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Anoxic injury accelerates phosphatidylcholine degradation in cultured cardiac myocytes by phospholipase C

Nabil Nachas, Arié Pinson

Laboratory for Myocardial Research, The Hebrew University - Hadassah Medical School, P.O.Box 1172, Jerusalem 91010, Israel

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In neonatal cultured cardiac myocytes under normoxic conditions, $^{32}P_i$ incorporation pattern into various phospholipids, and double-labeling experiments with $^{32}P_i$ and [^{3}H]methyl choline, suggest that phosphatidylcholine and phosphatidylinositol are turned over rapidly, whereas the turnover of phosphatidylethanolamine is probably much slower. While increased levels of the corresponding lysophospholipids were not found under anoxia, release of diacylglycerol and phosphorylcholine was observed. These data strongly suggest that phospholipase C, and not phospholipase A_2, is involved in phospholipid degradation in cultured cardiomyocytes under anoxic conditions.

Cultured cardiomyocyte; Anoxia; Phospholipid degradation; Phospholipase C

1. INTRODUCTION

Previous studies on the effect of oxygen restriction or deprivation on myocardium showed that the shift from aerobic to anaerobic metabolism under anoxic conditions led to ATP depletion [1] accompanied by increased lactic acid formation [2]. Moreover, increases in arachidonic acid [2–6] and cellular Ca^{2+}[7] levels have also been implicated in the process leading to irreversible cell injury during anoxia and ischemia. The leakage of cellular enzymes during oxygen deprivation is believed to be the result of sarcolemmal damage caused by impaired cellular metabolism.

It was also shown that a decrease in cellular phospholipid levels, particularly in phosphatidylcholine and phosphatidylethanolamine, occurs concomitantly with the increase in arachidonic acid concentration [3,4,6]. Based on these results, it was suggested that phospholipid degradation causes disruption of plasmalemma and loss of membrane integrity and function, leading to leakage of cellular enzymes. Despite the intensive research on phospholipid degradation and arachidonic acid mobilization under ischemic conditions, the exact mechanism underlying this process is still unclear. Several investigators suggested the involvement of phospholipase A_2 in the degradation of phospholipids [8,9], whilst others failed to find an increase of lysophosphoglycerides during oxygen deprivation [4,10]. Various laboratories reported that metabolic inhibitors causing ATP depletion can closely mimic ischemic conditions [11,12], and that ATP depletion occurs prior to arachidonic acid accumulation during ischemic insult [12]. These results suggest that depletion of ATP stores under ischemic conditions is the key step in the sequence of biochemical events leading to plasmalemmal damage and cell death.

Recent reports have implicated the involvement of phospholipses C and D in phosphatidylcholine degradation promoted by hormones, growth factors, neurotransmitters and related agonists in a broad variety of tissues [13,14].

In this study, we show that phosphatidylcholine in neonatal cardiac myocytes has the highest turnover among the phospholipid classes, is most affected by anoxic conditions, and that its degradation under anoxic conditions is promoted by phospholipase C.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; PA, phosphatidic acid; PIP, phosphatidylinositol monophosphate; PIP2, phosphatidylinositol diphosphate; LPC, lysophosphatidylcholine; TAG, triacylglycerol; DAG, diacylglycerol; AA, arachidonic acid; CE, cholesterol ester.

Correspondence address: A. Pinson, Laboratory for Myocardial Research, The Hebrew University - Hadassah Medical School, P.O.Box 1172, Jerusalem 91010, Israel. Fax: (9722) 784010.

2. MATERIALS AND METHODS

2.1. *Materials*

[13–^{14}C]arachidonic acid (45 mCi/mmol) was obtained from CEA (Saclay, France), [^{3}H]methyl choline chloride (75 mCi/mmol) from Amersham (England), and $^{32}P_i$ from the Negev Radiochemistry Research Centre (Israel). Organic solvents and the silica gel G thin layer chromatography plates were obtained from Merck (Germany). All the other chemicals were purchased from Sigma (Israel).

2.2. *Cell culture*

Cell cultures from 1-day-old rats were prepared as previously described [15,16]. Approximately 2×10^6 cells were seeded onto 35

mm-diameter Petri dishes (Falcon 3001). On the fifth day of culture, over 80% of the culture consisted of beating cardiomyocytes [16].

2.3. *Incubation and anoxic conditions*

The cells were used after 5 days in culture. The growth medium was discarded and cells were immediately incubated at 37°C with either F-10 culture medium containing 0.8% bovine albumin Fraction V, fatty acid free and 200 μM [13-^{14}C]arachidonic acid (2,000–3,000 dpm/nmol) or $^{32}P_i$ in isotonic Tyrode's buffer (pH 7.4) without phosphate, establishing isotonic conditions by increasing NaCl concentration. [^{3}H]choline and $^{32}P_i$ were added for double labeling of phosphatidylcholine, in the same incubation conditions. After incubation with labeled substances for various time intervals, the cells were washed with 2 ml Tyrode's buffer without phosphate and 1 ml of the same buffer was then added. The cells were immediately scraped off into tubes and the lipids were extracted according to the method of Bligh and Dyer [17]. To extract phosphoinositides, 1 ml of 1 N HCl was added to the extraction medium (acidic Bligh and Dyer). To study the effect of anoxia on phospholipid degradation, following incubation with radioactive substrates, the cells were washed with Tyrode's buffer without phosphate, which had been pre-equilibrated with 95% N_2/5% CO_2 for at least 20 min. Anoxic conditions were achieved in 1 ml of the pre-equilibrated buffer using a special incubation device in an atmosphere of 95% N_2/5% CO_2 saturated with water at 37°C, as described previously [18]. Simultaneously, control incubations under normoxic conditions were carried out. The incubations were terminated by adding 1 ml methanol, the cells scraped off into separate tubes, and the lipids extracted, as described above.

2.4. *Lipid separation and analysis*

The chloroform phase from the lipid extraction was dried under a stream of nitrogen and the lipids were dissolved in 100 μl C/M (2:1). Neutral lipids and fatty acids were separated by spotting the extract onto thin layer plates and developed in hexane/diethyl ether/methanol/acetic acid (90:20:5:2) with the appropriate markers (17). The lipid spots were visualized by a brief exposure to iodine vapour, scraped off into scintillation vials, and counted for radioactivity using Packard scintillation counter. The phospholipids were separated by a monodimensional thin layer chromatography using either chloroform/methanol/ammonia (65:25:5) or chloroform/methanol/4 N ammonia (9:7:2) as the solvent system. The second solvent system was mainly used for separating $^{32}P_i$ labeled phosphoinositides. Two-dimensional chromatography was carried out with chloroform/methanol/ammonia/water (50:50:9:5, by volume) in the the first dimension, and chloroform/methanol/formic acid (11:5:1, by volu me) in the second. The phospholipid spots were visualized by autoradiography using 18 × 24 cm Agfa X-ray films. The various phospholipid spots were scraped off into scintillation vials and counted for radioactivity.

2.5. *Separation of choline and choline phosphate*

Choline and choline phosphate were found in the aqueous phase of the lipid extract. The aqueous phase was dried under a stream of N_2 at 37°C and the residue dissolved in 50 μl water. Choline and choline phosphate were then analyzed by thin layer chromatography using the solvent system methanol/0.6% NaCl/ammonia (50:50: 5) with choline and choline phosphate as markers. In this system the R_f values for choline and choline phosphate were 0.17 and 0.48, respectively. The corresponding spots were scraped off and counted for radioactivity.

3. RESULTS

Time-dependent $^{32}P_i$ incorporation into various phospholipids as well as the incorporation of $^{32}P_i$ and [^{3}H]choline into phosphatidylcholine are shown in Fig. 1. The incorporation of $^{32}P_i$ into various phospholipids followed a biphasic pattern. The first slow phase of 1 h in duration was followed by a second rapid phase,

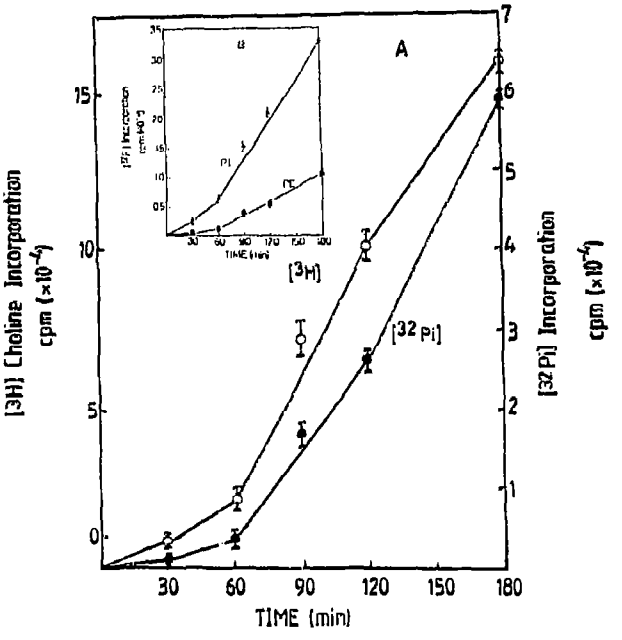

Fig. 1. Time-dependent incorporation of [^{3}H]choline and $^{32}P_i$ into phospholipids. The cells were incubated with [^{3}H]choline (3.5 × 10^5 cpm/ml) and $^{32}P_i$ (1.9 × 10^6 cpm/m) for 1 h at 37°C in 1 ml Tyrode's buffer (pH 7.4) without inorganic phosphate. At various time intervals, the incubation was stopped and the cell lipids were extracted. The lipid extract from each incubation was analyzed using a monodimensional thin layer chromatography with chloroform/methanol/4 N ammonia (9:7:2). The various phospholipids were visualized by autoradiography. The corresponding spots were scraped off and counted for radioactivity using a ^{3}H/^{32}P double-labeling counting program. (A) incorporation of [^{3}H]choline and $^{32}P_i$ into PC; (B) incorporation of $^{32}P_i$ into PE and PI.

which lasted for at least the next 2 h. The incorporation of [^{3}H]choline and $^{32}P_i$ given simultaneously to cardiac myocytes (double labeling) shows similar and parallel incorporation of these radiolabeled compounds into phosphatidylcholine (Fig. 1A), the latter being more heavily labeled than phosphatidylinositol and phosphatidylethanolamine. However, under anoxic conditions, the $^{32}P_i$ incorporation into phosphatidylcholine was 23% and 11% of the control value after incubation for 2 h and 4 h, respectively.

Degradation of myocardial cell phospholipids under anoxic conditions was assessed from the extent of radioactivity loss from each class of phospholipids after prelabeling of cardiac myocyte phospholipids with $^{32}P_i$. Table I shows that incubation of cardiac myocytes for 1 h under anoxic conditions results in the degradation of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate. Depletion of phosphatidylcholine and phosphatidylethanolamine was 62% and 36%, respectively, as compared to 11–22% for the other phospholipids. The radioactivity found in sphingomyelin, which was poorly labeled with $^{32}P_i$, was

Table I

Degradation of phospholipids in myocardial cells during anoxia

Phospholipid class	Normoxia (cpm)	Anoxia
PC	42,131	16,206
PE	4,459	2,887
PI	18,296	14,470
PS	n.d.	n.d.
SM	496	958
PA	748	716
PIP	3,076	2,408
PIP_2	8,726	7,736
LPC	1,510	1,372

Myocardial cells were incubated for 1 h at 37°C wtih $^{32}P_i$ (4.26×10^6 cpm/ml) in 1 ml Tyrode's buffer (pH 7.4) without phosphate, as described in section 2. At the end of incubation, the buffer was discarded and replaced with 1 ml of the same buffer. For anoxic conditions, the buffer was pre-equilibrated for 20 min with N_2/CO_2 (95:5%). Incubations were cotinued either under anoxic or normoxic conditions for 1 h and were stopped by adding 1 ml methanol, the cells scraped off, and the lipids were extracted according to Bligh and Dyer using 1 ml of 1 N HCl instead of water (acid extraction). The lower phase was dried under a stream of N_2 and phospholipids were separated using two-dimensional thin layer chromatography, as described in section 2. The phospholipids were visualized by autoradiography, scraped off, and counted for radioactivity. The results are given for one representative experiment out of three similar incubations.

almost doubled following anoxia. Under the anoxic conditions employed, the amount of phosphatidic acid and lysophosphatidylcholine were not affected by oxygen deprivation. Lysophosphatidylethanolamine could not be detected under the experimental conditions employed.

Since phosphatidylcholine was the main phospholipid depleted under anoxic conditions with no increase in the accumulation of lysophosphatidylcholine or phosphatidic acid, the possible involvement of phospholipase C in the degradation of phosphatidylcholine

Table II

Changes in neutral lipids and arachidonic acid in myocardial cells during anoxia

Neutral lipid	Normoxia (dpm)	Anoxia
TAG	3,768 ± 124	3,468 ± 163
DAG	1,172 ± 98	3,110 ± 86
AA	1,038 ± 53	2,078 ± 102
CE	826 ± 86	806 ± 77

Cardiac myocytes were incubated with 200 μM [^{14}C]arachidonic acid (3,490 dpm/nmol) in 1 ml F-10 medium with 0.8% human serum albumin (HSA) at 37°C for 2 h. At the end of incubation, the medium was discarded and replaced with 1 ml Tyrode's buffer without glucose. For anoxia, the buffer was pre-equilibrated with N_2/CO_2 (95:5%) for at least 20 min. Incubation was then carried out either in anoxic or normoxic conditions, as described in section 2. At the end of incubation, the lipids were extracted, and the neutral lipids were analyzed using thin layer chromatography. The results are expressed as the mean ± average deviation of two experiments carried out in duplicate.

Table III

Effect of anoxia on the accumulation and phosphorylation of choline

	Normoxia (dpm)	Anoxia
Choline	16,900 ± 826	14,153 ± 1,348
Phosphorylcholine	31,709 ± 2,026	105,249 ± 6,172

The myocytes were incubated with [^{3}H]choline in 1 ml Tyrode's buffer (2.7×10^6 dpm/ml) at 37°C for 2 h. At the end of incubation, the medium was discarded and replaced with Tyrode's buffer without [^{3}H]choline. For anoxic conditions, the buffer was pre-equilibrated with a mixture of N_2/CO_2 (95/5%) for 20 min at room temperature. The incubation was continued for 1 h at 37°C either under anoxic or normoxic conditions and stopped by adding 1 ml methanol. The cells were then scraped off into tubes and the cellular lipids extracted. The aqueous phase was removed for analysis of choline and phosphorylcholine. The chloroform phase was analyzed by TLC and peaks collected and the radioactivity measured as described in Section 2. The radioactivities of phosphatidylcholine in the lipid extract under anoxic and normoxic conditions were 240,827 ± 5,394 and 359 ± 7,148 dpm, respectivelyl The results are the mean ± S.E.M. of an experiment carried out in triplicate.

was postulated. To test this hypothesis, cells were labeled with [^{14}C]arachidonic acid (Table II) or with [^{3}H]choline (Table III) and the accumulation of the degradation products of phosphatidylcholine by the action of phospholipase C (i.e. diacylglycerol and phosphorylcholine) was followed. As shown in Table II, anoxic conditions resulted in the accumulation of arachidonic acid and diacylglycerol. Triacylglycerol and cholesterolester were not affected by anoxia. Table III shows that phosphorylcholine was accumulated in myocytes under anoxic conditions, while choline levels were slightly reduced. The amount of radioactivity in the accumulated phosphorylcholine under anoxic conditions closely reflects the loss of radioactivity found in phosphatidylcholine.

4. DISCUSSION

The results presented in Fig. 1 show that $^{32}P_i$ incorporation into PC is approximately 4- and 15-fold higher than into PI and PE, respectively. This suggests a high PC turnover, as compared to the other two major PLs. However, since PC is the major PL in cardiac myocytes and the PC/PI and PC/PE molar ratios are 4 and 1.5, respectively [20]. Therefore, expressed as relative amounts, the labeling of PC and PI is the same. The low P_i incorporation into PE therefore indicates a real slower turnover of PE and/or its rapid methylation to PC.

It has long been known that oxygen deprivation in myocardial cells causes a marked depletion of ATP and sarcolemmal phospholipids and accumulation of arachidonic acid and intracellular Ca^{2+} [1–7]. The shift from aerobic to anaerobic metabolism giving rise to a dramatic decrease in the energy state of the cell preceding arachidonic acid accumulation (i.e. phospholipid

degradation). Moreover, the use of metabolic inhibitors of glycolysis and oxidative phosphorylation causes changes in heart cell metabolism similar to those found under anoxic conditions [12,21]. It has been suggested that phospholipid degradation, especially phosphatidylcholine and phosphatidylethanolamine, plays an important role in the process of ischemic cell injury through modifications in the sarcolemmal structure, the mechanism underlying phospholipid depletion is still in dispute [4,8–10].

The present study demonstrates that the decreased intracellular phospholipids under anoxic conditions was not accompanied by an increase in the corresponding lysophospholipids, and thus cannot be attributed to phospholipase A_2 activity. Phospholipase A_2 was ruled out by other investigators [8,10], however, the mechanism of phospholipid degradation under anoxic conditions remained unresolved.

As shown in this manuscript phosphatidylcholine undergoes rapid degradation followed by resynthesis, which is reflected in the rapid and simultaneous incorporation of $^{32}P_i$ and [^{3}H]choline into this phospholipid in normoxic cells. This result is substantiated by the increased accumulation of diacylglycerol and phosphorylcholine found in myocytes under anoxic conditions. Contrary to the negligible degradation of phospholipids and arachidonic acid accumulation at 3 h of ischemia reported by others [4], our results clearly show a marked degradation of phospholipid and accumulation of arachidonic acid and diacylglycerol, thus demonstrating that the degradation of phosphatidylcholine in neonatal cardiac myocytes under anoxic conditions is promoted by phospholipase C. This degradation of phosphatidylcholine is similar to polyphosphoinositide degradation in many tissues, which has been implicated in many cell stimuli involving prostaglandin biosynthesis and intracellular Ca^{2+} mobilization [22,23]. These results do not rule out the possibility of phospholipase A_2 involvement in phospholipid degradation during the late stages of ischemic damage. However, the involvement of phospholipase C in the degradation of phosphatidylcholine and possibly polyphosphoinositides might be one of the main initial steps leading to phospholipid degradation, arachidonic acid mobilization and cell damage during the early stages of anoxia.

Phosphatidylcholine degradation under anoxic conditions could not be attributed to increased turnover since $^{32}P_i$ incorporation was markedly reduced under anoxic, as compared to normoxic, conditions. The increased labeling of sphingomyelin with $^{32}P_i$ during anoxia lends further support to the involvement of phospholipase C in the degradation of phosphatidylcholine: the phosphorylcholine released under anoxic conditions serves as a precursor for sphingomyelin biosynthesis. Involvement of phospholipase D in phosphatidylcholine degradation under anoxic conditions could not be substantiated since increased labeling of phosphatiditic acid and choline was not found.

Degradation of diacylglycerol by diacylglycerol lipase is also thought to be involved in the process of arachidonic acid release [24]. Since phosphoinositides are also degraded in myocytes under anoxic conditions, it is reasonable to assume that phospholipase C may be involved in this reaction. In other tissues, it has been shown that the degradation of phosphatidylinositol 4,5 bisphosphate by phospholipase C causes accumulation of diacylglycerol and inositol triphosphate; the latter is an intracellular second messenger responsible for Ca^{2+} release from the endoplasmic reticulum [22]. If phospholipase A_2 plays a role in ischemic damage, since it has a high K_m for Ca^{2+}, this step might only occur subsequently to an increase in intracellular Ca^{2+} concentration.

In conclusion, the present study shows that in normoxic cultured cardiac myocytes, there is a high phosphatidylcholine turnover, as indicated by the rapid concomitant $^{32}P_i$ and [^{3}H]choline incorporation. Phosphatidylcholine degradation by phospholipase C seems to be among the major events induced by anoxic injury.

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