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Acute effects of insulin on the activity of mitochondrial GPAT1 in primary adipocytes

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Abstract

The mitochondrial enzyme 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (mtGPAT1) catalyzes a rate-limiting step in triacylglycerol and glycerophospholipid biosynthesis, which can be modulated by protein kinases in cell free analyses. We report that treatment of primary rat adipocytes with insulin acutely affects the activity of mtGPAT1 by increasing V_{MAX} and K_M for the substrates glycerol-3-phosphate and palmitoyl-CoA. Proteolytic cleavage of isolated mitochondrial membranes and mass spectrometric peptide sequencing identify *in vivo* phosphorylation of serine 632 and serine 639 in mtGPAT1. These phosphorylation sites correspond to casein kinase-2 consensus sequences and are highly conserved in chordate animal, but not fly, fungal or plant, mtGPAT1.

Keywords: Insulin control; Triacylglycerol; Obesity; Kinetics; Phosphorylation; Mass spectrometry

With the prevalence of obesity taking pandemic proportions, and diabetes and cardiovascular disease as common co-morbidities, much interest focuses on adipose tissue, the pathways of triacylglycerol biosynthesis and their regulation. The enzyme 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (GPAT) catalyzes the conversion of glycerol-3-phosphate to lysophosphatidic acid, which is a rate-limiting step in triacylglycerol and glycerophospholipid biosynthesis in cells. In mammals there are two major iso-forms of the enzyme: mitochondrial and microsomal [1,2]. The mitochondrial sulfhydryl reagent-insensitive enzyme (mtGPAT1) [3–5] prefers saturated fatty acyl-CoA as a substrate and comprises about 1/10 of total GPAT activity in most tissues, whereas in liver it represents up to half the total activity [2]. Despite a dominance of the microsomal activity, the mtGPAT1 was found important for triacylglycerol synthesis from exogenous fatty acids in CHO cells overexpressing the mtGPAT1 [6] or in liver of mtGPAT$^{-/-}$ mice [7]. Mitochondrial GPAT1$^{-/-}$ mice were protected from high-fat diet-induced insulin resistance [8,9], while transgenic overexpression resulted in hepatic steatosis [10]. Hepatic knockdown of mtGPAT1 improved the metabolic profile in ob/ob mice [9,11], whereas hepatic overexpression induced insulin resistance in liver [12]. Mitochondria also contain some minor *N*-ethylmaleimide-sensitive GPAT activity (mtGPAT2) with kinetic properties similar to the microsomal enzyme [13]. Recently two splice variants of mtGPAT1, which differ in their expression pattern, were also identified [14].

Insulin stimulates transcription of the mtGPAT1 gene and glucagon antagonizes the insulin effect. Transcription of mtGPAT1 is enhanced by re-feeding a carbohydrate diet to fasted mice or by insulin administration to diabetic animals, whereas adrenalectomy or administration of anti-insulin antibodies significantly reduced the liver mtGPAT1 [1,2,15,16]. Effects on the activity of mtGPAT1 are largely due to transcriptional regulation and changes in levels of the mtGPAT1 protein [17]. However, a discordance

Abbreviations: GPAT, 1-acyl-*sn*-glycerol-3-phosphate acyltransferase.

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between the amount of mtGPAT1 protein and activity was found in the heart of re-fed rats [16], indicating that the enzyme activity may also be regulated at a post-translational level. Moreover, the activity of mtGPAT1 in cell free systems was affected by protein kinases, particularly, casein kinase-2 [18]. In this paper we report acute effects of insulin on the kinetic properties of mtGPAT1 in adipocytes and identify two *in vivo* phosphorylation sites in this enzyme.

Materials and methods

Materials. L-[U-^{14}C]glycerol-3-phosphate (2000–4000 cpm/nmol) and iminodiacetic acid-coupled Sepharose (for IMAC) were from Amersham, UK. E64, bestatin, cantharidin, cypermethrin, and palmitoyl-CoA were from Sigma Chem. Co. All other reagents were from Sigma unless otherwise indicated.

Isolation and incubation of adipocytes. Adipocytes were isolated from the epididymal fat pads of Sprague–Dawley rats (140–160 g, B&K Universal, Sollentuna, Sweden; animals were treated according to Swedish animal care regulations) by collagenase digestion [19]. Cells (100 μl packed cell volume per ml) in Krebs–Ringer solution (0.12 M NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 1.2 mM KH_2PO_4) containing 20 mM Hepes, pH 7.40, 1% (w/v) fatty acid-free bovine serum albumin, 100 nM phenylisopropyladenosine, 0.5 U mL^{-1} adenosine deaminase with 2 mM glucose, were incubated with or without 5 nM insulin for 20 min before homogenization.

Isolation of mitochondria. Cells were homogenized in 5 vol of homogenization buffer (0.25 M sucrose, 10 mM NaH_2PO_4, pH 7.4, 1 mM EDTA, 0.5 mM EGTA, 1 mM $Na_2P_2O_7$, 4 mM iodoacetate, 5 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, containing, the additional protease and phosphatase inhibitors (2 mM orthovanadate, 1.5 μM E64, 3 μM bestatin, 20 μM cantharidin, and 25 nM cypermethrin) at room temperature, with five strokes in loose-fitting motor-driven teflon/glass homogenizer. Homogenates were centrifuged at 1000*g*, 10 min, 4 °C, and the supernatant further at 16,000*g*, 20 min. The pellet was resuspended and layered onto a 0.75 mL cushion of 1.12 M sucrose and centrifuged 1 h, 140,000*g* (Beckman TLS-55) [20]. The pellet was again resuspended and pelleted to constitute purified mitochondria (stored at −20 °C).

Glycerol-3-phosphate acyltransferase assay. For kinetic analysis purified mitochondria were suspended at 2 mg protein/mL by bath-sonication 10 mM Tris–Cl, pH 7.4, 0.25 mM sucrose, 1 mM EDTA, 0.2 mM dithiothreitol, containing protease and phosphatase inhibitors. The mitochondrial homogenate was pre-incubated on ice with 2 mM *N*-ethylmaleimide, 20 min, to inactivate microsomal GPAT.

The enzyme was assayed for transfer of palmitate from palmitoyl-CoA to L-[U-^{14}C]glycerol-3-phosphate to generate the lysophosphatidate product in 250 μL of 100 mM Tris–HCl, pH 7.4, 10 mM NaF, 0.7 mM dithiothreitol, 1.75 mg/mL bovine serum albumin (fatty acid-free), and protease and phosphatase inhibitors, at 30 °C for 15 min. Reactions were terminated by extraction of the product with 1-butanol and radioactivity determined by liquid scintillation counting [3]. The assay was linear for at least 15 min.

From plots of initial rate of lysophosphatidic acid formation versus substrate concentration we determined K_M and V_{MAX} by fitting data to Michaelis–Menten or Hill equations using non-linear least squares fit. With Hill coefficient of 1 ± 0.05 Michaelis–Menten Eq. (1) was used. When the Hill coefficient deviated from 1 by >0.05 Hill Eq. (2) was used

$$V_{MAX}/(1 + K_M/[S]), \quad (1)$$

$$V_{MAX}/(1 + (K_M/[S])^h). \quad (2)$$

For analysis of dose–response curves with allosteric inhibition, when substrate binds at two different sites, one catalytic and one allosteric inhibitory (bell-shaped curves), Eqs. (3)–(5) were used [21,22]:

$$V_{MAX} - V_{MAX}/(1 + IC_{50}/[S]), \quad (3)$$

$$V_{MAX}/(1 + K_M/[S]) - V_{MAX}/(1 + IC_{50}/[S]), \quad (4)$$

$$V_{MAX}/(1 + K_M/[S]) - V_{MAX}/(1 + (IC_{50}/[S])^h). \quad (5)$$

Sub-fractionation of mitochondria, tryptic digestion and phosphopeptide isolation. Purified mitochondria were resuspended at 2 mg/mL protein in 25 mM NH_4HCO_3, pH 8.0, 0.5 mM EDTA, containing protease and phosphatase inhibitors. The suspension was sonicated for 3×30 s, diluted to 0.5 mg/mL and centrifuged 200,000*g*, 20 min (Beckman TLA-110). The pellet was suspended in 150 μl, re-sonicated (2×30 s), diluted with 500 μl of above buffer, and re-centrifuged 200,000*g*, 20 min. The pellet, consisting of submitochondrial particles, was suspended in 50 μl 25 mM NH_4HCO_3, pH 8.0, containing phosphatase inhibitors. The suspension was alkylated with iodoacetamide and treated with trypsin at 1 μg per 25 μg protein for 20 h at 37 °C. After centrifugation at 200,000*g*, 20 min, the peptide-containing supernatant was dried in a vacuum centrifuge. The dried peptide pellet was methylated twice and phosphopeptides enriched by immobilized-metal-affinity-chromatography [20].

Mass spectrometry. Phosphopeptides were analyzed on a hybrid mass spectrometer API Q-STAR *Pulsar i* (Applied Biosystems, Foster City, CA, USA) equipped with nanoelectrospray ion-source (MDS Protana, Odense, Denmark). Desalted peptides in 50% acetonitrile, 1% formic acid in water were analyzed. Mass spectra of phosphopeptides fragmented by collision-induced dissociation were acquired with instrument settings recommended by Applied Biosystems with manual control of collision energy.

Results

Adipose tissue mitochondrial activity and activity of mtGPAT1

The abundance of mitochondria and mitochondrial DNA in adipose tissue correlates with the activities of citrate synthase and cytochrome *c* oxidase [23]. We thus examined these activities in epididymal fat and mixed-fiber quadriceps muscle [24]. The specific activity of the citrate synthase was comparable in adipose tissue and in skeletal muscle homogenates (79 ± 10 and 96 ± 8 nmol/min/mg homogenate protein, respectively, mean ± SE, $n = 6$ preparations), demonstrating a mitochondrial density in adipose tissue that is comparable to skeletal muscle. The specific activity of cytochrome *c* oxidase (295 ± 54 and 454 ± 13 nmol/min/mg protein, in adipose and muscle, respectively, $n = 6$ preparations) was 4- to 5-fold higher than that of citrate synthase, indicating metabolically active mitochondria of high quality in the adipose preparations used herein.

We purified mitochondria from isolated rat adipocytes using sucrose density gradient centrifugation. We have earlier demonstrated the purity of adipocyte mitochondria prepared by this method [20]. In the mitochondrial fraction we assayed the activity of mtGPAT1. There was very little effect by the sulfhydryl reagent *N*-ethylmaleimide on the enzyme activity (⩽10% inhibition, most likely representing the sulfhydryl-sensitive mtGPAT2). This also indicated that there was very little interference from contamination with the *N*-ethylmaleimide-sensitive microsomal GPAT3 enzyme. However, we preincubated all preparations with *N*-ethylmaleimide prior to further analysis to inactivate traces of the microsomal enzyme activity and the mtGPAT2 activity [25].

Effects of insulin on the kinetic properties of mtGPAT1

We next examined the effect of insulin treatment of adipocytes on the activity of mtGPAT1 in the purified mitochondria. Varying the concentration of glycerol-3-phosphate and keeping the other substrate palmitoyl-CoA at 80 μM, we observed that the response curves for the enzyme activity changed after insulin treatment (Fig. 1). The apparent V_{MAX} and K_M for glycerol-3-phosphate were increased 3- and 4-fold, respectively, in response to insulin (Table 1). The enzyme kinetics deviated from ordinary Michaelis–Menten kinetics. When we used the Hill equation for non-linear regression of data, a Hill coefficient of 1.12 (Table 1) was determined for the control preparation, indicating existence of a site where glycerol-3-phosphate binding can lead to partial inhibition of enzyme activity. After incubation of the cells with insulin the enzyme exhibited a Hill coefficient of 0.87 (Table 1), suggesting the presence of two or more active forms of the enzyme.

Varying the concentration of glycerol-3-phosphate in the presence of a lower concentration (20 μM) of palmitoyl-CoA demonstrated a biphasic effect on the mtGPAT1 activity in mitochondria from both control and insulin-treated cells. At low concentrations of glycerol-3-phosphate the enzyme activity exhibited a typical Michaelis–Menten behavior with $K_M \sim 0.5$ mM, whereas higher concentrations of glycerol-3-phosphate had an inhibitory effect with $IC_{50} \sim 12$ mM (Table 1). This inhibition is compatible with the existence of an inhibitory glycerol-3-phosphate binding site with an apparent Hill coefficient

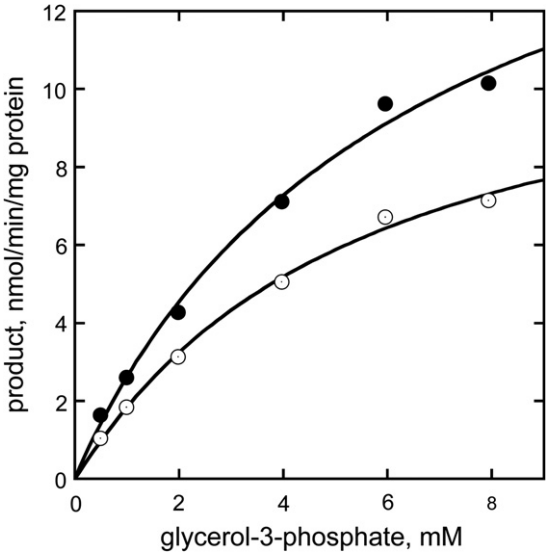

Fig. 1. Effects of insulin on the activity of mtGPAT1 at varying concentrations of glycerol-3-phosphate. Adipocytes were incubated without (open) or with insulin (closed), when cells were homogenized and mitochondria prepared. After disruption of mitochondria by sonication, mtGPAT1 activity was analyzed at 80 μM palmitoyl-CoA and 0.5–8 mM glycerol-3-phosphate. Values are the mean of two determinations from one representative experiment.

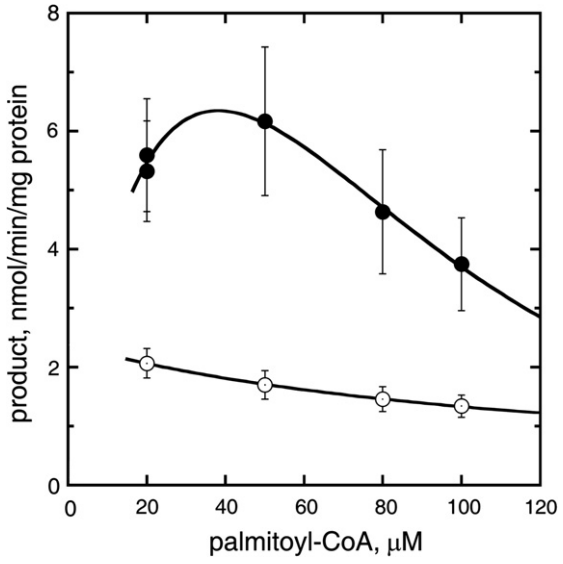

Fig. 2. Effects of insulin on the activity of mtGPAT1 at varying concentrations of palmitoyl-CoA. Adipocytes were incubated without (open) or with insulin (closed), when cells were homogenized and mitochondria prepared. After disruption of mitochondria by sonication mtGPAT1 activity was analyzed at 2 mM glycerol-3-phosphate and 20–100 μM palmitoyl-CoA. Experimental data are mean ± SE of three (without insulin) or four (with insulin) independent analyses.

Table 1
Summary of kinetic parameters of the acyltransferase reaction determined with the two substrates glycerol-3-phosphate and palmitoyl-CoA in mitochondria isolated from adipocytes incubated with (+) or without (−) insulin

Fixed substrate concentration	Insulin	K_M (mM) glycerol-3-phosphate	K_M (μM) palmitoyl-CoA	V_{MAX} (nmol/min/mg) glycerol-3-phosphate	IC_{50} (mM) glycerol-3-phosphate	IC_{50} (μM) palmitoyl-CoA	Hill coefficient	*n*
Palmitoyl-CoA 80 μM	−	3.9 ± 0.6		6.1 ± 1.6			1.12 ± 0.04	7
	+	16.4 ± 4.9*		16.6 ± 3.7*			0.87 ± 0.01*	7
Palmitoyl-CoA 20 μM	−	0.42 ± 0.04		7.2 ± 0.3	12.0 ± 1.1		1#	4
Glycerol-3-phosphate	−		⩽7	2.40 ± 0.02		126.0 ± 3.5	1#	3
2 mM	+		26.4 ± 7.3	14.1 ± 2.4*		94.5 ± 2.2*	2##	4

#Eqs (3) and (4) (Materials and methods) were used (Hill coefficients = 1).
##Eq. (5) gave Hill coefficient = 1.94 and, as the nearest integer, 2 was used in calculations.
*The effect of insulin was statistically significant ($P < 0.05$) by Student's *t*-test.

>1 seen in the control mitochondria at high palmitoyl-CoA concentrations.

We next varied the concentration of palmitoyl-CoA against a fixed 2 mM concentration of glycerol-3-phosphate. Mitochondrial GPAT 1 also exhibited a biphasic response to increasing concentrations of this substrate, with increased inhibition at higher concentrations of palmitoyl-CoA (Fig. 2). However, with insulin-treated cells the response curve was shifted to the right, requiring higher concentrations of palmitoyl-CoA for inhibition, with IC_{50} at ~130 and ~90 μM palmitoyl-CoA in mitochondria from control and insulin-treated cells, respectively (Table 1).

The calculated V_{MAX} after insulin stimulation was ~6-fold higher than in mitochondria from control cells (Table 1). The K_M for palmitoyl-CoA was calculated to 26 μM (Table 1) in insulin-treated cells. K_M was not determined in mitochondria from control cells as the enzyme was saturated already at 20 μM palmitoyl-CoA, but it was estimated to be <5–7 μM which is in agreement with earlier estimates (~3–7 μM) [4]. Hence, the insulin treatment of

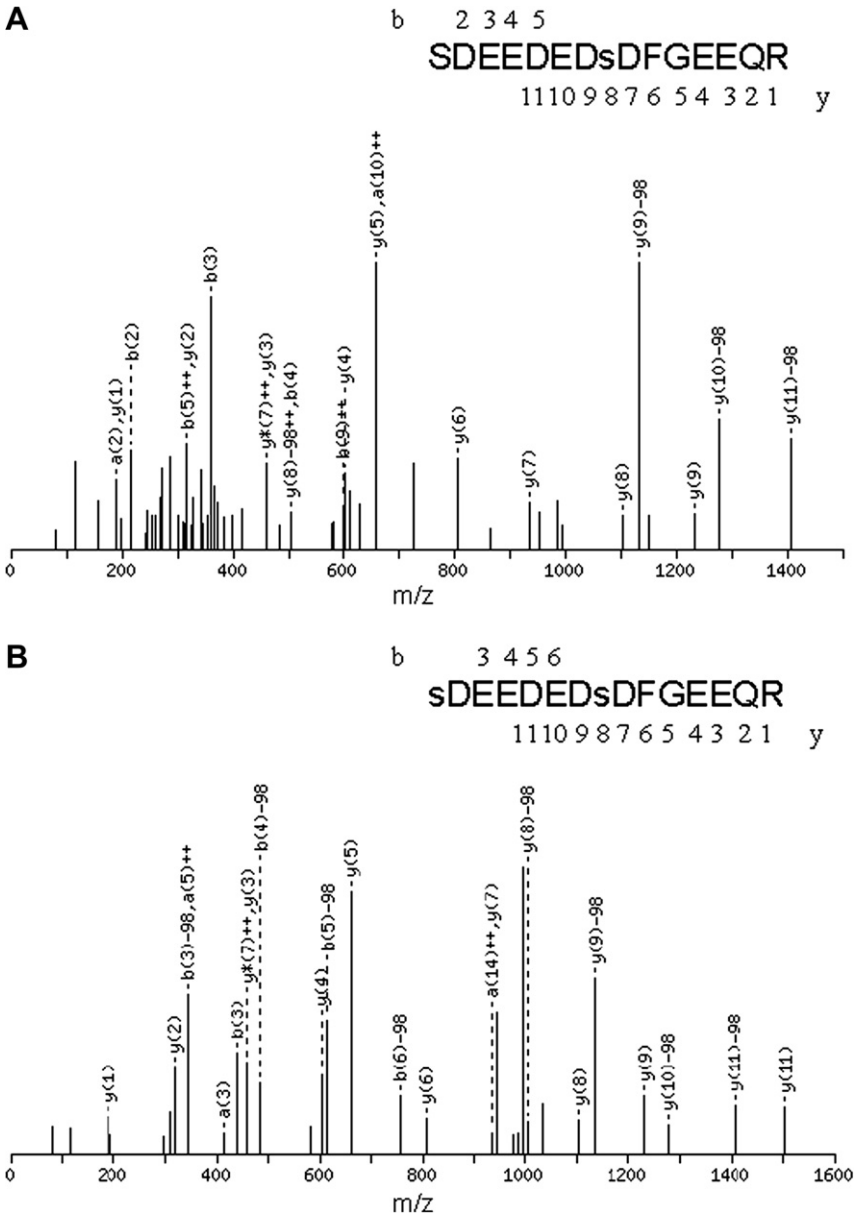

Fig. 3. Mapping of phosphorylation sites in mtGPAT1. Collision-induced fragmentation ion spectra of doubly protonated ions with $m/z = 1004.0$ (A) and $m/z = 1043.9$ (B) corresponding to mono- (A) and doubly-phosphorylated (B) peptide from the C-terminal part of mtGPAT1 (accession AAB39470: residues from 632 to 646). The major detected *y*(C-terminal) and *b*(N-terminal) fragment ions are indicated in the spectra and in corresponding amino acid sequences. Ions that underwent neutral loss of phosphoric acid (H_3PO_4, 98 Da) are indicated as *y*(*n*)-98 or *b*(*n*)-98. (A) The fragment ions from *b*(2) to *b*(5) and from *y*(1) to *y*(7) do not contain phosphate, while the fragments from *y*(8) to *y*(11) contained phosphate and/or underwent neutral loss of 98, which defines the phosphorylation site at position 8 from the peptide N-terminus. (B) The series of *y* ions like in (A) defines one phosphorylation site at position 8, while the *b* ions, particularly *b*(3) and *b*(3)-98, reveal additional phosphorylation of N-terminal serine residue. The phosphorylated serine residues are indicated by lower case "s".

adipocytes also increased the K_M for palmitoyl-CoA binding to the mtGPAT1 active site. The allosteric sites, where palmitoyl-CoA binding induced mtGPAT1 inhibition, appeared to be similar after insulin treatment as the ratio IC_{50}(+insulin)/IC_{50}(−insulin) for palmitoyl-CoA inhibition was 0.73. A Hill coefficient of 2 after insulin treatment indicates the existence of at least two sites where palmitoyl-CoA binding caused inhibition with similar IC_{50}.

Identification of phosphorylated serine residues in mtGPAT1

As the activity of mtGPAT1 is under control by insulin we examined the protein for phosphorylation. Submitochondrial particles of isolated mitochondria were prepared and treated with trypsin to digest exposed regions of membrane proteins [26]. Phosphopeptides enriched by immobilized metal affinity chromatography [20] were analyzed by peptide-sequencing using electrospray ionization quadrupole time-of-flight mass spectrometry. We identified two phosphorylated sites in the C-terminal domain of mtGPAT1. Sequencing of one phosphopeptide revealed phosphorylation at serine 639 (Fig. 3A) and a second doubly phosphorylated peptide was phosphorylated on serines 632 and 639 (Fig. 3B). The phosphorylated peptides were found in mitochondria from the cells treated with insulin as well as from control cells.

Both phosphorylation sites in mtGPAT1 correspond to consensus sequences for phosphorylation by casein kinase-2 (http://scansite.mit.edu). Alignment of this part of the protein sequence from rat with the homologous C-terminal regions of mtGPAT1 from other organisms (Fig. 4) reveals that phosphorylated serine residues are conserved in the enzymes from mammals, frog and fish. However, no serine residues can be found in the corresponding C-terminal regions of mtGPAT1 from Drosophila or *C. elegans* (Fig. 4). Moreover, the mtGPAT1 from plants or yeast are shorter than animal mtGPAT1 and do not contain the C-terminal sequence corresponding to that found phosphorylated in the rat enzyme.

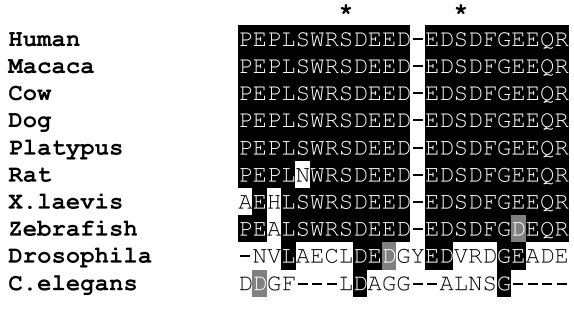

Fig. 4. Sequence alignment of the phosphorylated C-terminal segment of mtGPAT1 (residues from 625 to 646 in the rat enzyme) from different organisms. The serine residues found phosphorylated in the rat mtGPAT1 and conserved in mtGPAT1 from other mammals as well as frog and zebra fish are marked by asterisks over the alignment. Black and gray boxes highlight identical and similar amino acids, respectively.

Discussion

Our main finding is that insulin acutely affects the activity of the mtGPAT1 in adipocytes, which is also compatible with our identification of two *in vivo* phosphorylated serine residues in the enzyme. These findings are of potential importance as mtGPAT1 appears to catalyze a rate-limiting step in triacylglycerol synthesis. While regulation of mtGPAT1 at the transcriptional level in different cells and under different conditions, including insulin treatment, has been thoroughly documented, very little was known about control of the inherent enzyme activity. It was, however, reported that the activity of mtGPAT1 was increased, without a proportional increase of the protein, in the hearts of re-fed rats [16]. As re-feeding involves a surge of circulating insulin, those findings may be explained by the acute effects of insulin demonstrated herein.

We demonstrated acute effects of insulin on the mtGPAT1 activity for the first time, likely because earlier studies of the kinetics or hormonal effects on this enzyme have examined crude cellular mixtures of microsomal and mitochondrial enzymes or isolated and reconstituted mitochondrial enzyme [3,4,27]. We found that insulin treatment of adipocytes increased the V_{MAX} of mtGPAT1 for substrates, glycerol-3-phosphate and palmitoyl-CoA. A parallel increase of K_M for glycerol-3-phosphate in combination with an intracellular concentration of glycerol-3-phosphate (<1 mM) that is below the measured K_M, makes it difficult to accurately predict the impact of insulin on substrate flow *in situ*. This was further emphasized by the inhibitory effect of glycerol-3-phosphate found at low concentrations of palmitoyl-CoA. Interestingly, palmitoyl-CoA was inhibitory at all measured concentrations in the control cells, but only at higher concentrations after insulin treatment. Taken together the kinetic analysis of mtGPAT1 in mitochondria indicate that after insulin action, acyl-CoA at <50 μM will be converted to glycerolipids more or less in proportion to its concentration, but at higher concentrations acyl-CoA will increasingly be targeted to mitochondrial oxidation. In contrast, in non-insulin stimulated control cells increasing concentrations of acyl-CoA will increasingly inhibit glycerolipid synthesis and target fatty acids for oxidation. Such a scheme where mtGPAT1 competes with the mitochondrial carnitine palmitoyl transferase I has been demonstrated in rat hepatocytes that overexpress mtGAPT1, which synthesized more triacylglycerol and oxidized less fatty acids than normally expressing hepatocytes [28,29].

We found that mtGPAT1 protein in mitochondria of adipocytes can be phosphorylated at serine residue 639 as well as doubly phosphorylated at the closely located serine residues 632 and 639. Thus, the enzyme can exist in several differently phosphorylated forms, which potentially could explain some of its kinetic properties revealed in our analyses. It is reasonable to suggest that insulin affects the enzyme activity through control of its phosphorylation state. Casein kinase 2 was identified as a potential protein

kinase for phosphorylation of both serines 632 and 639. This protein kinase has indeed been shown to directly phosphorylate mtGPAT, although the phosphorylated residues have not been identified [18]. Interestingly, casein kinase 2 has also been demonstrated to enhance the activity of mtGPAT1 in mitochondria from Jurkat cells [30] and from rat liver [18]. It will be an important future issue to examine whether insulin affects mtGPAT1 in adipocytes through casein kinase 2. Analysis of the mtGPAT1 sequence around the phosphorylated sites shows that the phosphorylated residues and the surrounding amino acids are highly conserved in the enzyme from mammals, amphibia and fish, but not in mtGPAT1 from fly, worm, yeast or plants. It is tempting to suggest that phosphorylation in the C-terminus of mtGPAT1 could be a specific trait in regulation of triacylglycerol synthesis by insulin in chordate animals.

Acknowledgments

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