Biochimica et Biophysica Acta, 941 (1988) 176–186
Elsevier

BBA 74015

Sarcolemmal phospholipid fatty acid composition and permeability

R. Bester and A. Lochner

MRC Centre for Molecular and Cellular Biology, Department of Medical Physiology and Biochemistry, University of Stellenbosch Medical School, Tygerberg (Republic of South Africa)

(Received 7 December 1987)
(Revised manuscript received 22 February 1988)

Key words: Sarcolemma; Ischemia; Phospholipid bilayer; (Rat heart)

In this study, the mechanism of ischaemia-induced increased sarcolemmal permeability, as manifested by release of intracellular enzymes, was investigated. The role of changes in the sarcolemmal phospholipid bilayer in this process was evaluated by experimental modulation of the phospholipid fatty acid composition. The isolated perfused rat heart subjected to low-flow hypoxia, was used as a model of global ischaemia. Glucose as well as saturated (palmitate) and unsaturated (linoleate) long-chain fatty acids were used as substrates. Hearts perfused with palmitate or linoleate (1.5 mM, fatty acid / albumin ratio, 3.4) showed a significantly higher rate of lactate dehydrogenase release in both control and ischaemic conditions than hearts perfused with glucose (10 mM). Lactate dehydrogenase release in the fatty acid-perfused hearts was associated with a significant increase in the percentage unsaturation of the sarcolemmal phospholipid fatty acids. Glucose-perfused hearts, on the other hand, showed only minor changes in the sarcolemmal phospholipid fatty acid composition. Attempts to correlate enzyme release directly with an increase in the percentage unsaturation of phospholipid fatty acids failed, since enzyme release was also stimulated in control fatty-acid-perfused hearts which (when compared with glucose) contained a higher percentage saturated phospholipid fatty acids. The results suggest that myocardial ischaemia, apart from changes in the sarcolemmal phospholipid fatty acid composition, also induces several other changes in sarcolemmal composition (e.g., cholesterol loss) which may affect is permeability for macromolecules.

Introduction

Increased sarcolemmal permeability, as manifested by release of macromolecules such as intracellular enzymes, is one of the characteristic changes occurring during myocardial ischaemia [1]. Although the exact mechanism of this phenomenon has not yet been established, recent studies indicated that rupture of the sarcolemmal membrane is not a prerequisite for this phenomenon [2–5]. There has been increasing evidence that alterations in myocardial membrane phospholipids play an important role in the pathogenesis of ischaemic cell injury [6,7]. However, phospholipid loss is a relatively late event in myocardial ischaemia (1–3 h) [6,8] and the indications are that more subtle changes in membrane structure are probably involved in enzyme release.

Evidence has been obtained that permeability of membranes other than myocytes can be markedly influenced by the fatty acid composition of phospholipids and their degree of unsaturation

Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine.

Correspondence: A. Lochner, P.O. Box 63, Tygerberg, 7505, Republic of South Africa.

[9–12]. Previous studies from this laboratory are indicative of significant changes in the physical properties of the sarcolemma in myocardial ischaemia, viz. changes in phospholipid fatty acid composition, cholesterol loss and increased microviscosity. These changes occurred early in the ischaemic process and preceded the onset of irreversible cell damage [8]. The observation that these compositional changes in sarcolemmal structure were associated with the release of significant amounts of enzymes during reperfusion (our unpublished observations) suggested a possible causal relationship. To evaluate this hypothesis, it was attempted to modulate the sarcolemmal phospholipid fatty acid composition experimentally by perfusing the isolated rat heart with different substrates (e.g., glucose or long-chain fatty acids) and to correlate enzyme release with the sarcolemmal phospholipid fatty acid composition. Due to the small size of the rat heart, it was decided to concentrate on the two main sarcolemmal phospholipids, viz. phosphatidylcholine (PC) and phosphatidylethanolamine (PE). These two phospholipid species comprise approx. 70% of the total sarcolemmal phospholipid content [13,14].

Materials and Methods

Animals

Male Wistar rats (200–300 g) were used in all experiments. Animals were fed ad libitum before experimentation and anaesthetized by intraperitoneal injection of Sagatal (pentobarbitone sodium, 30 mg/kg body weight).

Perfusion technique

The hearts were removed rapidly, arrested in ice-cold saline and perfused in a recirculating Langendorff perfusion system [15], using 20 ml Krebs-Henseleit bicarbonate solution at 37°C. The composition of the Krebs-Henseleit solution was as follows (in mM): NaCl, 124; $NaHCO_3$, 26.1; KCl, 4.93; KH_2PO_4, 1.23; $MgSO_4$, 1.62; Na_2SO_4, 0.6; $CaCl_2$, 2.5. Glucose (10 mM) as well as long-chain fatty acids (palmitate and linoleate) (free fatty acid/albumin ratio, 3.4) were used as substrates. (The albumin was 'Fraction V, fatty acid poor', obtained from Sigma, St. Louis, MO, U.S.A.) Fatty acid containing perfusates were prepared as follows: a 0.441 mM solution of albumin in Krebs-Henseleit buffer was prepared at 37°C. The correct volume of the fatty acid stock was added very slowly with constant stirring to a final concentration of 1.5 mM. Before use, the solution was filtered using a Millipore filter (0.45 μm).

Control hearts were perfused for 60 or 90 min at a perfusion pressure of 80 mmHg with 95% O_2 : 5% CO_2 as gas phase.

Before induction of ischaemia, each heart was perfused retrogradely for a control period of 15 min (perfusion pressure 80 mmHg, gas phase 95% O_2 : 5% CO_2). Global ischaemia was induced by hypoxic, low-flow perfusion (perfusion pressure 40 mmHg, gas phase 95% N_2 : 5% CO_2).

Following a 60 min period of hypoxic low-flow perfusion, reperfusion was initiated by restoring the coronary perfusion pressure to the control level of 80 mmHg and changing the gas phase to 95% O_2 : 5% CO_2. The duration of reperfusion was 30 min.

Freeze-clamping

In some experiments, the hearts were freeze-clamped with prechilled Wollenberger tongs and stored in liquid nitrogen until sarcolemmal isolation. In other experiments, the sarcolemma was prepared immediately after cessation of the experiment without prior freeze-clamping.

Sarcolemmal isolation

The sarcolemma was isolated according to a modification of the technique of Philipson et al. [14]. To obtain enough material for the subsequent lipid analysis, at least two hearts were pooled for each sarcolemmal preparation. The hearts were placed into approx. 36 ml ice-cold sarcolemmal isolation medium (250 mM sucrose/20 mM Tris-maleate/1 mM dithiothreitol, pH adjusted to 7.6 at 4°C with Tris base), cut finely with scissors and homogenized with a Polytron PT10 homogenizer (2 × 4 s, setting 4) before the homogenate was filtered through nylon gauze (pore size 200 μm). 4 ml of 3 M KCl/0.25 M sodium pyrophosphate was then added to 36 ml homogenate (final concentration 0.3 M KCl/25 mM sodium pyrophosphate) and the whole was centrifuged for 60 min at 177000 × g in a Damon IEC/B-60 ultracentrifuge. The supernatant was discarded and the

sides of the tubes were washed carefully with double distilled H_2O to remove excess KCl. The pellets were resuspended in 20 ml isolation medium. DNAase I (30000 Kunitz units, Sigma, No. D0876, DN 25) dissolved in 8 ml isolation medium (sucrose/ Tris-maleate/ dithiothreitol) was added to the resuspended pellet and incubated for 30 min at 22° C.

Both the purity and yield were improved by the DNAase treatment. After homogenization (2 × 7 s, setting 5), the suspension was centrifuged for 10 min at 12000 × *g*. The supernatant was carefully removed and centrifuged for 60 min at 220000 × *g*. The resulting pellets were resuspended in 1 ml Tris-maleate buffer (20 mM Tris-maleate/1 mM dithiothreitol, pH adjusted to 7.6 at 4° C with Tris base). The final purification step was modified to employ a 25% isopycnic metrizamide (2-(3-acetamido-5-*N*-methyl-acetamido-2,4,6-triiodo-benzamido)-2-deoxy-D-glucose) gradient. Gradients were centrifuged for 18 h at 260000 × *g*.

The sarcolemmal enzymes Na^+/K^+-ATPase and Ca^{2+}-ATPase, were used as markers for identification of the sarcolemmal fraction, using techniques described by Philipson and co-workers [14]. Mitochondrial and sarcoplasmic reticulum contamination of the final preparation was negligible, as indicated by the NAD–cytochrome-*c* reductase and glucose-6-phosphatase activity, respectively. The sarcolemmal fraction was finally centrifuged for 60 min at 70000 × *g* to obtain a pellet, which was suspended in 0.6 ml Tris-maleate buffer. A portion (0.1 ml) of this suspension was used for the determination of sarcolemmal protein content using the technique of Lowry et al. [16]. The rest of the suspension (0.5 ml) was used for phospholipid analysis.

Phospholipid extraction and separation

Sarcolemmal phospholipids were extracted according to the acid-butanol technique of Bjerve et al. [17]. After addition of 0.5 ml 1 M HCl and 0.5 ml butanol (containing the anti-oxidant, butylated hydroxytoluene) to 0.5 ml sarcolemmal suspension, the tubes were shaken and centrifuged for 10 min at 755 × *g* (4° C). The butanol phase was subsequently removed and kept on ice. The residue was re-extracted three times with 0.5 ml butanol saturated with double-distilled H_2O and the washings were combined.

Water-soluble impurities were removed by washing the butanol phase twice with 1 vol. of butanol-saturated H_2O. Lipid residues were dried at 40° C under a stream of nitrogen and dissolved in 50 µl chloroform/ methanol/ 0.9% NaCl (86 : 14 : 1, v/v). The phospholipid fractions were separated by two-dimensional thin-layer chromatography as described by Bowyer and King [18]. Plates (10 × 10 cm), coated with Kieselgel (Type 60H) were used. After activation of the plates (10 min at 110° C), 50 µl extract was spotted on each plate. Chromatograms were run in tanks lined with Whatman glass microfibre paper GF/A, containing chloroform/ methanol/ acetic acid/ H_2O (55 : 35 : 3 : 2, v/v) and chloroform/ acetone/ methanol/ acetic acid/H_2O (45 : 16 : 15 : 11 : 6, v/v), respectively. A few grains of BBOT (2,5-bis-2-(5-*t*-butylbenzoxazolyl)thiophene) were added to the separation phases to obtain fluorescence in ultraviolet light. The different phospholipid species were localized under ultraviolet light. For determination of phospholipid inorganic phosphate content, the spots were scraped off and digested in perchloric acid (100 µl) for about 1 h at 180° C. After cooling, 1 ml H_2O was added to each sample and the inorganic phosphate content was determined as described previously [19]. Recoveries of phospholipid standards added to sarcolemmal extracts averaged 97%.

Phospholipid fatty acid composition

The sarcolemmal phospholipid fatty acid composition was quantified as described by Victor et al. [19]. After separation of the phospholipids, PC and PE spots were scraped off and 2 ml methanol/ sulphuric acid (95 : 5) was added. The fatty acid moieties of the two phospholipids were transmethylated at 70° C for 2 h. The fatty acid methyl esters were extracted with 4 ml hexane/H_2O (1 : 1, v/v) (the H_2O double-distilled). The hexane layer was removed and dried under a stream of nitrogen at 37° C. Residues were dissolved in CS_2 and the fatty acid methyl ester mixtures analysed by gas chromatography. A Becker model 417 gas chromatograph with flame ionization detectors was used, together with a 2 mV potentiometric recorder. Glass U-columns, 2

m long and with 3 mm internal diameter, were packed with 10% (by weight) SP 2330 on 100–120 mesh Chromosorb WAW. DMCS. The following conditions were employed: initial oven temperature, 150 °C; temperature rise, 4 C° · min^{-1}; maximum oven temperature 210 °C; flow rate of carrier gas (nitrogen), 25 ml · min^{-1}; flow rate of hydrogen 25 ml · min^{-1}. The gas chromatograph was linked by means of an analogue-to-digital converter to a Hewlett-Packard (Model 3352B) Laboratory Data System. For standardization of the gas chromatograph, a standard containing 100 nmol/ml of each of the following fatty acids was prepared from stock solutions: 12 : 0, 14 : 0, 14 : 1, 16 : 0, 16 : 1, 18 : 0, 18 : 1, 18 : 2, 20 : 3, 20 : 4, 22 : 0 and 22 : 6. Heptadecanoic acid (20 nmol/ml) was used as internal standard. The concentrations of the sarcolemmal phospholipid fatty acids were expressed as a percentage of the total fatty acids detected.

Measurement of [1-^{14}C]palmitate incorporation into sarcolemmal phospholipids

Retrograde perfusion of the isolated rat heart (under control and hypoxic, low-flow conditions) as described before, was used. The perfusate was Krebs-Henseleit bicarbonate buffer containing 1.5 mM [1-^{14}C]palmitate (0.5 μCi/ml) (fatty acid/albumin molar ratio, 3.4). The hearts were perfused for a period of 60 min and then freeze-clamped with Wollenberger tongs and stored in liquid nitrogen pending isolation of the sarcolemmal membranes. The sarcolemmal phospholipids were extracted and separated as described above. The individual PC and PE phospholipid fractions were scraped off into 2 ml methanol (containing 3% H_2O). The samples were incubated for 2 h at 55 °C, 1 ml Soluene (Packard Instruments) and 8 ml scintillation fluid added and counted.

Enzyme release

Hearts were perfused retrogradely by the Langendorff technique in a recirculating manner as described above. In control hearts, samples (exactly 1 ml) of the perfusate were taken after 15, 60 and 90 min of perfusion. In ischaemic hearts, aliquots were taken after 15 and 60 min of hypoxic low-flow perfusion and again after a 30 min reperfusion period. Samples were stored at 4 °C and assayed within 24 h.

Lactate dehydrogenase release in the perfusate was measured by the method of Wroblewski et al. [20]. Enzyme activity was expressed as U/g wet weight present in the perfusion fluid after 15, 60 or 90 min.

Statistical analysis

Results are expressed as means ± S.E. for the number of observations, n. P values were determined by the non-paired Student's t-test, and values of $P < 0.05$ are regarded as statistically significant.

Results

Incorporation of [1-^{14}C]palmitate into sarcolemmal phosphatidylcholine and phosphatidylethanolamine of control and hypoxic, low-flow perfused hearts

To determine whether ischaemia-induced changes in the phospholipid composition of the sarcolemmal membrane play a role in increased membrane permeability, it was attempted to modulate the sarcolemmal phospholipid fatty acid composition experimentally by perfusing with different substrates. To determine the feasibility of such a study, a pilot study was performed in which control and hypoxic hearts were perfused with radiolabelled palmitate (1.5 mM [1-^{14}C]palmitate/0.441 mM albumin) to determine the degree of incorporation of fatty acids into this particular membrane. The results obtained showed that, although [1-^{14}C]palmitate was incorporated into all eight phospholipid fractions of the sarcolemma of both control and hypoxic hearts, it was found mainly in PC and PE. Hypoxic perfusion stimulated the incorporation of palmitate into both these fractions to a significant degree ($P < 0.005$) (Table I).

The effect of different substrates on sarcolemmal phospholipid fatty acid composition and membrane permeability

Glucose (10 mM) as well as saturated and unsaturated long-chain fatty acids (palmitate, linoleate) were used as substrates. These substrates

TABLE I

EFFECT OF HYPOXIC, LOW-FLOW PERFUSION ON THE INCORPORATION OF [1-^{14}C]PALMITATE INTO SARCOLEMMAL PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE

Results are expressed as nmol [^{14}C]palmitate incorporated/mg protein per 60 min. Numbers in parentheses indicate number of hearts. *P* indicates significance of difference between control and hypoxic hearts.

	Control	Hypoxic	*P*
PC	20.3 ± 0.9 (6)	31.3 ± 1.7 (8)	< 0.005
PE	15.2 ± 2.1 (6)	38.8 ± 3.2 (8)	< 0.005

had no effect on the phospholipid class composition of sarcolemmal preparations isolated from both control and hypoxic, low-flow perfused hearts (results not shown).

Control hearts

Phospholipid fatty acid composition. The results obtained clearly showed that the sarcolemmal phospholipid fatty acid composition of control perfused hearts can be affected significantly by

TABLE II

THE SARCOLEMMAL PHOSPHATIDYLCHOLINE FATTY ACID COMPOSITION IN CONTROL (60 MIN) HEARTS PERFUSED WITH GLUCOSE (10 mM), PALMITATE OR LINOLEATE (1.5 mM BOUND TO 0.441 mM ALBUMIN) AS SUBSTRATE

The fatty acids are expressed as a percentage of the total fatty acid content of PC. Numbers in parentheses indicate number of hearts. *P* indicates significance of difference between glucose-perfused hearts and fatty-acid-perfused hearts.

Fatty acid	Glucose (8)	Palmitate (4)	Linoleate (6)
16:0	18.4 ± 0.5	24.6 ± 0.04 *P* < 0.001	15.4 ± 2.4
18:0	24.5 ± 0.8	31.3 ± 0.2 *P* < 0.001	30.5 ± 2.0 *P* < 0.05
18:1	8.7 ± 0.2	10.0 ± 0.1	9.1 ± 0.2
18:2	15.4 ± 0.7	10.1 ± 0.7 *P* < 0.001	21.2 ± 2.4
20:3	0.7 ± 0.2	0.8 ± 0.05	1.1 ± 0.07
20:4	22.5 ± 1.1	16.3 ± 0.2 *P* < 0.005	11.8 ± 3.9 *P* < 0.05
22:6	9.7 ± 2.4	6.4 ± 0.3	9.4 ± 2.7
% saturated	43.3 ± 1.2	56.1 ± 0.1	46.5 ± 3.3
% unsaturated	56.9 ± 1.2	43.9 ± 0.2 *P* < 0.001	52.7 ± 2.8

TABLE II

THE SARCOLEMMAL PHOSPHATIDYLETHANOLAMINE FATTY ACID COMPOSITION IN CONTROL (60 MIN) HEARTS PERFUSED WITH GLUCOSE (10 mM), PALMITATE OR LINOLEATE (1.5 mM BOUND TO 0.441 mM ALBUMIN) AS SUBSTRATE

The fatty acids are expressed as a percentage of the total fatty acid content of PE. Numbers in parentheses indicate number of hearts. *P* indicates significance of difference between glucose-perfused hearts and fatty-acid perfused hearts.

Fatty acid	Glucose (6)	Palmitate (4)	Linoleate (6)
16:0	9.1 ± 0.1	13.8 ± 0.1 *P* < 0.001	16.1 ± 4.1
18:0	29.8 ± 0.3	46.5 ± 0.4 *P* < 0.001	24.6 ± 5.9
18:1	6.4 ± 0.2	7.0 ± 0.3	9.1 ± 1.5
18:2	13.8 ± 0.2	6.4 ± 0.1 *P* < 0.001	19.3 ± 8.2
20:3	0.5 ± 0.05	1.0 ± 0.5	3.4 ± 1.0 *P* < 0.05
20:4	14.6 ± 0.1	9.1 ± 1.2 *P* < 0.05	13.0 ± 2.7
22:6	25.7 ± 0.5	16.1 ± 1.4 *P* < 0.01	14.0 ± 7.5
% saturated	39.0 ± 0.3	60.4 ± 0.6 *P* < 0.001	40.9 ± 9.5
% unsaturated	61.0 ± 0.2	39.6 ± 0.6 *P* < 0.001	58.3 ± 9.3

the substrate employed. The sarcolemmal PC and PE composition of hearts perfused with palmitate or linoleate (1.5 mM bound to 0.441 mM albumin)

TABLE IV

LACTATE DEHYDROGENASE RELEASE BY CONTROL HEARTS PERFUSED WITH GLUCOSE (10 mM), PALMITATE OR LINOLEATE (1.5 mM BOUND TO 0.441 mM ALBUMIN)

Results expressed as U/g wet weight present in the perfusion fluid after 15, 60 or 90 min of perfusion. Numbers in parentheses indicate number of hearts. *P* indicates significance of difference between glucose-perfused hearts and fatty-acid-perfused hearts. n.s., not significant.

Time (min)	Glucose (6)	Palmitate (6)	Linoleate (6)
15	1.032 ± 0.166	0.744 ± 0.190 *P* = n.s.	0.784 ± 0.160 *P* = n.s.
60	1.156 ± 0.169	2.431 ± 0.476 *P* < 0.05	1.417 ± 0.267 *P* = n.s.
90	1.210 ± 0.236	3.325 ± 0.603 *P* < 0.01	3.562 ± 0.460 *P* < 0.005

differed significantly from those of hearts perfused with glucose alone (10 mM) (Tables II and III). In the case of PC, perfusion with palmitate caused an increase in 16:0 and 18:0, as well as reduction in 18:2 and 20:4 when compared to glucose-perfused hearts. In linoleate-perfused hearts, a significant increase in PC 18:0, with a concomitant decline in the unsaturated fatty acid, 20:4 were observed (Table II).

The fatty acid composition of sarcolemmal PE of control hearts perfused with fatty acids also changed when compared to that of glucose-perfused hearts. In the case of palmitate, the changes observed were very similar to those observed in PC, viz. a significant increase in 16:0 and 18:0 while the percentages of 18:2, 20:4 and 22:6 were reduced. The changes in PE-fatty acid composition were less marked in linoleate-perfused hearts and an increase in 20:3 was the only significant change observed (Table III).

When compared to glucose-perfused hearts, palmitate as substrate caused a significant overall increase ($P < 0.001$) in the percentage saturated fatty acids of both sarcolemmal phospholipids, PC and PE. The percentage unsaturated fatty acids decreased ($P < 0.001$) accordingly (Tables II and III).

Another interesting observation was that the fatty acid composition of PC and PE differed significantly ($P < 0.001$) in the case of glucose- and palmitate-perfused hearts: with both substrates the percentages of 16:0 and 20:4 were higher, while those of 18:0 and 22:6 were lower in PC, when compared to PE. In linoleate-perfused hearts there was, apart from a few minor changes, no significant difference between the fatty acid profiles of PC and PE.

TABLE V

GLUCOSE-PERFUSED HEARTS: THE SARCOLEMMAL PHOSPHATIDYLCHOLINE FATTY ACID COMPOSITION OF CONTROL (60 MIN) AND HYPOXIC (60 MIN) HEARTS

The fatty acids are expressed as a percentage of the total fatty acid content of PC. Numbers in parentheses indicate number of hearts. P values were in all cases not significant.

Fatty acid	Control (8)	Hypoxic (6)
16:0	18.4±0.5	20.5±2.0
18:0	24.5±0.8	26.9±2.3
18:1	8.7±0.2	8.8±0.4
18:2	15.4±0.7	12.9±1.3
20:3	0.7±0.2	0.9±0.1
20:4	22.5±1.1	19.7±1.7
22:6	9.7±2.4	10.1±1.9
% saturated	43.3±1.2	47.6±4.3
% unsaturated	56.9±1.2	52.4±4.3

TABLE VI

GLUCOSE-PERFUSED HEARTS: THE SARCOLEMMAL PHOSPHATIDYLETHANOLAMINE FATTY ACID COMPOSITION OF CONTROL (60 MIN) AND HYPOXIC (60 MIN) HEARTS

Fatty acids are expressed as a percentage of the fatty acid content of PE. Numbers in parentheses indicate number of hearts. P values were in all cases not significant.

Fatty acid	Control (6)	Hypoxic (6)
16:0	9.1±0.1	9.8±1.1
18:0	29.8±0.3	33.7±2.1
18:1	6.4±0.2	6.9±0.5
18:2	13.8±0.2	13.8±1.02
20:3	0.5±0.05	0.8±0.15
20:4	14.6±0.1	14.0±1.4
22:6	25.7±0.5	20.7±2.9
% saturated	39.0±0.3	43.7±3.5
% unsaturated	60.0±0.2	56.2±3.3

Enzyme release. Table IV shows the cumulative release of lactate dehydrogenase by control hearts perfused over a period of 90 min. Hearts perfused with glucose as substrate released a small amount of enzyme during the first 15 min of perfusion (probably due to injury during mounting) – thereafter, no significant release occurred. With both fatty acids, lactate dehydrogenase was released throughout the perfusion period, resulting in a significant increase (compared to glucose) after 90 min.

Effect of ischaemia on sarcolemmal phospholipid fatty acid composition and membrane permeability

Glucose. When compared to control hearts, hypoxic perfusion of hearts with glucose as substrate had, apart from a few minor changes, no significant effect on the fatty acid profile of the two major phospholipids, PC and PE. Although both PC and PE showed an increase in the percentage saturated fatty acids with a concomitant decline in

TABLE VII

PALMITATE-PERFUSED HEARTS: THE SARCOLEMMAL PHOSPHATIDYLCHOLINE FATTY ACID COMPOSITION IN CONTROL (60 MIN) AND HYPOXIC (60 MIN) HEARTS

The fatty acids are expressed as a percentage of the total fatty acid content of PC. Numbers in parentheses indicate number of hearts. *P* indicates significance of difference between control and hypoxic hearts; n.s., not significant.

Fatty acid	Control (4)	Hypoxic (6)	*P*
16:0	24.6±0.04	17.1±0.8	< 0.005
18:0	31.3±0.2	24.9±0.7	< 0.005
18:1	10.0±0.1	8.4±0.4	n.s.
18:2	10.1±0.7	14.4±0.9	< 0.05
20:3	0.8±0.05	0.9±0.2	n.s.
20:4	16.3±0.2	21.6±1.5	< 0.025
22:6	6.4±0.3	12.4±0.8	< 0.01
% saturated	56.1±0.1	42.2±1.2	< 0.001
% unsaturated	43.9±0.2	57.8±1.2	< 0.001

TABLE IX

LINOLEATE-PERFUSED HEARTS: THE SARCOLEMMAL PHOSPHATIDYLCHOLINE FATTY ACID COMPOSITION IN CONTROL (60 MIN) AND HYPOXIC (60 MIN) HEARTS

The fatty acids are expressed as a percentage of the total fatty acid content of PC. Numbers in parentheses indicate number of hearts. *P* indicates significance of difference between control and hypoxic hearts; n.s., not significant.

Fatty acid	Control (6)	Hypoxic (6)	*P*
16:0	15.4±2.4	14.6±0.9	n.s.
18:0	30.5±2.04	24.8±1.2	n.s.
18:1	9.1±0.2	9.0±0.7	n.s.
18:2	21.2±2.4	11.2±2.04	< 0.05
20:3	1.1±0.07	5.8±1.2	< 0.02
20:4	11.8±3.9	22.0±2.2	n.s.
22:6	9.4±2.7	9.9±0.8	n.s.
% saturated	46.5±3.3	39.4±1.0	n.s.
% unsaturated	52.7±2.8	58.1±3.4	n.s.

the percentage unsaturated fatty acids, these changes were insignificant (Tables V and VI).

Palmitate. Hypoxic perfusion of hearts with palmitate (1.5 mM bound to 0.441 mM albumin) as substrate, induced marked changes in the sarcolemmal phospholipid-fatty acid composition (Tables VII and VIII) when compared to those of the corresponding control hearts. Significant changes in the percentage composition of the PC-fatty acids occurred, viz. a reduction in the percentages of 16:0 and 18:0 associated with increases in 18:2, 20:4 and 22:6. Consequently, hypoxic low-flow perfusion resulted in a significant ($P < 0.001$) increase in the total percentage unsaturated fatty acid in PC (control, 43.9 ± 0.2;

TABLE VIII

PALMITATE-PERFUSED HEARTS: THE SARCOLEMMAL PHOSPHATIDYLETHANOLAMINE FATTY ACID COMPOSITION IN CONTROL (60 MIN) AND HYPOXIC (60 MIN) HEARTS

The fatty acids are expressed as a percentage of the total fatty acid content of PE. Numbers in parentheses indicate number of hearts. *P* indicates significance of difference between control and hypoxic hearts; n.s., not significant.

Fatty acid	Control (4)	Hypoxic (6)	*P*
16:0	13.9±0.1	8.3±0.4	< 0.001
18:0	46.5±0.4	34.7±3.1	< 0.05
18:1	7.0±0.3	6.2±0.2	n.s.
18:2	6.4±0.1	11.1±0.8	< 0.01
20:3	10.0±0.5	0.8±0.2	n.s.
20:4	9.1±1.2	14.3±0.7	< 0.05
22:6	16.1±1.4	24.4±2.3	n.s.
% saturated	60.4±0.6	43.1±3.5	< 0.001
% unsaturated	39.6±0.6	56.9±3.4	< 0.001

TABLE X

LINOLEATE-PERFUSED HEARTS: THE SARCOLEMMAL PHOSPHATIDYLETHANOLAMINE FATTY ACID COMPOSITION OF CONTROL (60 MIN) AND HYPOXIC (60 MIN) HEARTS

The fatty acids are expressed as a percentage of the total fatty acid content of PE. Numbers in parentheses indicate number of hearts. *P* values were in all cases not significant.

Fatty acid	Control (6)	Hypoxic (6)
16:0	16.1±4.1	13.1±2.4
18:0	24.6±5.9	29.6±1.2
18:1	9.1±1.5	7.3±0.6
18:2	19.3±8.2	15.4±1.9
20:3	3.4±1.0	3.4±0.3
20:4	13.0±2.7	17.5±1.6
22:6	14.0±7.5	13.4±2.3
% saturated	40.9±9.5	42.9±1.4
% unsaturated	58.3±9.3	57.1±1.4

hypoxic, 57.8 ± 1.2) with a concomitant reduction in the total percentage saturated fatty acid (control, 56.1 ± 0.1 hypoxic, 42.2 ± 1.2) (Table VII).

The sarcolemmal PE-fatty acid composition of hypoxic hearts showed similar changes. As with PC, PE exhibited significant decreases in the saturated fatty acids, 16 : 0 and 18 : 0, associated with concomitant increases in the unsaturated fatty acids 18 : 2 and 20 : 4, causing an overall increase ($P < 0.001$) in percentage unsaturation (control, 39.6 ± 0.6; hypoxic, 56.9 ± 3.43). The total percentage saturated PE-fatty acid decreased accordingly (control 60.4 ± 0.6; hypoxic 43.1 ± 3.5) (Table VIII).

Linoleate. Hypoxic perfusion of hearts with linoleate (1.5 mM bound to 0.441 mM albumin) also induced significant changes in the percentage composition of sarcolemmal PC-fatty acid, when compared to controls. The percentage of 18 : 2 decreased, while 20 : 3 increased (Table IX). In contrast with PC, the sarcolemmal PE-fatty acid composition of hypoxic hearts perfused with linoleate as substrate did not change significantly when compared to controls (Table X). Although PC showed an increase in the total percentage unsaturated fatty acids with a concomitant decline in the percentage saturated fatty acids, these changes were not significant (Table IX).

Enzyme release. To determine whether changes in the sarcolemmal phospholipid-fatty acid composition had any effect on membrane permeability the release of lactate dehydrogenase was measured under similar control and hypoxic low-flow conditions. Control hearts were perfused for 90 min, whereas the hypoxic low-flow perfused hearts were perfused for a period of 60 min followed by 30 min reperfusion.

Small amounts of lactate dehydrogenase were released from glucose-perfused hearts and no significant difference could be observed between the accumulated lactate dehydrogenase release from control (90 min) hearts and hearts perfused under hypoxic conditions for 60 min followed by 30 min reperfusion. The release of lactate dehydrogenase from both control and hypoxic hearts was significantly higher with linoleate and palmitate as substrates than with glucose (10 mM). In linoleate- and palmitate-perfused hearts, there were also

TABLE XI

LACTATE DEHYDROGENASE-RELEASE BY CONTROL AND HYPOXIC HEARTS PERFUSED WITH GLUCOSE (10 mM), LINOLEATE OR PALMITATE (1.5 mM BOUND TO 0.441 mM ALBUMIN) AS SUBSTRATE

Results are expressed as U/g wet weight present in the perfusion fluid after 15, 60 or 90 min of perfusion. (reperf. = reperfusion.). Numbers in parentheses indicate number of hearts. P indicates significance of difference between control and hypoxic hearts. P_1 indicates significance of difference between glucose- and fatty-acid perfused hearts.

Substrate	Time (min)	Control (6)	Time (min)	Hypoxic (6)	P
Glucose	15	1.032 ± 0.166	15	0.873 ± 0.252	n.s.
	60	1.156 ± 0.169	60	1.231 ± 0.356	n.s.
	90	1.210 ± 0.236	30 (reperf.)	2.302 ± 0.489	n.s.
Palmitate	15	0.744 ± 0.190 P_1 = n.s.	15	0.659 ± 0.189 P_1 = n.s.	n.s.
	60	2.431 ± 0.476 $P_1 < 0.05$	60	3.728 ± 0.274 $P_1 < 0.001$	< 0.05
	90	3.325 ± 0.603 $P_1 < 0.01$	30 (reperf.)	8.140 ± 0.923 $P_1 < 0.001$	< 0.005
Linoleate	15	0.784 ± 0.160 P_1 = n.s.	15	1.696 ± 0.219 $P_1 < 0.05$	< 0.01
	60	1.417 ± 0.207 P_1 = n.s.	60	3.076 ± 0.488 $P_1 < 0.02$	< 0.002
	90	3.562 ± 0.460 $P_1 < 0.005$	30 (reperf.)	8.807 ± 1.086 $P_1 < 0.001$	< 0.005

marked differences between the lactate dehydrogenase release of control and hypoxic hearts (Table XI), particularly during the reperfusion period.

Discussion

Despite the widespread use of enzyme release as an indicator of the severity of ischaemic damage [5,21], surprisingly little is known about the basic changes occurring in the cell membrane leading to increased permeability for macromolecules. Phospholipid loss does not occur during the 60 min hypoxic, low-flow condition and can be ruled out as a possible causal factor (see Results). ATP loss, particularly of that fraction generated during glycolysis, has been shown to be of particular importance in the development of impaired membrane integrity [22,23]. Evidence has also been obtained that permeability of cells other than myocytes can be markedly influenced by the fatty acid composition of membrane phospholipids and particularly their degree of unsaturation [9,10,12].

To evaluate whether changes in sarcolemmal composition have an effect on its permeability, the sarcolemmal fatty acid composition was modulated experimentally by perfusing hearts with different substrates and correlated with enzyme release. The choice of glucose and long-chain fatty acids as substrates was based on the observations of Bricknell and Opie [23] that glucose-perfused ischaemic hearts had the lowest rate of lactate dehydrogenase release, while palmitate-perfused hearts showed the greatest release of lactate dehydrogenase. Furthermore, fatty acids are highly hydrophobic, intercalate readily into the membrane bilayer and can produce significant changes in the packing of lipid molecules [24].

Technique for modulation of sarcolemmal phospholipid fatty acid composition

In view of the possibility that relatively long-term exposure of both control and ischaemic hearts to the different substrates might be required to induce changes in phospholipid fatty acid, the hypoxic low-flow perfused heart was chosen as a model of global ischaemia. This model has many characteristics in common with the working rat heart model of global ischaemia as well as regional ischaemia [25].

A prerequisite for our hypothesis is that the long-chain fatty acids should be incorporated into sarcolemmal phospholipids. Using [1-^{14}C]palmitate as substrate, it was found that under both control and hypoxic perfusion conditions significant amounts of the radiolabelled compound were incorporated preferentially into PC and PE (Table I). Vasdev and Kako [26] also reported that the formation of radiolabelled PC was the highest when perfusing with linoleic acid, followed by palmitic acid.

Subcellular distribution of labelled fatty acids should be interpreted with great caution, since radioactive material can be transferred from one fraction to another during ultracentrifugation [27,28]. However, this process is greatly diminished once these fatty acids are incorporated into phospholipids. This process has been shown to be very rapid and will counteract 'cross-fire' between cell fractions [27].

Due to the small size of the rat heart and consequently the relatively low yield of sarcolemmal membranes, it was decided to concentrate on the two major phospholipids, viz. PC and PE. Not only do PC and PE comprise approx. 70% of the total sarcolemmal phospholipid content [8,13,29,30], but in addition, exogenous fatty acids are incorporated mainly into these two phospholipids (Table I).

Sarcolemmal phospholipid fatty acid composition

Control hearts. The results obtained showed that the fatty acid composition of both PC and PE is dependent on the substrate used during perfusion. Another interesting observation was that the fatty acid composition of PC and PE differed significantly in glucose- and palmitate-perfused hearts.

Hypoxic, low-flow perfused hearts. Two noteworthy observations were made in these experiments:

(i) In contrast to the results obtained in control hearts, the substrate used had no effect on the fatty acid composition of both PC and PE in hypoxic low-flow perfused hearts.

(ii) Comparison of the sarcolemmal phospholipid-fatty acid composition of control and hypoxic, low-flow perfused hearts showed that when glucose was used as substrate, no significant

change occurred (Tables V and VI). On the other hand, fatty acid perfused hypoxic hearts showed marked changes in the fatty acid composition of both PC and PE when compared to those of the corresponding control hearts (Tables VII–X). The most significant changes were obtained with palmitate as substrate, viz. an increase in the total percentage unsaturated fatty acids (18:2, 20:4 and 22:6) with a concomitant reduction in saturated fatty acids (16:0, 18:0). These results suggest a significant degree of chain elongation and desaturation occurring simultaneously, since [1-^{14}C]palmitate incorporation into PC and PE was increased under these experimental conditions.

Correlation between enzyme release and phospholipid fatty acid composition

The finding that glucose-perfused hearts had the lowest rate, while palmitate-perfused hearts had the highest rate of enzyme release under both control and hypoxic low-flow perfusion conditions, confirm the earlier findings of Bricknell and Opie [23].

The observation by these workers that acetate- and palmitate-perfused hearts showed the greatest release of lactate dehydrogenase suggested that it was the metabolic and not the detergent effects of palmitate that were operating, and glycolytically derived ATP was suggested to be an important factor in the action of glucose on hypoxic tissue. Higgins and Bailey [22] suggested three possible mechanisms for ATP in the maintenance of membrane integrity, viz. (i) involvement in the biosynthesis of new membrane components, (ii) maintenance of ionic homeostasis by driving ion pumps and (iii) maintenance of the cell membrane in a highly phosphorylated state.

Although the evidence for the role of glycolytically produced ATP in the maintenance of membrane integrity is convincing, a substantial amount of evidence has recently accumulated on the physical disturbance caused by fatty acid–membrane interactions [31]. Fatty acids have long been known to affect a variety of membrane-related metabolic reactions [32–37] and can exert a biphasic effect on membrane permeability and stability – e.g., low concentrations of fatty acids can reduce membrane permeability while higher fatty acid concentrations, by their detergent-like actions, abolish the ability of the membranes to serve as a permeability barrier [38].

It is possible that the membrane effects of fatty acids could be due to both (i) the presence of these compounds in the free form, intercalating into cell membranes and producing local lipid disorder and (ii) exerting its effects via rapid incorporation into phospholipids and changing of the phospholipid fatty acid composition.

The observation that the relatively unchanged phospholipid fatty acid composition in hypoxic low-flow glucose-perfused hearts (Tables V and VI) was associated with minimal enzyme release (Table XI), while the significant increase in phospholipid fatty acid unsaturation in fatty-acid-perfused hearts was accompanied by higher rates of enzyme release (Tables VII–X), was suggestive of a possible link between increased membrane permeability and a perturbation in phospholipid fatty acid composition.

Attempts to directly correlate enzyme release with the percentage unsaturation of phospholipid fatty acid failed due to two factors:

(i) Enzyme release was also stimulated in control fatty-acid perfused hearts. Such hearts, when compared with glucose as substrate, contained a higher percentage saturated phospholipid fatty acid (Table II).

(ii) Despite the marked differences in enzyme release, the sarcolemmal phospholipid fatty acid composition of hypoxic hearts was similar regardless of the substrate used (Tables V–X). Both these observations make a positive correlation between the degree of phospholipid fatty acid unsaturation and increased membrane permeability unlikely.

However, in both control and low-flow hypoxic conditions, changes in phospholipid fatty acid composition were associated with enzyme release. For example, in both palmitate- and linoleate-perfused hearts, a change to a higher percentage unsaturation was associated with a higher rate of enzyme release.

Therefore, although the results obtained in this study could not demonstrate a direct correlation between sarcolemmal phospholipid fatty acid composition and permeability, it should be kept in

mind that ischaemia also induces several other compositional changes in the sarcolemmal phospholipid bilayer, e.g., loss of cholesterol [19] as well as a reduction in PC biosynthesis (Lochner, A. and De Villiers, M., unpublished data). A combination of the above changes, all of which have membrane effects, may play an important role in increasing membrane permeability in myocardial ischaemia.

Acknowledgements

We wish to thank Prof. J. Lochner for reading the manuscript and for many helpful discussions. We wish to thank Miss Anemie Jordaan, Mrs. Leonore Griessel and Mr. Tommie Victor for their expert technical assistance. The South African Medical Research Council is thanked for financial support.

References

1 Hearse, D.J. (1978) in Enzymes in Cardiology: Diagnosis and Research (Hearse, D.J. and De Leiris, J., eds.) pp. 1–19, John Wiley & Sons, Chichester.
2 Sakai, K., Gebhard, M.M., Spieckermann, P.G. and Bretschneider, H.J. (1975) J. Mol. Cell. Cardiol. 7, 827–840.
3 Sobel, B.E., Roberts, R. and Larson, K.B. (1976) Circ. Res. 38, 99.
4 Spieckermann, P.G., Nordbeck, H. and Preusse, C.J. (1979) in Enzymes in Cardiology: Diagnosis and Research (Hearse, D.J. and De Leiris, J., eds.), pp. 81–95, John Wiley & Sons, Chichester.
5 De Leiris, J. and Hearse, D.J. (1984) in Methods in Studying Cardiac Membranes, Vol. 1 (Dhalla, N.S., ed.), pp. 253–278. CRC Press, Boca Raton, FL.
6 Chien, K.R., Reeves, J.P., Buja, L.M., Bonte, F., Parkey, R.W. and Willerson, J.T. (1981) Circ. Res. 48, 711–719.
7 Sen, A., Buja, L.M., Willerson, J.T. and Chien, K.R. (1987) Basic Res. Cardiol. 82, Suppl. 1, 121–125.
8 Lochner, A., Sanan, D., Victor, T., Bester, R., Kotzé, J.C.N., Van der Merwe, N. and Shabort, I. (1985) in Membrane and Muscle (Berman, M.C., Gevers, W. and Opie, L.H., eds.), pp. 309–325, IRL Press, Oxford.
9 Holmes, R.P., Mahfouz, M., Travis, B.D., Yoss, N.L. and Keenan, M.J. (1983) N.Y. Acad. Sci. 414, 44–56.
10 Kummeron, F.A. (1983) N.Y. Acad. Sci. 414, 29–43.
11 Rothman, J.E. and Lenard, J. (1977) Science 195, 743–753.
12 Stubbs, C.D. and Smith, A.D. (1984) Biochim. Biophys. Acta 779, 89–137.
13 Tibbits, G.F., Sasaki, M., Ikeda, M., Shimada, K., Tsuruhara, T. and Nagatomo, T. (1981) J. Mol. Cell. Cardiol. 13, 1051–1061.
14 Philipson, K.D., Bers, D.M. and Nishimoto, A.Y. (1980) J. Mol. Cell. Cardiol. 12, 1159–1173.
15 Langendorff, O. (1885) Pflügers Arch. 61, 291–332.
16 Lowry, A.O., Rosebrough, N.J., Farr, A.L. and Randall, R.J., (1951) J. Biol. Chem. 193, 265–275.
17 Bjerve, K.S., Daae, L.N.W. and Bremer, J. (1974) Anal. Biochem. 58, 238–245.
18 Bowyer, D.E. and King, J.P. (1977) J. Chromatogr. 143, 473–490.
19 Victor, T., Van der Merwe, N., Benade, A.J.S., La Cock, C. and Lochner, A. (1985) Biochim. Biophys. Acta 834, 215–223.
20 Wroblewski, F. and La Due, J.S. (1955) Proc. Soc. Exp. Biol. Med. 90, 210–213.
21 Kjeksus, J.K. and Sobel, B.E. (1970) Circ. Res. 27, 403–414.
22 Higgins, T.J.C. and Bailey, P.J. (1983) Biochim. Biophys. Acta 762, 67–75.
23 Bricknell, O.L. and Opie, L.H. (1978) Circ. Res. 43, 102–115.
24 Klausner, R.D., Kleinfeld, A.M., Hoover, R.L., Karnovsky, M.J. (1980) J. Biol. Chem. 255, 1286–1295.
25 De Kock, A., Lochner, A., Kotzé, J.C.N. and Gevers, W. (1978) Basic Res. Cardiol. 73, 506–527.
26 Vasdev, S.C. and Kako, K.J. (1977) J. Mol. Cell. Cardiol. 9, 617–631.
27 Fields, M., Gloster, J.G. and Harris, P. (1975) J. Mol. Cell. Cardiol. 7, 970–916.
28 Ward, B. and Harris, P. (1984) J. Mol. Cell. Cardiol. 16, 765–770.
29 Feldman, D.A. and Weinhold, P.A. (1977) Biochemistry 16, 3470–3475.
30 Panagia, V., Lamers, J.M.J., Singal, P.K. and Dhalla, N.S. (1982) Int. J. Biochem. 14, 387–397.
31 Katz, A.M. and Messineo, F.C. (1981) Circ. Res. 48, 1–16.
32 Pressman, B.C. and Lardy, M. (1956) Biochim. Biophys. Acta 21, 458–466.
33 Pande, S.V. and Mead, J.F. (1968) J. Biol. Chem. 243, 6180–6185.
34 Philipson, K.D. and Ward, R. (1985) J. Biol. Chem. 260, 9666–9671.
35 Philipson, K.D. and Ward, R. (1987) Biochim. Biophys. Acta 897, 152–158.
36 Katz, A.M., Nash-Adler, P., Watras, J., Messineo, F.C., Takenska, H. and Louis, C.F. (1982) Biochim. Biophys. Acta 687, 17–26.
37 Messineo, F.C., Rathier, M., Favreau, C., Watras, J. and Takenaka, H. (1984) J. Biol. Chem. 259, 1336–1343.
38 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79.