notes on methodology

A comparison of procedures to determine free fatty acids in rat heart

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Summary The level of free fatty acids in intact tissues has been found to be low but is known to rise in proportion to the extent of autolysis. Therefore, the high levels of free fatty acid reported in the cardiac lipids of rats fed rapeseed oil were reinvestigated using two different procedures for homogenization. Quick freezing and pulverization at dry ice temperature followed by lipid extraction was found to give lower values of free fatty acids (200 μg/g of wet heart tissue) than the more commonly used technique of employing rotating blade-type homogenizers (>1700 μg/g of wet heart tissue). The amount of diglycerides was found to be 3 times greater when the latter method was used. The high levels of free fatty acid and diglyceride suggest that extensive autolysis occurs during homogenization with a rotating blade-type homogenizer. Freezing and pulverization at dry ice temperature is therefore recommended for determining intact lipid classes in rat heart.

Supplementary key words cardiac lipids · autolysis · diglycerides

The need to minimize postmortem lipolytic activity during lipid extraction is well-known (1–7). Fairbairn (2) demonstrated that the presence of FFA was found to be very low in normal tissues when proper conditions of extraction were employed; however, even these values should be regarded as maximum because autolysis cannot be prevented entirely. The method devised to obtain results that would reflect actual tissue levels of FFA was a quick freezing of the tissue and pulverization with dry ice followed immediately by solvent extraction (1, 2). At present, however, the lipid extraction procedures most commonly used (8, 9) employ a Potter-Elvehjem or a rotating blade-type homogenizer to prepare tissues for lipid extraction, and only for certain tissues rich in connective tissue is a prior grinding with sand or grinding at dry ice temperature recommended (10). The fact remains that the freezing process may disrupt cells and thus cause lipolytic activity even on storage at −20°C (7). Enzymatic degradation may also occur during the homogenization process prior to inactivation of the enzymes by the extracting solvent.

The purpose of this communication is to reinvestigate recent reports (Table 1) that, in the cardiac lipids of rats fed diets containing rapeseed oil high in erucic acid, TG and FFA levels increase dramatically (11–14), while phospholipids remain constant (12–15), with the exception of small changes in sphingomyelin (16). Of particular concern were the reported high levels of FFA in animals fed the control diets and whether the significant increases reported in rats fed rapeseed oil were real (**Table 1**). Considering the fact that lipolytic activity has been shown to be extensive in the cardiac tissue of rats fed rapeseed oil (14), these high values may represent extensive autolysis.

Materials and methods

Thirty Sprague-Dawley male rats, 3 weeks of age, were randomly placed into three groups of 10 each, and for 0, 3, and 7 days were fed a diet containing rapeseed oil. The compositions of the diet and oil have been reported previously (17). Animals were anesthetized lightly with CO_2 and killed by decapitation. Hearts were removed immediately, weighed, and frozen between blocks of dry ice.

The tissue was homogenized by one of two methods. Method A involved pulverizing the heart tissue at dry ice temperature using a steel mortar (5 cm ID × 1.3 cm deep) and pestle (4.9 cm diameter). The powdered tissue was placed into $CHCl_3$–CH_3OH 2:1 at room temperature, thoroughly dispersed with a Virtis 45 homogenizer (The Virtis Co., Gardiner, NY), and allowed to stand at room temperature for 1 hr. Method B involved cutting the frozen heart into small pieces, placing these pieces into $CHCl_3$–CH_3OH 2:1 kept at 0°C, and then homogenizing them with a Virtis 45 homogenizer. This homogenate was then allowed to warm up to room temperature (approximately 1 hr). The subsequent filtration through sintered glass funnels, the re-extraction of the residue with the same solvent, and the removal of the solvent from the combined filtrates were common to both methods. Total lipids were taken up directly in $CHCl_3$–CH_3OH 2:1. To prevent partitioning of lipid material into the CH_3OH–H_2O phase, the lipids were not washed (8, 9); thus a quantitative recovery was obtained (18).

Abbreviations: FFA, free fatty acid; TG, triglyceride; DG, diglyceride; CE, cholesteryl ester; PL, phospholipids; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

TABLE 1. Quantities of free fatty acid and triglyceride reported in rats fed diets containing rapeseed oil or synthetic oils containing long chain monoenes

Reference	Age of Rat	Diet (%, Monoene)	Time on Diet	FFA	TG
	weeks		*days*	*μg/g*	*μg/g*
12	8	Control	0	1,900	6,200
		RSO (45%, 22:1)	3	4,500	62,100
13, 15	3	Control (72%, 18:1)	7	2,900	3,800
		Synthetic oil (66%, 20:1)	7	1,900	10,500
		Synthetic oil (73%, 22:1)	7	8,300	47,500
14	6–7	Control (Rat Chow)	3	1,500	3,200
		Corn oil	3	1,600	3,700
		RSO (22.3%, 22:1)	3	2,600	19,700

Several neutral lipid classes (TG, DG, FFA, and CE) and total phospholipids (PL) were isolated from the total lipid extract by TLC using hexane–diethyl ether–acetic acid 85:15:1 as developing solvent. After the addition of methyl heptadecanoate as internal standard, each of the lipid classes was transesterified; TG, DG, FFA, and PL were reacted with 5% HCl in anhydrous methanol (19) and CE was reacted with $NaOCH_3$–CH_3OH (20). The resultant methyl esters were purified by TLC using hexane–diethyl ether 90:10 as developing solvent, and analyzed by GLC using a column packed with 5% butanediol succinate on Chromosorb G (high performance) 80/100 mesh operated at 190°C. Quantitation of the lipid classes was made according to the method of Christie, Noble, and Moore (21).

Results and discussion

The amounts of several cardiac neutral lipids per gram of wet tissue as determined by methods A and B are presented in **Table 2**. As determined by either method, all measured neutral lipid classes increased significantly ($P < 0.01$) on feeding the rats a diet containing rapeseed oil for 3 days (except CE, method B). There were no significant changes between day 3 and day 7 (except TG, methods A and B). Preparation of the heart homogenate by method B resulted in significantly higher levels of FFA (day 0, 2.3×; day 3, 8.8×; day 7, 7.9×) and DG (day 0, 3.5×; day 3, 3.7×; day 7, 3.0×) and similar levels of TG and CE, as compared to the results obtained by method A. The TG and FFA values obtained using method B were similar to those obtained in a previous study (14), when method B was also used and where the same rapeseed oil was fed for 3 days, even though the ages of the rats differed. However, such was not the case with the control rats; weanling rats contained lower levels of both TG (900 μg/g, method B, Table 2) and FFA (100 μg/g, method B, Table 2) compared to 6 to 7-week-old rats fed a commercial chow diet (TG: 3,200 μg/g; FFA: 1,500 μg/g, ref. 14, Table 1). Although Houtsmuller, Struijk, and Van der Beek (12) and Beare-Rogers, Nera, and Craig (13) did not indicate their method of homogenization and extraction, their results suggest that a method similar to method B was employed.

The fatty acid composition of the neutral lipid classes isolated from the total lipids and of total

TABLE 2. Quantitative analysis[a] of cardiac neutral lipids of 3-week-old rats fed diets containing rapeseed oil.[b] Comparison of two methods of homogenization

Lipid Class	Method[c]	Time on Diet (days) 0	3	7
Diglyceride	A	72 ± 15[1]	260 ± 20[2]	340 ± 60[2]
	B	250 ± 20[2]	980 ± 70[3]	1,000 ± 260[3]
Free fatty acid	A	40 ± 15[1]	210 ± 60[3]	210 ± 50[3]
	B	100 ± 30[2]	1,800 ± 280[4]	1,700 ± 180[4]
Triglyceride	A	670 ± 210[1]	14,400 ± 4,300[2]	29,300 ± 2,200[3]
	B	900 ± 130[1]	16,100 ± 2,600[2]	29,400 ± 4,200[3]
Cholesteryl ester	A	70 ± 20[1]	210 ± 90[2,3]	260 ± 30[3]
	B	100 ± 13[1]	140 ± 40[1,2]	230 ± 60[2,3]

[a] Given as μg/g of wet tissue. All values are means of five animals ± SD. Means within a lipid class with different superscript numbers are significantly different at the 1% level ($P < 0.01$).

[b] The repeseed oil contained 22.3% 22:1 and 12.3% 20:1.

[c] Method A consisted of pulverizing the tissue at dry ice temperature, followed by extracting the lipids with $CHCl_3$–CH_3OH 2:1. Method B consisted of homogenizing the tissue in $CHCl_3$–CH_3OH 2:1 at 0°C using a Virtis homogenizer.

TABLE 3. Fatty acid composition[a] of cardiac neutral lipids[b] and total phospholipids[b] of rats fed rapeseed oil for 7 days. Comparison of two methods of homogenization[c]

Fatty Acid[d]	FFA			DG			TG			CE			PL		
	A	B	LSD[e]	A	B	LSD	A	B	LSD	A	B	LSD	A	B	LSD
14:0	1.0	0.3	0.8	0.4	0.3	0.2	0.2	0.3	0.2	1.2	1.5	0.8	0.1	0.1	0.05
16:0	8.1	4.3	1.9	5.6	5.9	1.1	5.0	6.0	1.8	9.3	10.6	2.0	7.3	6.3	1.3
16:1	1.3	2.2	0.7	0.8	1.0	0.2	1.7	0.8	0.2	2.3	3.0	1.1	0.4	0.4	0.2
18:0	4.6	3.9	1.8	6.4	5.2	1.5	2.6	2.8	0.4	2.7	3.0	1.2	28.7	25.0	4.7
18:1	18.6	37.5	8.2	29.2	40.1	4.3	36.7	35.6	3.8	25.8	24.6	3.2	16.0	14.3	2.2
18:2	6.2	16.9	2.7	9.4	13.9	2.4	9.4	8.6	1.8	8.7	8.9	1.4	18.0	18.9	3.3
18:3	1.4	2.8	1.3	1.1	1.2	0.5	1.2	1.0	0.5	0.4	0.4	0.2	0.3	0.6	0.3
20:1	7.5	8.4	3.3	13.1	9.3	1.6	14.4	14.0	1.3	9.5	8.1	1.3	6.4	5.6	1.2
20:4	0.8	3.7	1.2	2.7	3.0	1.5	0.4	0.2	0.2	5.1	5.6	1.6	13.0	17.0	3.0
22:1	42.5	16.1	9.4	26.3	15.3	4.3	25.3	26.9	4.6	32.6	30.4	4.0	5.1	4.7	1.5
24:1	3.8	1.1	1.1	1.5	1.2	0.5	1.5	1.4	0.5	trace	trace		trace	trace	

[a] Given as weight percent. All values are the mean of five animals.
[b] FFA, free fatty acids; DG, diglycerides; TG, triglycerides; CE, cholesteryl ester; PL, phospholipid.
[c] Methods A and B are described in Materials and Methods.
[d] Number of carbon atoms: number of double bonds. Polyunsaturates 22:4, 22:5, and 22:6 were present in phospholipids only.
[e] LSD signifies least significant difference based on pooled error estimates. Means differing by more than the LSD are significantly different at the 1% level.

phospholipids at day 7 using methods A and B are shown in **Table 3**. The fatty acid compositions of the common tissue constituents TG, CE, and PL were in general not significantly different between methods. On the other hand, many significant differences were observed in the compositions of the trace constituents FFA and DG. The higher levels of 18:2 and 20:4 in FFA using method B suggest extensive phospholipid hydrolysis, since phospholipids are rich in these acids (see PL, Table 3). Further evidence that PL autolysis occurs during extraction when using method B was indicated by higher levels of lysophosphatidylcholine and ethanolamine as judged by TLC. That triglycerides also appeared to be hydrolyzed was indicated by the increased levels of 18:1 in FFA using method B and by increased levels of DG. The higher levels of DG could be related to a higher lipase activity (22); method B produced consistently higher levels of DG than did method A. However, not all of the accumulated DG could be attributed to degradative processes. The increase in the level of DG between day 0 and days 3 and 7 using method A could, in part, be due to an increase in the steady state concentration of this biosynthetic intermediate. An increase in the biosynthetic activity of TG in the hearts of rats fed rapeseed oil has been demonstrated (23).

Changes in the absolute amounts of TG and PL due to autolysis could not readily be detected quantitatively because of the large amounts of these lipid classes normally present (see TG, Table 2), nor could qualitative changes in their fatty acid composition be detected because autolysis is not selective with regard to the fatty acids hydrolyzed. However, any increase in the level of trace constituents (FFA and DG), resulting from hydrolysis or increased formation, was easily detected (Table 2), and the increase was accompanied by significant changes in the fatty acid composition (Table 3).

As suggested by Fairbairn (2), even the FFA levels obtained by the technique of quick freezing, pulverization, and extraction should be regarded as maximum values for the tissue studied. He obtained FFA values of about 440 μg/g of wet mouse tissue (calculated on the basis of 80% water in mouse tissue). Results from the present study indicated that the endogenous levels (μg/g of wet heart tissue) in 3-week-old rats were FFA, 40; DG, 70; TG, 700; and CE, 70; thus, it is evident from the results presented in Table 2 that the values previously reported for FFA in the cardiac lipids of rats fed control diets or diets containing rapeseed oil (12–14) were very much exaggerated (about 8 times greater) as a direct result of the inappropriate method of homogenization that permitted extensive autolysis. However, the 5-fold increase of FFA due to feeding rapeseed oil (200 vs. 40 μg/g) observed in our study was probably real. Furthermore, the results of Table 3 showed that the 22:1 content of FFA and DG was significantly ($P < 0.01$) lower as a result of dilution by the FFA and DG produced from autolysis of PL and TG, which contained much lower levels of this acid. The fatty acid composition of FFA, as determined using method A, consisted largely of 22:1 with considerable amounts

of 24:1, probably resulting from an accumulation of these acids.

In order to understand structural and functional relationships of a tissue known to be affected by dietary fat, it is essential that methods be used that give accurate and reliable results both of lipid classes and of their compositions. The results reported here clearly show that a quick pulverization of the tissue at dry ice temperature followed by extraction is the preferred method for obtaining intact total lipids of rat heart rich in connective tissues.

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