Division of Medicine
Dana-Farber Cancer Institute and
Harvard Medical School
Boston, MA 02115, U.S.A.

ROBERT J. FRAM*
DONALD W. KUFE†

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* Current address: Division of Oncology, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01605.

† To whom reprint request should be addressed at: Dana-Farber Cancer Institute, 44 Binney Street, D1610, Boston, MA 02115.

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Distinctive responses of heart muscle and non-muscle cells to oxygen and glucose deprivation as regards phospholipid fatty acids

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Heart cells in culture allow metabolic and pharmacological investigations to be performed excluding the systemic reactions and interactions challenged in the whole heart by experimental conditions and environmental factors [1].

Moreover, they offer the possibility of distinguishing the particular responses between muscle and non-muscle cells, which coexist in heart tissue, by growing them separately. Compared to non-muscle cells, one of the distinctive features of muscle cell and mixed cell cultures is their beating activity, which is correlated to a higher level of β-oxidation [2].

In the last few years, cardiac cell cultures served also as an experimental model to several authors who studied the effects of oxygen deprivation upon heart tissue (see for instance [1–7]). In our laboratory, we considered fatty acid oxidation and non-esterified fatty acid composition [8], energy metabolism [9], hypoxanthine and enzyme leakage [10]. The present work was designed to characterize the ischemia-induced modifications in membrane phospholipids in the same preparation.

Rat heart cells were cultured either mixedly (H cells) or separately (M = muscle cells, F = non-muscle cells). Ischemia was mimicked by imposing simultaneously to the cultures three conditions related to it: partial oxygen deprivation, deficiency of energy-yielding substrates, especially glucose, and accumulation of metabolic end-products. Phospholipids were scrutinized with regard to their fatty acid composition.

Materials and methods

Heart cell cultures. These were prepared from 3-day-old rats (Sprague–Dawley) according to Harary and Farley [11] with some modifications. Cells were isolated from the minced hearts by trypsinization at 23°. The enzyme, twice crystallized from pig pancreas (Labor. Choay, Paris) was used at a 0.5–1.0 g/l concentration in Dulbecco's buffered Ca^{2+}- and Mg^{2+}-free salt solution. Repeated 5-min incubations with trypsin were done until the tissue was almost completely dispersed. To obtain mixed cell cultures (H), the cells were plated into Corning culture flasks at a density of $1.2.10^5/cm^2$ in Eagle's minimum essential medium (MEM) (4.2 ml/1.10^6 cells) containing Earle's salts, glucose 5.55 mM, antibiotics and 10% calf serum. Muscle and non-muscle cells were separated by means of a differential attachment technique, based on the fact that non-muscle cells attached faster to the substrate of the culture flask. To establish F cultures, the trypsinized cells were plated at a density of $3.2.10^5/cm^2$ in MEM (1.1ml/1×10^6 cells) and incubated at 37°. Thirty-five to 40 min later, F cells interspersed with 5–10% M cells were attached to the flask. The medium, which contained floating M cells interspersed with 10% F cells, was recovered, replaced in F cell flasks by fresh medium and centrifuged. The cell pellet (M cells) was resuspended in MEM and plated at a density of $1.61.10^5/cm^2$ in MEM (3.1 ml/1.10^6 cells). M, F and H cell cultures were grown for 6 days at 37°. The gas phase consisted of air without addition of CO_2. The pH was adjusted every

other day with a 4.2% (w/v) $NaHCO_3$ sterile solution in the presence of phenol red as an indicator. The medium was not renewed in order to allow the cells to achieve complete uptake of the energy-yielding substrates, especially glucose, and to release metabolic end-products. This procedure, on the other hand, protected H cultures against F overgrowth, so that the proportion of M cells was about 55% [12]. In M cultures, the proliferation of the residual F cells was limited to 10% by two additions, 48 and 72 hr after the plating, of arabino-furanosyl-cytosine at a final concentration of 10^{-6} M [12].

Induction of oxygen deprivation. The experiments were carried out with 6-day-old cells remaining in the medium where they were grown. First, glucose concentration was verified to be < 0.03 mM by the glucose-oxydase method (glucose enzymatique color, Biotrol, Paris). Then half of the flasks were gassed with nitrogen + CO_2 (95% + 5%), the other half, used as the control, with air + CO_2 (N_2 75% + O_2 20% + CO_2 5%). The temperature was maintained at 37°. Gassing was stopped after 55 min, so that the pO_2 values, as measured with a Radiometer polarograph were 110 ± 5 mm Hg in the culture medium of the air-gassed flasks, 27 ± 4 mm Hg in the medium of the N_2-gassed flasks. These values remained steady up to the end of the experiment lasting 20 hr (first series) or 3 hr (second series) [8]. As described previously [8], M and H cells stopped beating during nitrogen-gassing.

Analysis of the fatty acids of the phospholipids. At the end of the experiments, the cells were chilled, scrapped up and homogenized in buffered salt solution. Thereafter, aliquot of the cell suspension was used for the estimation of cellular protein content according to Hartree [13]. The lipids of another aliquot, corresponding to 1 mg protein, were extracted according to Folch *et al.* [14] and dried off. Lipids were fractionated by means of column chromatography, performed with a glass column (internal diameter 0.5 cm), packed with 0.5 g of silicic acid (silicAR CC-4, Mallinckrodt Inc.) Unpolar lipids were eluted first with 15 ml of a mixture of hexan/ether 90:10 (v/v) [15] and discarded. Then phospholipids were eluted with 15 ml methanol 100% [16]. The eluates were dried off under a stream of nitrogen and stored under nitrogen at −20°. The phospholipid residue was redissolved in 1 ml methanol 100%; after addition of 3 μg of margaric acid as an internal standard and of 1.0 ml of BF_3 methanolic solution (14%, v/v), it was submitted to transmethylation for 10 min at 65°. Fatty acid methyl esters were extracted with hexan 100% (5 × 1.5 ml) and, after adequate concentration, analysed by means of a gas chromatograph (Carlo Erba 4100) equipped with a flame ionization detector and connected to an integrator (Spectra Physics). Glass capillary column (0.3 mm × 25 m) coated with Carbowax 20 M was used. The temperature of the oven was 200°, of the detector 240° and of the injector 240°. Among the 15 identified fatty acids, the ones which represent ≤ 1% of the total mass were not taken in account. They were: C 18:3, C 20:0, C 20:1, C 20:3, C 22:0, C 22:1 and C 24:0.

Statistics. The results, expressed as percentages of the total amount, and as μg/mg protein, were submitted to variance analysis according to Snedecor and Cochran [17].

Results

Comparison of normally oxygenated F, M and H cell cultures (Tables 1a and 2a). As regards the fatty acid

Table 1. Phospholipid fatty acids of mixed (H), separate muscle (M) and non-muscle (F) heart cell cultures 20 hr after the onset of the gassing

a: Control cultures (pO_2 = 112 ± 5 mm Hg)

Fatty acids	H cells (10)		M cells (12)		F cells (6)	
	%	μg/mg protein	%	μg/mg protein	%	μg/mg protein
C14:0	1.9 ± 0.4	0.47† ± 0.09	0.6 ± 0.1	0.12 ± 0.02	0.8 ± 0.3	0.32 ± 0.09
C16:0	19.5† ± 1.1	4.58* ± 0.25	16.3 ± 0.5	3.55 ± 0.15	14.6 ± 0.7	5.56† ± 0.24
C16:1	5.0 ± 0.6	1.13† ± 0.12	2.3 ± 0.3	0.49 ± 0.06	3.8* ± 0.5	1.47† ± 0.07
C18:0	20.9† ± 1.0	4.89† ± 0.19	28.6 ± 0.8	6.24 ± 0.40	26.6 ± 0.6	10.24† ± 0.61
C18:1	17.4* ± 0.8	4.09† ± 0.25	11.0 ± 0.7	2.39 ± 0.09	26.1† ± 1.0	9.99† ± 0.24
C18:2	7.5 ± 1.2	1.75 ± 0.18	7.0 ± 0.4	1.50 ± 0.07	9.2* ± 0.6	3.58† ± 0.40
C20:4	20.6† ± 1.6	4.83 ± 0.44	28.1 ± 0.9	6.11 ± 0.39	14.0† ± 1.1	5.40 ± 0.67
C22:6	5.4 ± 0.7	1.24 ± 0.07	5.2 ± 0.4	1.14 ± 0.11	3.0† ± 0.2	1.18 ± 0.16
Total amount		23.60 ± 1.60		21.76 ± 0.86		38.45† ± 1.98

* $P < 0.05$; † $P < 0.01$ compared to M cells.

b: Oxygen-deprived cultures (pO_2 = 28 ± 5 mm Hg)

Fatty acids	H cells (10)		M cells (12)		F cells (6)	
	%	μg/mg protein	%	μg/mg protein	%	μg/mg protein
C14:0	2.6 ± 0.7	0.74 ± 0.09	1.8* ± 0.4	0.19 ± 0.03	1.4 ± 0.3	0.53 ± 0.12
C16:0	21.4 ± 1.4	6.10* ± 0.37	21.1† ± 1.0	2.47† ± 0.26	15.5 ± 0.4	5.69 ± 0.29
C16:1	3.2 ± 0.3	0.91 ± 0.13	2.9 ± 0.4	0.33 ± 0.05	4.1 ± 0.4	1.49 ± 0.15
C18:0	23.4 ± 0.7	6.67† ± 0.29	29.1 ± 1.4	3.39† ± 0.37	26.7 ± 0.8	9.77 ± 0.44
C18:1	14.4 ± 1.0	4.10 ± 0.27	14.8† ± 0.4	1.71* ± 0.14	26.5 ± 0.8	9.72 ± 0.51
C18:2	8.3 ± 0.9	2.37 ± 0.19	10.0 ± 1.1	1.16 ± 0.08	8.9 ± 0.4	3.27 ± 0.25
C20:4	21.5 ± 1.4	6.13 ± 0.33	17.2† ± 0.9	2.01† ± 0.24	12.9 ± 1.3	4.78 ± 0.62
C22:6	3.6 ± 0.4	1.03 ± 0.10	2.2† ± 0.3	0.25† ± 0.05	2.8 ± 0.3	1.04 ± 0.16
Total amount		28.50 ± 1.84		11.63† ± 0.78		36.72 ± 1.94

* $P < 0.05$; † $P < 0.01$ compared to control cultures

Each value represents the mean ± S.E.M. Number of experiments in parentheses.

Table 2. Phospholipid fatty acids of separate muscle (M) and non-muscle (F) cell cultures 3 hr after the onset of the gassing

a: Control cultures ($pO_2 = 105 \pm 6$ mm Hg)

Fatty acids	M cells (6)		F cells (5)	
	%	μg/mg protein	%	μg/mg protein
C14:0	1.1 ± 0.1	0.26 ± 0.04	1.7 ± 0.5	0.65† ± 0.06
C16:0	18.1 ± 1.0	4.04 ± 0.43	22.7* ± 0.6	8.45† ± 0.70
C16:1	2.9 ± 0.4	0.62 ± 0.11	6.1† ± 0.6	2.25† ± 0.25
C18:0	28.2 ± 1.6	6.21 ± 0.50	21.0 ± 2.2	7.81 ± 0.98
C18:1	9.1 ± 0.9	2.00 ± 0.28	26.3† ± 1.6	9.74† ± 0.49
C18:2	8.6 ± 0.9	1.92 ± 0.52	12.2 ± 1.6	4.52† ± 0.61
C20:4	26.5 ± 0.9	5.83 ± 0.33	6.9† ± 0.6	2.60† ± 0.30
C22:6	3.8 ± 0.6	0.81 ± 0.15	0.9† ± 0.1	0.34† ± 0.02
Total amount		22.10 ± 1.59		37.10† ± 2.22

* $P < 0.05$; † $P < 0.01$ compared to M cells.

b: Oxygen deprived cultures ($pO_2 = 25 \pm 5$ mm Hg)

Fatty acids	M cells (6)		F cells (5)	
	%	μg/mg protein	%	μg/mg protein
C14:0	1.9 ± 0.3	0.28 ± 0.03	0.8 ± 0.2	0.35† ± 0.04
C16:0	17.4 ± 1.0	2.61 ± 0.47	21.2 ± 2.0	8.40 ± 1.47
C16:1	3.9 ± 0.3	0.57 ± 0.08	5.0 ± 0.9	1.99 ± 0.47
C18:0	26.3 ± 2.0	3.96 ± 0.77	24.2 ± 1.0	9.57 ± 0.73
C18:1	10.3 ± 1.3	1.58 ± 0.15	25.2 ± 3.0	9.96 ± 0.79
C18:2	10.7 ± 0.6	1.62 ± 0.20	10.1 ± 1.1	3.99 ± 0.54
C20:4	23.8 ± 1.0	3.55† ± 0.39	8.7 ± 1.0	3.48 ± 0.38
C22:6	4.0 ± 0.5	0.58 ± 0.06	1.6 ± 0.4	0.65* ± 0.03
Total amount		15.0* ± 1.93		39.60 ± 3.06

* $P < 0.05$; † $P < 0.01$ compared to control cultures.
Each value represents the mean ± S.E.M. Number of experiments in parentheses.

composition of the phospholipids as expressed as the percentages of the total amount, the most striking differences between M and F cells affected oleic acid (C 18:1), arachidonic acid (C 20:4) and docosahexaenoic acid (C 22:6). In F cells, the proportion of oleic acid was 2.5–3 times as high as in M cells ($P < 0.01$). Palmitoleic acid (C 16:1) and linoleic acid (C 18:2) proportions were also higher ($P < 0.05$), however not to the same degree. Inversely, in M cells, the proportions of arachidonic acid and docosahexaenoic acid were about 2–4 times as high as in F cells ($P < 0.01$). When cells were grown as mixed M and F cells, they exhibited percentages of oleic, linoleic and arachidonic acids near to the average values of M and F cells separate cultures. The percentage of the other fatty acids were more erratic. When pooling the fatty acids according to their saturation degree, it appeared that the saturated fatty acids represented about 45% of the whole, whatever was the type of cells. Mono- and dienoic acids were 39–44% in F cells, 20% in M cells and the mean value, that is 30%, in H cells. The pool of the polyenoic acids was 7–17% in F cells, 30–33% in the M cells and the mean value, that is 26%, in H cells. When the total amount, expressed as μg/mg proteins was considered, the most striking result was that in F cells, the value was almost twice as high as compared H and M cells ($P < 0.01$). High values were not found for every individual fatty acid, but only for the saturated, mono- and diunsaturated ones; the quantities of polyenoic acids however, were of the same order in the three types of cells.

Effects of oxygen deprivation upon phospholipid fatty acids (Tables 1b and 2b). On F cells, oxygen deprivation showed no effect, neither on the percentage distribution nor on the quantities. In H cells, the variations regarding proportions of the different fatty acids were discrete. The pool of saturated fatty acids however was enhanced from 42 to 48% ($P < 0.05$), mono and dienoic acids were decreased from 30 to 26%, whereas polyenoic acids were not modified. The total amount was increased by 23%. This increase was distributed between the individual fatty acids, except palmitoleic, oleic and docosahexaenoic acids, which did not vary. M cells exhibited important disturbances at the end of 20 hr of oxygen deprivation. Except for stearic acid, the percentages of saturated, mono- and dienoic acids were enhanced, while the polyenoic acids were drastically reduced ($P < 0.01$). The total amount of phospholipid fatty acids was reduced by 47%, which loss affected the polyenoic fatty acids especially. At the end of 3 hr of oxygen deprivation, the percentages of the phospholipid fatty acids remainded unchanged compared to the controls. Nevertheless, the total amount had decreased by 32% ($P < 0.05$) and the individual quantities were reduced, although to a lesser extent than after 20 hr. Here also, polyenoic fatty acids had decreased most markedly ($P < 0.01$).

Discussion

The main differences between the normally oxygenated F and M cells were: 1. the total amount of phospholipid fatty acids, and 2. the proportions of the individual fatty acids, essentially of the unsaturated fatty acids. The reasons why the total amounts differ are unknown. The differences observed in the fatty acid composition of the phospholipids could be related to the functional specificity of the two cell types. M cells which exhibit contractile activity, need polyenoic fatty acids, as suggested by Gudbjarnason *et al.* [18]. Moreover, in a previous study [8], we showed that a high percentage of non-esterified arachidonic acid is correlated with beating frequency of heart cell cultures. In F cells, the high proportion of oleic acid is noteworthy,

although not easily explainable. Possibly, the high percentage of oleic acid in F cells could balance the low proportion of arachidonic acid, so establishing a percentage of unsaturated fatty acids of about 53% in F cells as well as in M cells. A definite steady state between saturated and unsaturated molecules could be a requisite for membrane fluidity and function [19].

In oxygen deprived conditions, glucose functions as the major energy-yielding substrate, by synthesis of ATP through the glycolytic pathway. In the present study, oxygen and glucose deprivation together are responsible for the modifications observed in the fatty acid composition of the phospholipids of H, M and F cell cultures. They induce different responses according to the types of cell which is submitted to them. F cultures, which were the least sensitive, maintained normal amounts and proportions of fatty acids in phospholipids. Inversely, M cells were utterly affected by the "ischemic" condition. They undergo a progressive loss of phospholipids, similar to the one observed by Chien *et al.* [20] in ischemic hearts. This loss may be considered as a manifestation of membrane injury, probably due to phospholipase activation [20, 21] and to impaired resistance of sarcolemma to enzymatic attack [22]. The reason why F cells were not susceptible to lytic phenomena would call for additional studies. Nevertheless, a difference in phospholipase activity seems to be present notwithstanding the difference found by Chajek *et al.* [23] between M and F cells as regards lipoproteinlipase.

In M cells, the most important loss of phospholipid fatty acids was observed in the polyenoic acids, thereby modifying fatty acid proportions. Arachidonic and docosahexaenoic acid proportions are reduced to levels which approximate the specific values of F cells. Otherwise as M cells stop beating under oxygen and glucose deprivation, they tend to reach the polyenoic profile of non-beating F cells. Myristic, palmitic and oleic acids, although reduced absolutely, were increased in percentages. This could be a consequence of the leakage of arachidonic acid, which could promote the reinsertion of saturated and monoenoic fatty acids [24].

The response of H cells to "ischemic" condition resemble those of F cells more closely than those of M cells: the fatty acid proportions shift to a disproportion between the percentages of monoenoic acids (which are reduced) and saturated acids (which are increased); the normal proportion of polyenoic fatty acids is maintained, although beatings stop. H cells do not undergo any phospholipid loss; on the contrary, they exhibit a 24% increase in total amount of fatty acids. These facts could suggest that, in mixed cell cultures, F cells tend to stabilize M cells.

In summary, heart muscle and non muscle cells in culture exhibited different proportions of phospholipid fatty acids: in M cells, polyenoic fatty acid proportions were higher, and mono- and dienoic fatty acid proportions were lower than in F cells. Moreover, M cells showed responses to simulated ischemia more sensitiviely. The model studied here appears a useful tool to investigate the effects of drugs on membrane composition in two different types of heart cells.

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Institut de Pharmacologie
Laboratoire Associé du C.N.R.S.
n°206, 15, rue de l'Ecole de Médecine
75006 Paris, France

MARIANNE FREYSS-BEGUIN
ELISABETH MILLANVOYE-VAN BRUSSEL
GENEVIÈVE GRIFFATON
PAUL LECHAT

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Inhibition of bovine brain monoamine oxidase by lead

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One of the clinical symptoms of lead poisoning is encephalopathy characterized by seizures and coma [1, 2]. Although the biochemical basis of this neurotoxic effect remains to be completely elucidated, it is known that the concentrations of several neurotransmitters are altered in the brains of animals exposed to lead [3, 4]. Of the catecholamines, norepinephrine is elevated after lead treatment [5, 6]. One possible explanation for this finding is that lead alters the activities of enzymes involved in norepinephrine metabolism. Recent studies in this laboratory have shown that lead inhibits phenylethanolamine-*N*-methyltransferase, the enzyme responsible for the conversion of