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t-Butyl hydroperoxide alters fatty acid incorporation into erythrocyte membrane phospholipid

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Because the ability of cells to replace oxidized fatty acids in membrane phospholipids via deacylation and reacylation in situ may be an important determinant of the ability of cells to tolerate oxidative stress, incorporation of exogenous fatty acid into phospholipid by human erythrocytes has been examined following exposure of the cells to t-butyl hydroperoxide. Exposure of human erythrocytes to t-butyl hydroperoxide (0.5–1.0 mM) results in oxidation of glutathione, formation of malonyldialdehyde, and oxidation of hemoglobin to methemoglobin. Under these conditions, incorporation of exogenous [9,10-^{3}H]oleic acid into phosphatidylethanolamine is enhanced while incorporation of [9,10-^{3}H]oleic acid into phosphatidylcholine is decreased. These effects of t-butyl hydroperoxide on [9,10-^{3}H]oleic acid incorporation are not affected by dissipating transmembrane gradients for calcium and potassium. When malonyldialdehyde production is inhibited by addition of ascorbic acid, t-butyl hydroperoxide still decreases [9,10-^{3}H]oleic acid incorporation into phosphatidylcholine but no stimulation of [9,10-^{3}H]oleic acid incorporation into phosphatidylethanolamine occurs. In cells pre-treated with $NaNO_2$ to convert hemoglobin to methemoglobin, t-butyl hydroperoxide reduces [9,10-^{3}H]oleic acid incorporation into phosphatidylcholine by erythrocytes but does not stimulate [9,10-^{3}H]oleic acid incorporation into phosphatidylethanolamine. Under these conditions oxidation of erythrocyte glutathione and formation of malonyldialdehyde still occur. These results indicate that membrane phospholipid fatty acid turnover is altered under conditions where peroxidation of membrane phospholipid fatty acids occurs and suggest that the oxidation state of hemoglobin influences this response.

Introduction

Peroxidation of the polyunsaturated fatty acids of membrane phospholipids alters membrane physical properties and disrupts the function of membrane proteins by (i) causing the release of acyl chain fragments such as malonyldialdehyde which can react with and crosslink amino groups in membrane phospholipids and proteins [1–6], and (ii) generating phospholipids containing carbonyl, epoxy, peroxy- and hydroxyl fatty acids [7,8] These altered fatty acids differ in physical properties from native fatty acids and could disrupt lipid-lipid and lipid-protein interactions in the cell membrane Initiation of these free radical-mediated processes occurs under both physiological conditions and during exposure of cells to elevated p_{O_2}, nitrogen oxides, aromatic xenobiotics and radiation [1,2] Free radicals are also involved in cellular injury occurring as a

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consequence of inflammation and ischemia [1,9]

The ability of cells to remove oxidized fatty acids from membrane phospholipids and to restore normal phospholipid fatty acid composition via deacylation and reacylation in situ may be one determinant of the ability of cells to tolerate oxidative stress The role of phospholipid fatty acid turnover in situ in the response of cells to oxidative stress has not been thoroughly studied Alteration in the turnover of membrane phospholipid fatty acids may be one of earliest changes in cellular function occurring after oxidant exposure and precede significant accumulation of lipid peroxides [10] Recent studies in isolated membrane systems have suggested that phospholipases have greater activity toward phospholipids containing peroxidized fatty acids than intact fatty acids and that removal of peroxidized fatty acids from phospholipids by phospholipase A_2 precedes reduction of these compounds by glutathione peroxidase [11,12]

In this study the human erythrocyte has been employed as a model system to assess the effects of oxidative stress induced by treatment with *t*-butyl hydroperoxide (*t*BuOOH) on the pathway for in situ phospholipid fatty acid turnover Use of the erythrocyte as a model system is physiologically relevant because the erythrocyte is normally exposed to high intracellular and extracellular pO_2 in vivo The erythrocyte is also directly exposed to ingested drugs, inhaled gases and activated neutrophils which are capable of generating free radicals in the circulation [2,9] Because the erythrocyte lacks the enzymes for synthesis of phospholipid and fatty acid de novo, renewal of membrane phospholipid fatty acids is limited to the pathway for deacylation and reacylation in situ utilizing exogenous fatty acid [13–16]

Exposure of human erythrocytes to *t*BuOOH has been shown to cause oxidation of hemoglobin (Hb) and peroxidation of membrane lipid by a heme-catalyzed free radical-mediated process involving *t*-butoxy radicals [17–21] Formation of these oxidation products has been shown to result in increased crosslinking of membrane proteins and reduced membrane deformability [21,22] In this study treatment of human erythrocytes with *t*BuOOH has been shown to stimulate incorporation of exogenous fatty acids into phosphatidylethanolamine (PE) and to inhibit incorporation of exogenous fatty acids into phosphatidylcholine (PC) The relationship between these alterations in fatty acid incorporation, *t*BuOOH-induced peroxidation of membrane lipid and the oxidation state of Hb are examined In addition, since alterations in cellular calcium and potassium homeostasis have been implicated in the pathogenesis of cell injury following lipid peroxidation in erythrocytes and other systems [23–26] and have been shown to influence erythrocyte phospholipid fatty acid turnover directly [27–29], the effects of altered transmembrane gradients for both potassium and calcium on the activity of the acylation-deacylation pathway following *t*BuOOH exposure have been examined

Experimental procedures

Erythrocyte preparation Heparinized venous blood was obtained from normal human donors Erythrocytes were separated from plasma and leukocytes by centrifugation at $800 \times g$ for 7 min at room temperature and washed four times with five volumes of a standard buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM NaH_2PO_4, 5 mM glucose, 10 mM Tris (pH 7 4) Methemoglobin (MetHb) containing erythrocytes were prepared as described [18–20] by suspending washed erythrocytes (20% v/v) in a solution composed of equal volumes of 29 mM $NaNO_2$ and standard buffer for 10 min at room temperature The cells were then washed five times with 10 volumes of standard buffer and resuspended (5% v/v) in that buffer

Incorporation of [9,10-^{3}H]oleic acid by erythrocytes Erythrocytes (5% v/v) were incubated at 37°C under air with [9,10-^{3}H]oleic acid (10 μM, 1 Ci/mol) (Amersham) complexed to fatty acid-free bovine serum albumin (1 65 mg/ml) (Sigma) [16] in the presence or absence of *t*BuOOH (Aldrich) After incubation duplicate aliquots of the cell suspensions were washed three times with five volumes of buffer containing 1% bovine serum albumin and lysed by treating the cell pellet with an equal volume of water Lipids were then immediately extracted three times with five volumes of methanol and chloroform as described [16]

Following removal of aliquots for determination of lipid phosphorus and total radioactivity [16], extracts were concentrated under nitrogen and applied to a thin layer of Silica H prepared with 1 mM Na_2CO_3 Thin-layer plates were developed first in $CH_3COOC_2H_5/CH_3COOH$ (98 2, v/v) to separate neutral lipids and phospholipids, dried in air and then developed in $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (50 20 5 2, v/v) to separate individual phospholipid classes Lipids were visualized by brief exposure to I_2 vapor and identified by co-chromatography with standards Radioactivity was determined by liquid scintillation counting after scraping gel fractions into scintillation vials and adding scintillation fluid consisting of toluene/ethylene glycol monomethyl ether (5 3, v/v) with PPO (5 mg/l) and POPOP (62 5 mg/l) [16] All measurements were corrected for quenching using an external standard (Beckman LS2800) Calculations were based on the specific activity of exogenous fatty acid Isolation of [9,10-^{3}H]oleic acid incorporated into phospholipids in the presence or absence of *t*BuOOH by acidic methanolysis and gas chromatography [30] showed that its radiopurity was unaltered *t*BuOOH did not affect the recovery of phospholipid from erythrocytes

Malonyldialdehyde release from erythrocytes Lipid peroxidation was assessed by measuring the appearance of malonyldialdehyde in erythrocyte suspensions incubated with unlabeled oleic acid complexed to bovine serum albumin Aliquots of erythrocyte suspension (5% v/v) were treated with one-half volume of 28% w/v trichloroacetic acid/0 1 M sodium arsenite to precipitate protein One volume of 0 1 M thiobarbituric acid in 1 M NaOH was added to four volumes of the acid supernatant and the mixture heated in a boiling water bath for 15 min Samples were then cooled rapidly in an ice bath and absorbance measured at 532 nm with correction for a reagent blank [31]

Reduced glutathione content of erythrocytes Reduced glutathione (GSH) content of erythrocytes was determined by measuring acid-soluble sulfhydryl groups in the same trichloroacetic acid supernatant used for malonyldialdehyde determination Sulfhydryl content was determined by adding one volume of acid supernatant to two volumes 1 M Tris (pH 8 0), adding 0 2 volume of 0 01 M 5,5'-dithiobis(2-nitrobenzoic acid) (pH 7 0) and measuring absorbance at 412 nm with correction for a reagent blank [32]

Hb content of erythrocyte suspensions Total Hb content of erythrocyte suspensions was measured by the cyanomethemoglobin method of Drabkin and Austin [33]

Results

GSH following tBuOOH

Exposure of human erythrocytes to tBuOOH (0 1–1 mM) in the presence of glucose induces a transient depletion of GSH as *t*BuOOH is reduced via glutathione peroxidase to *t*-butyl alcohol (*t*BuOH) (Fig 1) The duration of the depletion and the rate of restoration of GSH are dependent on the concentration of *t*BuOOH These results are similar to those obtained previously with human and mouse erythrocytes using slightly different incubation conditions [19,34,35]

Lipid peroxidation following tBuOOH

Treatment of human and mouse erythrocytes with *t*BuOOH also results in the production of malonyldialdehyde derived primarily from per-

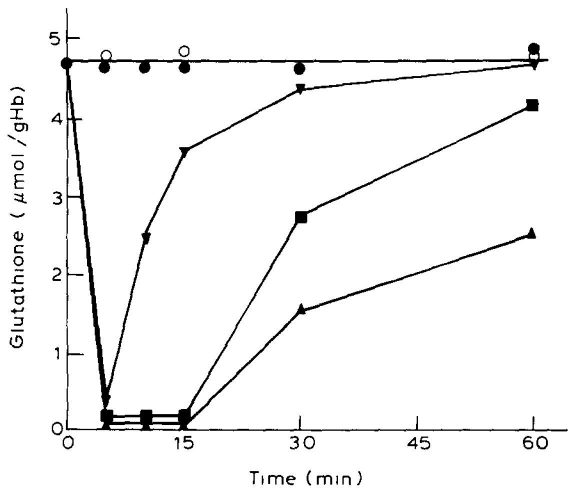

Fig 1 GSH content of human erythrocyte suspensions following addition of *t*BuOOH Erythrocytes (5% v/v) in standard buffer containing oleic acid (10 μM) complexed to fatty acid free bovine serum albumin (1 65 mg/ml) were treated with 0 (●), 0 1 (▼), 0 5 (■), or 1 0 mM (▲) *t*BuOOH or *t*BuOH (1 0 mM) (0) at $t = 0$ and then incubated at 37°C for the times indicated GSH was measured in duplicate aliquots of each suspension as described in Experimental Procedures

oxidation of membrane phospholipid fatty acids [17–19,35] Measurement of the release of free malonyldialdehyde does not quantitatively reflect total lipid peroxidation because the amount formed is a function of the polyunsaturated fatty acid composition of the membrane [36] and because some of the malonyldialdehyde formed reacts with other membrane components and is not released [1,2] However, when comparisons of the amount of lipid peroxidation induced by an oxidant such as *t*BuOOH are made within the same membrane system, as in this study, measurement of malonyldialdehyde relase is a simple and widely used method to assess the degree of lipid peroxidation [17–21,31] Under the conditions employed in this study, treatment of erythrocytes with *t*BuOOH (0 5–1 0 mM) results in release of malonyldialdehyde and depletion of GSH The amount of malonyldialdehyde produced increases with the extent and duration of GSH depletion, suggesting that sustained depletion of GSH must occur before significant peroxidation of membrane lipid occurs (Fig 2) [19] Incubation with 0 1 mM *t*BuOOH, which is rapidly reduced by glutathione peroxidase in the presence of glucose, does not result in detectable release of malonyldialdehyde

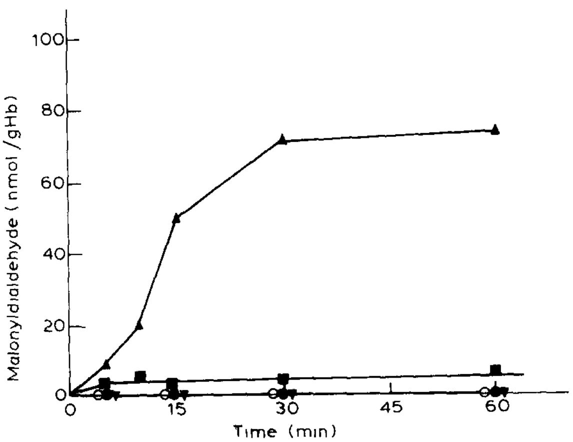

Fig 2 Formation of Malonyldialdehyde by human erythrocyte suspensions following addition of *t*BuOOH Erythrocytes (5% v/v) were incubated with *t*BuOOH or *t*BuOH as in Fig 1 Malonyldialdehyde production was measured in duplicate aliquots of each suspension as described in Experimental Procedures Symbols are as in Fig 1

Effect of tBuOOH on [9,10-^{3}H]oleic acid incorporation into phospholipid

Since the erythrocyte lacks the ability to synthesize fatty acid or phospholipid de novo, repair of oxidant injury to membrane phospholipid fatty acids in erythrocytes is limited to replacement of oxidized fatty acids via deacylation and reacylation in situ using exogenous fatty acids [13–16] In this study oleic acid has been employed as the exogenous fatty acid substrate because it is less susceptible to peroxidaton than polyunsaturated fatty acids and is incorporated primarily into the 2-position of phospholipids, the site at which polyunsaturated fatty acids are esterified [14] Thus, changes in the incorporation of exogenous oleic acid into phospholipid could reflect changes in turnover occurring primarily at the 2-position of membrane phospholipids following oxidation of the polyunsaturated fatty acids esterified there

Exposure of human erythrocytes to *t*BuOOH increases the rate of incorporation of [9,10-^{3}H]oleic acid into PE (Fig 3) This change in the rate of [9,10-^{3}H]oleic acid incorporation begins following a brief lag period similar to that for malonyldialdehyde production The rate of [9,10-^{3}H]oleic acid incorporation into PE remains elevated even though the concentration of *t*BuOOH declines as it is reduced by GSH [18] The stimulation of [9,10-^{3}H]oleic acid incorporation into PE and malonyldialdehyde production show a parallel dependence on initial *t*BuOOH concentration (Fig 4) Thus, no stimulation of [9,10-^{3}H]oleic acid incorporation into PE is observed following exposure to 0 1 mM *t*BuOOH while progressively greater enhancement of incorporation occurs with 0 5 –1 0 mM *t*BuOOH Release of malonyldialdehyde shows a similar dependence on *t*BuOOH concentration (Fig 4), but no direct stoichiometric relationship between malonyldialdehyde production and [9,10-^{3}H]oleic acid incorporation is observed The ratio of malonyldialdehyde released to [9,10-^{3}H]oleic acid incorporated is approx 40 1 Thus, malonyldialdehyde production greatly exceeds the amount by which [9 10-^{3}H]oleic acid incorporation is increased following *t*BuOOH In contrast to the effect of *t*BuOOH on [9,10-^{3}H]oleic acid incorporation into PE, incorporation of [9,10-^{3}H]oleic acid into PC decreases following treatment with *t*BuOOH (Fig

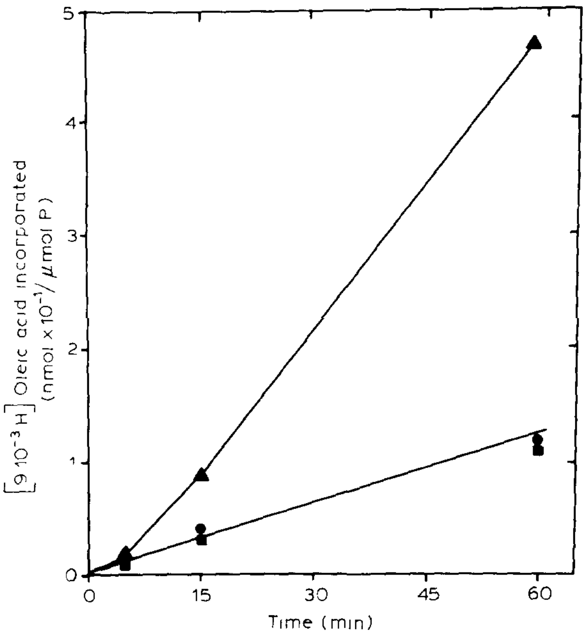

Fig 3 Incorporation of [9,10-^{3}H]oleic acid into PE by human erythrocytes following addition of *t*BuOOH Erythrocytes (5% v/v) in standard buffer containing [9,10-^{3}H]oleic acid (10 μM) complexed to fatty acid-free bovine serum albumin (1 65 mg/ml) were incubated in the presence (▲) or absence (●) of *t*BuOOH (1 0 mM) or *t*BuOH (1 0 mM) (■) At the times indicated, cells were isolated from duplicate aliquots of each suspension, washed with 1% bovine serum albumin in buffer, extracted with $CHCl_3$ and CH_3OH and phospholipids separated by thin-layer chromatography as described in Experimental Procedures

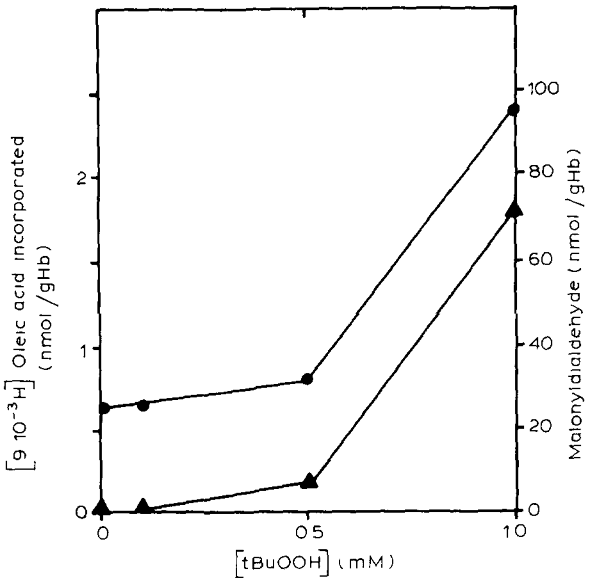

Fig 4 The effect of [*t*BuOOH] on incorporation of [9,10-^{3}H]oleic acid into PE and malonyldialdehyde production by human erythrocytes Erythrocytes (5% v/v) in standard buffer containing [9,10-^{3}H]oleic acid complexed to bovine serum albumin as in Fig 3 were incubated with varying concentrations of *t*BuOOH for 30 min at 37°C Malonyldialdehyde production (▲) and [9,10-^{3}H]oleic acid incorporation into PE (●) were determined as described in Experimental Procedures

5) with inhibition becoming greater as the initial concentration of *t*BuOOH is increased (Table I) *t*BuOOH exposure has similar effects on [9,10-^{3}H]palmitic acid and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid incorporation into PE and PC Thus, *t*BuOOH (1 mM) increases [9,10-^{3}H]palmitic acid incorporation into PE from 0 14 to 1 09 nmol/μmol P per h while incorporation into PC decreases from 0 32 to 0 26 nmol/μmol P per h [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid incorporation into PE is increased from 0 020 to 0 026 nmol/μmol P per h by *t*BuOOH (1 mM) while incorporation into PC decreases from 0 034 to 0 014 nmol/μmol P per h (means of duplicate experiments) Exposure of erythrocytes to *t*BuOH (1 mM), which accumulates in the cells and medium as *t*BuOOH is reduced by glutathione peroxidase [17], did not alter the incorporation of [9,10-^{3}H]oleic acid into phospholipid by erythrocytes nor lead to production of malonyldialdehyde or a decrease in GSH (Figs 1–3, 5)

Role of lipid peroxidation in tBuOOH-induced alterations in [9,10-^{3}H]oleic acid incorporation

The relationship of the *t*BuOOH-induced alterations in [9,10-^{3}H]oleic acid incorporation into phospholipid to the peroxidation of membrane lipid was assessed by examining the effect of *t*BuOOH on [9,10-^{3}H]oleic acid incorporation under conditions where lipid peroxidation is inhibited Ascorbic acid has been shown to prevent *t*BuOOH-induced lipid peroxidation in erythrocytes without preventing *t*BuOOH-induced oxidation of Hb to MetHb [18] Addition of ascorbic acid (0 5 mM) prevents both the *t*BuOOH-induced formation of malonyldialdehyde and the selective stimulation of [9,10-^{3}H]oleic acid incorporation into PE without preventing the *t*BuOOH-induced decrease in GSH or [9,10-^{3}H]oleic acid incorporation into PC (Table II)

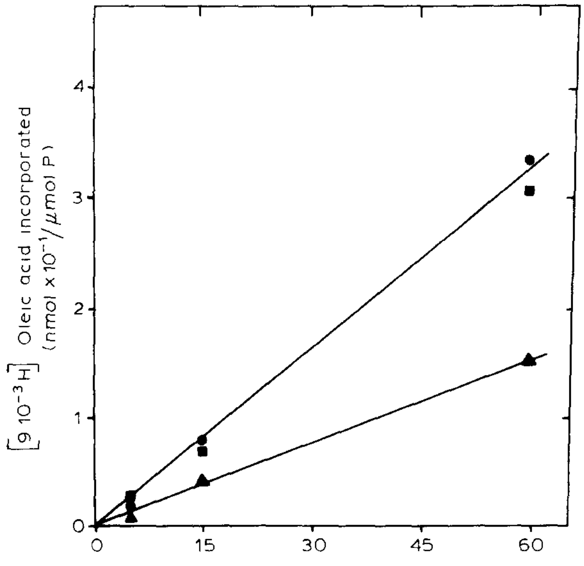

Fig 5 Incorporation of [9 10-^{3}H]oleic acid into PC by human erythrocytes following addition of *t*BuOOH Erythrocytes were incubated with *t*BuOOH or *t*BuOH and phospholipids extracted and separated as described in Fig 3 Symbols are as in Fig 3

Addition of ascorbic acid (0 5 mM) alone does not cause release of malonyldialdehyde nor alter the rate of incorporation of [9,10-^{3}H]oleic acid into phospholipid

Effects of Hb oxidation on the tBuOOH-induced alterations in [9,10-^{3}H]oleic acid incorporation

The effect of alterating the oxidation state of Hb on the *t*BuOOH-induced perturbations of phospholipid fatty acid turnover was assessed by examining the effects of *t*BuOOH on erythrocytes pretreated with $NaNO_2$ to convert Hb to MetHb [18] Treatment of MetHb containing erythrocytes with *t*BuOOH results in release of malonyldialdehyde and a decrease in GSH similar to that observed in Hb containing cells (Table III) However, no stimulation of [9,10-^{3}H]oleic acid incorporation into PE occurs in MetHb containing cells following *t*BuOOH exposure Incorporation of [9,10-^{3}H]oleic acid into PC, which is decreased by *t*BuOOH in Hb containing cells, is decreased even further in MetHb containing cells following exposure to *t*BuOOH

Effect of altered transmembrane gradients for calcium and potassium on tBuOOH-induced alterations in [9,10-^{3}H]oleic acid incorporation

Changes in erythrocyte calcium and potassium permeability induced by ionophores are associated with a selective stimulation of fatty acid incorporation into PE [27–29] Because changes in both calcium and potassium permeability and the func-

TABLE I

THE EFFECT OF [*t*BuOOH] ON INCORPORATION OF [9 10-^{3}H]OLEIC ACID INTO PHOSPHATIDYLCHOLINE (PC) BY HUMAN ERYTHROCYTES

Erythrocytes were incubated as in Fig 4 with [9 10-^{3}H]oleic acid and varying concentrations of *t*BuOOH for 30 min at 37°C Phospholipids were extracted and separated as in Fig 4

[*t*BuOOH] (mM)	PC (nmol/µmol P)
0	0 39
0 1	0 39
0 5	0 36
1 0	0 24

TABLE II

THE EFFECT OF ASCORBIC ACID ON MALONYLDIALDEHYDE PRODUCTION, GSH CONTENT AND INCORPORATION OF [9,10-^{3}H]OLEIC ACID INTO PHOSPHATIDYLETHANOLAMINE (PE) AND PHOSPHATIDYLCHOLINE (PC) FOLLOWING ADDITION OF *t*BuOOH

Erythrocytes suspended in standard buffer (5% v/v) in the presence and absence of ascorbic acid (0 5 mM) were incubated with [9,10-^{3}H]oleic acid complexed to bovine serum albumin as in Fig 3 in the presence and absence of *t*BuOOH (1 mM) Following incubation for 15 min at 37°C, duplicate aliquots were removed from each suspension for determination of malonyldialdehyde production, GSH content and incorporation of [9 10-^{3}H]oleic acid into PE and PC as described in Experimental Procedures

Addition	GSH (µmol/g Hb)	Malonyldialdehyde (nmol/g Hb)	PE (nmol / µmol P)	PC (nmol/ µmol P)
None	4 4	2 5	0 035	0 073
Ascorbate	3 8	4 0	0 038	0 069
*t*BuOOH	0 4	71 0	0 087	0 022
*t*BuOOH + ascorbate	0 4	15 0	0 039	0 029

TABLE III

THE EFFECT OF *t*BuOOH ON MALONYLDIALDEHYDE PRODUCTION, GSH CONTENT AND INCORPORATION OF [9,10-^{3}H]OLEIC ACID INTO PHOSPHATIDYLETHANOLAMINE (PE) AND PHOSPHATIDYLCHOLINE (PC) IN MetHb-ERYTHROCYTES

Erythrocytes were pretreated with $NaNO_2$ as described in Experimental Procedures to convert Hb to metHb MetHb and Hb containing erythrocytes (5%v/v) were then incubated at 37°C in standard buffer containing [9 10-^{3}H]oleic acid- bovine serum albumin as in Fig 3 and treated with *t*BuOOH(1 0 mM) After 15 min duplicate aliquots of each suspension were removed for measurement of malonyldialdehyde production, glutathione content and incorporation of [9,10-^{3}H]oleic acid into PE and PC as described in Experimental Procedures

Pretreatment	Addition	GSH (μmol/g Hb)	Malonyldialdehyde (nmol/g Hb)	PE (nmol/ μmol P)	PC (nmol/ μmol P)
None	None	5 1	0	0 035	0 085
$NaNO_2$	None	5 2	5 3	0 032	0 072
None	*t*BuOOH	0 5	67 5	0 106	0 035
$NaNO_2$	*t*BuOOH	0 8	70 0	0 029	0 010

tion of the transport mechanisms for these ions occur following exposure of erythrocytes to oxidants [23,24], it is possible that the effect of *t*BuOOH on [9,10-^{3}H]oleic acid incorporation into PE is a consequence of a *t*BuOOH-induced alteration in cellular calcium or potassium content To address this question the effects of *t*BuOOH on lipid peroxidation and [9,10-^{3}H]oleic acid incorporation were examined under conditions where no net transmembrane movement of calcium or potassium can occur because the transmembrane gradients for these ions are dissipated Incubation of erythrocytes in a buffer containing 125 mM K^+, which would prevent net efflux of K^+ from

TABLE IV

EFFECT OF ALTERED TRANSMEMBRANE GRADIENTS FOR POTASSIUM AND CALCIUM ON MALONYLDIALDEHYDE PRODUCTION AND INCORPORATION OF [9,10-^{3}H]OLEIC ACID INTO PHOSPHATIDYLETHANOLAMINE (PE) BY HUMAN ERYTHROCYTES FOLLOWING ADDITION OF *t*BuOOH

Erythrocytes (5% v/v) were incubated in either standard buffer or a modified buffer In Experiment 1, the standard buffer was modified by raising [KCl] from 5 mM to 125 mM and reducing [NaCl] from 145 mM to 25 mM In Experiment 2 1 1 mM EGTA was added to completely chelate extracellular Ca^{2+} Malonyldialdehyde production and [9,10-^{3}H]oleic acid incorporation were determined on duplicate aliquots of each suspension after incubation for 30 min in the presence and absence of tBuOOH (1 mM) [9,10-^{3}H]Oleic acid incorporation into PE is expressed as the ratio of (incorporation in the modified buffer)/(incorporation in the standard buffer in the absence of *t*BuOOH) × 100

Buffer Modification	*t*BuOOH (1 mM)	Malonyldialdehyde (nmol/g Hb)	[9,10-^{3}H]Oleic acid incorporation into PE (% of control)
Experiment 1			
None	−	3	100
	+	57	490
125 mM KCl	−	3	103
	+	53	467
Experiment 2			
None	−	0	100
	+	101	650
1 1 mM EGTA	−	1	96
	+	95	714

the cell following any *t*BuOOH-induced increase in K^+ permeability [28], had no effect on *t*BuOOH-induced malonyldialdehyde production or the *t*-BuOOH-induced stimulation of [9,10-^{3}H]oleic acid incorporation into PE (Table IV) Addition of 1 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetracetic acid (EGTA) which is sufficient to chelate all extracellular calcium and would thus prevent calcium influx into the cells following any *t*BuOOH-induced increase in calcium permeability [28], also had no effect on the *t*BuOOH-induced increase in malonyldialdehyde production or [9,10-^{3}H]oleic acid incorporation into PE

Discussion

The importance of the in situ acylation-deacylation pathway in the response of normal erythrocytes to oxidant stress has not been fully evaluated Earlier studies showed that fatty acid incorporation into PE increased following exposure of vitamin E deficient erythrocytes to H_2O_2 in vitro [37] More recently, alterations in cell volume, filterability and cation permeability have been detected in vitamin E deficient erythrocytes exposed to H_2O_2 under identical conditions [38] In the present study the effects of oxidant stress on the pathway for deacylation and reacylation of membrane phospholipid in situ have been studied by examining the effects of *t*BuOOH on the incorporation of exogenous [9,10-^{3}H]oleic acid into phospholipid by human erythrocytes

Exposure of normal human erythrocytes to *t*BuOOH results in depletion of GSH [18,34] (Fig 1) oxidation of Hb to MetHb [18] and release of malonyldialdehyde, a lipid peroxidation product [18] (Fig 2) Exposure of erythrocytes to *t*BuOOH also simultaneously increases incorporation of exogenous [9,10^3-H]oleic acid into PE (Fig 3) Increased incorporation of [9,10-^{3}H]oleic acid into PE continues even after reduction of *t*BuOOH to *t*BuOH is completed (Figs 1 and 3) [18] This increase in the rate of [9,10-^{3}H]oleic acid incorporation into PE shows a dependence on initial *t*BuOOH concentration which parallels that for malonyldialdehyde formation (Fig 4), suggesting that the stimulation of phospholipid fatty acid turnover is associated with the peroxidation of membrane phospholipid fatty acids However, no stoichiometric relationship between these two *t*BuOOH induced processes has been demonstrated The amount of malonyldialdehyde formed exceeds the amount of [9,10^3-H]oleic acid incorporated by a ratio of 40 1 (Fig 4) The lack of a direct stoichiometric relationship suggests that the capacity of the acylation-deacylation pathway to replace all of the fatty acid oxidized with oleic acid is limited by either the incomplete removal of oxidized fatty acids from their glycerol ester linkages or the inability of oleic acid to substitute for all of the oxidized fatty acids hydrolyzed It may also reflect the contribution of oxidation products derived from other lipid and non-lipid cellular components to malonyldialdehyde formation

Addition of *t*BuOOH to cells suspended in buffer containing ascorbic acid prevents the release of malonyldialdehyde and the stimulation of [9,10^3-H]oleic acid incorporation into PE (Table II) Since ascorbic acid scavenges *t*-butoxyl radicals and prevents *t*BuOOH-mediated lipid peroxidation but not *t*BuOOH-mediated-Hb oxidation in erythrocytes [19], these observations suggest that the stimulation of [9,10-^{3}H]oleic acid incorporation into PE following *t*BuOOH exposure is related to the degree of lipid peroxidation (Figs 3 and 4) However when erythrocytes are pre-treated with $NaNO_2$ to convert Hb to MetHb [19], *t*BuOOH Still causes release of malonyldialdehyde but there is no enhancement of [9,10-^{3}H]oleic acid incorporation into PE (Table III) Thus, the stimulation of [9,10-^{3}H]oleic acid incorporation into PE occurs only under conditions where lipid peroxidation can occur but is not directly related to the formation and release of malonyldialdehyde These results suggest that this stimulation may be triggered by the presence of lipid peroxides or decomposition products within the membrane whose formation is inhibited when Hb is completely converted to MetHb

The mechanisms by which *t*BuOOH alters the activity of the pathway for fatty acid incorporation into PE have not yet been elucidated Since availability of lysophospholipid has been shown to be the rate-limiting step for acyl-CoA-lysophospholipid acyltransferase activity in a variety of systems [39] the *t*BuOOH-induced stimulation of [9,10-^{3}H]oleic acid incorporation into PE may reflect increased availability of lyso-PE formed by either direct oxidation of unsaturated fatty acids or the action of an endogenous phospholipase A_2 In vitro studies have shown that oxidized phospholipids are better substrates for phospholipase A_2 than native phospholipids [11,12], In the erythrocyte, phospholipase A_2 activity cannot be assayed directly because reacylation cannot be completely inhibited so that accumulation of detectable levels of lysophospholipid does not occur Use of phospholipase inhibitors such as mepacrine is also not feasible in the erythrocyte since it has been shown previously that mepacrine interacts directly with membrane PE [40] Thus, it is not yet possible to determine whether the increase in incorporation of exogenous [9,10-^{3}H]oleic acid into PE following tBuOOH exposure is due to activation of an endogenous phospholipase or to direct stimulation of acyl-CoA-lysophosphatidylethanolamine acyltransferase

Alterations in both the potassium and calcium permeability of erythrocyte membranes have been observed following exposure to oxidants [23,24] Since similar alterations in calcium and potassium permeability induced by exposure of erythrocytes to ionophores have been associated with stimulation of the incorporation of exogenous fatty acids into PE [27–29], the possibility that the effects of tBuOOH on [9,10-^{3}H]oleic acid incorporation into PE are due to alterations in potassium or calcium pemeability was examined The lack of effect of alterations in the transmembrane gradients for potassium or calcium on the tBuOOH-induced alterations in [9,10-^{3}H]oleic acid incorporation or malonyldialdehyde production (Table IV) indicate that tBuOOH exposure influences the pathway for acylation and deacylation of PE directly rather than by merely causing net transmembrane movement of calcium or potassium

In contrast to the effect of tBuOOH on [9,10-^{3}H]oleic acid incorporation into PE, incorporation of exogenous [9,10-^{3}H]oleic acid into PC decreases following tBuOOH exposure (Fig 5) This decrease in [9,10-^{3}H]oleic acid incorporation is not prevented by addition of ascorbic acid (Table II) and is greater in MetHb containing erythrocytes (Table III) The inhibition of [9,10-^{3}H]oleic acid incorporation into PC thus does not require the peroxidation of membrane lipid but rather is associated with the formation of MetHb and oxidation of GSH These results suggest that the inhibition of [9,10-^{3}H]oleic acid incorporation into PC reflects oxidation of a sensitive sulfhydryl group on one of the enzymes in the pathway Such a mechanism has been proposed to account for altered phospholipid fatty acid turnover in isolated liver mitochondria following exposure to tBuOOH [41] These results also indicate that tBuOOH affects the turnover of [9,10-^{3}H]oleic acid in PC and PE by different mechanisms and that there are separate pathways for acylation and deacylation of these two phospholipid classes in the erythrocyte The step(s) in the pathway for acylation and deacylation of PC at which inhibition occurs has not yet been elucidated The inhibition of [9,10-^{3}H]oleic acid turnover within membrane PC pool(s) would permit oxidized fatty acids to accumulate in the membrane following exposure to tBuOOH and could interfere with membrane function by either perturbing the hydrophobic lipid bilayer or by reacting covalently with membrane proteins and/or lipids [1,2,7,8] Recent studies have shown that inhibition of fatty acid incorporation into PC is associated with cellular injury in ischemic myocardium [42] and also occurs in erythrocytes obtained from sheep after exposure of the animals to normobaric hyperoxia [43] Thus, it is possible that the inhibition of [9,10-^{3}H]oleic acid incorporation into PC observed in human erythrocytes following exposure to tBuOOH reflects an early step in the pathogenesis of oxidant-induced cellular injury

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References

1 Freeman B A and Crapo, J D (1982) Lab Invest 47 412–426

2 Chiu D Lubin B and Shohet S B (1982) in Free Radicals in Biology (Pryor W A ed), pp 115–160 Academic Press, New York
3 Pfafferott C, Meiselman, H J and Hochstein, P (1982) Blood 59, 11–15
4 Jain S K, Mohandas N, Clark, M R and Shohet, S B (1983) Br J Haematol 53, 247–255
5 Jain, S K (1984) J Biol Chem 259 3391–3394
6 Van Duijn G, Verkleij A J and De Kruijff B (1984) Biochemistry 23 4969–4977
7 Benedetti A Fulceri, R and Comporti M (1984) Biochim Biophys Acta 793, 489–493
8 Esterbauer H, Cheeseman, K H, Dianizan, M U, Poli, G and Slater T F (1982) Biochem J 208, 129–140
9 Claster, S Chiu, D T, Quintanilha, A and Lubin B (1984) Blood 64, 1079–1084
10 Slater T F (1984) in Methods in Enzymology Vol 105 (Packer L ed), pp 283–293, Academic Press New York
11 Sevanian, A, Stein, R A and Mead, J F (1981) Lipids 16, 781–789
12 Sevanian A Muakkassah-Kelly, S F and Montestruque S (1983) Arch Biochem Biophys 223 441–452
13 Shohet, S B, Nathan, D G and Karnovsky, M L (1968) J Clin Invest 47, 1096–1107
14 Mulder, E and Van Deenen, L L M (1965) Biochim Biophys Acta 106, 348–356
15 Shohet, S B and Nathan, D G (1970) Biochim Biophys Acta 202, 202–205
16 Dise, C A, Goodman, D B P and Rasmussen, H (1980) J Lipid Res 21, 292–300
17 Trotta, R J, Sullivan, S G and Stern, A (1981) Biochim Biophys Acta 679, 230–237
18 Trotta, R J, Sullivan, S G and Stern, A (1982) Biochem J 204, 405–415
19 Trotta, R J Sullivan, S G and Stern, A (1983) Biochem J J 212, 759–772
20 Thornalley, P J Trotta, R J and Stern A (1983) Biochim Biophys Acta 759, 16–22
21 Rice-Evans, C Baysal E Dachby, D P and Hochstein P (1985) Biochim Biophys Acta 815, 426–432
22 Corry W D, Meiselman H J and Hochstein, P (1980) Biochim Biophys Acta 587 224–234
23 Orringer E P (1984) Am J Hematol 16, 355–366
24 Shaler, O, Leida, M N, Hebbel, R P, Jacob, H S and Eaton, J W (1981) Blood 58, 1232–1235
25 Farber, J L (1982) Lab Invest 47, 114–123
26 Thor, H, Hartzell, P and Orrenius S (1984) J Biol Chem 259 6612–6615
27 Dise, C A, Lake, W C, Goodman, D B P and Rasmussen H (1976) J Biol Chem 251 4162–4164
28 Dise, C A Goodman, D B P and Rasmussen H (1980) J Biol Chem 255 5201–5207
29 Kaya, K and Miura, T (1982) Biochim Biophys Acta 688 305–315
30 Goodman D B P Wong, M and Rasmussen H (1975) Biochemistry 14 2803–2809
31 Stocks J, Offerman, E L, Modell, C B and Dormandy T L (1972) Br J Haematol 23, 713–724
32 Beutler E, Duran, O and Kelly B M (1963) J Lab Clin Med 61, 882–890
33 Drabkin D L and Austin J H (1935) J Biol Chem 112, 51–58
34 Srivastava, S K, Awasthi, Y C and Beutler E (1974) Biochem J 139, 289–295
35 Clark I A, Hunt, N H Cowden, W B, Maxwell L E and Mackie, E J (1984) Clin Exp Immunol 56, 524–530
36 Yamamoto Y Niki E, Kamiya, Y and Shimasaki H (1984) Biochim Biophys Acta 795, 332–340
37 Lubin B H, Shohet S B and Nathan, D G (1972) J Clin Invest 51 338–344
38 Lubin, B H and Chiu, D (1982) Pediatr Res 16, 928–932
39 Walsh, C E De Chatelet, L R, Chilton F H, Wykle, R L and Waite, M (1983) Biochim Biophys Acta 750, 32–40
40 Dise, C A, Burch, J W, and Goodman D B P (1982) J Biol Chem 257, 4701–4704
41 Beatrice, M C, Stiers D L and Pfeiffer D R (1984) J Biol Chem 259 1279–1287
42 Chien K R, Hon A Sen, A, Buja, L M and Willerson J T (1984) Circ Res 54 313–322
43 Dise, C A Hansen-Flaschen J, Lanken P and Goodman, D B P (1985) J Lab Clin Med 105, 89–93