BBA 66026

THE EFFECT OF COLLAGENASE PREPARATIONS CONTAMINATED WITH PHOSPHOLIPASE C ACTIVITY ON ADIPOSE TISSUE LECITHIN

P ELSBACH AND M A RIZACK

Department of Medicine, New York University School of Medicine, and Rockefeller University, New York, N Y (U S A)

(Received July 10th, 1969)
(Revised manuscript received October 3rd, 1969)

SUMMARY

Crude commercial collagenase (clostridiopeptidase, EC 3 4 4 19) used for preparing suspensions of isolated adipose tissue cells hydrolyse large portions of cellular [*Me*-^{14}C]choline lecithin

By use of lecithin labeled biosynthetically with [*Me*-^{14}C]choline, ^{32}P or [1-^{14}C]-labeled fatty acid, the phospholipase activity that contaminates the crude collagenase preparation was identified as phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3 1 4 3), in line with the bacterial source *(Clostridium histolyticum)* of the enzyme preparation

Purified commercial collagenase contains little phospholipase C activity

INTRODUCTION

Since RODBELL[1] introduced collagenase (clostridiopeptidase, EC 3 4 4 19) treatment of adipose tissue as a means of obtaining suspensions of free fat cells, this technique has been widely used for the study of metabolism of homogeneous populations of isolated adipocytes

While studying phospholipid metabolism by fat cells isolated from rat epididymal fat pads by collagenase treatment, we observed degradation of cellular phospholipid Since collagenase is prepared from Clostridia, a bacterial species rich in phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3 1 4 3)[2], we examined several commercial preparations of collagenase for the presence of phospholipase activity

The findings indicate that crude collagenase preparations, while exceedingly effective in producing suspensions of isolated fat cells, manifest striking phospholipase C activity capable of hydrolysing most of prelabeled cellular lecithin during incubation for 1 h

MATERIALS AND METHODS

Epididymal fat pads were obtained from Sprague–Dawley rats (150–200 g) maintained on a regular laboratory chow diet Crude and purified bacterial collagenase *(Clostridium histolyticum)* was purchased from Worthington Biochemical Corp (Freehold, N J), bovine serum albumin (Fraction V) from Armour Co , [*Me*-^{14}C]choline chloride (specific activity, 51 8 mC/mmole) from Nuclear Chicago Co , and phospholipase A *(Crotalus adamanteus)* from Lights and Co , Colnbrook, Bucks, England

Preparation of various radioisotopically-labeled lecithins

Lecithin was labeled biosynthetically by incubating rat-liver slices with $^{32}P_i$ or [*Me*-^{14}C]choline as previously described[3]

Lecithin labeled with either [1-^{14}C]palmitate or [1-^{14}C]linoleate was isolated from the urinary bladder of the toad *(Bufo marinus)*, after previous incubation of the tissue with the (1-^{14}C)-labeled fatty acid[4]

Adipose tissue lipids were labeled with [*Me*-^{14}C]choline by incubating epididymal fat pads for 1 h in Krebs–Ringer bicarbonate solution, containing 1 μC of [*Me*-^{14}C]choline After 1 h the tissues were rinsed in nonradioactive choline Ringer's solution and reincubated in this medium for 20 min to reduce the pools of radioactive water-soluble precursors In experiments in which the effect of collagenase on cellular choline-lipids was examined, pieces of epididymal fat pads were incubated in Krebs–Ringer bicarbonate (0 5 ml per piece) with collagenase at a concentration comparable to the one used to isolate adipocytes (0 3 mg/0 5 ml of incubation medium) for varying periods of time At the end of incubation 10 ml of chloroform–methanol (2 1, v/v)[5] were added to the reaction mixture After lipid extraction in this solvent at room temperature for 48 h, the extracts were quantitatively transferred with chloroform–methanol (2 1, v/v) through filter paper to 40-ml glass centrifuge tubes Extracts were washed with 0 2 vol of 7 mM $CaCl_2$ (ref 5) Where appropriate the washes were transferred to counting vials, evaporated under a stream of air and counted in a liquid scintillation counter using 2,5-bis-2-(5-*tert* -butylbenzoxazolyl)-thiophene (BBOT) (4 g/l of toluene) as scintillator

The lower (chloroform rich) phase was evaporated and phospholipids in the residue were separated from the bulk of triglycerides by three extractions with methanol The methanol extracts were evaporated and transferred to a silica gel G thin-layer plate Only small amounts of triglycerides appeared on these plates

The triglyceride content of the original whole lipid extracts was determined on aliquots of the chloroform-soluble lipid remaining after methanol extraction[6] The radioactivity in the neutral lipid fraction was less than 1% of the radioactivity in the methanol extract

Assay of phospholipase activity

Collagenase preparations were tested for phospholipase activity against various radioisotopically labeled lecithins Reaction mixtures contained 1 mg of deoxycholate, 2 mM $CaCl_2$, 0 3 mg of collagenase and labeled lecithin (280 nmoles [^{14}C]choline-labeled or 4 nmoles [^{14}C]linoleic acid-labeled lecithin + 100 nmoles carrier lecithin, or 5 nmoles [^{14}C]palmitic acid-labeled lecithin + 100 nmoles carrier lecithin, or 100 nmoles ^{32}P-labeled lecithin) in a total volume of 0 5 ml of Krebs–Ringer's bicarbonate

solution at pH 7 25 The lecithin in the mixture was dispersed by vigorous agitation on a Vortex mixer Incubation was carried out for 1 h and reactions were terminated by addition of 10 ml of chloroform–methanol (2 1, v/v) Extracts were washed with 0 2 vol of 7 mM $CaCl_2$ when the label was [*Me*-^{14}C]choline Aliquots of water washes were counted as described above and the radioactive water-soluble products were identified by paper chromatography in propanol–NH_4OH–water (60 30 10, by vol) and in phenol–water (50 20, v/v)[7,8] Radioactive lipid fractions were separated by thin-layer chromatography and counted as previously described[3] Triglycerides, fatty acids, and diglycerides were allowed to ascend high on the plate and a line was drawn under the diglyceride spot before phospholipids were separated

TABLE I

INCORPORATION OF [*Me*-^{14}C]CHOLINE INTO LECITHIN, SPHINGOMYELIN AND LYSOLECITHIN OF ADIPOSE TISSUE

Epididymal fat pads of one rat were cut into halves and the 4 pieces incubated in 1 0 ml of Krebs–Ringer's bicarbonate buffer containing 1 0 μC of [*Me*-^{14}C]choline and 3 2 mg of glucose After the indicated periods of time tissues were rinsed 3 times in unlabeled choline Ringer's solution and then extracted with 20 ml of chloroform–methanol (2 1, v/v) Extracts were washed with 0 2 vol of 7 mM $CaCl_2$ (ref 5), and the washes were discarded Phospholipids were separated and triglycerides determined as described in the text

Incubation time (min)	*Triglyceride content (mg)*	*Total counts/min*	*Counts/min per mg triglyceride*	*% of total counts/min in*		
				Lecithin	*Sphingomyelin*	*Lysolecithin*
30	130	6 590	50	91 6	3 7	4 7
60	200	52 540	260	91 4	5 3	3 3
90	112	43 675	390	93 0	3 0	4 0
120	139	78 650	565	94 2	2 2	3 6

RESULTS

Table I shows the incorporation of [*Me*-^{14}C]choline into lecithin, sphingomyelin and lysolecithin of epididymal fat pads incubated for a 2-h period More than 90% of lipid radioactivity appears in lecithin and the remainder is approximately equally distributed between sphingomyelin and lysolecithin Incorporation, after an initial lag period of 30 min continued in roughly linear fashion during 2-h incubation period

The effect of a crude collagenase preparation on the distribution of radioactivity among fractions of adipose tissue previously incubated with [*Me*-^{14}C]choline is shown in Table II In the absence of collagenase adipose tissue lecithin exhibits a small loss of radioactivity during the first 2 h of incubation, followed by a more appreciable degradation during the 3rd h Radioactivity lost from lecithin was recovered in the 7 mM $CaCl_2$ washes of the lipid extracts

In the presence of collagenase, almost 80% of the radioactivity was lost from lecithin in 1 h and after 3 h more than 85% of the lecithin radioactivity was recovered in the water-soluble fraction These findings indicate a rapid and almost complete breakdown of labeled cellular lecithin

TABLE II

EFFECT OF COLLAGENASE TREATMENT ON DISTRIBUTION OF RADIOACTIVITY AMONG FRACTIONS OF ADIPOSE TISSUE PREVIOUSLY INCUBATED WITH [*Me*-^{14}C]CHOLINE

Lecithin of epididymal fat pads was labeled with [*Me*-^{14}C]choline as described in MATERIALS AND METHODS. After labeling and washing of the tissue to remove labeled choline, the fat pads were cut into 8 approximately equal pieces and incubated with and without collagenase (0.3 mg per 0.5 ml). At the end of the indicated periods of time 10 ml of chloroform–methanol (2:1, v/v) were added as described in the text. Lecithin, lysolecithin + sphingomyelin and water-soluble radioactivity were separated by thin-layer chromatography on silica gel G in chloroform–methanol–acetic acid–water (100:56:20:10, by vol.).

Incubation (h)		*% of total counts/min in*		
		Lecithin	*Lysolecithin + sphingomyelin*	*Water-soluble fraction*
Without collagenase	0	67.0	8.0	25.0
	1	64.6	8.4	27.0
	2	62.1	8.9	29.0
	3	52.9	8.8	38.3
With collagenase	0	66.5	7.2	26.3
	1	14.3	5.2	80.5
	2	16.9	4.6	78.5
	3	9.6	5.0	85.4

That the crude collagenase preparation used in these experiments was indeed contaminated by phospholipase activity was established by use of various lecithins incubated with collagenase in the same amounts as employed for preparation of suspensions of adipocytes. Table III contains the results of phospholipase assays of crude and "pure" collagenase preparations. The crude collagenase rapidly hydrolysed lecithins labeled with ^{32}P, [[1-^{14}C]palmitate, [1-^{14}C]linoleate or [*Me*-^{14}C]choline. The radioactive products of hydrolysis were phosphorylcholine (approx. 50%) and choline (approx. 50%) when the label was in the choline moiety of lecithin.

The radioactive product of the degradation of lecithin labeled with [1-^{14}C]-palmitate or [1-^{14}C]linoleate was almost exclusively in the diglyceride fraction providing further evidence that the crude collagenase preparation contained phospholipase C activity.

Incubation of the various substrates with the "pure" collagenase preparations resulted in little accumulation of radioactive breakdown products. For comparison the lecithin labeled with ^{14}C-labeled fatty acid was also treated with snake venom phospholipase A (*Crotalus adamanteus*). Almost complete hydrolysis was obtained, with lysolecithin and free fatty acids as sole radioactive products. Since the snake venom phospholipase A acts specifically on the 2-ester position[9], it may be concluded that the dienoic acid occurs predominantly in the 2-position of the toadbladder lecithin. Palmitic acid is approximately equally distributed between the 1- and 2-position.

TABLE III

EFFECT OF CRUDE OR PURIFIED COLLAGENASE PREPARATIONS ON VARIOUS ISOTOPICALLY LABELED LECITHINS

Extracts of assay mixtures that contained [*Me*-^{14}C]choline or ^{32}P-labeled lecithin were subjected to thin-layer chromatography in chloroform–methanol–acetic acid–water (100 50 20 10, by vol.) to obtain the indicated fractions. Extracts of assay mixtures that contained lecithin labeled with (1-^{14}C)-labeled fatty acid were subjected to thin-layer chromatography in two solvent systems, first in light petroleum–ethyl ether–acetic acid (65 25 1, by vol.) to move fatty acids and diglycerides towards the front and then in chloroform–methanol–acetic acid–water (100 50 20 10, by vol.) to separate lecithin, lysolecithin and radioactivity at the origin.

Lecithin labeled with	*Incubation time (min)*	*% of total counts/min in*				
		Lecithin	*Lysolecithin*	*Water-soluble fraction*	*Free fatty acids*	*Diglycerides*
^{32}P						
+ crude collagenase*	0	95.1	2.2	2.7		
	30	34.8	1.8	63.4		
	60	17.9	1.8	80.3		
+ purified collagenase I*	60	91.2	4.2	4.6		
+ purified collagenase II*	60	92.6	4.0	3.4		
No addition	60	95.6	3.8	0.6		
[1-^{14}C]*Palmitate*	0	97.6	1.1	1.1		
+ snake venom	240	7.8	48.5	2.6	37.7	
+ crude collagenase	60	31.5	1.3	1.3	1.3	64.5
+ purified collagenase	60	91.4	1.1	1.8	3.2	2.5
[1-^{14}C]*Linoleate*	0	98.1	1.5	0.4		
+ snake venom	240	4.1	12.0	2.0	78.7	
+ crude collagenase	60	25.9	1.8	1.1	3.8	67.4
+ purified collagenase	60	93.1	2.1	1.4	2.1	1.4
[*Me*-^{14}C]*Choline*	0	97.0	2.6	0.5		
+ crude collagenase	60	25.0	2.0	72.4		

* Crude collagenase (batch CLS-65109). Purified collagenase I (batch CLS 8 GF). Purified collagenase II (batch CLS PA 8 GA).

DISCUSSION

These findings indicate that crude collagenase from *Clostridium histolyticum* contains substantial phospholipase C activity capable of hydrolysing most of the lecithin of adipose tissue cells within 1 h. The finding that [*Me*-^{14}C]choline also accumulated cannot be attributed to the additional presence of phospholipase D in the collagenase preparation, since no labeled phosphatidic acid was recovered with any of the labeled lecithins. No attempt has been made to establish the possible presence in the collagenase preparation of an enzymatic activity that splits the phosphorylcholine ester.

Adipocytes treated with crude collagenase have been reported to retain a "normal" appearance on microscopy even though their fragility is increased and they must be handled in siliconized or plastic rather than glass vessels[1]. We have found that fat pads treated with crude preparations break up more rapidly than those treated with purified collagenase. The adipocytes used in this study, though prepared

with collagenase containing high phospholipase C activity, remained intact with a normal appearance even in electron micrographs taken in connection with a study on pinocytosis in which the same collagenase preparation was used[10,11] These cells also converted glucose to CO_2 and fatty acids and responded to insulin and epinephrine The properties of adipocytes prepared with collagenase are affected not only by phospholipase, but also by insulinase and a lipoprotein lipase inhibitor identified in both crude and purified preparations[12]

Not only phosphatidylcholine but also other phosphoglycerides may be lost when phospholipase C is present during the preparation of adipocytes[2] This attack on membrane phospholipids confirms the mechanism postulated in previous reports to account for the insulin-like effect of phospholipase C on adipocytes[13,14] Since the degree of contamination of collagenase preparations with phospholipase C is variable this may be a factor in the variability in insulin response of different preparations of adipocytes[12,15] Alteration of the plasma membrane by phospholipase could also be a factor in the failure of prostaglandin E_1 to cause a rise in the cyclic-AMP levels of adipocytes, even though the level does increase when intact adipose tissue is treated with this hormone[16]

ACKNOWLEDGMENTS

The excellent technical assistance of Corazon de Leon and Sandra Farrow is gratefully acknowledged

Supported by grants from the U S Public Health Service (AM 05472) and the National Science Foundation (GB 6035) One of the authors (P E) is a career investigator of the Health Research Council of the City of New York (Contract I-379).

REFERENCES

1 M RODBELL, *J Biol Chem*, 239 (1964) 375
2 M KATES, in K BLOCH, *Lipid Metabolism*, John Wiley, New York, 1960, p 214
3 P ELSBACH, *Biochim Biophys Acta*, 125 (1966) 510
4 A A ROSENBLOOM AND P ELSBACH, *J Lipid Res*, 10 (1969) 406
5 J FOLCH, M LEES AND G H SLOANE STANLEY, *J Biol Chem*, 226 (1957) 497
6 F T HATCH AND R S LEES, *Advan Lipid Res*, 6 (1968) 59
7 J OLLEY AND R M C DAWSON, *Biochem J*, 62 (1956) 5P
8 R M C DAWSON, *Biochem J*, 75 (1960) 45
9 G H DE HAAS, F J M DAEMEN AND L L M VAN DEENEN, *Biochim Biophys Acta*, 65 (1962) 260
10 S W CUSHMAN AND M A RIZACK, *Federation Proc*, 28 (1969) 280
11 S W CUSHMAN, Thesis, The Rockefeller University, 1969
12 A SCHREIBMAN, D E WILSON AND R A ARKY, *Life Sci*, 7 (1968) 1295
13 M RODBELL, *J Biol Chem*, 241 (1966) 130
14 M BLECHER, *Biochem Biophys Res Commun*, 21 (1965) 202
15 R B GOLDRICK, B C E ASHLEY AND M L LLOYD, *J Lipid Res*, 10 (1969) 253
16 R W BUTCHER AND C E BAIRD, *J Biol Chem*, 243 (1968) 1713