain this threshold effect.

Because the heart is known to be sensitive to injury by peroxides [14,25], we examined the effect of 15-HPETE on functional morphology and cell viability. Loss of the typical rod shape and of ability to exclude trypan blue were both time- and concetration-dependent with 15-HPETE (not shown). The oxidation of Mb preceded these changes, and loss of rod shape preceded loss of the ability to exclude trypan blue (fig. 4). Thus, the temporal sequence of changes, Mb oxidation, loss of functional morphology and finally loss of membranal integrity, indicates that Mb oxidation may be an early indicator of oxidative (peroxidative) injury in heart cells.

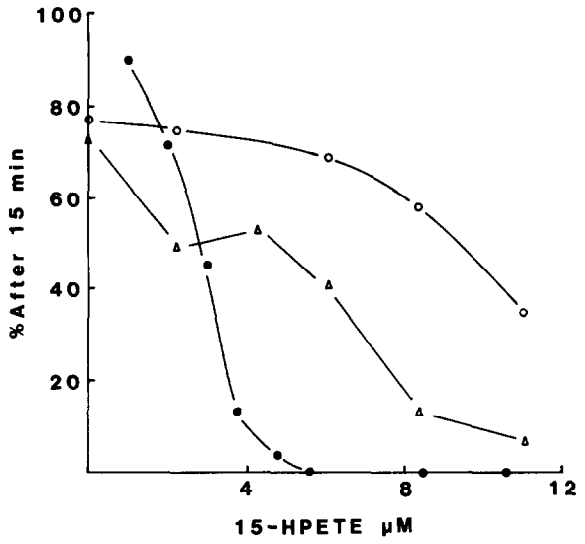

Fig. 4. Comparison of sensitivity of Mb oxidation by 15-HPETE with change in morphology and loss in viability of cardiac myocytes. Loss of MbO_2 (●) as shown by this plot of remaining Mb preceded loss in viability (○) of myocytes. Cells lost the normal rod shape (△) before cell death occurred which suggests damage to structural elements before membranal damage. Cell concentration was 4×10^5 cells/ml, incubated for 15 min at 37°C in a Hepes-supplemented modified Krebs-Henseleit buffer (pH 7.4). Data are from two experiments matched for initial viability and are representative of experiments from 7 cell preparations.

Many previous studies of peroxidative injury have focussed on the derangement of membrane lipids which occurs as a result of a free-radical process [26]. Howeve, major changes in membrane permeability do not occur until substantial destruction of membrane lipids occurs and, therefore, the site of lethal injury may be specific proteins with critical functional groups which are particularly sensitive to peroxides or other reactive species generated during peroxidative processes [27]. In liver microsomes, we found that Ca^{2+} sequestration is very sensitive to oxidative injury and that this appears to be due to the susceptibility of the Ca^{2+} ATPase [28]. Contractile systems in heart are sensitive to thiol reagents [29] and, therefore, are likely to be sensitive to oxidation. Mb oxidation may be a useful in situ indicator of such a process.

The accessibility of exogenous peroxy fatty acids to the cytosolic fraction indicates that critical oxidative lesions are not limited to membranal systems and can occur in subcellular compartments distinct from the site of peroxide generation. This is of particular interest with regard to the peroxide intermediates in the synthesis of prostaglandin and prostaglandin-like molecules. Although tissue concentrations of these compounds are typically very low, pathologic alterations in their production could result in regions of relatively high concentrations. Production of peroxy fatty acids by neutrophils or platelets could thereby damage adjacent cells.

Because of the presence of systems to prevent study injury (e.g. glutathione peroxidase [24]) and peroxide inhibition of cyclooxygenase [30] additional studies are required to determine the quantitative role of peroxy fatty acids in pathologic processes. The current studies establish that exogenous peroxy fatty acids are accessible to the cytosolic region of myocytes and indicate that oxidation of Mb may be an early functional change during oxidative stress.

ACKNOWLEDGEMENTS

This research was supported by NIH grants GM 28176 and HL 30286.

REFERENCES

[1] Vliegenthart, J.F.G. and Veldink, G.A. (1982) in: Free Radicals in Biology, vol. 5 (Pryor, W.A. ed) pp. 29–64, Academic Press, New York.
[2] Herman, A.G. (1982) in: Cardiovascular Pharmacology of the Prostaglandins (Herman, A.G. et al., eds) pp. 1–5, Raven Press, New York.
[3] Borgeat, P. and Samuelsson, B. (1979) Proc. Natl. Acad. Sci. USA 76, 2448–2452.
[4] Kuehl, F.A., Himes, J.L., Egan, R.W., Ham, E.A., Beverage, G.C. and Van Arman, C.G. (1977) Nature 265, 170–173.
[5] Moncada, S., Gryglewski, R.J., Bunting, S. and Vane, J.R. (1976) Prostaglandins 12, 715–733.
[6] Willis, R.J. (1979) Fed. Proc. 39, 3134–3137.
[7] Wenger, N.K., Abelmann, W.H. and Roberts, W.C. (1982) in: The Heart (Hurst, J.W. et al. eds) pp. 1278–1299, McGraw-Hill, New York.
[8] Haft, J.I. and Fani, K. (1973) Circulation 47, 353–358.

[9] Mustard, J.F. and Packham, M.A. (1969) Circulation (Suppl. 40), 17–20.
[10] Millikan, G.A. (1939) Physiol. Rev. 19, 503–523.
[11] Wittenberg, J.B. and Wittenberg, B.A. (1981) in: Oxygen and Living Processes (Gilbert, D.L. ed) pp. 177–199, Springer-Verlag, New York.
[12] Jones, D.P. and Kennedy, F.G. (1982) Biochem. Biophys. Res. Commun. 105, 419–424.
[13] Cole, R.P. (1982) Science 216, 523–525.
[14] Tamura, M., Oshino, N., Chance, B. and Silver, I.A. (1978) Arch. Biochem. Biophys. 191, 8–22.
[15] Wittenberg, B.A., Wittenberg, J.B. and Caldwell, P.R.B. (1975) J. Biol. Chem. 250, 9038–9043.
[16] Hirsch, J. and Ahrens, E.H. (1958) J. Biol. Chem. 233, 311–320.
[17] Zenser, T.V., Mattammal, M.B. and Davis, B.B. (1980) J. Pharmacol. Exp. Ther. 214, 312–317.
[18] Ohkawa, H., Ohishi, W. and Yagi, K. (1978) J. Lipid Res. 19, 1053–1057.
[19] Wittenberg, B.A. (1979) in: Biochemical and Clinical Aspects of Oxygen (Caughey, W.S. ed) pp. 35–50, Academic Press, New York.
[20] Nicholls, P. (1961) Biochem. J. 81, 374–383.
[21] Berzofsky, J.A., Peisach, J. and Blumberg, W.E. (1971) J. Biol. Chem. 246, 3367–3377.
[22] Berzofsky, J.A., Peisach, J. and Blumberg, W.E. (1971) J. Biol. Chem. 246, 7366–7372.
[23] Smith, M.T., Thor, H., Hartzell, P. and Orrenius, S. (1982) Biochem. Pharmacol. 31, 19–26.
[24] Lawrence, R.A. and Burk, R.F. (1978) J. Nutr. 108, 211–215.
[25] Shattock, M.J., Manning, A.S. and Hearse, D.J. (1982) Pharmacology 24, 118–122.
[26] Recknagel, R.O., Glende, E.A. and Hruszkewycs, A.M. in: Free Radicals in Biology (Pryor, W.A. ed.) pp. 97–132, Academic Press, New York.
[27] Hruszkewycz, A.M., Glende, E.A. and Recknagel, R.O. (1978) Tox. Appl. Pharmacol. 46, 695–702.
[28] Jones, D.P., Thor, H., Smith, M.T., Jewell, S.A. and Orrenius, S. (1983) J. Biol. Chem., 258, 6390–6393.
[29] Srivastava, S. and Wikman-Coffett, J. (1982) Eur. J. Biochem. 121, 457–461.
[30] Cook, H.W. and Lands, W.E.M. (1976) Nature 260, 630–632.