A novel pathway for lipid biosynthesis: the direct acylation of glycerol

Douglas P. Lee, Andrew S. Deonarine, Martin Kienetz, Quansheng Zhu, Monika Skrzypczak, Monroe Chan, and Patrick C. Choy[1]

Department of Biochemistry and Medical Genetics, Faculty of Medicine, University of Manitoba, 770 Bannatyne Ave., Winnipeg, Manitoba, Canada R3E 0W3

Abstract The acylation of glycerol-3-phosphate by acyl-CoA is regarded as the first committed step for the synthesis of the lipoidal moiety in glycerolipids. The direct acylation of glycerol in mammalian tissues has not been demonstrated. In this study, lipid biosynthesis in myoblasts and hepatocytes was reassessed by conducting pulse-chase experiments with [1,3-^{3}H]glycerol. The results demonstrated that a portion of labeled glycerol was directly acylated to form monoacylglycerol and, subsequently, diacylglycerol and triacylglycerol. The direct acylation of glycerol became more prominent when the glycerol-3-phosphate pathway was attenuated or when exogenous glycerol levels became elevated. Glycerol:acyl-CoA acyltransferase activity, which is responsible for the direct acylation of glycerol, was detected in the microsomal fraction of heart, liver, kidney, skeletal muscle, and brain tissues. The enzyme from pig heart microsomes displayed optimal activity at pH 6.0 and the preference for arachidonyl-CoA as the acyl donor. The apparent K_m values for glycerol and arachidonyl-CoA were 1.1 mM and 0.17 mM, respectively. The present study demonstrates the existence of a novel lipid biosynthetic pathway that may be important during hyperglycerolemia produced in diabetes or other pathological conditions.—Lee, D. P., A. S. Deonarine, M. Kienetz, Q. Zhu, M. Skrzypczak, M. Chan, and P. C. Choy. **A novel pathway for lipid biosynthesis: the direct acylation of glycerol.** *J. Lipid Res.* 2001. **42**: 1979–1986.

Supplementary key words monoacylglycerol • arachidonyl-CoA • acyltransferase • lipid biosynthesis • cardiac myoblast • hepatocyte

Lipids are an integral component of the biological membrane. They are also involved in the generation of secondary messengers for signal transduction (1), modulation of enzyme activity (2), eicosanoid production (3), and the storage of metabolic energy. Phosphatidylcholine and phosphatidylethanolamine are major phospholipids in the mammalian membrane. The metabolism of both phospholipids has been extensively studied since the discovery of their biosynthetic pathways in the early 1950s by Kennedy and co-workers (4). The activated form of choline or ethanolamine condenses with diacylglycerol for the formation of the appropriate phospholipid. Diacyl-glycerol is produced from the glycerol-3-phosphate pathway (5) (**Fig. 1**). In this pathway, endogenous glycerol or glycerol transported into the cell from an extracellular source is converted to glycerol-3-phosphate by glycerokinase (6). Alternatively, glycerol-3-phosphate is produced when dihydroxyacetone phosphate is reduced by glycerol-3-phosphate dehydrogenase (7). Acylation of glycerol-3-phosphate with acyl-CoA at the *sn*-1 position by acyl-CoA: glycerol-3-phosphate acyltransferase results in the formation of lysophosphatidate (8). A second acylation step converts the lysophosphatidate to phosphatidate (9). Phosphatidate is converted to diacylglycerol in a reaction catalyzed by phosphatidate phosphatase.

The study of glycerol metabolism dates back to the early 1960s, when the uptake of glycerol was studied in hepatocytes (10), liver (11), kidney (12), mammary cells (13), adipose tissue (14), pneumocytes (15), aorta (16), heart (17), cardiac myocytes (18), and skeletal muscle (19). These studies were exemplified by the work of Kinsella in which the incorporation of [^{14}C]glycerol into the lipid fraction of bovine mammary cells was examined (13). After incubation, the specific radioactivities of mono-, di-, triacylglycerol, and other various lipids were determined. More than any other lipid, the monoacylglycerol pool was found to have the highest specific radioactivity immediately after pulse labeling. The labeling profiles clearly indicated monoacylglycerol as an early intermediate in lipid biosynthesis. Because the direct acylation of glycerol had not been previously established, the production of mono-acylglycerol was instead attributed to the catabolism of the labeled diacylglycerol or lysophosphatidate. Remarkably, little is known about the direct acylation of glycerol in vivo and its incorporation into the glycerolipid pool.

The acylation of glycerol-3-phosphate has been comprehensively studied (8). To a lesser extent, the direct acylation of glycerol has also been documented (20, 21). Glycerol was found to compete with water as a nucleophile at
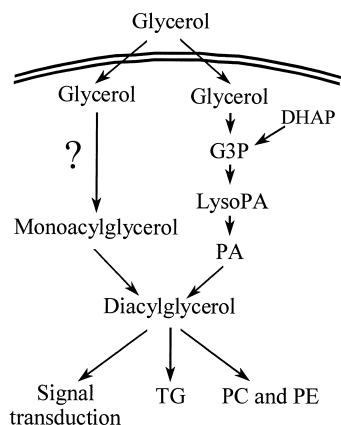

Fig. 1. Glycerol metabolism. The glycerol-3-phosphate pathway (right) and the direct acylation pathway (left) are illustrated. DHAP, dihydroxyacetone-3-phosphate; G3P, glycerol-3-phsophate; LysoPA, lysophosphatidate; PA, phosphatidate; TG, triacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

the active site of the enzyme, resulting in the formation of monoacylglycerol. Monoacylglycerol was also produced from glycerol by a 60 kDa lysophospholipase-transacylase with lysophosphatidylcholine as the acyl donor (21). In each case, a high concentration of glycerol was required to detect product formation. Glycerol, ethanol, and several other alcohols formed esters with [14C]palmitate in the presence of adipose tissue microsomes (22). An 85 kDa recombinant phospholipase A_2 was also reported to transfer an acyl group from phosphatidylcholine to glycerol.

Diacylglycerol can also be synthesized from the acylation of monoacylglycerol (23). The reaction is catalyzed by acyl-CoA:monoacylglycerol acyltransferase, which has been characterized in the epithelial cells of the intestine and the liver. Monoacylglycerol acyltransferase activity has also been detected in cardiac myocytes (24), hepatocytes (25), and adipose tissue (26). Studies have previously demonstrated that radiolabeled monoacylglycerol can serve as a precursor for diacylglycerol, triacylglycerol, and phosphatidylcholine (27). In Swiss 3T3 cells, monoacylglycerol is preferentially incorporated into phophatidylinositol (28).

Transient amounts of diacylglycerol are produced in the phospholipase C-mediated hyrdolysis of phopshatidylinositol 4,5-bisphosphate. The diacylglycerol may activate protein kinase C (29) and regulate the Ras and Rho family of proteins (30). Within the lipid biosynthetic pathway, diacylglycerol occupies a central position in the synthesis of triacylglycerides and major phosphopholipids such as phosphatidylcholine and phosphatidylethanolamine.

In this study, we demonstrated that glycerol is directly acylated in mammalian tissues in a pathway that we identified as the direct acylation pathway (Fig. 1). Pulse-chase experiments conducted with [1,3-3H]glycerol revealed a precursor-product relationship between glycerol, mono-, di-, and triacylglycerol. The direct acylation pathway became prominent when the glycerol-3-phosphate pathway was attenuated or when the extracellular glycerol concentration was elevated. The enzyme that catalyzed the direct

acylation of glycerol was detected in the microsomal fraction of mammalian tissues. Characterization of the acyl-CoA:glycerol acyltrasnsferase activity from the pig heart microsomes demostrated that the enzyme preferred arachidonyl-CoA as the substrate, although other long-chain acyl-CoAs could also be employed as acyl donors.

EXPERIMENTAL PROCEDURES

Materials

All lipid standards were obtained from Serdary Research Laboratories (London, Ontario, Canada). TLC plates (K6 Silica gel 60A) and DE81 (2.5 cm) filter discs were purchased from Whatman, Inc. (Clifton, NJ). The radiolabeled compounds, [1-14C]arachidonyl-CoA (51.6 mCi/mmol) and [1,3-3H]glycerol (3.5 Ci/mmol), were obtained from NEN (Boston, MA). Protease inhibitor cocktail tablets were purchased from Roche Diagnostics Corporation (Indianapolis, IN). The BCl3-methanol kit was purchased from Supelco, Inc. (Bellefonte, PA). All other chemicals were of analytical grade and obtained from Sigma Chemical Company (St. Louis, MO). Bicinchoninic acid protein assay reagents were purchased from Pierce (Rockford, IL).

Tissue culture

H9c2 cells (a rat myoblast cell line) and Chang liver cells were obtained from the American Type Culture Collection. They were cultured in DMEM containing 10% FBS, 100 U/ml of penicillin G, 10 μg/ml of streptomycin, and 0.25 μg/ml amphotericin B. The cells were incubated at 37°C in an atmosphere of 95% humidified air/5% carbon dioxide until 90% confluency was achieved.

Preparation of subcellular fractions and confirmation of monoacylglycerol production

Pig hearts were obtained fresh from a local abattoir. A 10% homogenate (w/v) was prepared in a buffer containing 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, and 0.1 mM PMSF. Subcellular fractions were obtained as previously described (31). The cross-contamination of each subcellular fraction was assessed by enzyme markers. Fumarase (32) and succinate dehydrogenase (33) activities were used as mitochondrial markers. Glucose-6-phosphatase (34), 5′-nucleotidase (35), and K+-stimulated p-nitrophenylphosphatase (35) activities were employed as microsomal markers. From the determination of these enzyme activities, the microsomal fraction was contaminated by 5% of the mitochondrial material, whereas the mitochondral fraction was contaminated by 9% of the microsomal material. The cytosolic fraction was contaminated by less than 1% of mitochondrial or microsomal materials. The protein content in each fraction was determined by the bicinchoninic acid method (36).

Rigorous analysis was conducted to confirm the identity of monoacylglycerol formed by the glycerol acyltransferase reaction. Pig heart microsomes were incubated in a buffer containing 50 mM Tris-succinate (pH 6.0), 32.2 μM [1-14C]arachidonyl-CoA (51.6 mCi/mmol), 0.1 mM EDTA, and 5% (v/v) glycerol in a total volume of 60 μl. The reaction was terminated by addition of 0.75 ml chloroform–methanol 2:1 (v/v). Water (0.25 ml) was added to mixture, and centrifugation was used to speed up phase separation. The organic phase was recovered and analyzed by TLC. The radioactivity associated with monoacylglycerol was determined by scintillation counting. Counts recovered from a control assay mixture lacking glycerol were subtracted from experimental values. TLC methods were used employing over a

TABLE 1. Solvent systems for the identification of monoacylglycerol by TLC

Chromatographic System	Solvent System	R_f Value of MG
One-dimensional TLC	Diethyl ether−toluene−ethanol−acetic acid 40:50:1:0.5	0.08
	Hexane−diethyl ether−acetic acid 70:30:1	0.02
	Bezene−diethyl ether−ethanol−acetic acid 50:40:2:0.2	0.23
	Hexane−diethyl ether−acetic acid 70:30:1	0.02
	Petroleum ether−diethyl ether−acetic acid 50:50:1	0.08
	Heptane−diethyl ether−methanol−acetic acid 90:20:2:3	0.05
	Hexane−isopropyl ether−diethyl ether−acetone−acetic acid 85:12:1:4:1	0.01
	Heptane−isopropyl ether−acetic acid 60:40:4	0.04
	Chloroform−acetone 96:4	0.05 (1-MG)
	(Boric acid-impregnated plate used)	0.1 (2-MG)
Multi one-dimensional TLC	Diethyl ether−hexane 60:40 7 cm	0.03
	Diethyl ether−petroleum ether−formic acid (90:10:1) 18cm	
	Isopropyl ether−acetic acid 90:10:1 13 cm	0.15
	Petroleum ether−diethyl ether−acetic acid 85:12:1:4:1 18 cm	
	Diethyl ether−toluene−ethanol−acetic acid 45:50:1:0.5 12 cm	0.16
	Diethyl ether−hexane 8:92 18 cm	
	Chloroform−methanol−acetic acid−water 70:30:4:2 9 cm	0.68
	Diethyl ether−hexane−ethanol−acetic acid 40:50:2:0.2 18 cm	
	Chloroform−methanol−acetic acid−water 70:30:4:2 13 cm	
Two-dimensional TLC	Benzene−diethyl ether−ethanol−acetic acid 50:40:2:0.2	
	Benzene−diethyl ether−ethanol−ammonium hydroxide 50:40:2:0.2	
	Benzene−diethyl ether−ethanol−acetic acid 50:40:2:0.2	
	Diethyl ether−hexane−acetic acid 60:40:1	

MG, monoacylglycerol.

dozen solvent systems for the analysis. In every case, the radiolabeled product migrated identically with the authentic monoacylglycerol standard (**Table 1**). The monoacylglycerol isomers were analyzed by boric acid-impregnated TLC (37). After sample application, the plate was developed in chloroform−acetone 96:4 (v/v). The radioactivity associated with each isomer was determined, and the analysis indicated that 10% of the monoacylglycerol produced was of the 2-isomer and 90% was of the 1(3)-isomer. Incubation of radioactive monoacylglycerol with pig heart microsomes resulted in the production of radioactive arachidonate and diacylglycerol, which were determined by TLC (data not shown). The conversion of monoacylglycerol to diacylglycerol was concurrent with the presence of monoacylglycerol acyltransferase in cardiomyocytes (24).

Enzyme assays

The glycerol acyltransferase activity was determined by monitoring the formation of radiolabeled monoacylglycerol as described in the preceding section. Glycerokinase activity was determined using a modified method described by Westergaard, Madsen, and Lundgren (38). The enzyme activity was determined by measuring the rate of [1,3-^{3}H]glycerol-3-phosphate production. H9c2 or Chang liver cells were scraped from the petri dish and suspended in a PBS solution, pH 7.4. A cocktail of protease inhibitors was added to the cell suspension, and a homogenate (~0.1 mg protein/ml) was produced by sonication. A 10 μl aliquot of cell homogenate was incubated with a reaction mixture containing 25 mM HEPES buffer (pH 7.4), 3 mM ATP, 2.5 mM MgCl$_2$, and 32.3 μM [1,3-^{3}H]glycerol (3.5 Ci/mmol) in a total volume of 100 μl for 10 min at 37°C. The reaction was terminated by incubating the mixture at 100°C for 5 min. After the mixture was centrifuged for 5 min at 10,000 g, the supernatant was applied onto a DE3-81 Whatman filter disc. The disc was placed onto a scintered glass filter under reduced pressure and washed slowly with 25 ml 80% ethanol. The radioactive glycerol-3-phosphate associated with the filter after washing was determined by scintillation counting. The radioactivity obtained from a control assay mixture without ATP was subtracted from the experimental value.

Pulse-chase analysis

H9c2 cells or Chang liver cells were cultured in 35 mm dishes or 24-well plates until they became 90% confluent. Culture medium containing [1,3-^{3}H]glycerol (3.5 Ci/mmol) was added to each dish for the prescribed time. The medium containing the label was removed from the dish, and the cells were incubated in culture medium containing nonradiolabeled glycerol for various times. Subsequently, the dishes were rinsed three times with ice-cold PBS solution, pH 7.4. Cells were scraped into a test tube with 1 ml methanol−HCl 100:1 (v/v), and then chloroform (1.3 ml) and water (0.7 ml) were added to the tube. The lipid fraction was recovered in the organic phase and analyzed by TLC.

Separation of lipids

The simultaneous separation of radiolabeled neutral lipids and phospholipids was performed by one-dimensional TLC using multiple development systems. The TLC plate (20 cm × 20 cm) was activated by incubating at 135°C for at least 1 h, and allowed to cool to room temperature before sample application. The plate was developed in a solvent containing chloroform−methanol−water−acetic acid 70:30:4:2 (v/v/v/v) until the solvent front reached 11 cm from the origin. After drying, the plate was fully developed in a second solvent containing benzene−diethyl ether−ethanol−acetic acid 50:40:2:0.2 (v/v/v/v). Again, the plate was dried and then developed in the first solvent to 12.5 cm for further separation of phospholipids. Lipid fractions were visualized by exposure to iodine vapor. The bands on the TLC plate corresponding to authentic lipid standards were scraped into scintillation vials, and their radioactivities were determined.

Lipid determination

For the determination of monoacylglycerol, diacylglycerol, triacylglycerol, lysophosphatidate, phosphatidate, and phosphatidylcholine, the acyl groups were converted to the respective methyl esters by reaction with BCl$_3$-methanol (39). The total fatty acid methyl esters were quantified by gas-liquid chromatography using a Hewlett Packard HP 5890A gas chromatograph equipped with a Supelcowax 10 30 m × 0.25 mm, 0.25 μm fused

silica capillary column. Heptadecanoic acid methyl ester was used as a standard for quantification.

Statistical analysis

Data were analyzed using the paired Students' t-test unless otherwise indicated. The level of statistical significance was defined as $P < 0.05$. The data presented without statistical analysis were performed in duplicate. The values from duplicate experiments were always within 10% of each other and were reproducible in at least three separate experiments.

RESULTS

The acylation of glycerol

A pulse-chase study was conducted to determine the pathway for the acylation of glycerol. H9c2 cells were preincubated with the culture medium containing 0.1 mM [1,3-³H]glycerol (3.5 Ci/mmol) for 1 min and then incubated in culture medium containing 1 mM nonradiolabeled glycerol for various time periods. The specific radioactivities of monoacylglycerol, diacylglycerol, lysophosphatidate, and phosphatidate were determined at each time point. As depicted in **Fig. 2**, the monoacylglycerol pool was immediately radiolabeled at the beginning of the chase period, and its specific radioactivity rapidly diminished within 3 min. The specific radioactivity of diacylglycerol peaked after 5 min of incubation. The labeling of lysophosphatidate was almost linear, and its specific radioactivity remained low at all time points. The labeling of phosphati-
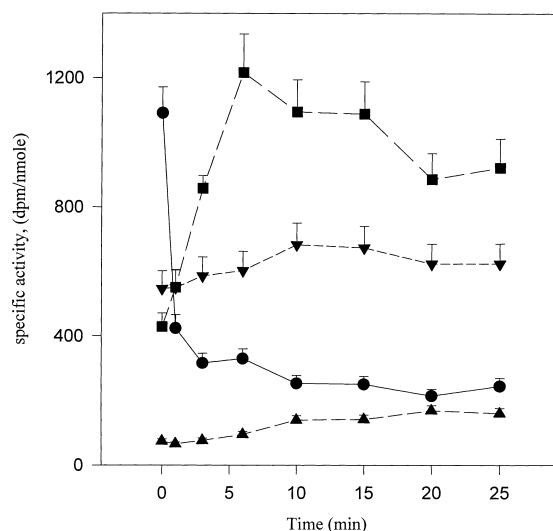

Fig. 2. Pulse labeling and chase analysis on glycerol metabolism in H9c2 cells. H9c2 in 35 mm dishes were preincubated with culture medium containing 0.1 mM [1,3-³H]glycerol for 1 min as described in Experimental Procedures. The preincubation medium was removed, and the cells were further incubated in culture medium containing 1 mM nonradiolabeled glycerol for different periods. The specific radioactivities of monoacylglycerol (closed circles), diacylglycerol (closed squares), lysophosphatidate (up closed triangles), and phosphatidate (down closed triangles) were determined as described in Experimental Procedures. Values are expressed as means ± SEM of three separate experiments done in triplicate.

date was not clearly defined, but appeared to peak after 10–15 min of incubation. Taken together, our results indicate that a considerable portion of the radiolabeled glycerol taken up by the cell was directly acylated to form monoacylglycerol. Some of the newly formed monoacylglycerol was converted to diacylglycerol and might subsequently have been converted to phosphatidate. The importance of diacylglycerol kinase for the conversion of diacylglycerol to phosphatidate is well documented (30), and the presence of this enzyme in cardiac tissues has been reported (40). Because the direct acylation of glycerol appeared to be rapid, the specific activity of glycerol acyltransferase was determined and compared with the specific activity of glycerokinase. Glycerol acyltransferase activity was 310 ± 18 pmol/min/mg protein, whereas the glycerokinase activity was found to be 8.3 ± 1.8 pmol/min/mg protein. The low glycerokinase activity may account for the low amount of [1,3-³H]glycerol incorporation into lysophosphatidate during the pulse-chase study.

The inhibition of glycerokinase

Monobutyrin is a known inhibitor of glycerokinase (41), and its ability to enter the cell has been demonstrated (38). The compound was employed to study the contribution of the direct acylation of glycerol by attenuating the glycerol-3-phosphate pathway. Our approach was to select a cell line with a relatively high degree of glycerokinase activity that could be attenuated by monobutyrin without inhibition to the glycerol acyltransferase activity. To determine whether monobutyrin could inhibit cellular glycerokinase in vivo, Chang liver cells were incubated for 1 h in the growth medium in the presence and absence of 10 mM monobutyrin. After the incubation, the cells were removed from the dish and suspended in a PBS solution, pH 7.4, containing a cocktail of protease inhibitors. The cells were disrupted by sonication, and the glycerol acyltransferase and glycerokinase activities were determined. Monobutyrin reduced the glycerokinase activity by 43%, from 119.5 ± 10 nmol/min/mg protein to 68 ± 10 nmol/min/mg protein. In contrast, glycerol acyltransferase activity was stimulated by 38%, from 92.7 ± 4 pmol/min/mg protein to 128 ± 9 pmol/min/mg protein. It is clear that monobutyrin has the ability to selectively attenuate the glycerol-3-phosphate pathway.

Attenuated glycerol-3-phosphated pathway and monoacylglycerol production

The effect of monobutyrin on the acylation of glycerol in the Chang liver cells was examined by a pulse-chase experiment. Cells were preincubated with or without 10 mM monobutryin and then incubated with a medium containing 0.1 mM [1,3-³H]glycerol (3.5 Ci/mmol) for 2 min. The media containing the label was removed from the dish, and the cells were subsequently incubated in culture media containing 1 mM nonradiolabeled glycerol for different time periods. The specific radioactivities of monoacylglycerol, diacylglycerol, lysophosphatidate, and phosphatidate were determined, and the results are depicted in **Fig. 3**. In the absence of monobutyrin, lysophosphati-
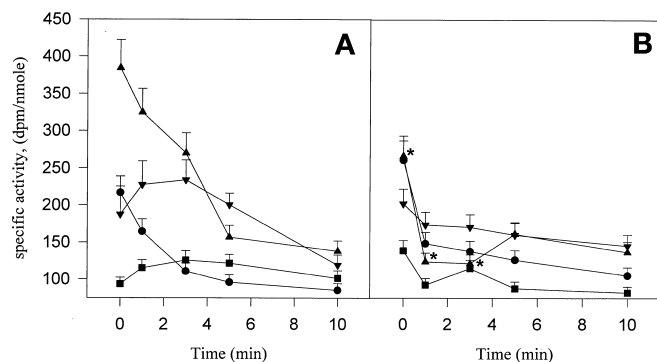
Fig. 3. Attenuation of the glycerol-3-phosphate pathway in Chang liver cells. Chang cells in 35 mm dishes were incubated with culture medium without (A) or with (B) 10 mM monobutyrin for 1 h. Subsequent to the monobutyrin exposure, the cells were incubated in a medium containing 0.1 mM $[1,3-^3H]$glycerol for 1 min. The medium was removed, and the cells were further incubated with medium containing 1 mM nonradiolabeled glycerol for different periods. The specific radioactivities of monoacylglycerol (closed circles), diacylglycerol (closed squares), lysophosphatidate (up closed triangles), and phosphatidate (down closed triangles) were determined and expressed as means ± SEM, n = 4; * $P < 0.05$ compared with the corresponding value in Fig. 3A.

date was immediately labeled (Fig. 3A). The specific radioactivity of lysophosphatidate decreased as the incubation time progressed, whereas the specific radioactivity of phosphatidate peaked after 3 min of incubation. The labeling profile of monoacylglycerol was parallel to that of lysophosphatidate, but never exceeded the latter in specific radioactivity. The diacylglycerol profile closely resembled the phosphatidate profile but was never higher in specific radioactivity. In concurrence with the specific activities of the enzymes, our results demonstrate that the majority of glycerol was metabolized through the glycerol-3-phosphate pathway in Chang liver cells. After the uptake of glycerol, lysophosphatidate was synthesized and subsequently converted to phosphatidate. Alternatively, a smaller percentage of glycerol was directly acylated to form monoacylglycerol, and acylated again to form diacylglycerol.

When Chang cells were preincubated with 10 mM monobutyrin (Fig. 3B), the specific radioactivity profile for lysophosphatidate was reduced during the first minute of incubation compared with cells not treated with monobutyrin (Fig. 3A). In contrast, the specific radioactivity in monoacylglycerol increased by 20% initially and as much as 32% midway during the chase period. Our results indicate that the direct acylation pathway could function as a shunt when the glycerol-3-phosphate pathway was attenuated.

The direct acylation pathway at high exogenous glycerol concentration

To determine whether the direct acylation of glycerol could be affected by exogenous glycerol concentration, pulse-chase experiments using H9c2 cells at different glycerol concentrations were conducted. The cells were grown in a 24-well plate to reduce the amount of radiolabel required. The cells were pulse labeled for 5 min with either

0.2 or 2.0 mM $[1,3-^3H]$glycerol (3.5 Ci/mmole) in a serum-free culture medium. Serum, which contained glycerol, was excluded from the labeling media to avoid diluting the specific radioactivity of glycerol. The cells were subsequently incubated in culture medium containing 1 mM or 10 mM glycerol, respectively, for different times, and the specific radioactivities of the lipids were determined after each incubation. In comparison with the first pulse-chase experiment (Fig. 2), the labeling profiles for mono- and diacylglycerol were noticeably altered (**Fig. 4A**). The difference may be due to the absence of serum in the labeling medium and the consequent reduced uptake of glycerol (38, 42). At 0.2 mM glycerol, the specific radioactivity curves for monoacylglycerol, triacylglycerol, and phosphatidylcholine were low and remained linear throughout the incubation. Lysophosphatidate was immediately labeled at the beginning of the chase period, and the specific radioactivity rapidly decreased within 3 min. The specific radioactivity of phosphatidate peaked after 3 min and decreased as the incubation progressed. The specific radioactivity of diacylglycerol peaked after 12 min of incubation. The labeling profile indicates that under low glycerol concentrations, a small amount of glycerol is converted to lysophosphatidate and, subsequently, to phosphatidate and diacylglycerol in H9c2 cells.

When H9c2 cells were pulse labeled with higher concentrations of glycerol, the labeling profiles of phosphatidylcholine, mono-, di-, and triacyglcyerol were significantly altered (Fig. 4B). Monoacylglycerol was immediately labeled, and its specific radioactivity rapidly decreased within 1 min. Diacylglycerol and triacylglycerol were also labeled, and their specific radioactivity curves peaked after 6 min and 12 min of incubation, respectively. The specific radioactivity of phosphatidylcholine gradually increased as the incubation progressed. These results indicate that at phys-
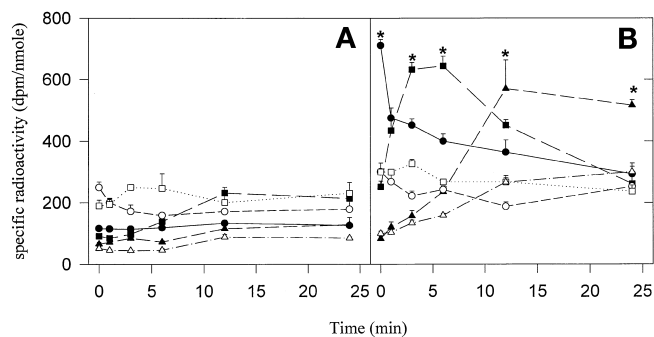

Fig. 4. The direct acylation pathway is regulated by substrate availability. H9c2 cells in 24-well plates were pulse labeled with 0.2 mM (A) or 2.0 mM (B) $[1,3-H]$glycerol in serum-free medium for 5 min. The medium was removed and the cells were further incubated with medium containing 1 mM (A) or 10 mM (B) nonradiolabeled glycerol for different time periods. The specific radioactivities of monoacylglycerol (closed circles), diacylglycerol (closed squares), triacylglycerol (closed triangles), lysophosphatidate (open circles), phosphatidate (open squares), and phosphatidylcholine (open tirangles) were determined. Values are expressed as means ± SEM of two separate experiments done in quadruplicate; * $P < 0.05$ compared with the corresponding value in Fig. 4A.

TABLE 2. Glycerol acyltransferase activity in microsomal rat and porcine tissue

	Specific Activity	
Tissue	Rat	Pig
	nmoles/min/mg	
Heart	0.56	3.88
Liver	1.06	1.04
Skeletal muscle	0.86	3.78
Kidney	0.35	2.16
Brain	ND	0.96

The reaction mixture contained 10 μg of protein, 64.4 μM [1-^{14}C]-arachidonyl-CoA (51.6 mCi/mmol), and 10% (v/v) glycerol in 50 mM Tris-HCl, pH 7.8. The reaction mixture was incubated at 37°C for 30 min, and the product was separated by TLC using benzene−diethyl ether−ethanol−acetic acid 50:40:2:0.2 (v/v/v/v) as the solvent system. ND = not determined.

iological levels, glycerol is metabolized through the glycerol-3-phosphate pathway in H9c2 cells. In contrast, a considerable amount of glycerol is shunted toward the direct acylation pathway for lipid biosynthesis when glycerol levels become elevated.

Characterization of the acyl-CoA:glycerol acyltranseferase

The characterization of acyl-CoA:glycerol acyltransferase activity has not been previously documented. Hence, the enzyme activity was determined in various tissues. As shown in **Table 2**, the distribution of the enzyme appears to be tissue and species specific. Because of the high activity of glycerol acyltransferase in pig heart, this tissue was chosen for subsequent studies. The heart was selected also because a substantial amount of glycerol is produced during the lipolysis of triacylglycerol (43). Glycerokinase activity is nearly undetectable in mammalian cardiac tissues (44) and, consequently, a lesser amount of glycerol is recycled for the glycerol-3-phosphate pathway.

The majority of glycerol acyltransferase activity was localized in the microsomal fraction. The microsomal enzyme was active over a broad pH range, with the optimal activity at pH 6.0. Enzyme activity was optimal at 55°C, but incubation at 60°C resulted in an irreversible inactivation. The enzyme had the highest specificity toward arachidonyl-CoA (100%), but with a lower activity toward palmitoyl-CoA (25%), stearoyl-CoA (40%), and oleoyl-CoA (60%). No activity was observed when arachidonic acid was used

as the acyl donor. The apparent K_m values of the enzyme for glycerol and acyl-CoA were determined by varying one substrate concentration while maintaining the other substrate concentration constant (**Fig. 5**). From the Lineweaver-Burke plots, the apparent K_m for glycerol was 1.1 mM, whereas the apparent K_m for arachidonyl-CoA was 0.17 mM.

DISCUSSION

In the synthesis of glycerolipids, it is well established that glycerol has to be phosphorylated to glycerol-3-phosphate before acylation occurs. Consequently, results from previous studies on glycerol metabolism might have been misinterpreted, especially when other metabolic routes for glycerol are present. The metabolism of glycerol was re-examined in the current study, and there are several lines of evidence supporting a novel pathway for lipid biosynthesis. First, pulse-chase studies with both H9c2 and Chang liver cells demonstrated that glycerol could be directly converted to monoacylglycerol and, subsequently, other lipids. Second, we detected and characterized a novel enzyme for the direct acylation of glycerol. The glycerol acyltransferase has the ability to transfer various long-chain acyl-CoA species, but displays a high degree of specificity for arachidonyl-CoA.

Although the physiological importance of the direct acylation pathway has not been completely established, we produced evidence that suggests that the pathway may serve as a shunt for glycerol metabolism. Glycerol is present in the mammalian tissues at a concentration of about 0.1 mM (45). In human serum, the level of glycerol fluctuates between 0.04 and 0.4 mM (46). Kinetic studies on the glycerol acyltransferase revealed that it has an apparent K_m value of 1.1 mM for glycerol, indicating the rate of acylation should be directly proportional to the intracellular glycerol concentration. As such, the direct acylation of glycerol may occur only in limited capacity during normal physiological conditions.

Alternatively, the intracellular glycerol concentration is dramatically increased in certain forms of muscular dystrophy, in diabetes, and during fasting, extreme cold, and ischemia of the heart (46, 47). Because the acylation of glycerol is regulated by its availability, the direct acylation pathway may be potentiated during hyperglycerolemia.
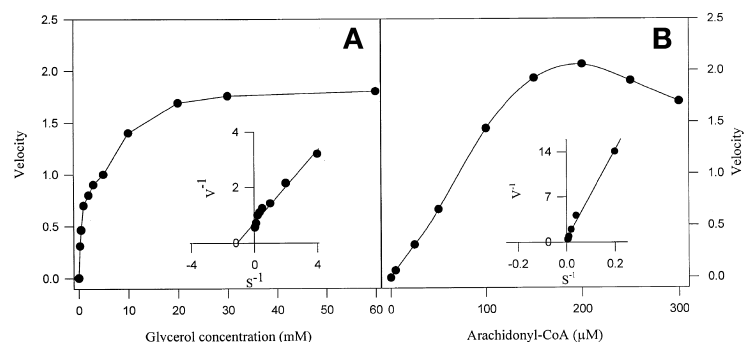

Fig. 5. Kinetic analysis of glycerol acyltransferase. The apparent K_m values for glycerol (A) and arachidonyl-CoA (B) were determined by varying one substrate concentration while maintaining the other substrate concentration constant. Enzyme assays were conducted as described in Experimental Procedures. The arachidonyl-CoA concentration was held at 200 μM while the glycerol concentration was varied (A). The glycerol concentration was held at 5% (v/v; 0.68 M) while the arachidonyl-CoA concentration was varied (B). Inset: Lineweaver-Burk plots. Velocity is expressed as nmoles/min/mg protein.

The importance of the direct acylation pathway under such pathological condition is evident in our pulse-chase studies on glycerol metabolism. In addition, the inhibition of glycerol kinase in Chang liver cells caused glycerol metabolism to be shunted toward the direct acylation pathway. Consequently, the enhanced direct acylation of glycerol resulted in elevated monoacylglycerol production. Incubation of H9c2 cells in medium containing high glycerol levels also caused the direct acylation pathway to become more prominent. These studies clearly establish the direct acylation pathway as an alternative route for glycerol metabolism. ⬛

REFERENCES

1. Brindley, D. N., and D. W. Waggoner. 1996. Phosphatidate phosphohydrolase and signal transduction. *Chem. Phys. Lipids.* **80:** 45–57.
2. Exton, J. 1994. Phosphtidylcholine breakdown and signal transduction. *Biochem. Biophys. Acta.* **1212:** 26–42.
3. Janssen-Timmen, U., I. Tomic, E. Specht, B. U. Beileke, and A. J. Habenicht. 1994. The arachidonic acid cascade, eicosanoids, and signal transduction. *Ann. NY Acad. Sci.* **733:** 325–334.
4. Kennedy, E. P. 1989. Discovery of the pathways for the biosynthesis of phosphatidylcholine, *In* Phosphatidylcholine Metabolism. D. E. Vance, editor. CRC Press, Boca Raton, FL. 1–8.
5. Lands, W. E., and P. Hart. 1965. Metabolism of glycerolipids. *J. Biol. Chem.* **240:** 1905–1911.
6. Thorner, J. W. 1975. Glycerol kinase. *Methods Enzymol.* **42:** 148–156.
7. Hajra, A. K., L. K. Larkins, A. K. Das, N. Hemati, R. L. Erickson, and O. A. MacDougald. 2000. Induction of the peroxisomal glycerolipid-synthesizing enzymes during differentiation of 3T3–L1 adipocytes. Role in triacylglycerol synthesis. *J. Biol. Chem.* **275:** 9441–9446.
8. Wilkison, W. O., and R. M. Bell. 1997. sn-Glycerol-3-phosphate acyltransferase from Escherichia coli. *Biochim. Biophys. Acta.* **1348:** 3–9.
9. Dircks, L., and H. S. Sul. 1999. Acyltransferases of de novo glycerophospholipid biosynthesis. *Prog. Lipid Res.* **38:** 461–479.
10. Carrasco, M. P., J. L. Segovia, and C. Marco. 1998. Incorporation of exogenous precursors into neutral lipids and phospholipids in rat hepatocytes: effect of ethanol in vitro. *Biochem. Pharmacol.* **56:** 1639–1644.
11. Akesson, B., J. Elovson, and G. Arvidson. 1970. Initial incorporation into rat liver glycerolipids of intraportally injected (3H) glycerol. *Biochim. Biophys. Acta.* **210:** 15–27.
12. Wirthensohn, G., A. Vandewalle, and W. Guder. 1981. Renal glycerol metabolism and the distribution of glycerol kinase in rabbit nephron. *Biochem. J.* **198:** 543–549.
13. Kinsella, J. E. 1968. The incorporation of [14C3]glycerol into lipids by dispersed bovine mammary cells. *Biochim. Biophys. Acta.* **164:** 540–549.
14. Kasemsri, S., L. L. Bernardis, C. Chlouverakis, and J. D. Schnatz. 1972. The incorporation of 14 C-glycerol into adipose tissue lipids of weanling rats with hypothalamic obesity. *Proc. Soc. Exp. Biol. Med.* **141:** 38–42.
15. Uhal, B. D., and W. J. Longmore. 1988. Glycerol as a substrate for phospholipid biosynthesis in type II pneumocytes isolated from streptozotocin-diabetic rats. *Biochim. Biophys. Acta.* **961:** 122–128.
16. Chung, A. C. 1978. Incorporation of labelled glucose and glycerol into phospholipids and triacylglycerides by rat and rabbit aorta. *Atherosclerosis.* **29:** 277–290.
17. Wong, J. T., R. Y. Man, and P. C. Choy. 1996. The effects of lidocaine and hypoxia on phospholipid biosynthesis in the isolated hamster heart. *Lipids.* **31:** 1059–1067.
18. Fotheringham, J., F. Y. Xu, M. Nemer, E. Kardami, P. C. Choy, and G. M. Hatch. 2000. Lysophosphatidylethanolamine acyltransferase activity is elevated during cardiac cell differentiation. *Biochim. Biophys. Acta.* **1485:** 1–10.
19. Guo, Z., and M. D. Jensen. 1999. Blood glycerol is an important precursor for intramuscular triacylglycerol synthesis. *J. Biol. Chem.* **274:** 23702–23706.
20. Hanel, A. M., and M. H. Gelb. 1995. Multiple enzymatic activities of the human cytosolic 85kDa phopholipase A2: hydrolytic reactions and acyl transfer to glycerol. *Biochemistry.* **34:** 7807–7818.
21. Sugimoto, H., and S. Yamashita. 1999. Characterization of the transacylase activity of rat liver 60-kDa lysophospholipase-transacylase. Acyl transfer from the sn-2 to the sn-1 position. *Biochim. Biophys. Acta.* **1438:** 264–272.
22. Margolis, S., and M. Vaughan. 1962. Glycerophosphate synthesis and breakdown in homogenates of adipose tissue. *J. Biol. Chem.* **237:** 44–48.
23. Coleman, R., and E. Haynes. 1985. Subcellular location and topography of rat hepatic monoacylglycerol acyltransferase activity. *Biochem. Biophys. Acta.* **834:** 180–187.
24. Swanton, E., and E. Saggerson. 1997. Effects of adrenaline on triacylglycerol synthesis and turnover in ventricular myocytes from adult rats. *Biochem. J.* **328:** 913–922.
25. Coleman, R. 1993. Hepatic monoacylglycerol acyltransferase activity in HA1 and HA7 hepatoma/hepatocyte hybrid cells: regulation by insulin and dexamethasone and by cell density. *Biochem. Biophys. Acta.* **1165:** 306–313.
26. Mostafa, N., B. Bhat, and R. Coleman. 1994. Adipose monoacylglycerol:acyl-coenzyme A acyltransferase activity in the white-throated sparrow (Zonotrichia albicollis): characterization and function in a migratory bird. *Lipids.* **29:** 785–791.
27. Lehner, R., and A. Kuksis. 1992. Utilization of 2-monoacylglycerols for phophatidylcholine biosynthesis in the intestine. *Biochim. Biophys. Acta.* **1125:** 171–179.
28. Simpson, C. M. F., H. Itabe, C. N. Reynolds, W. C. King, and J. A. Glomset. 1991. Swiss 3T3 cells preferentially incorporate sn-2-arachidonoyl monoacylglycerol into sn-1-stearoyl-2-arachidonoyl phosphatidylinositol. *J. Biol. Chem.* **266:** 15902–15009.
29. Wakelam, M. 1998. Diacylglycerol—when is it an intracellular messenger? *Biochim. Biophys. Acta.* **1436:** 117–126.
30. Topham, M., and S. M. Prescott. 1999. Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions. *J. Biol. Chem.* **274:** 11447–11450.
31. Arthur, G., L. Page, T. Mock, and P. C. Choy. 1986. The catabolism of plasmenylcholine in the guinea pig heart. *Biochem. J.* **236:** 475–480.
32. Hill, R. C., and R. A. Bradshaw. 1969. Fumarase. *Methods Enzymol.* **13:** 91–99.
33. Pennington, R. J. 1961. Biochemistry of dystrophic muscle. *Biochem. J.* **80:** 649–654.
34. Morre, J. D. 1971. Isolation of golgi apparatus. *Methods Enzymol.* **22:** 130–148.
35. Bers, D. M. 1979. Isolation and characterization of cardiac sarcolemma. *Biochim. Biophys. Acta.* **555:** 131–146.
36. Kessler, R. J., and D. D. Fanestil. 1986. Interference by lipids in the determination of protein using bicinchoninic acid. *Anal. Biochem.* **159:** 138–142.
37. Bisogno, T., N. Sepe, D. Melck, S. Maurelli, L. De Petrocellis, and V. Di Marzo. 1997. Biosynthesis, release and degradation of the novel endogenous cannabimimetic metabolite 2-arachidonylglycerol in mouse neuroblastoma. *Biochem. J.* **322:** 671–677.
38. Westergaard, N., P. Madsen, and K. Lundgren. 1998. Characterization of glycerol uptake and glycerol kinase activity in rat hepatocytes cultured under different hormonal conditions. *Biochim. Biophys. Acta.* **1402:** 261–268.
39. Metcalfe, L. D., and A. A. Schmitz. 1961. The rapid preparation of fatty acid esters for gas chromatographic analysis. *Anal. Chem.* **33:** 363–364.
40. Yamada, K., and H. Kanoh. 1988. Occurrence of immunoreactive 80 kDa and non-immunoreactive diacylglycerol kinases in different pig tissues. *Biochem. J.* **255:** 601–608.
41. Tisdale, M. J., and M. D. Threadgill. 1984. (+/−)2,3,-dihydroxy-

propyl dichloroacetate, an inhibitor of glycerol kinase. *Cancer Biochem. Biophys.* **7:** 253–259.

42. Dominguez, M. C., and E. Herrera. 1976. The effect of glucose, insulin and adrenaline on glycerol metabolism in vitro in rat adipose tissue. *Biochem. J.* **158:** 183–190.

43. Trach, V., E. Buschmans-Denkel, and W. Schaper. 1986. Relation between lipolysis and glycolysis during ischemia in the isolated rat heart. *Basic Res. Card.* **81:** 454–464.

44. Robinson, J., and E. A. Newsholme. 1967. Glycerol kinase activities in rat heart and adipose tissue. *Biochem. J.* **104:** 2C–4C.

45. Robergs, R. A., and S. E. Griffin. 1998. Glycerol—bio-chemistry and clinical and practical applications. *Sports Med.* **26:** 145–167.

46. Lin, E. C. 1977. Glycerol utilization and its regulation in mammals. *In* Annual Review of Biochemistry. E. E. Snell, P. D. Boyer, A. Meister, and C. C. Richardson, editors. Annual Reviews, Palo Alto, CA. 765–795.

47. Kohlschutter, A., H. P. Willig, D. Schlamp, K. Kruse, E. R. McCabe, H. J. Schafer, G. Beckenkamp, and R. Rohkamm. 1987. Infantile glycerol kinase deficiency—a condition requiring prompt identification. Clinical, biochemical, and morphological findings in two cases. *Eur. J. Pediatr.* **146:** 575–581.