ORIGINAL ARTICLE

# Evidence for a Novel Key Regulatory Step in the Triglyceride Synthetic Pathway of Human Adipose Tissue: Partial Purification of Diacylglycerol Acyltransferase Kinase

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## ABSTRACT

**The triglyceride (TG) synthetic pathway is believed to be regulated at the level of the rate-limiting enzyme acyl-CoA:1,2-diacylglycerol O-acyltransferase (DGAT). Recent reports using rat hepatic tissue suggest a kinase-dependent mechanism for the regulation of DGAT activity. To examine this process further, the present study investigates the regulatory mechanisms involved in the modulation of DGAT in human adipocytes. Adipocytes were fractionated into a microsomal fraction containing DGAT and a cytosolic fraction containing a putative regulatory kinase. DGAT activity was determined by measuring the incorporation of $^{14}$C-oleoyl-CoA into TG with exogenously supplied 1,2-dioleoyl-sn-glycerol. Kinase activity was assayed by addition of the cytosolic fraction in the presence of $Mg^{2+}$ and ATP. The results indicate a significant inhibition of human adipose tissue DGAT activity by as much as 43% (avg: 17.5% ± 10.4%, $p < 0.01$) via a mechanism consistent with a phosphorylation event. Partial purification of the putative cytosolic kinase was achieved by multidimensional chromatography. This study thus provides evidence for a novel and key regulatory step in the human TG biosynthetic pathway. Further research is necessary to determine whether the model outlined here is a physiologic conduit through which extracellular hormones exert a regulatory influence on TG synthesis.**

## INTRODUCTION

Considerable attention has recently focused on the hypothesis that defects in the intracellular triglyceride (TG) biosynthetic pathway may play a role in the pathogenesis of such pervasive conditions as obesity, NIDDM, and atherosclerosis. Although much is known about the metabolic regulation of TG lipolysis, the regulatory mechanisms underlying intracellular TG lipogenesis in adipocytes have yet to be fully elucidated

(1-4). The addition of fatty acid to diacylglycerol by acyl-CoA:1,2-diacylglycerol O-acyltransferase (DGAT) is the only step in TG synthesis unique to this biosynthetic pathway, and strong evidence exists for its regulation in rat liver and adipose tissue by phosphorylating-dephosphorylating activities present in a cytosolic fraction (5,6). Thus, the present study addresses the possibility that human adipose tissue DGAT is regulated by cytosolic kinases.

Although there is some controversy as to whether the rate-determining step for de novo synthesis of TG is catalyzed by diacylglycerol acyltransferase (DGAT) or phosphatidate phosphohydrolase (7,8), much of the convincing evidence favors DGAT (9,10). Using a specific inhibitor of DGAT in lysolecithin-permeabilized cells, Mayorek et al. have demonstrated that the overall flux of substrate through the TG pathway is rate-limited by DGAT (11,12). Furthermore, an upregulation in DGAT activity has been correlated with increased demand on the TG pathway following acute ethanol ingestion challenge (13). Long-term regulation of the TG pathway also appears to be mediated through changes in DGAT activity during adipocyte differentiation (14).

DGAT is a membrane enzyme located principally on the endoplasmic reticulum (15) and has been recently purified to near homogeneity by Andersson et al. (16). Haagsman et al. have shown that TG synthesis can be regulated independently of phospholipid synthesis by modulation of DGAT activity (17). These investigators have also presented strong evidence indicating that rat liver DGAT exists in two catalytic states, interconvertible by phosphorylating-dephosphorylating activities present in a cytosolic fraction.

Thus, the investigation of the molecular mechanisms involved in the regulation of TG synthesis by pharmacological agents (18) and by novel effectors, such as Acylation Stimulating Protein (ASP) (19), is of considerable interest. ASP has been purified from human plasma, characterized, and found to be secreted in the post-prandial state (20). This protein markedly stimulates de novo TG synthesis in human skin fibroblasts and adipocytes (21,22), and is believed to increase the TG synthetic capacity of adipocytes by two distinct mechanisms. ASP stimulates the translocation of glucose transporters from an intracellular pool to the cell surface membrane (23), thus increasing the availability of glycerol-3-phosphate to the cell. Secondly, ASP increases the flux of substrate through the triglyceride synthetic pathway, apparently by increasing the $V_{max}$ of the enzyme DGAT (24). Interestingly, ASP modulates TG synthesis via intracellular phosphorylation events, so that it is conceivable that the stimulatory effect of ASP on the instrinsic activity (Vmax) of the enzyme DGAT is mediated by a change in the phosphorylation state of this enzyme (25).

In the current investigation, the hypothesized inhibition of adipocyte TG storage via DGAT phosphorylation in the presence of cytosol, ATP, and cofactors was studied. Furthermore, using standard and affinity chromatography, the purification of a putative kinase regulating DGAT activity was sought.

## MATERIALS AND METHODS

### Tissue Preparation and Fractionation

Human adipose tissue was obtained with informed consent from women undergoing mammoplasty at the Royal Victoria Hospital. At the time of surgery, the adipose tissue was rid of connective tissue by manual excision with a scalpel, rinsed with 0.9% ice-cold saline, and weighed. Between 50 and 150 g of adipose tissue was typically harvested. The tissue was homogenized in 3:1 (v/w, buffer:tissue) ice-cold homogenization buffer (100 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.25 M sucrose, 50 mM NaCl, and 50 $\mu$g/mL PMSF) using a Waring blender at the highest setting for 30 s. The homogenate was filtered through cotton gauze and centrifuged at 2000 rpm for 15 min in 500 mL bottles at 4°C. The homogenate, consisting of the infranate between the floating fat and the pellet, was transferred to 35 mL plastic Oak Ridge tubes (Fisher Scientific, Montreal, Canada) and centrifuged at 20K x *g* for 10 min at 4°C. The supernatant was transferred to 35 mL Polyallomer Quick Seal tubes (Beckman, Mississauga, Ontario, Canada) tubes and ultracentrifuged

at 100K x *g* for 1 hr at 4°C using a TFT 50.38 rotor (Beckman). The process yielded a microsome pellet and cytosol supernatant. The cytosol was passed through a 0.22 $\mu$m filter and then concentrated by ultrafiltration in a stirred cell using a YM-3 ultrafiltration membrane (series 8400, Amicon, Beverly, MA, USA). The concentrated cytosol was dialyzed for 24 hrs against four 1 L changes of Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 7.5, 150 mM NaCl). The microsomes were resuspended in 1 mL of homogenization buffer by repeated passage through a 26.5-gauge insulin syringe and washed by dilution with homogenization buffer to a volume of approximately 9 mL. This washing was followed by recentrifugation at 100K x *g* for 1 hr at 4°C. The pellet was resuspended in a minimal volume of homogenization buffer, aliquoted, and stored at -80°C. Protein determinations were performed with a commercial kit (Bio-Rad, Hercules, CA, USA) as previously described (26), using bovine serum albumin (BSA) as a standard.

### Diacylglycerol Acyltransferase Kinase Assay

DGATK activity was assayed by modification of the method outlined by Haagsman et al. (5). Microsomes (source of DGAT) were incubated with ATP, cofactors, and cytosol in an attempt to phosphorylate and thereby inactivate DGAT. The sample was then assessed for the phosphorylation-dependent inactivation of DGAT by in situ assay. Briefly, the reaction was carried out in
12 x 75 mm glass test tubes in a total volume of 400 $\mu$L. The following were added to each assay tube in an ice bath (control group): 50 $\mu$L of sample (cytosol or column fraction) to be assayed for DGATK activity, 5-100 $\mu$g of microsomes in 50 $\mu$L TBS, and 100 $\mu$L of 2X DGATK assay buffer (100 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1.5 mM DTT, 10 $\mu$M bovine serum albumin (BSA)-essentially fatty acid free). For DGAT inactivation (experimental group), 2mM ATP was added to the assay buffer. The tubes were transferred to a 37°C shaker bath for 10 min. Following ATP-dependent inactivation, DGAT activity was assayed by addition of 200 $\mu$L of 2X DGAT substrate buffer (100 mM Tris-HCl, 1 mM DTT, 50 mM NaF, 5 $\mu$M BSA-essentially fatty acid free, 300 $\mu$M 1,2-dioleoyl-sn-glycerol, 40 $\mu$M $^{14}$C-oleoyl-CoA at 0.1 $\mu$Ci/tube). The reaction was stopped after 10 min by addition of 1.25 mL of a 1:1 isopropanol:heptane solution. A 600 $\mu$L aliquot of the organic phase was transferred to a new tube and dried in a centrifuge-evaporator (Jouan, Canberra-Packard, Mississauga, Ontario, Canada). TGs were separated from other lipids by thin-layer chromatography and quantified by scintillation counting as previously described (20). Background radioactivity was determined by addition of the stop solution prior to addition of the DGAT substrate buffer, and was subtracted from the sample counts. Data is expressed as the mean ± standard deviation in pmol of $^{14}$C-oleoyl CoA incorporated into TG in 10 min per 25 $\mu$g of microsome.

### Purification of DGATK

Concentrated and dialyzed human adipose tissue cytosol was fractionated by anion exchange chromatography on a Mono-Q HR 5/5 FPLC column (Pharmacia, Uppsala, Sweden), with the following specifications: flow rate 1 mL/min, fraction size 1 mL, detector * 280 nm (model 486 detector, Waters Chromatography, Milford, MA, USA). The column was equilibrated in buffer A (20 mM Tris-HCl, pH 7.5), and 10-20 mg of total protein in 1-2 mL was typically loaded. Following a 10-min wash in buffer A, proteins were eluted with a 0-100% gradient of buffer B (20 mM Tris-HCl, pH 7.5, 1 M NaCl) in 40 min. DGATK activity was assayed as described above from an aliquot of each column fraction and was found repeatedly to elute from the column between 230 and 300 mM NaCl (23% - 30% buffer B).

Tubes corresponding to the active fraction from several elutions were pooled (fraction A) and analyzed by chromatograph on a Vydac Protein C4 HPLC column (Separations Group, Hesperia, CA, USA), with the following specifications: flow rate 2 mL/min, fraction size 1 mL, detector * 280 nm. The column was equilibrated in solvent A (water/0.1% TFA), and 1-4 mg of protein from fraction A was typically loaded. Following a 10-min wash in solvent A, proteins were eluted from the column with a 0-100% gradient of solvent B (80% acetonitrile/20% water/0.1% TFA) in 50 min. DGATK activity was assayed from a dried

aliquot of each fraction reconstituted in 50 mL TBS, and was found repeatedly to elute between 54% and 58% acetonitrile (67% - 72% solvent B).

Purification of DGATK activity was attempted by affinity chromatography on Cibachrom Blue, as described by the supplier (Pharmacia). The cytosolic fraction, consisting of the 100K x *g* centrifugation supernatant, was diluted 1:1 or dialyzed in buffer C (5 mM sodium phosphate, pH 6.8, 10 mM $MgCl_2$, 1 mM DTT), and 10-30 mg of total protein was typically loaded on an HR 5/20 column packed with 4 mL of Blue-Sepharose CL-6B media (Pharmacia) previously equilibrated in buffer C. Following a 10-min buffer C wash, proteins were eluted from the column with a 0-100% gradient of buffer D (5 mM sodium phosphate, pH 6.8, 10 mM $MgCl_2$, 1 mM DTT, 1 M NaCl) in 40 min. The eluent was then analyzed by chromatography according to the following specifications: flow rate 1 mL/min, fraction size 1 mL, detector * 280 nm. DGATK activity was assayed from an aliquot of each fraction and was found repeatedly to elute from the Blue-Sepharose column between 400 mM and 600 mM NaCl (40% - 60% buffer D).

### Polyacrylamide Gel Electrophoresis

Purifity of DGATK was assessed by polyacrylamide gel electrophoresis as previously described (27) using a mini-gel electrophoresis apparatus (Bio-Rad). An aliquot of the pooled active fraction from each chromatographic step was loaded on a 15% polyacrylamide discontinuous gel and run concurrently with molecular weight standards (Bio-Rad, broad range molecular weight standards) using 0.1% Coomassie Brillant Blue R-250 stain.

### Statistical Analysis

Significant differences between control and sample groups were determined by paired Student's *t*-test using the SigmaStat statistical software (Sigma, St. Louis, MO, USA).

## RESULTS

For confirmation of the validity of the DGAT assay, all parameters--enzyme concentration, incubation time, and substrate concentration--were determined and optimized to fall within the linear range of the assay. Varying amounts of microsomes were used for a 10-min DGAT activity assay in Figure 1A, a time-course DGAT assay was performed using 25 $\mu$g/tube of microsomal protein in Figure 1B, and DGAT activity was measured at varying concentrations of oleoyl CoA substrate in Figure 1C. The results of linear regression show that the concentration of 25 $\mu$g/tube of microsomal protein, the assay time of 10 min, and the concentration of 20 $\mu$M oleoyl CoA used for the assay all lie within the linear range of the assay ($r^2$=0.999, y=0.285x+0.00676; $r^2$=0.96, y=1.440x+1.117; and $r^2$=0.979, y=0.936x+3.472, respectively). These results indicate that substrate availability is not limiting at a microsomal protein concentration of 25 $\mu$g/tube, and substrate is not exhaused by 10 min.

Figure 2 demonstrates that the adipocyte cytosolic fraction (100K x *g* supernatant) inhibits DGAT in the presence of ATP. The data shown represent 11 independent experiments with each determination performed in triplicate. In each case, tissue was obtained from a different subject. The inter-experimental variation is thus explained by the differences in basal specific activity of the individual microsomal tissue preparations. Paired analysis of the data indicates, on average, a 17.5% ± 10.4% inhibition of DGAT by ATP in the presence of cytosol as compared to control (microsomes with cytosol but no ATP). This difference is highly statistically significant ($p < 0.01$).

Figure 3 depicts a representative purification scheme from among those performed in the study. After elution from the column, each fraction was tested for DGATK activity. The fractions showing DGATK activity (darkened areas on the chromatograms) represent the pooled fractions capable of significantly inhibiting basal

DGAT activity. In Figure 3A, concentrated and dialyzed human adipose tissue cytosol (15.8 mg) was resolved on a Mono-Q anion exchange column, and the fraction showing the least DGAT activity (i.e., the greatest DGAT inhibition) was pooled and resolved on a reverse-phase Vydac C4 column (Figure 3B). Figure 3C shows a separate experiment in which concentrated and dialyzed human adipose tissue cytosol was analyzed by chromatograph using a Blue-Sepharose affinity column.

Partial purification of DGATK was assessed by polyacrylamide gel electrophoresis. For either purification scheme, when a 13 $\mu$g aliquot of the pooled active fraction from each chromatographic step was loaded on a the gel, an apparent enrichment was observed in a band running at approximately 97 kD (Figure 4, lanes 4 and 5).

## DISCUSSION

The results from the reconstitution experiments, in which the microsomal and cytosolic fractions of human adipose tissue were incubated together, indicate a significant inhibition of DGAT in the presence of ATP and $Mg^{2+}$. These findings are consistent with the existence of an intracellular kinase, present in the cytosol of adipocytes, capable of phosphorylating, and thus inhibiting, the activity of the enzyme DGAT. These results, from experiments using human adipose tissue, support previous suggestions of kinase-mediated inhibition of DGAT obtained using rat liver and adipose tissue (5,6). Furthermore, electrophoretic analysis of the active fractions obtained from the two purification schemes indicates an apparent enrichment of a band at 97 kD. Interpretation of these findings is, however, guarded. Other proteins that have co-purified are visible on the stained gel. In addition, it is unlikely that an intracellular regulatory kinase would be present in sufficient amounts this early in a purification process to be visible by Coomasie blue staining. Taken together, however, these findings provide preliminary evidence of a putative putative kinase (DGATK) involved in a novel regulatory step of TG synthesis in humans.

Nevertheless, the discovery of a novel key regulatory step in the triglyceride biosynthetic pathway of human adipose tissue has substantial physiologic relevance. Two fates exist for fatty acids released into the peripheral tissue's microenrivoment by the hydrolytic action of lipoprotein lipase on TG-rich lipoproteins: either (i) the fatty acids are taken up by the tissues, or (ii) under a fatty acid load exceeding the peripheral tissue's metabolic needs or storage capacity, the fatty acids are liberated into the circulation and absorbed downstream by the liver. It has been proposed that a defect in the rate at which adipose tissue mobilizes fatty acids in the post-prandial state may play a significant role in the pathogenesis of atherosclerosis (28).

Indeed, an impairment in the clearance of post-prandial lipidemia by peripheral tissue would result in increased delivery of substrate to the liver and increased hepatic production of atherogenic lipoproteins, culminating in the establishment of an atherosclerotic disease process. This impaired clearance is believed to be the principal mechanism underlying hyperapo- betalipoproteinemia, one of the most common dyslipoproteinemias associated with coronary artery disease. It is possible that this pathologic process results from an intracellular dysregulation at the level of DGAT. In the diseased state, this rate-limiting enzyme may be unable to convert to an active conformation
in response to a post-prandial stimulus. Such dysregulation could lead to a decreased ability of peripheral tissues to store free fatty acids, the products of vascular hydrolysis. Build-up of free fatty acids could then result in end-product inhibition of lipoprotein lipase at the endothelial surface, thus increasing the return flux of lipoproteins to the liver and establishing the pathogenic pathway outlined above (29,30).

In addition to atherosclerosis, dysregulation of intracellular TG synthesis may be involved in obesity and diabetes (NIDDM). In a recent study utilizing an in vitro muscle cell model (31), exogenous TG was found to decrease the capacity of muscle cells to respond to insulin-stimulated glucose uptake. These and other findings corroborate the view that a reduced ability of peripheral tissue to store exogenous fatty acids as intracellular TG may play an important role in the development of NIDDM.

In conclusion, the findings presented in this report point to a novel regulatory mechanism involved in lipid metabolism in humans. Future studies must determine whether the molecular changes described here represent the physiologic conduit through which extracellular hormones regulate intracellular TG synthesis.

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## BIOGRAPHY

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