Journal of Chromatography, 229 (1982) 455—459
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1192

Note

Lipids in human neutrophils determined by a microanalytical method

KAZUKO HIRAMATSU* and SHIGERU ARIMORI

The Fourth Department of Internal Medicine, School of Medicine, Tokai University, Isehara-City, Kanagawa-ken, 259-11 (Japan)

(First received October 8th, 1981; revised manuscript received December 9th, 1981)

Lipids in neutrophils have been investigated in de novo synthesis using precursors labelled by radioisotopes [1]. However, the lipid composition of neutrophils has not been reported except in one case [2]. Lipids in blood cells are usually analysed by thin-layer chromatography and each spot is quantitated either by densitometry [3] or by colorimetry of lipid eluted from the silica gel [4]. These methods are quite useful if sufficient sample can be obtained.

We established the microanalytical quantitation of lipids in platelets [5] and lymphocytes [6] using a Chromarod with thin-layer chromatography connected to a flame ionization detector. In this present paper, lipids in neutrophils separated from 20 ml of human peripheral blood were quantitated using this microanalytical technique.

MATERIALS AND METHODS

Subjects

Five females and six males, whose physical and laboratory examinations were determined to be within normal limits, were examined. Their ages, serum total cholesterol, triglyceride and phospholipid were, respectively, 25 ± 2 years, 162 ± 25 mg/dl, 75 ± 6 mg/dl and 169 ± 18 mg/dl.

Preparation of neutrophils

A 20-ml sample of heparinized venous blood was drawn from each subject after overnight fasting. Leukocyte-rich plasma obtained by the dextran (molecular weight 25,000; Nakarai Chemicals, Tokyo, Japan) sedimentation method was placed on Conray—Ficoll solution (density 1.077 at 25°C) and centrifuged at 400 *g* for 40 min at 20°C to remove mononuclear cells [7].

Residual red blood cells in amongst the neutrophils were lysed by hypotonic saline [8]. Erythrocyte ghosts and hemoglobin were removed by centrifugation at 400 *g* for 10 min with 0.15 *M* sodium chloride solution containing 1 mg/dl EDTA (Wako Chemicals, Osaka, Japan) and the number of neutrophils was adjusted to $1 \cdot 10^6$ per ml. More than 95% of the cells collected were neutrophils. The number of neutrophils obtained from 20 ml of peripheral blood ranged between $1.5 \cdot 10^7$ and $3.0 \cdot 10^7$. The neutrophils were filled with nitrogen and stored at —70° C until the lipid analysis.

Lipid extraction from neutrophils and lipid analysis by thin-layer chromatography in combination with flame ionization detection

Lipid extraction from neutrophils and lipid analysis by thin-layer chromatography in combination with flame ionization detection were performed by the method reported previously for lymphocytes [6].

Statistical evaluation

Student's *t*-test was used for the statistical evaluation.

RESULTS

Quantitation of neutral lipids and phospholipids

Phospholipid, 54.4% and 18.1 μg per 10^6 neutrophils, was the major lipid component of neutrophils. Triglyceride, 21.9% and 4.5 μg per 10^6 neutrophils, was the major component of the neutral lipids. Cholesteryl ester, 10.9% and 3.3 μg per 10^6 cells, and free cholesterol, 13.4% and 3.4 μg per 10^6 cells, were nearly equal in the lipids of neutrophils. The molar ratio of free cholesterol to phospholipid was 0.47. No sex difference existed for cholesteryl ester, free cholesterol, phospholipid and the molar ratio of free cholesterol to phospholipid except for triglyceride, which was slightly higher in male than in female neutrophils ($0.05 < P < 0.1$) (Table I).

Quantitation of phospholipid fractions

Phosphatidylethanolamine (40.4%) and phosphatidylcholine (36.8%) were two major components of the phospholipid fractions. The third component was sphingomyelin (15.2%); phosphatidylinositol plus phosphatidylserine (7.4%) was a minor component. There was no sex difference in any of these phospholipid fractions (Table II).

Quantitation of free fatty acid

$C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ were each determined as approximately 20% of the free fatty acids using gas chromatography; $C_{20:4}$ was 13.5%; $C_{14:0}$, $C_{16:1}$ and $C_{18:2}$ ranged between 7.4 and 8.9%. No sex difference was observed (Table III).

DISCUSSION

Freshly isolated human neutrophils can be used to estimate the activities of the insulin receptor and of the low density lipoprotein receptor which regulate

TABLE I

LIPID COMPOSITION AND THE MOLAR RATIO OF FREE CHOLESTEROL TO PHOSPHOLIPID IN MALE AND FEMALE NEUTROPHILS

Lipids were qualitated and quantitated by thin-layer chromatography with a flame ionization detector and expressed as weight per cent and μg per 10^6 neutrophils. Figures in parentheses are the number of experiments. All values are expressed as mean ± S.D.

	Total (11)	Female (5)	Male (6)
Cholesteryl ester (weight per cent)	10.9 ± 5.1	10.1 ± 4.9	11.7 ± 5.4
(μg per 10^6 cells)	3.3 ± 2.1	2.9 ± 2.3	3.8 ± 1.9
Triglyceride (weight per cent)	21.9 ± 14.8	16.6 ± 11.4	30.4 ± 14.3
(μg per 10^6 cells)	4.5 ± 2.1	3.6 ± 2.1	6.2 ± 0.9
Free cholesterol (weight per cent)	13.4 ± 3.4	14.7 ± 2.3	12.2 ± 4.0
(μg per 10^6 cells)	3.4 ± 1.7	2.8 ± 1.5	3.9 ± 1.9
Phospholipid (weight per cent)	54.4 ± 14.7	58.6 ± 13.7	50.7 ± 15.6
(μg per 10^6 cells)	18.1 ± 9.8	13.5 ± 5.2	21.4 ± 11.1
Molar ratio of free cholesterol to phospholipid	0.47 ± 0.08	0.48 ± 0.03	0.47 ± 0.11

TABLE II

PHOSPHOLIPID DISTRIBITION IN MALE AND FEMALE NEUTROPHILS

The phospholipid distribution was analysed by thin-layer chromatography with a flame ionization detector and expressed as weight per cent. Figures in parentheses are the number of experiments. All values are expressed as mean ± S.D.

	Total (11)	Female (5)	Male (6)
Phosphatidylethanolamine	40.0 ± 6.2	43.2 ± 4.7	37.9 ± 7.3
Phosphatidylinositol plus phosphatidylserine	7.4 ± 1.9	7.8 ± 2.7	7.6 ± 1.9
Phosphatidylcholine	36.8 ± 6.9	36.3 ± 8.3	37.2 ± 6.4
Sphingomyelin	15.2 ± 4.6	14.0 ± 3.1	18.4 ± 6.8

TABLE III

FATTY ACID COMPOSITION OF MALE AND FEMALE NEUTROPHILS

Fatty acid composition was analysed by gas chromatography and expressed as weight per cent. Figures in parentheses are the number of experiments. All values are expressed as mean ± S.D.

	Total (11)	Female (5)	Male (6)
$C_{14:0}$	8.9 ± 3.3	7.5 ± 1.9	10.4 ± 4.1
$C_{16:0}$	20.9 ± 3.5	21.6 ± 3.9	20.2 ± 3.3
$C_{16:1}$	8.5 ± 1.7	9.2 ± 1.0	7.6 ± 1.9
$C_{18:0}$	18.9 ± 2.9	19.5 ± 2.7	18.4 ± 3.4
$C_{18:1}$	22.1 ± 5.1	23.1 ± 4.2	21.2 ± 6.3
$C_{18:2}$	7.4 ± 2.0	7.8 ± 1.7	7.0 ± 2.4
$C_{20:4}$	13.5 ± 3.5	12.0 ± 3.5	14.9 ± 3.2

the intracytoplasmic lipid metabolism of cells [9, 10]. The relationship between these receptor activities and lipid metabolism needs to be clarified beyond analysis of adenylate cyclase and the cyclic AMP system. Studies on the lipids of neutrophils have been performed using a radioisotopic method [1]. The lipid composition of neutrophils has been reported in only one paper,

which was published in 1967 [2]. Gottfried separated his neutrophils on glass-bead columns from 250 ml of peripheral blood. However, it is now known that both glass-bead and nylon-wool columns cause adherence of not only neutrophils but also monocytes, B lymphocytes, some T lymphocytes and platelets [11]. We have prepared much purer neutrophils using gradient sedimentation methods [7]. This combination of dextran, Ficoll—Conray, and hypotonic lysis yielded over 95% pure neutrophils.

Extraction of lipids from the nuetrophils was performed by the Folch method [12]. However, the solvent system for chromatographic development on thin-layer plates was hexane—diethyl ether—acetic acid (80:20:1) for neutral lipids [2], while we used *n*-hexane—diethyl ether (9:1) with the Chromarod. Gottfried's [2] results were for cholesteryl ester — triglyceride could not be located — and he reported that cholesterol was 5.33 ± 0.70 X 10^{-15} moles/cell and 10% of the total lipids. This agrees well with our determination. Furthermore, cholesteryl ester, triglyceride and free cholesterol were clearly identified in our system, indicating that thin-layer chromatography with a Chromarod connected to an ionization detector is superior to classical thin-layer chromatography with silica gel plates.

The mobile phase for the phospholipids was chloroform—methanol—water (60:30:3.5) for our thin-layer chromatography with flame ionization detection, while classical two-dimensional thin-layer chromatography depended on chloroform—methanol—acetic acid—water (25:15:4:2) as solvent for the first direction and diisobutyl ketone—acetic acid—water (40:25:5) as solvent for the second dimension. We examined which of these and several other solvent systems would provide a clear fraction of phospholipids on the Chromarods. The best separation of phosphatidylinositol and phosphatidylserine, which were very difficult to identify as two spots even by two-dimensional thin-layer chromatography [1], was obtained with a chloroform—methanol—water (60:30:3.5) system. Even so, clear separation of these two phospholipids was obtained by chance. This is the reason why they are expressed as phosphatidylinositol plus phosphatidylserine.

We used cholesterol acetate as an internal standard for calibration of relative amounts of lipid fractions and also for absolute amounts in combination with calibration curves for cholesteryl ester, triglyceride and phosphatidylcholine. For the determination of phospholipid, this method using phosphatidylcholine as a standard presents something of a problem because of the difference in molecular sizes of total phospholipids and phosphatidylcholine; however, a good correlation was obtained between this method and the colorimetric method reported previously [6].

Calibrations of weight percentages of the fractionated lipids were performed automatically by a densitometer together with a flame ionization detector. It takes only 50 min from development to densitometry.

Thus, this newly developed microanalytical method can be used as a clinical technique to quantitate neutral lipids and fractions of phospholipids in neutrophils as well as in lymphocytes [6] and platelets [5]. Fatty acids in the extracted neutrophil lipids were detected by ordinary gas chromatography. A sex difference among these lipids and fatty acids was noted only for the triglycerides, but it was not statistically significant.

REFERENCES

1 E.L. Gottfried, in G.J. Nelson (Editor), Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism, Wiley-Interscience, New York, 1972, p. 387.
2 E.L. Gottfried, J. Lipid Res., 8 (1967) 321.
3 J.L. Hojnacki, R.J. Nicolosi and K.C. Hayes, J. Chromatogr., 128 (1976) 133.
4 D.E. Bowyer and J.P. King, J. Chromatogr., 143 (1977) 473.
5 K. Hiramatsu, H. Nozaki and S. Arimori, J. Chromatogr., 182 (1980) 301.
6 K. Hiramatsu and S. Arimori, J. Chromatogr., 227 (1982) 423.
7 A. Böyum, Scand. J. Clin. Invest. Suppl., 21 (1968) 97.
8 H.J. Fallon, E. Frei, III, J.D. Davidson, T.S. Trier and D. Burk, J. Lab. Clin. Med., 59 (1962) 779.
9 K. Hiramatsu, H. Sakai and S. Arimori, Tokai J. Exp. Clin. Med., 4 (1976) 205.
10 Y.K. Ho, R.G. Smith, M.S. Brown and J.L. Golstein, Blood, 52 (1978) 1099.
11 L. Chess and S.F. Schlossmann, Advan. Immunol., 25 (1977) 213.
12 J. Folch, M. Lees and G.H. Sloane-Stanley, J. Biol. Chem., 226 (1957) 497.