Biochimica et Biophysica Acta, 688 (1982) 305–315
Elsevier Biomedical Press

BBA 71202

SELECTIVE CHANGES IN FATTY ACID COMPOSITION OF PHOSPHATIDYLSERINE IN RAT ERYTHROCYTE MEMBRANE INDUCED BY NITRATE

KUNIMITSU KAYA and TAKASHI MIURA *

Basic Medical Sciences Division, The National Institute for Environmental Studies, Yatabe, Tsukuba, Ibaraki 305 (Japan)

(Received November 2nd, 1981)
(Revised manuscript received February 2nd, 1982)

Key words: Phosphatidylserine; Nitrate induction; Fatty acid composition; (Rat erythrocyte membrane)

The relationship between nitrate which is formed from inhaled nitrogen dioxide, a common air pollutant, and changes in fatty acid metabolism of phosphatidylserine in rat erythrocytes has been examined. When erythrocytes were incubated at 37°C for 60 min with fatty acid, the incorporation rate of [1-^{14}C]arachidonic acid and [9,10-^{3}H]palmitic acid into phosphatidylserine was 15% (80 pmol/h per μmol lipid phosphorus) and 20% (12 pmol/h per μmol lipid phosphorus) of those into phosphatidylethanolamine, respectively. By the addition of 1.0 mM sodium nitrate or 0.5 μM ionophore A23187 to the incubation mixture, the rate of incorporation of both arachidonic acid and palmitic acid into phosphatidylethanolamine was stimulated 1.45-fold. On the other hand, the incorporation of palmitic acid into phosphatidylserine was little affected, while that of arachidonic acid was stimulated 1.35-fold. An increase in arachidonic acid of phosphatidylserine was also found by the addition of nitrate or ionophore A23187. This increase was dependent on the concentration of extracellular calcium and observed by the addition of other chaotropic anions in the order $SCN^- > ClO_4^- > NO_3^-$. It seems likely, therefore, that nitrate causes changes in erythrocyte membranes to facilitate calcium uptake. Increasing the concentration of intracellular calcium may cause stimulation of acyl-CoA:lysophospholipid acyltransferase and/or endogenous phospholipase A_2.

Introduction

Nitrate and nitrite ions are possible products of inhaled nitrogen dioxide (NO_2), which is present in cigarette smoke and is a common air pollutant. NO_2 has been known to induce pulmonary damage in mammals [1]. Entry of inhaled NO_2 into the bloodstream was first suggested by the estimation of the nitrate and nitrite levels in the blood and urine following inhalation of NO_2 [2]. It was subsequently confirmed by the observation that dissemination of inhaled $^{15}NO_2$ to extrapulmonary sites occurred via the bloodstream [3]. Recently, it has been reported that, in in vivo and in vitro experiments, nitrite was converted to nitrate by hemoglobin in erythrocytes and the nitrate level of the blood reached several-fold that of the control [4]. In the preceding papers [5,6], we have shown that NO_2 inhalation resulted in alterations of the compositions of phospholipid and its fatty acid in erythrocyte membranes. On these bases, we have focused our attention on the effect of an anion, nitrate, on fatty acid metabolism of erythrocyte phospholipid.

The fatty acid composition of erythrocyte phos-

* To whom correspondence should be addressed.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; lysoPS, lysophosphatidylserine; 16:0, palmitic acid; 18:0, stearic acid; 20:4, arachidonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetric acid.

0005-2736/82/0000–0000/$02.75

pholipid is known to be controlled by two processes, as follows: one is exchange of intact phosphatidylcholine, sphingomyelin and lysophosphatidylcholine molecules with corresponding plasma phospholipids [7,8], and the other is acylation of endogenous lysophospholipids with exogenous fatty acids, because erythrocytes cannot alter fatty acid chain length or degree of unsaturation or synthesize phospholipid de novo [9,11]. Rasmussen and co-workers [11] have extensively investigated fatty acid turnover of membrane phospholipids using human erythrocytes. They have demonstrated that exogenous free fatty acids are incorporated into the membrane and then activated to acylcoenzyme A thioesters. The acyl group is transferred from acylcoenzyme A thioester to lysophospholipid. This system has been confirmed in the incorporation of exogenous fatty acid into PE and PC. However, little work has dealt with the mechanism of fatty acid turnover of PS in erythrocytes or with factors regulating such fatty acid turnover.

In the present study, we have shown evidence that nitrate causes alteration in the fatty acid metabolism of erythrocyte phospholipid. An increase in the arachidonic acid (20:4) content of PS was noted when rat erythrocytes were treated with sodium nitrate. Incorporation of exogenous fatty acids into endogenous phosphatidylserine was also stimulated by sodium nitrate. This stimulation was specific for 20:4 but not for palmitic acid (16:0), while incorporation of 20:4 and 16:0 into PE were equally stimulated by sodium nitrate.

Experimental procedures

Erythrocyte preparation

Heparin-treated blood was obtained from male Wistar rats by exsanguination via the carotid artery. Erythrocytes were separated from plasma and buffy coat by centrifugation (Tomy CD-100R, Tomy Seiko Co., Tokyo, Japan) at $1000 \times g$ for 10 min [12] and washed three times with the incubation medium. The incubation medium was prepared according to Nakao et al. [13] as follows; 100 mM trisodium citrate, 4 mM NaH_2PO_4, 10 mM glucose, 2 mM adenine and 20 mM inosine, pH 7.5.

Incubation of erythrocytes and fatty acid incorporation

Erythrocytes were resuspended in 3 vol. of the incubation medium containing radioactive fatty acid, 4 mM $CaCl_2$ and sodium nitrate at the concentration indicated. [1-^{14}C]Arachidonic acid (55.8 mCi/mmol, New England Nuclear) or [9,10-^{3}H]palmitic acid (11.8 Ci/mmol, New England Nuclear) complexed to bovine serum albumin (Sigma) in the incubation medium before use. Incubation was performed at 37°C for 60 min.

Extraction of lipids from erythrocytes

After incubation, cells were washed with 10 vol. of 0.9% NaCl and lysed with 15 vol. 10 mM Tris buffer (pH 7.4) at 2°C [14]. Erythrocyte membranes were isolated by centrifugation ($28000 \times g$ for 20 min) and washed twice with 30 vol. of the same buffer. Immediately after preparation, lipids were extracted from erythrocyte membranes according to a modified procedure of the method as described by Ways and Hanahan [15], in which 7 vol. methanol were added to erythrocyte membranes followed after 20 min by 14 vol. chloroform. 20 min after the addition of chloroform, the extract was filtered and the residue was washed twice with chloroform/methanol (2:1, v/v). Filtrates were washed with 0.2 vol. 0.9% NaCl to form a two-phased system. The lower phase was concentrated under nitrogen and used for fractionation of phospholipids.

Purification of PS

Phospholipids were separated from concentrated lower phase of lipid extracts by the thin-layer chromatography. HPTLC silica-gel plates (E. Merck) were developed in diethyl ether/acetic acid (100:1, v/v) to separate neutral lipids and phospholipids, dried in air for 10 min, and then developed in the same direction in chloroform/methanol/acetic acid/water (67:33:4:2, v/v) to separate individual phospholipid fractions As a result four fractions, PE, PC, sphingomyelin and PS plus phosphatidylinositol (PI) were obtained. PS could not be separated from PI by this solvent system and was purified as follows: the fraction containing PS and PI was scraped from the plate and was extracted with chloroform/methanol (1:4, v/v). PS in the extracts was re-

acted with 2,4,6-trinitrobenzene sulfonate in a solution containing chloroform/methanol/5% bicarbonate (1:4:0.25, v/v) [16]. 20 min after incubation at room temperature, water and chloroform were added to the reaction mixture to form a two-phased system. The lower phase was concentrated under nitrogen and applied to an HPTLC silica-gel plate. The plate was developed in chloroform/methanol/ammonium hydroxide/water (70 : 30 : 2.5 : 2.5, v/v) to separate 2,4,6-trinitrophenyl-PS from PI. The yield of 2,4,6-trinitrophenyl-PS was 85% of PS in the reaction mixture. When the authentic PS purified from bovine brain (P-L Biochemicals) was reacted with 2,4,6-trinitrobenzene sulfonate, the fatty acid compositions of reacted and unreacted PS were the same as that of the original PS.

Hydrolysis of 2,4,6-trinitrophenyl-PS

Aliquots of 2,4,6-trinitrophenyl-PS (approx. 0.6 μmol) were dissolved in 2 ml diethyl ether containing 2.5 mg butylated hydroxytoluene [17]. To this solution were added 0.1 ml of snake venom phospholipase A_2 (1 mg/ml in 0.2 M Tris-HCl, pH 8.0) (P-L Biochemicals) and 0.05 ml 0.2 M $CaCl_2$ containing 0.24 mg of cetyltrimethylammonium bromide [18]. 6 h after incubation, the reaction mixture was dried under nitrogen and was applied to an HPTLC silica-gel plate. The plate was developed in chloroform/methanol/ammonium hydroxide/water (67:33:2.5:2.5, v/v) for separation of free fatty acid, 2,4,6-trinitrophenyl-PS and 2,4,6-trinitrophenyl-lysoPS.

Determination of fatty acid composition

Fatty acid compositions of phospholipids were determined by gas-liquid chromatography after methylation of the isolated phospholipids. Each phospholipid was methylated using boron trifluoride/methanol by the method of Metcalfe and Schmitz [19] and resulting methyl esters were analyzed at 230°C in Shimadzu GC-7AG (Shimadzu Corp., Kyoto, Japan) using a flame ionization detector and a glass column (4.1 m × 0.5 cm) packed with 5% Shinchrom E-71 on 80–100 mesh Shimalite (AW) (Wako Pure Chemicals Industries Ltd., Osaka, Japan). Areas of the peaks and percentage composition of fatty acid methyl esters were computed using a Shimadzu Chromatopack R1-A Data System (Shimadzu Corp.).

Measurement of exogenous fatty acid incorporation

The radioactivity present in each phospholipid fractions was determined in a toluene scintillator containing 2,5-diphenyloxazole (3 mg/l) and 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (5 g/l) by using liquid scintillation counter (Packard Model 2355) with a computer. Counting efficiency was determined with an external standard. Calculations were based on the specific activity of exogenous fatty acid. Phospholipids were determined as phosphate after digestion with $HClO_4$ [20]. Ionophore A23187 was obtained from Calbiochem-Behring Corp. (La Jolla, CA, U.S.A.). The free calcium concentration in the medium was determined by calcium electrode (Orion Research Inc., Cambridge, MA, U.S.A.).

Results

Effect of nitrate on fatty acid composition of phospholipids in erythrocyte membranes

In rat erythrocyte membranes, the major species of phospholipids are PC, PE, PS and sphingomyelin [21], and 20:4 is the most abundant species of the fatty acids of PE and PS [22]. When rat erythrocytes were incubated with 1.0 mM nitrate, the fatty acid composition of PS in erythrocyte membranes altered significantly (Table I). Incubation of erythrocytes without nitrate did not affect the fatty acid composition of PS, PE and PC. The most notable alteration was found in the percentage of 20:4, which increased by 4.5% from control value of 52.6 ± 0.1%. Simultaneously, the percentages of 16:0 and stearic acid (18:0) of PS decreased by 2.6% and 2.5% from control values of 6.0 ± 0.1% and 23.9 ± 0.3%, respectively. Changes in other fatty acids of PS were much slighter and less significant. On the other hand, no appreciable alteration was found in fatty acid composition of PE and PC upon incubation with nitrate. This specific increase in the 20:4 of PS as well as decrease in the 16:0 plus 18:0 was dependent on the nitrate concentration added to the incubation medium. Fig. 1 shows the typical dependency on the concentration of sodium nitrate. Alterations in percentages of these fatty acids were observed

TABLE I

FATTY ACID COMPOSITIONS OF PHOSPHATIDYLSERINE, PHOSPHATIDYLETHANOLAMINE AND PHOSPHATIDYLCHOLINE OF RAT ERYTHROCYTES INCUBATED WITH NITRATE

Rat erythrocytes were incubated with 1.0 mM sodium nitrate in an incubation medium containing 4 mM $CaCl_2$ at 37°C for 60 min. Purification of phospholipids and determination of their fatty acid compositions were performed as described under Experimental procedures. Values were expressed as percentages (mean ± S.D., $n = 3$) of the total fatty acid recovered. The significance of the difference between control and nitrate-treated values is shown: [b] $P < 0.01$; [c] $P < 0.001$.

	Phosphatidylserine		Phosphatidylethanolamine		Phosphatidylcholine	
	Control	$NaNO_3$	Control	$NaNO_3$	Control	$NaNO_3$
16:0	6.0 ± 0.1	3.4 ± 0.1 [c]	29.5 ± 0.2	29.7 ± 0.3	44.8 ± 0.6	45.8 ± 0.6
18:0	23.9 ± 0.3	21.4 ± 0.4 [b]	3.0 ± 0.2	2.8 ± 0.1	16.3 ± 0.2	16.5 ± 0.2
18:1	5.1 ± 0.2	5.6 ± 0.3	8.0 ± 0.1	7.9 ± 0.1	7.7 ± 0.1	7.9 ± 0.1
18:2	5.9 ± 0.1	5.8 ± 0.2	4.4 ± 0.1	4.3 ± 0.1	14.6 ± 0.1	14.2 ± 0.2
20:4	52.6 ± 0.1	57.1 ± 0.4 [c]	38.1 ± 0.2	38.0 ± 0.2	14.5 ± 0.2	14.2 ± 0.2
20:5	0.6 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	–	–
22:4	–	–	4.1 ± 0.1	4.2 ± 0.1	–	–
22:5	–	–	6.8 ± 0.1	6.9 ± 0.1	–	–
22:6	5.8 ± 0.1	6.1 ± 0.2	5.3 ± 0.2	5.4 ± 0.2	–	–

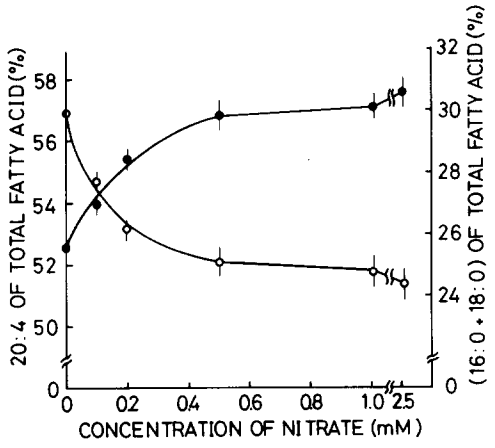

Fig. 1. Effect of nitrate on fatty acid composition of phosphatidylserine (PS) in erythrocyte membranes. The incubation media comprised 100 mM trisodium citrate/4 mM NaH_2PO_4/2 mM adenine/20 mM inosine/10 mM glucose (pH 7.5). The media were supplemented with 4 mM $CaCl_2$ and sodium nitrate at the concentrations indicated. The incubation was performed at 37°C. 60 min after incubation, erythrocytes were washed with 0.9% NaCl and erythrocyte membranes were prepared. Purification of PS and determination of fatty acid composition were performed as described in Experimental procedures. ●——●, arachidonic acid (20:4); ○——○, palmitic acid (16:0) plus stearic acid (18:0). Each point is the mean of three samples and error bars represent ± S.D.

clearly at a concentration of 0.1 mM. Both the increase in 20:4 and the decrease in 16:0 plus 18:0 reached a plateau at a concentration around 0.5 mM followed by a slight change up to 2.5 mM. Simultaneously with changes in fatty acid composition of PS, the 20:4 of the free fatty acid fraction of the erythrocyte membranes decreased and the 16:0 plus 18:0 increased (Kaya, K. and Miura, T., unpublished data).

Fatty acid composition of 1- and 2-positions of PS

In order to clarify the effect of nitrate on the fatty acid metabolism of PS, changes in fatty acid composition of the 1- and 2-positions of PS were determined. Rat erythrocytes were incubated with 1.0 mM nitrate and the PS of erythrocyte membranes were purified as a form of 2,4,6-trinitrophenyl-PS as described in Experimental procedures. Fatty acids at the 2-position of 2,4,6-trinitrophenyl-PS were released by snake venom phospholipase A_2 and those at the 1-position were obtained from 2,4,6-trinitrophenyl-lysoPS. Table II shows the fatty acid composition of the 1- and 2-positions of PS purified from control and nitrate-treated erythrocytes. In control erythrocytes the percentages of 18:0 and oleic acid (18:1) at the 1-position were 4.9- and 3.5-fold at the

TABLE II

FATTY ACID COMPOSITIONS OF 1- AND 2-POSITIONS OF PHOSPHATIDYLSERINE IN RAT ERYTHROCYTES INCUBATED WITH NITRATE

Rat erythrocytes were incubated with 1.0 mM sodium nitrate. Incubation, purification of PS and fatty acid analyses were performed as described in Table I and under Experimental procedures. PS was purified as a form of 2,4,6-trinitrophenyl-PS and 2,4,6-trinitrophenyl-PS was treated with snake venom phospholipase A_2. The resulting products, 2,4,6-trinitrophenyl-lysoPS and free fatty acid, were separated by TLC. Fatty acid compositions of 2,4,6-trinitrophenyl-lysoPS and free fatty acid were expressed as those of the 1- and 2-positions, respectively. 'Whole' refers to fatty acid composition of untreated trinitrophenyl-PS. Values were expressed as percentages (mean ±S.D., $n=3$) of the total fatty acid recovered. The significance of the difference between control and nitrate-treated values is shown: [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$.

	Control			$NaNO_3$		
	whole	1-position	2-position	whole	1-position	2-position
16:0	6.2±0.1	5.0±1.2	7.3±1.3	3.2±0.1 [c]	5.1±1.5	2.2±1.7 [a]
18:0	24.0±0.3	41.0±1.9	8.4±1.7	20.9±0.4 [c]	38.7±1.0	6.2±1.7
18:1	5.0±0.2	8.0±0.4	2.3±0.2	5.3±0.3	9.5±0.9	1.9±0.1
18:2	5.9±0.1	3.8±0.2	7.9±0.3	5.8±0.2	4.8±0.4	6.7±0.5
20:4	52.3±0.1	37.5±1.7	65.9±1.5	57.4±0.4 [c]	36.2±1.7	75.7±2.7 [b]
20:5	0.6±0.1	–	1.0±0.4	0.6±0.2	–	0.9±0.4
22:6	6.0±0.1	4.6±0.2	7.3±0.3	6.6±0.2	5.8±0.4	6.4±0.5

2-position, respectively. Other fatty acids were lower at the 1-position than at the 2-position. The percentage of 20:4 in the 2-position was 1.8-fold that in the 1-position. When erythrocytes were incubated with nitrate, notable changes in fatty acid composition were found in the 2-position. The percentage of 16:0 was decreased to 0.30-fold the control and that of 20:4 was increased to 1.15-fold. The changes in fatty acid composition of the 1-position were slight, except for those of 16:0 and 22:6, and the percentage of 20:4 in the 1-position was somewhat decreased by incubation with nitrate.

Effect of nitrate on fatty acid incorporation into phospholipids of erythrocyte membranes

When rat erythrocytes were incubated with [1-^{14}C]20:4 complexed to bovine serum albumin, this fatty acid was incorporated into PE, PC and PS at the rates of 530, 350 and 80 pmol/h per μmol of corresponding phospholipid, respectively (Fig. 2). Addition of 1.0 mM nitrate to the incubation mixture reproducibly resulted in enhanced incorporation of radioactive 20:4 into PE and PS, which were increased to 1.45- and 1.35-fold the control, respectively. On the other hand, 20:4 incorporation into PC was not affected by incubation with nitrate. Fig. 3 shows incorporation of [9,10-^{3}H]16:0 complexed to bovine serum albumin into phospholipid of erythrocyte membranes. This fatty acid was incorporated into PE, PC and PS at the rates of 61, 58 and 12 pmol/h per μmol corresponding phospholipid. Addition of 1.0 mM nitrate to the incubation mixture stimulated the incorporation into PE. The rate of 16:0 incorporation into PS was almost the same as that of the control, while that into PE was raised to 1.45-fold the control. The incorporation into PC did not show any appreciable change. These results suggest that stimulation of fatty acid incorporation into PS by nitrate may be preferential to 20:4. In order to determine the position of 20:4 incorporated into PS, rat erythrocytes were incubated with [1-^{14}C]20:4 in the presence of nitrate. Incorporation of radioactive 20:4 into the 1- and 2-positions of PS was examined using phospholipase A_2. 94% of 2,4,6-trinitrophenyl-PS was hydrolyzed by phospholipase A_2 and almost 90% of the radioactivity incorporated into hydrolyzed 2,4,6-trinitrophenyl-PS was recovered in the spot of free fatty acid. 8% of radioactivity was detected in 2,4,6-trinitrophenyl-lysoPS.

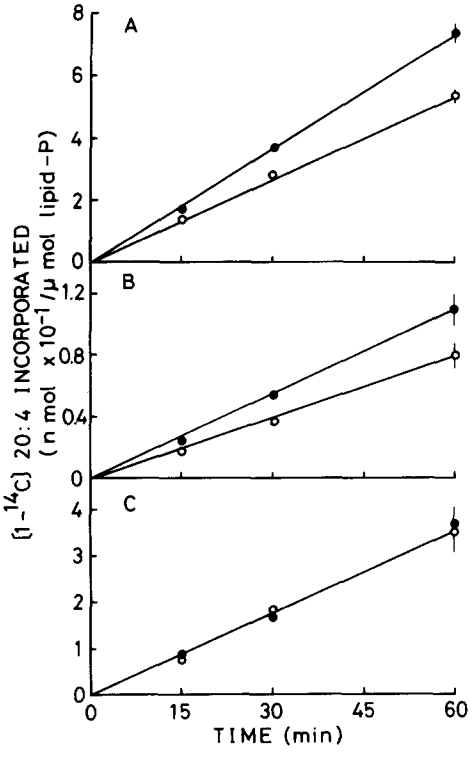

Fig. 2. Effect of nitrate on incorporation of [1-^{14}C]arachidonic acid (20:4) into phosphatidylethanolamine (A), phosphatidylserine (B) and phosphatidylcholine (C) of erythrocyte membranes. Erythrocytes were incubated with 1.0 mM sodium nitrate in an incubation medium supplemented with 4 mM $CaCl_2$ and [1-^{14}C]20:4 (3.6 μM/2.7 μCi) complexed to bovine serum albumin. For the incubation conditions see the legend of Fig. 1. Purification of each phospholipid and determination of radioactivity incorporated into phospholipid were performed as described in Experimental procedures. O——O, −nitrate; ●——●, +nitrate. Each point is the mean of three samples and error bars represent ±S.D.

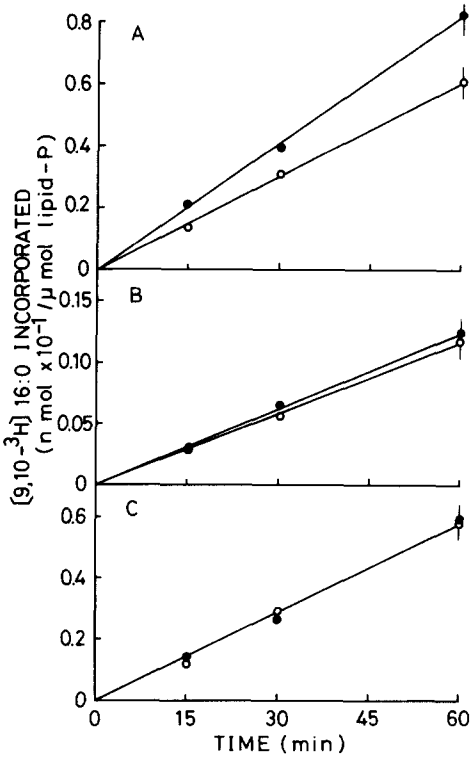

Fig. 3. Effect of nitrate on incorporation of [9,10-^{3}H]palmitic acid (16:0) into phosphatidylethanolamine (A), phosphatidylserine (B) and phosphatidylcholine (C) of erythrocyte membranes. The media were supplemented with 4 mM $CaCl_2$ and [9,10-^{3}H]16:0 (3.6 μM/27 μCi) complexed to bovine serum albumin. Other conditions were the same as in Fig. 2. Each point is the mean of three samples and error bars represent ±S.D.

Effects of anions on increase in 20:4 of PS

Nitrate ion is one of the chaotropic agents which disturb membrane structure [23,24]. Enhancement of fatty acid incorporation into PE and PS will lead to the assumption that chaotropic action of nitrate causes stimulated incorporation of 20:4 into PS. In order to examine this assumption, rat erythrocytes were incubated with chaotropic agents, thiocyanate, perchlorate and nitrate at the concentration of 1.0 mM. Table III shows effects of various species of anions on the increase in the percentage of 20:4 of PS. The effects of chaotropic anions were highly significant compared to that of the control. The percentage of 20:4 of PS was increased to 1.13-, 1.11- and 1.09-fold the control by the addition of thiocyanate, perchlorate and nitrate, respectively. The magnitude of increase in the percentage of 20:4 reproducibly followed the series $SCN^- > ClO_4^- > NO_3^-$. On the other hand, other anions, such as bicarbonate and sulfate, resulted in only slight change in the percentage of 20:4 of PS.

Factors influencing the increase in 20:4 of PS and fatty acid incorporation into PS

It has been shown that treatment of human erythrocytes with the divalent cation ionophore, A23187, results in selective stimulation of fatty acid incorporation into PE simultaneously with

TABLE III

EFFECT OF ANIONS ON INCREASE IN ARACHIDONIC ACID CONTENT OF PHOSPHATIDYLSERINE

Rat erythrocytes were incubated at 37°C for 60 min with sodium thiocyanate, sodium perchlorate, sodium nitrate, sodium acetate, sodium bicarbonate or sodium sulfate at a final concentration of 1.0 mM in an incubation medium containing 4.0 mM $CaCl_2$. The 20:4 content of PS in erythrocyte membranes was determined as described in Fig. 1. Values were expressed as percentage (mean ±S.D., $n=3$) of the total fatty acid recovered. The significance of the difference between control and anion-treated values is shown: [c] $P<0.001$.

Addition	Arachidonic acid content of PS (%)	Percentage of control
None	52.4±0.1	(100)
Na_2SO_4	52.8±0.2	101
$NaHCO_3$	53.1±0.3	101
CH_3COONa	53.8±0.2 [c]	103
$NaNO_3$ [d]	57.3±0.4 [c]	109
$NaClO_4$ [d]	58.1±0.4 [c]	111
NaSCN [d]	59.2±0.7 [c]	113

[d] Chaotropic ions.

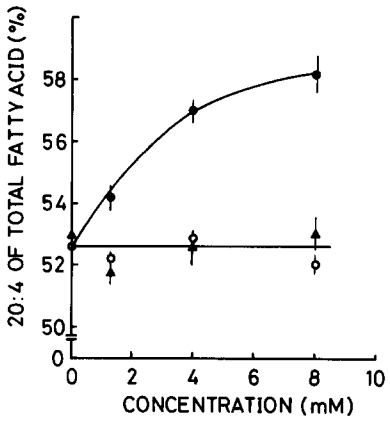

Fig. 4. Effects of calcium and magnesium on the increase in arachidonic acid (20:4) of phosphatidylserine of erythrocyte membranes. The incubation media were supplemented with 1.0 mM sodium nitrate and $CaCl_2$ or $MgCl_2$ at the concentrations indicated. For other experimental conditions see the legend of Fig. 1. ●——●, +calcium; ▲——▲, +calcium+9 mM EGTA; ○——○, +magnesium. Each point is the mean of three samples and error bars represent ±S.D.

net uptake of calcium and a calcium-dependent decrease in cellular potassium content [25]. In order to clarify factors influencing fatty acid incorporation into PS of rat erythrocytes, the fatty acid turnover of PS was examined in the presence of cations. Fig. 4 shows effects of calcium and magnesium ions on the percentage of 20:4 of PS. The percentage of 20:4 of PS was increased by raising the extracellular calcium concentration to 8 mM. The free calcium concentrations in the citrate medium supplemented with 1, 4 and 8 mM $CaCl_2$ were 4.1, 16.5 and 33.0 μM, respectively. This increment was inhibited completely by the addition of 9 mM EGTA. On the other hand, the extracellular magnesium concentration did not affect the percentage of 20:4 of PS.

In order to examine the effect of extracellular potassium on the percentage of 20:4 of PS and 20:4 incorporation into PE and PS, the trisodium citrate (sodium medium) in the incubation medium was replaced by tripotassium citrate (potassium medium), the rate of 20:4 incorporation into PS in the presence of nitrate was 1.34- and 1.35-fold the control in the sodium and potassium media, respectively (Table IV). Simultaneously, the percentage of 20:4 of PS was increased slightly by the addition of nitrate to the potassium medium to the same extent as to the sodium medium. By contrast, 20:4 incorporation into PE was scarcely affected by the incubation with nitrate in the potassium medium, while that in the sodium medium was increased to 1.40-fold the control.

When rat erythrocytes were incubated with ionophore A23187, an increase in the percentage of 20:4 of PS occurred in the sodium medium (Table V). 20:4 incorporation into PS was also stimulated to 1.30-fold the control, while 16:0 incorporation into PS was little affected. Replacement of the sodium medium by a potassium one did not alter the magnitude of increases in the percentage and incorporation of 20:4. On the other hand, treatment with ionophore A23187 resulted in enhanced incorporation of both 20:4 and 16:0 into PE in the sodium medium. Increasing extracellular potassium blocked the stimulative effect of ionophore A23187 on the rate of incorporation of 16:0 and 20:4 into PE.

TABLE IV

EFFECTS OF EXTRACELLULAR POTASSIUM AND SODIUM ON INCREASE IN ARACHIDONIC ACID CONTENT OF PHOSPHATIDYLSERINE AND STIMULATION OF [1-^{14}C]ARACHIDONIC ACID INCORPORATION INTO PHOSPHATIDYLSERINE AND PHOSPHATIDYLETHANOLAMINE INDUCED BY INCUBATION WITH NITRATE

Rat erythrocytes were incubated with 1.0 mM sodium nitrate in a potassium (100 mM tripotassium citrate) or sodium medium (100 mM trisodium citrate) containing 4.0 mM $CaCl_2$ and the 20:4 content of PS was determined as described in Fig. 1. In experiments on [1-^{14}C]20:4 incorporation into PE and PS, erythrocytes were incubated with [1-^{14}C]20:4 complexed to bovine serum albumin in the presence of 1.0 mM sodium nitrate. Purification of PE and PS and determination of radioactivity incorporated into PE and PS were performed as described under Experimental procedures and in Fig. 2. For arachidonic acid content, values are percentages of the total fatty acid. For incorporations, values are nmol 20:4 incorporated/μmol lipid phosphorus per h ($\times 10^{-1}$). The significance between control and nitrate-treated values is shown: [a] $P < 0.05$; [c] $P < 0.001$ (mean ± S.D., $n = 3$).

		Extracellular monovalent cation	
		Sodium medium	Potassium medium
Arachidonic acid content in PS	Control	53.0 ± 0.1	52.7 ± 0.1
	$NaNO_3$	57.1 ± 0.4 [c]	57.3 ± 0.3 [c]
	$NaNO_3$/Control	1.08	1.09
20:4 incorporation into PS	Control	0.82 ± 0.09	0.83 ± 0.08
	$NaNO_3$	1.10 ± 0.10	1.12 ± 0.10 [a]
	$NaNO_3$/Control	1.34	1.35
20:4 incorporation into PE	Control	5.3 ± 0.4	5.1 ± 0.3
	$NaNO_3$	7.4 ± 0.6 [a]	5.3 ± 0.6
	$NaNO_3$/Control	1.40	1.04

Discussion

The object of this study was to examine the relationship between exposure of rat erythrocytes to nitrate and changes in fatty acid metabolism of PS in erythrocyte membranes. The results show that selective changes in fatty acid composition of PS were induced by incubation with nitrate; that is, an increase in the percentage of 20:4 and a decrease in that of 16:0 plus 18:0 (Table I). These changes in fatty acid composition were dependent on the concentration of nitrate added to the incubation mixture (Fig. 1). An increase in 20:4 of PS was also induced when rat erythrocytes were incubated with other species of chaotropic anions, such as thiocyanate and perchlorate (Table IV). The magnitude of this increase was in the order $SCN^- > ClO_4^- > NO_3^-$, that is, in the order opposite to the Hofmeister series [23,24]. These results provide tentative evidence that selective changes in fatty acid composition of PS are induced by the chaotropic action of nitrate at a concentration greater than 0.1 mM. As cited in the Introduction, nitrate is one of possible products of inhaled NO_2 in pulmonary systems [2,3]. Recently, Oda et al. [4] have shown that short-term exposure of mice to NO_2 at the air-borne concentrations resulted in elevated level of nitrate ion in the blood. 30 min after exposure to 5 ppm NO_2 the level of nitrate ion reached almost 0.1 mM, which was 4-fold the control. It is therefore probable that changes in the fatty acid composition of PS in erythrocyte membranes will occur in daily life.

The fatty acid metabolism of mammalian erythrocytes has been extensively investigated [7,8,11]. However, little work has dealt with fatty acid metabolism of PS. The incorporation rate of 16:0 and 20:4 into PS was 15% and 20% of those into PE, respectively (Figs. 2 and 3). Incubation of erythrocytes with nitrate caused stimulated incorporation of both 16:0 and 20:4 into PE with no change in the rate of their incorporation into PC

(Figs. 2 and 3). The incorporation of 20:4 into PS was also stimulated to 1.34-fold the control but that of 16:0 was little affected (Figs. 2 and 3). Thus, selective changes in the fatty acid composition of PS seem to be interpreted by this selective stimulation of 20:4 incorporation into PE being the same as that of 16:0 into PE, and no change in fatty acid composition of PE was observed. 20:4 was incorporated exclusively into the 2-position of PS. It seems, therefore, probable that 16:0 in the 2-position of PS is replaced by 20:4 when rat erythrocytes are incubated with nitrate. Simultaneously with these changes, 20:4 in free fatty acid fraction in erythrocyte membranes decreased and 16:0 and 18:0 increased in the absence of exogenous free fatty acid. These results suggest that 20:4 of the free fatty acid fraction in membranes may be selectively incorporated into PS when erythrocytes are incubated with nitrate without supplemented of exogenous fatty acid.

Recent works on human erythrocytes have shown that fatty acid incorporation into membrane phospholipids occurred in the inner leaflet of membranes via acylation of lysophospholipids [26,27]. It seems, therefore, arguable that stimulation of 20:4 incorporation into PS occurring upon incubation with nitrate may be due to stimulation of acyltransferase and/or phospholipase. Cellular phospholipase has been indicated to require calcium for optimal activity [28] and play an important role on regulation of fatty acid metabolism of membrane phospholipids in other systems [23]. A selective increase in 20:4 of PS required calcium (Fig. 4). When calcium incorporation was blocked by the addition of EGTA, this stimulation did not occur. In accord with this observation, incubation of rat erythrocytes with divalent cation ionophore, A23187, also resulted in stimulated incorporation of 20:4 into PS as well as an increase in the 20:4 of PS (Table V). Ionophore A23187 induces a selective increase in the permeability of the membrane to calcium [29]. Paysant et al. [30] have demonstrated endogenous phospholipase activity in human erythrocytes. Subsequently, Dise et al. [11] have demonstrated possible involvement of endogenous phospholipase in fatty acid turnover of human erythrocytes. Waku and Lands [31] have suggested the activity of endogenous phospholipase in cell membranes of rat erythrocytes. It seems therefore, likely that the calcium-dependent increase in 20:4 of PS which occurred upon incubation with nitrate is a response to increased availability of lysoPS resulting from activation of endogenous phospholipase by calcium. If this is the case, incubation with nitrate will cause activation of phospholipase A_2 because 20:4 was exclusively incorporated into 2-position of PS.

Dise et al. [25] have shown that treatment of human erythrocytes with ionophore A23187 and the monovalent cation ionophore, nigericin, resulted in net loss of intracellular potassium stimultaneously with stimulated incorporation of fatty acids into PE. In addition, the stimulation of fatty acid incorporation was prevented by increasing the concentration of extracellular potassium. When rat erythrocytes were incubated with ionophore A23187 in the sodium medium, incorporation of 16:0 and 20:4 into PE was stimulated to 1.44- and 1.45-fold the control (Figs. 2 and 3, and Table V). Increasing the concentration of extracellular potassium abolished this stimulation (Tables IV and V). These results are completely consistent with the observations on human erythrocytes. The effect of nitrate on the fatty acid turnover of PE appears to be similar to that of ionophore A23187. Likewise, ionophore A23187 and nitrate stimulated 20:4 incorporation into PS of rat erythrocytes. It should, however, be noted that increasing the concentration of extracellular potassium did not abolish the stimulated incorporation of 20:4 into PS (Table IV). Moreover, ionophore A23187 and nitrate did not stimulate 16:0 incorporation into PS (Fig. 2 and Table V). These results seem to lead to the assumption that transacylation to lysoPS is mediated by an enzyme different from that in the case of lysoPE.

The present observations have indicated that nitrate, one of the chaotropic ions, affects fatty acid turnover of PE and PS of rat erythrocytes. The effect of nitrate appears to be similar to that of ionophore A23187. Waisman et al. [32] have proposed that, in the human erythrocyte membrane, anions stimulate calcium uptake by neutralizing the positive potential due to calcium pumping. It is also to consider at least two mechanisms for the effect of nitrate: (1) nitrate causes changes of erythrocyte membranes to facilitate calcium uptake into erythrocytes, and (2) it is incorporated

TABLE V

EFFECT OF IONOPHORE A23187 ON ARACHIDONIC ACID CONTENT OF PS AND RATES OF [1-^{14}C]ARACHIDONIC ACID AND [9,10-^{3}H]PALMITIC ACID INTO PE AND PS

Rat erythrocytes were incubated at 37°C for 60 min with ionophore A23187 in potassium or sodium medium containing 4.0 mM $CaCl_2$. The 20:4 content of PS in erythrocyte membranes was determined as described in Fig. 1. In order to examine fatty acid incorporation into PE and PS, incubation was performed in the presence of either [1-^{14}C]arachidonic acid or [9,10-^{3}H]palmitic acid complexed to bovine serum albumin. Incorporation of radioactive fatty aid into PE and PS was determined as described in Figs. 2 and 3. The control incubation mixture received ethanol (0.5%). 'Ionophore A23187' comprises 0.5 μM A23187 in 0.5% ethanol. For arachidonic acid content, values are percentage of the total fatty acid in PS. For all incorporations, values are nmol fatty acid incorporated/μmol lipid phosphorus per h ($\times 10^{-1}$). The significance between control and nitrate-treated values is shown: [a] $P < 0.05$; [c] $P < 0.001$ (mean ± S.D., $n = 3$).

	Control	A23187	A23187/control
Arachidonic acid content in PS			
Sodium medium	52.4 ± 0.1	55.2 ± 0.4	1.05
Potassium medium	52.2 ± 0.1	55.4 ± 0.5 [c]	1.06
Arachidonic acid incorporation into PS			
Sodium medium	0.82 ± 0.08	1.07 ± 0.09 [a]	1.30
Potassium medium	0.83 ± 0.08	1.09 ± 0.09 [a]	1.31
Palmitic acid incorporation into PS			
Sodium medium	0.12 ± 0.01	0.13 ± 0.01	1.08
Potassium medium	0.12 ± 0.01	0.12 ± 0.01	1.00
Arachidonic acid incorporation into PE			
Sodium medium	5.3 ± 0.4	7.7 ± 0.6 [a]	1.45
Potassium medium	5.1 ± 0.3	5.2 ± 0.6	1.02
Palmitic acid incorporation into PE			
Sodium medium	0.61 ± 0.05	0.88 ± 0.06 [c]	1.44
Potassium medium	0.61 ± 0.05	0.60 ± 0.06	0.98

into erythrocytes, coupling with the influx of extracellular calcium by an electroneutralizing mechanism. Increasing the concentration of intracellular calcium may cause stimulation of acylcoenzyme A:lysophospholipid acyltransferase and/or endogenous phospholipase A_2.

References

1 Morrow, P.E. (1975) Environ. Res. 10, 92–112
2 Shvorcova, S. and Kant, V. (1971) Cesk. Hyg. 17, 71
3 FGoldstein, E., Peek, N.F., Parks, N.J., Hines, H.H., Steffey, G.P. and Tarkington, B. (1977) Am. Rev. Respir. Dis. 115, 403–411
4 Oda, H., Tsubone, H., Suzuki, A., Ichinose, T. and Kubota, K. (1981) Environ. Res. 25, 294–301
5 Kaya, K., Miura, T. and Kubota, K. (1980) Environ. Res. 23, 397–409
6 Kaya, K. and Miura, T. (1982) Environ. Res. 27, 24–35
7 Reed, C.F. (1968) J. Clin. Invest. 47, 749–760
8 Shohet, S.B. (1970) J. Clin. Invest. 49, 1668–1678
9 Shohet, S.B., Nathan, D.G. and Karnousky, M.L. (1968) J. Clin. Invest. 47, 1096–1108
10 Mulder, E. and Van Deenen, L.L.M. (1965) Biochim. Biophys. Acta 106, 106–118
11 Dise, C.A., Goodman, D.B.P. and Rasmussen, H. (1980) J. Lipid Res. 21, 292–300
12 Allan, D., Watts, R. and Michell, R.H. (1976) Biochem. J. 156, 225–232
13 Nakao, M., Fujii, Y., Hara, Y., Nomura, T., Nakao, T. and Komatsu, Y. (1980) J. Biochem. 88, 327–335
14 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130
15 Ways, P. and Hanahan, D.J. (1964) J. Lipid Res. 5, 318–328
16 Gordesky, S.E. and Marinetti, G.V. (1973) Biochim. Biophys. Res. Commun. 50, 1027–1031
17 Ferber, E., De Pasquale, G.G. and Resch, K. (1975) Biochim. Biophys. Acta 398, 364–376
18 Saito, K. and Sato, K. (1968) J. Biochem. 64, 293–200
19 Metcalfe, L.D. and Schmitz, A.A. (1961) Anal. Chem. 33, 363–364
20 Harris, W.D. and Popat, P. (1954) J. Am. Oil Chem. Soc. 31, 124–127
21 Nelson, G.J. (1967) Biochim. Biophys. Acta 144, 221–232
22 Rao, G.A., Siler, K. and Larkin, E.C. (1979) Lipids 14, 30–38
23 Hamaguchi, K. and Guduschek, E.P. (1962) J. Am. Chem. Soc. 84, 1329–1338

24 Davis, K.A. and Hatefi, Y. (1969) Biochemistry 8, 3355–3361
25 Dise, C.A., Goodman, D.B.P. and Rasmussen, H. (1980) J. Biol. Chem. 255, 5201–5207
26 Renooij, W., Van Golde, L.M.G., Zwaal, R.F.A., Roelofsen, B. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 363, 287–297
27 Renooij, W., Van Golde, L.M.G., Zwaal, R.F.A. and Van Deenen, L.L.M. (1976) Eur. J. Biochem. 61, 53–58
28 Shier, W.T. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 195–199
29 Dise, C.A., Lake, W.C., Goodman, D.B.P. and Rasmussen, H. (1976) J. Biol. Chem. 251, 4162–4164
30 Paysant, M., Bitran, R., Wald, R. and Polonouski (1970) Bull. Soc. Chim. Biol. 52, 1259–1269
31 Waku, K. and Lands, W.E.M. (1968) J. Lipid Res. 9, 12–18
32 Waisman, D.M., Gimble, J.M., Goodman, D.B.P. and Rasmussen, H. (1981) J. Biol. Chem. 256, 420–424