Submicromolar Concentrations of Palmitoyl-CoA Specifically Thioesterify Cysteine 244 in Glyceraldehyde-3-phosphate Dehydrogenase Inhibiting Enzyme Activity: A Novel Mechanism Potentially Underlying Fatty Acid Induced Insulin Resistance†

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ABSTRACT: The accumulation of fatty acids and their metabolites results in insulin resistance and reduced glucose utilization through a variety of complex mechanisms that remain incompletely understood. Herein, we demonstrate that submicromolar concentrations of palmitoyl-CoA inhibit glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) enzyme activity through the covalent thioesterification of palmitate to GAPDH. First, incubation of GAPDH with palmitoyl-CoA (0.5−5 μM) resulted in the dramatic concentration-dependent inhibition of GAPDH enzyme activity. Second, incubation of GAPDH with [^{14}C]-palmitoyl-CoA followed by SDS−PAGE and autoradiography identified a covalently radiolabeled adduct present at ∼35 kDa with a stoichiometry of one molecule of palmitoyl-CoA per GAPDH tetramer. Third, mass spectrometric analyses of intact GAPDH treated with palmitoyl-CoA demonstrated the covalent addition of palmitate to the GAPDH protein. Fourth, trypsinolysis of the modified protein revealed that the peptide ^{232}VPTPNVSVVDLTRC*R^{245} was covalently modified. Fifth, the site of palmitoylation was demonstrated to be Cys-244 by analyses of product ion mass spectra. These assignments were further substantiated using different molecular species of acyl-CoAs resulting in the anticipated changes in both the masses of adduct ions and their fragmentation patterns. Sixth, GAPDH palmitoylation was demonstrated to facilitate the translocation of GAPDH to either lipid vesicles or naturally occurring biologic membranes. Since the hallmark of lipotoxicity is the accumulation of fatty acids and their acyl-CoA metabolites in excess of a cell's ability to appropriately metabolize them, these results identify a novel mechanism potentially contributing to the insulin resistance, reduced glucose utilization, and maladaptive metabolic alterations underlying the lipotoxic state.

During the past decade, excessive consumption of fat in high caloric Western diets in conjunction with a sedentary life style has resulted in an epidemic of obesity in industrialized nations (*1*, *2*). Obesity is associated with insulin resistance, hypertension, dyslipidemia, type 2 diabetes, and atherosclerosis, which collectively constitute the metabolic syndrome (*3*−*5*). Despite the enormous proportions of this public health problem, the biochemical mechanisms underlying the metabolic syndrome and its end-organ sequelae are poorly understood.

The increased level of intracellular fatty acyl-CoAs[1] in patients with obesity and/or type 2 diabetes is well-known (*6*−*8*). This increase led to our consideration that fatty acyl-CoAs regulate metabolic pathways by interacting with, or modifying the activities of, critical enzymes or processes participating in intermediary metabolism. Protein fatty acylation is a reversible covalent modification of proteins by fatty acyl groups (typically myristate and palmitate although other fatty acids can be employed) through amide, oxyester, or thioester linkages (*9*). The regulatory effects of fatty acylation on numerous different protein functions have been well documented in the literature (*10*, *11*). A dramatic example of the importance of protein fatty acylation was recently identified by Rocks et al. through demonstration of the importance of a palmitoylation/depalmitoylation cycle in the regulation of localization and activity of Ras in MDCK cells (*12*).

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[1] Abbreviations: CoA, coenzyme A; CHCA, α-cyano-4-hydroxycinnamic acid; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MALDI-TOF/TOF, matrix-assisted laser desorption ionization−time of flight/time of flight; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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Both enzymatic mechanisms and mechanisms exploiting membrane-assisted orientation and/or hydrophobic effects have been implicated in the palmitoylation of proteins. Deacylation of palmitoylated proteins is currently believed to be mediated largely by acyl protein thioesterase 1 or acyl protein thioesterase 2 (*13*, *14*). The initial insights into the enzymes catalyzing the transfer of palmitoyl-CoA to their protein targets have demonstrated that the protein palmitoyl-CoA transferase family contains a DHHC consensus motif (*15*, *16*). A second type of protein thioesterification, autoacylation, suggests that hydrophobic forces promote the nucleophilic attack of a cysteine residue with acyl-CoA in a membrane environment leading to covalent thioesterification by orientation of a thiolate anion (*17*, *18*). Herein, we propose a third hybrid mechanism where the target protein has substantive binding interactions with, and affinity for, the ADP-ribose portion of the acyl-CoA molecule due to an endogenous internal nucleotide binding motif. This would increase the effective concentration of acyl-CoA at or near potential nucleophilic cysteine residues, thereby converting a bimolecular reaction to a pseudounimolecular reaction with concomitant increases in reaction velocity. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) plays a central role in glycolysis catalyzing the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate in a reaction accompanied by the reduction of NAD to NADH. We hypothesized that the internal NAD(H) binding site of GAPDH could facilitate the binding of GAPDH to acyl-CoA through recognition of the ADP-ribose moiety in the terminal portion of acyl-CoA and this binding could facilitate the acylation of GAPDH. The predicted palmitoylated GAPDH product is particularly attractive because a portion of GAPDH is known to actively translocate from the cytosol to membrane compartments by an unknown mechanism (*19*) and palmitoylation is a commonly employed biochemical mechanism to facilitate the translocation of cytosolic proteins to membrane compartments.

In this study, we demonstrate that the dehydrogenase activity of rabbit muscle GAPDH is inhibited by submicromolar concentrations of palmitoyl-CoA and that the loss of dehydrogenase activity is due to the covalent modification of GAPDH by palmitoyl-CoA at Cys-244. Palmitoylation of GAPDH also increases the protein's association to either artificial lipid vesicles or naturally occurring biologic membranes. Collectively, these findings implicate a novel mechanism through which fatty acid metabolites can modulate glucose flux and induce insulin resistance and suggest that acylation of GAPDH may play an important role in multiple cellular regulatory processes contributing to the maladaptive changes in cellular metabolism and function that occur in the lipotoxic state.

MATERIALS AND METHODS

Materials. Rabbit muscle GAPDH was purchased from Sigma-Aldrich (St. Louis, MO). Palmitoyl-CoA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) were from Avanti Polar Lipids (Alabaster, AL). 1-[^{14}C]Palmitoyl-CoA was from PerkinElmer Life and Analytical Sciences (Boston, MA). *methyl*-^{14}C-labeled human albumin was purchased from American Radiolabeled Chemicals (St. Louis, MO). Monoclonal antibody to GAPDH was obtained from Ambion (Austin, TX). Materials for western blotting, autoradiography (ECL western blotting detection reagent, sheep anti-mouse IgG−horseradish peroxidase, Amplify flurographic reagent and films), and two-dimensional electrophoresis were from Amersham Bioscience (Piscataway, NJ). Bradford protein assay dye reagent was from Bio-Rad (Hercules, CA). Solvents used for mass spectrometric analyses were from Burdick & Jackson (Muskegon, MI). Trypsin was purchased from Promega (Madison, WI). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO).

In Vitro Fatty Acylation of GAPDH. Rabbit muscle GAPDH was dissolved in reaction buffer (50 mM Tris-HCl, pH 7.0, 2 mM EGTA, and 1 mM DTT) to a final concentration of 0.2 μM. Palmitoyl-CoA was added to various final concentrations as indicated in the individual experiments. The reaction mixtures were incubated at 37 °C for 30 min, and the dehydrogenase activity of GAPDH was assayed.

In Vitro Fatty Acylation of GAPDH in the Presence of Lipid Vesicles. Lipid vesicles were prepared as previously described (*20*) with minor modifications. Briefly, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) were purified by HPLC and redissolved in chloroform. POPC was mixed with POPS at a molar ratio of 9 to 1. The solvent was evaporated with nitrogen, and the mixture was dried under vacuum for 1 h. The dried mixture was resuspended into 2 mL of 50 mM Tris-HCl, pH 7.0, and sonicated for 2 × 2 min using a 1 s pulse setting. GAPDH (0.4 μM) in 50 mM Tris-HCl, pH 7.0, 2 mM EGTA, and 1 mM DTT was prepared. Equal volumes of lipid vesicle resuspension and GAPDH solution were mixed to final concentrations of lipid vesicles and GAPDH as 100 and 0.2 μM, respectively. Palmitoyl-CoA was added to a final concentration of 0, 2, 5, or 10 μM. The reaction mixtures were incubated at 37 °C for 2 h, and the dehydrogenase activity of GAPDH was assayed.

Assay of GAPDH Dehydrogenase Activity. The dehydrogenase activity of GAPDH was measured as described by Glaser et al. (*20*). A 50 μL sample was added to a 1 cm path-length semimicrocuvette which contained 900 μL of 50 mM Tris-HCl (pH 7.5), 50 mM sodium arsenate (pH 8.8), 2.4 mM glutathione (reduced), 0.5 mM NAD, and water. The reaction was initiated by the addition of 50 μL of 10 mM D-glyceraldehyde 3-phosphate. The absorbance at 340 nm was monitored for 5 min. GAPDH activity was determined by the absorbance change within the first minute of the reaction.

Radiolabeling of GAPDH with 1-[^{14}C]Palmitoyl-CoA. One milliliter of GAPDH (0.2 μM) was incubated with selected concentrations of [^{14}C]palmitoyl-CoA in 50 mM Tris-HCl, pH 7.0, 2 mM EGTA, and 1 mM DTT at 37 °C for 30 min. The reaction was terminated by precipitating the protein with chloroform and methanol as described previously (*21*). The pellet was dried, resuspended in 1 × SDS−PAGE sample buffer, and electrophoresed on a 12% gel. The radiolabeled products were detected by autoradiography. For the purpose of quantitation, *methyl*-^{14}C-labeled human albumin of known specific activity (50 nCi/μg of protein) and concentration (50 nCi/μL) was used to generate a standard curve. Briefly, 1−10 nCi of *methyl*-^{14}C-labeled human albumin was loaded

onto the same gel as the [^{14}C]palmitoylated GAPDH. The autoradiograph of the gel was scanned using a Kodak Image Station 440CF. The intensities of the visualized bands were measured by Kodak 1D 3.5 software, from which a standard curve of radioactivity vs band intensity was derived. The incorporation of [^{14}C]palmitate to GAPDH was determined by comparing the intensities of the radiolabeled GAPDH bands to the ^{14}C-labeled human albumin standard curve. The stoichiometry of GAPDH palmitoylation was then calculated on the basis of the incorporation of [^{14}C]palmitate, the specific activity of the [^{14}C]palmitoyl-CoA, and the amount of GAPDH protein.

Detection of the Intact GAPDH by Electrospray Ionization Mass Spectrometry (ESI-MS). GAPDH (0.2 μM) was incubated with 0.5 μM fatty acyl-CoA (palmitoyl-CoA, myristoyl-CoA, or decanoyl-CoA) in 5 mM Tris-HCl, pH 7.0, 2 mM EGTA, and 1 mM DTT at 37 °C for 60 min. The reaction mixture (500 μL) was concentrated and desalted by centrifugation using a NanoSep centrifugal device with 3000 MWCO (Pall Corp., Ann Arbor, MI). The concentrated sample was diluted in 30% methanol/1% formic acid and analyzed by a quadrupole time-of-flight mass spectrometer (QSTAR XL, Applied Biosystems/MDS Sciex, Foster City, CA) equipped with a nanoelectrospray ion source (MDS Sciex, Concord, Canada). The instrument was externally calibrated in the positive ion mode using two fragment ion peaks (*m*/*z* 175.1190 and *m*/*z* 1285.5444) from the tandem mass spectrum of Glu-fibrinopeptide. The samples were directly infused into the ion source using a 25 μL syringe at a flow rate of 300 nL/min through fused silica tubing. The tubing was connected to a PicoTip nanospray emitter (10 μm i.d.; New Objective, Woburn, MA) by a stainless steel union (Valco Instrument, Houston, TX) mounted on the nanoelectrospray ion source. The spray voltage (2000 V) was applied to the emitter through the stainless steel union. The following parameter settings were used to acquire mass spectra from *m*/*z* 600−2000 in the positive ion TOF/MS mode: declustering potential (DP), 55 V; declustering potential 2 (DP2), 12 V; focusing potential (FP), 252 V; accumulation time, 1 s. Typically, spectra were averaged over 60 scans, and the series of multiply charged ion peaks were deconvoluted by the Bayesian protein reconstruct tool (in the Bioanalyst 1.1 software package) to determine the zero-charge masses of the intact proteins.

Peptide Mapping by MALDI-TOF/TOF Mass Spectrometry. GAPDH (0.4 μM) was incubated with 0.8 μM fatty acyl-CoA (palmitoyl-CoA, myristoyl-CoA, or decanoyl-CoA) in 5 mM Tris-HCl, pH 7.0, 2 mM EGTA, and 1 mM DTT at 37 °C for 1 h. The samples were concentrated and desalted by centrifugation using a NanoSep centrifugal device with 3000 MWCO (Pall Corp., Ann Arbor, MI). A small amount of 50 mM Tris-HCl, pH 7.5, was added to ensure that the protein digestion was carried out at pH 7.5. Trypsin was added at a ratio of 1:30 (w/w, trypsin/protein), and the mixture was incubated at 37 °C for 24 h. The mass and product ion mass spectra of the tryptic peptides were acquired on a MALDI-TOF/TOF mass spectrometer equipped with a Nd:YAG 200 Hz laser (4700 Proteomics Analyzer; Applied Biosystems, Foster City, CA). The instrument was externally calibrated in the positive ion reflector mode with a standard peptide mixture (4700 Proteomics Analyzer calibration mixture; Applied Biosystems) by following the manufacturer's instructions. The matrix solution was prepared by dissolving 5 mg of α-cyano-4-hydroxycinnamic acid (α-CHCA) in 1 mL of 50% 2-propanol/25% formic acid. An aliquot of the tryptic digest was mixed with the matrix solution at different volume ratios. Approximately 0.5 μL of the mixture was deposited onto a 192-well stainless steel MALDI target plate and allowed to air-dry. Before sample analysis by mass spectrometry, 1 μL of cold water was applied on the deposits and removed within 1 min to clean the samples. Mass spectra were recorded in the positive ion reflector mode over an *m*/*z* range of 1000−6000. The ions of interest were selected and subjected to product ion analyses with the CID (collision-induced decay) gas off, and the collision energy was set as 1 keV. Approximately 2000−6000 consecutive laser shots were applied to obtain satisfactory mass spectra.

Translocation of Palmitoylated GAPDH to Lipid Vesicles and Rabbit Brain Microsomal Membranes. Lipid vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesterol were prepared in the following manner. POPC and cholesterol were mixed at a molar ratio of 3 to 2, dried by flushing with nitrogen for 30 min, and resuspended in 2 mL of 50 mM Tris-HCl, pH 7.4, by vortexing vigorously. The final concentration of the total lipid was 500 μM. The lipid resuspension was sonicated for 6 min using a 1 s pulse setting with a 40% duty cycle.

Rabbit brain microsomes were prepared from New Zealand white rabbits (Charles River Laboratory, Wilmington, MA). The rabbits were sacrificed by asphyxiation with carbon dioxide. The brain was removed and washed briefly with phosphate-buffered saline. Homogenization buffer A (0.25 M sucrose, 50 mM Tris-HCl, 10 mM EGTA, 2 mM EDTA, and 1 mM DTT, pH 7.4) was added at a ratio of 2 mL of buffer/g of tissue. The brain was homogenized using three 10 s pulses from a Brinkman PT 10/35 Polytron apparatus at incremental output settings of 4.5, 5, and 5.5. The brain homogenate was initially centrifuged at 20000*g*, 4 °C, for 20 min. The supernatant was collected and centrifuged at 100000*g*, 4 °C, for 60 min. The pellet was resuspended in homogenization buffer B (0.25 M sucrose, 50 mM Tris-HCl, 2 mM EGTA, and 2 mM EDTA, pH 7.4), and the protein concentration was determined by the Bradford protein assay dye reagent from Bio-Rad.

GAPDH (0.4 μM) was palmitoylated by incubation with palmitoyl-CoA (1 μM) in 50 mM Tris-HCl, pH 7.0, 2 mM EGTA, and 1 mM DTT at 37 °C for 1 h. For experiments examining the translocation of GAPDH to lipid vesicles, 50 μL of GAPDH (0.4 μM) or GAPDH (0.4 μM) treated with palmitoyl-CoA was added to 2 mL of the lipid vesicle suspension. After incubation at 37 °C for 15 min, the mixtures were centrifuged at 200000*g* for 1 h to spin down lipid vesicles. For experiments with rabbit brain microsomes, 3.5 mL of GAPDH (0.4 μM) or GAPDH (0.4 μM) treated with palmitoyl-CoA was concentrated by centrifugation using a MicroSep centrifugal device with MWCO 3000 (Pall Corp., Ann Arbor, MI). The protein concentrations of the concentrated samples were measured using the Bradford protein assay dye reagent. Microsomal fractions containing 50 μg of protein were diluted into 1 mL of homogenization buffer B. Two micrograms of palmitoylated GAPDH or unmodified GAPDH was added. The mixtures were incubated at 37 °C

for 15 min and then centrifuged at 100000*g* for 60 min to spin down the microsomal fractions. For both experiments, the supernatants were saved, and 60 μL of each supernatant was mixed with an equal volume of 2 × SDS−PAGE sample loading buffer. Each pellet was resuspended into 100 μL of 1 × SDS−PAGE sample loading buffer, boiled for 5 min, electrophoresed on a 12% SDS−PAGE gel, and transferred to a PVDF membrane. The amount of GAPDH was then determined by western blotting.

SDS−PAGE and Western Blotting. Proteins were separated by SDS−PAGE (*22*) and transferred to immobilon-P membranes in 10 mM CAPS buffer (pH 11) containing 10% methanol. Powdered milk [5% (w/v)] was used to block the nonspecific binding sites prior to incubation with primary antibody. Horseradish peroxidase linked secondary antibody was used in combination with an ECL detection system to visualize immunoreactive bands.

Two-Dimensional Electrophoresis of Rabbit Muscle GAPDH. Rabbit muscle GAPDH (40 μg) was dissolved in 250 μL of rehydration buffer composed of 8 M urea, 0.5% (w/v) CHAPS, 0.2% (w/v) DTT, 0.5% (v/v) IPG buffer (pH 6−11), and 0.002% bromophenol blue. The rehydration buffer containing GAPDH was applied to rehydrate a 13 cm IPG strip (pH 6−11) overnight. The strip was then subjected to the isoelectric focusing following the manufacturer's instruction. After isoelectric focusing, the strip was equilibrated in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and bromophenol blue), and the second dimension was performed on a 12% SDS−PAGE gel. The proteins were visualized by silver staining.

RESULTS

Inhibition of Rabbit Muscle GAPDH Enzyme Activity by Palmitoyl-CoA. To examine the effect of palmitoyl-CoA on GAPDH enzyme activity, we incubated GAPDH (0.2 μM) in the absence or presence of palmitoyl-CoA (0.5, 1, 2, and 5 μM) for 30 min at 37 °C and measured the GAPDH enzyme activity. GAPDH enzyme activity was attenuated at low concentrations of palmitoyl-CoA in a dose-dependent manner. Approximately 30% of GAPDH enzyme activity was inhibited by 1 μM palmitoyl-CoA, and the activity was completely ablated at a concentration of 5 μM palmitoyl-CoA (Figure 1A). Next, we examined whether inhibition was affected by the presence of lipid vesicles which sequester acyl-CoA in the membrane microenvironment. A modest decrease in GAPDH activity (∼30%) was manifest in the presence of 100 μM lipid vesicles, but the potency of inhibition of GAPDH enzyme activity by palmitoyl-CoA was not substantially modified using palmitoyl-CoA as a guest in the host lipid vesicles. However, the dose dependence profile was shifted slightly to the right (Figure 1B). Half-maximal inhibition was manifest at 2 mol % palmitoyl-CoA with complete inhibition at 5 mol % palmitoyl-CoA when palmitoyl-CoA was present as a guest in the host lipid vesicles.

Covalent Modification of GAPDH by Palmitoyl-CoA. To identify the mechanisms underlying the inhibition of GAPDH enzyme activity by palmitoyl-CoA, we incubated GAPDH with selected concentrations of [^{14}C]palmitoyl-CoA and determined whether a covalent adduct was formed by separating GAPDH and putative reactants by SDS−PAGE and visualizing the radiolabeled products by autoradiography. The experimental conditions (the concentrations of GAPDH and [^{14}C]palmitoyl-CoA, incubation time, and temperature) were the same as those used for the GAPDH enzyme activity assay. Autoradiography showed a dose-dependent increase in the intensity of a radiolabeled band at ∼35 kDa in the samples treated with [^{14}C]palmitoyl-CoA, demonstrating that GAPDH was covalently modified by [^{14}C]palmitoyl-CoA (Figure 2). The band intensity increased as the concentration of [^{14}C]palmitoyl-CoA was increased from 1 to 5 μM but was substantially saturated at 5 μM [^{14}C]palmitoyl-CoA (i.e., no substantive increase in band intensity was present when the [^{14}C]palmitoyl-CoA concentration was increased from 5 to 10 μM). These results demonstrated that the incorporation of [^{14}C]palmitoyl-CoA into GAPDH correlated with a loss of enzyme activity and that acylation was a high-affinity process. By using ^{14}C-labeled human albumin as an internal standard, we calculated the molar ratio of the total GAPDH to [^{14}C]palmitoyl-CoA-modified GAPDH in each radiolabeled band (Table 1). At a concentration of 5 μM [^{14}C]-palmitoyl-CoA, where GAPDH enzyme activity was totally inhibited, the molar ratio of the total GAPDH to the [^{14}C]-palmitoyl-CoA-modified GAPDH was approximately 4 to 1. Therefore, we concluded that the enzyme activity was completely inhibited when ∼25% of GAPDH was modified

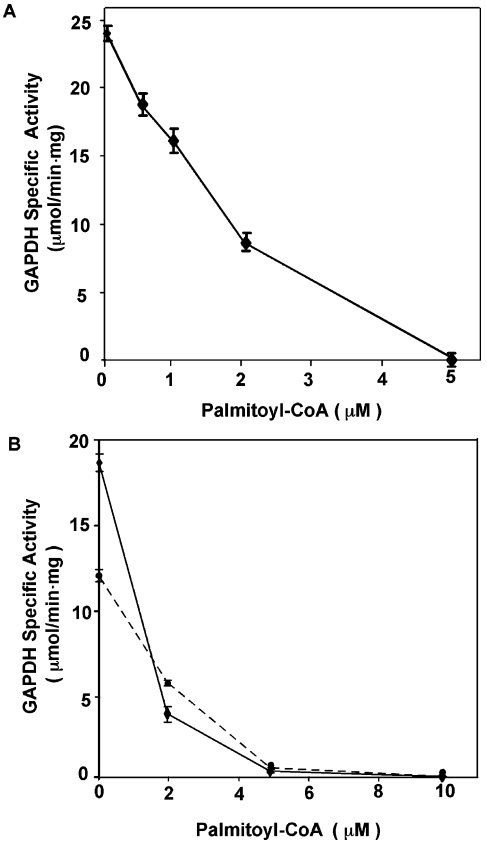

FIGURE 1: Inhibition of GAPDH activity by palmitoyl-CoA in vitro. The enzyme activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assayed spectrophotometrically as described in Materials and Methods. (A) GAPDH (0.2 μM) was incubated with 0−5 μM palmitoyl-CoA in 50 mM Tris-HCl, pH 7.0, 2 mM EGTA, and 1 mM DTT at 37 °C for 30 min. (B) GAPDH (0.2 μM) was incubated with 0−10 μM palmitoyl-CoA in the absence (—) or presence (···) of 100 μM lipid vesicles at 37 °C for 2 h. Each data point represents the average ± SD from at least three independent experiments.

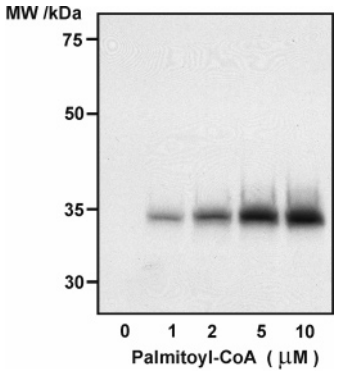

FIGURE 2: Modification of GAPDH by [^{14}C]palmitoyl-CoA. One milliliter of GAPDH (0.2 μM) was incubated with 0−10 μM [^{14}C]-palmitoyl-CoA in 50 mM Tris-HCl, pH 7.0, 2 mM EGTA, and 1 mM DTT at 37 °C for 30 min. Then the GAPDH protein was precipitated by the addition of chloroform/methanol, dried, resuspended in 1 × SDS−PAGE loading buffer, and electrophoresed on a 12% gel. The radiolabeled bands were visualized by autoradiography and quantified by densitometry as described in Materials and Methods. The results of the quantitative analysis are listed in Table 1.

Table 1: Molar Ratio of Total GAPDH to [^{14}C]Palmitoyl-CoA-Modified GAPDH[a]

conc of palmitoyl-CoA in reaction (μM)	1	2	5	10
total amount of protein in reaction (nmol)	0.2	0.2	0.2	0.2
total amount of modified protein in reaction (nmol)	0.015	0.02	0.04	0.05
molar ratio (total GAPDH:modified GAPDH)	16:1	10:1	5:1	4:1

[a] GAPDH (0.2 μM) was incubated with 1−10 μM [^{14}C]palmitoyl-CoA at 37 °C for 30 min. The radiolabeled products were analyzed by autoradiography (see Figure 2). The stoichiometry of palmitoylation was determined by comparing the intensities of the visualized bands to those of ^{14}C-labeled human albumin standards on the same film using a Kodak Image Station 440CF and Kodak 1D 3.5 software.

by palmitoyl-CoA, compatible with a single modification of the tetramer resulting in complete inhibition of enzyme activity (see Discussion).

Electrospray Ionization Mass Spectrometry (ESI-MS) of the Acylated GAPDH. To confirm that GAPDH was covalently modified by palmitoyl-CoA and to identify the nature of the covalent modification, we examined GAPDH and its palmitoyl-CoA-modified products by electrospray ionization mass spectrometry (ESI-MS). Although the purified protein was predominantly a single band on the silver-stained gel after SDS−PAGE electrophoresis (Figure 3A), further dissection of the endogenous heterogeneity of GAPDH by two-dimensional gel electrophoresis demonstrated multiple isoforms (Figure 3B), similar to previous results from our laboratory and others (*20*, *23*). The heterogeneity of GAPDH was further demonstrated by deconvoluted ESI mass spectra of the intact GAPDH, which showed multiple peaks corresponding to GAPDH containing mass differences of 16, or multiples of 16, between the major peaks (Figure 4A). Thus, the majority of heterogeneity in GAPDH was likely caused by the oxidation of labile amino acid residues such as methionine, tryptophan, or cysteine. A mass spectrum confirming the oxidation of tryptophan is shown in Figure 5. Comparison of the mass spectra of the palmitoyl-CoA-treated GAPDH to the native GAPDH identified a unique

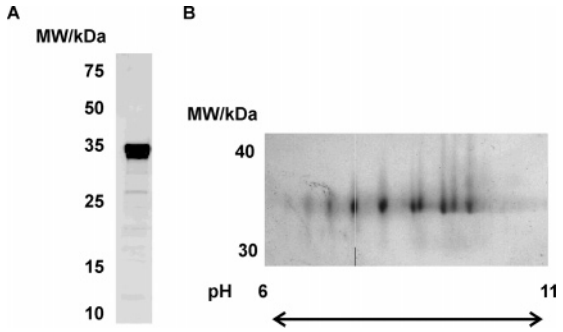

FIGURE 3: SDS−PAGE and two-dimensional electrophoresis of GAPDH. GAPDH was electrophoresed on a 12% gel (A) or separated by two-dimensional electrophoresis (B) as described in Materials and Methods. Proteins were visualized by silver staining.

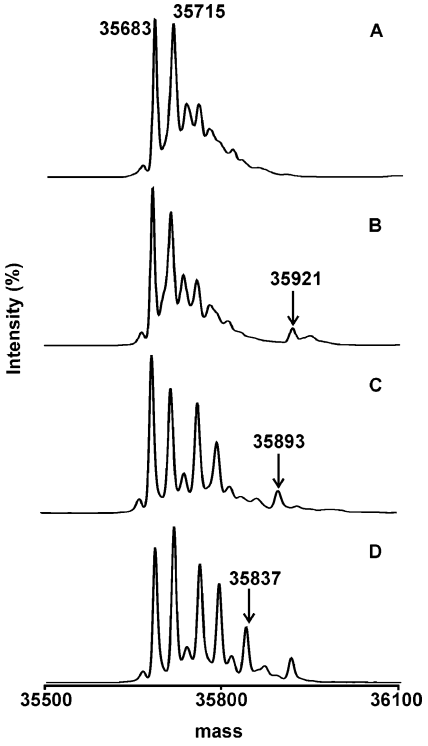

FIGURE 4: Deconvoluted ESI mass spectra of the intact native and fatty acyl-CoA-treated GAPDH. GAPDH (0.2 μM) was modified by 0.5 μM palmitoyl-CoA, myristoyl-CoA, or decanoyl-CoA. The samples were then concentrated and desalted by centrifugation using centrifugal devices (NanoSep, Pall Corp.). The resultant samples were mixed with 30% methanol/1% formic acid and infused into a quadrupole time-of-flight mass spectrometer equipped with a nanoelectrospray ion source. The positive ion mass spectra from *m*/*z* 600 to *m*/*z* 2000 were acquired and deconvoluted by the Bayesian protein reconstruct tool (in the Bioanalyst 1.1 software package) to get the zero-charge mass spectra. Key: (A) native GAPDH; (B) palmitoyl-CoA-treated GAPDH; (C) myristoyl-CoA-treated GAPDH; (D) decanoyl-CoA-treated GAPDH.

peak with mass of 35921 Da (Figure 4B). The mass difference between this unique peak and the major peak in the spectrum of the native GAPDH (i.e., 35683 Da) was 238 Da, which agreed well with the mass shift resulting from the addition of a single palmitate group (theoretical value = 238.23 Da). These results were consistent with the resolution (~12000) and mass accuracy (~100 ppm) of the instrument used in the experiment and, in conjunction with the autoradiographic studies, strongly suggested that the observed mass difference of 238 Da was caused by the addition of a palmitate group to GAPDH. Although we cannot make

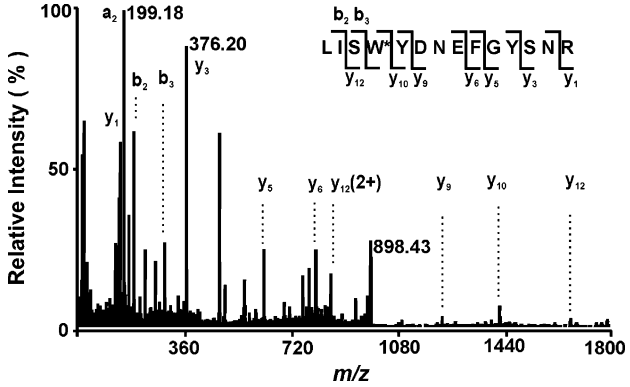

FIGURE 5: Electrospray ionization tandem mass spectrum of an oxidized GAPDH tryptic peptide. A doubly charged ion (898.43) was selected for product ion mass spectrometric analysis utilizing a quadrupole time-of-flight mass spectrometer equipped with a nanoelectrospray ion source. Manual interpretation of the spectrum identified the sequence of this peptide as ^{307}LISW*YDNEFGYSNR^{320} with Trp-310 being oxidized.

quantitative comparisons about the relative amounts of each isoform from mass spectral analysis alone due to the potential differences in ionization efficiencies of each species (although addition of a small noncharged hydrophobic molecule to a 35 kDa protein is not anticipated to result in marked differences in ionization efficiency), it is evident that only a small portion (approximately 25% of the major peak) of the native GAPDH was modified under the experimental conditions employed. These results suggest that modification of one residue per tetramer is sufficient to ablate enzyme activity or, alternatively, that only those tetramers manifesting bona fide enzyme activity can be acylated. These results were substantiated by both autoradiographic analysis and crude ESI mass analyses. Thus, acylation is highly specific, does not require a membrane hydrophobic surface to occur to organize reactants, and only one acyl group is added to each tetramer even at a concentration of 10 μM palmitoyl-CoA within the time frame of the experiment.

To determine whether GAPDH can be modified by other fatty acyl-CoA molecular species and to substantiate these assignments, we incubated GAPDH with myristoyl-CoA or decanoyl-CoA using the same conditions as were employed with palmitoyl-CoA and analyzed the reaction mixtures by mass spectrometry. After incubation with these acyl-CoA molecular species, unique peaks with the anticipated mass shifts (210 Da for myristoyl-CoA and 154 Da for decanoyl-CoA) were observed in the mass spectra (Figure 4C,D), indicating that GAPDH can also be modified by myristoyl-CoA and decanoyl-CoA. Collectively, the ESI mass spectra of intact GAPDH and its acyl adducts demonstrated that incubation of GAPDH with palmitoyl-CoA (or other acyl-CoA molecular species) at submicromolar concentrations resulted in the covalent linking of a palmitate group (or other acyl groups) to the protein.

Identification of the GAPDH Palmitoylation Site by MALDI-TOF/TOF Mass Spectrometry. To identify which amino acid residue(s) was (were) modified by fatty acyl-CoAs, we mapped the protein sequence of the modified GAPDH by MALDI-TOF/TOF mass spectrometry. We digested the fatty acyl-CoA-treated GAPDH with trypsin and analyzed the resultant tryptic peptides by MALDI-TOF mass spectrometry. In our efforts to detect the palmitoylated peptide by MALDI, we found that the solvent for dissolving the matrix (α-CHCA) was critical. When the matrix solution was prepared in 50% acetonitrile/0.1% trifluoroacetic acid (the most common solvent for α-CHCA), we could not detect the palmitoylated peptide. However, when we changed the solvent to 50% 2-propanol/25% formic acid, the palmitoylated peptide was routinely ionized and easily identified. A unique peak at *m*/*z* 1738.07 was detected in the mass spectrum of the tryptic peptides from palmitoyl-CoA-treated GAPDH (Figure 6B). The mass difference between this peak and the peak at *m*/*z* 1499.82 is 238.25 and is consistent with the calculated mass shift resulting from the addition of a palmitate group (theoretical value = 238.23). Product ion mass spectra revealed that the peak at *m*/*z* 1499.82 was a GAPDH tryptic peptide corresponding to amino acid residues 232−245 (VPTPNVSVVDLTCR). The unique peak at *m*/*z* 1738.07 was subjected to product ion analysis by MALDI-TOF/TOF to both substantiate the amino acid sequence and identify the site of acylation. The product ion mass spectrum of *m*/*z* 1738.07 authenticated the sequence of the tryptic peptide as ^{232}VPTPNVSVVDLTCR^{245} and identified cysteine 244 as the modification site by a robust series of y and b ions (Figure 7A). We next examined the tryptic digests of myristoyl-CoA-treated and decanoyl-CoA-treated GAPDH. The unique peaks (at *m*/*z* 1710.04 for myristoyl-CoA-treated GAPDH and at *m*/*z* 1653.73 for decanoyl-CoA-treated GADPH) (Figure 6C,D) and their product ion mass spectra (Figure 7B,C) also identified cysteine 244 as the site of these acyl covalent thioesterifications.

Association of Palmitoyl-CoA-Treated GAPDH to Synthetic Lipid Vesicles and Authentic Biologic Membranes. One common biologic consequence of palmitoylation of a cytosolic protein is that palmitoylation facilitates the translocation of the cytosolic protein to cellular membrane compartments. Despite prior expectations that GAPDH is exclusively a cytosolic enzyme, in many biological systems, a substantial portion of the GAPDH is membrane-associated and, importantly, can be induced to translocate to membranes during cellular activation by different stimuli through as yet unidentified chemical mechanisms (*19*). Accordingly, we prepared lipid vesicles composed of 60 mol % POPC and 40 mol % cholesterol and incubated them with either GAPDH preincubated with buffer alone or GAPDH preincubated with palmitoyl-CoA. Then, the lipid vesicles were spun down by centrifugation, and the resultant pellets were resuspended in SDS−PAGE sample buffer. Both the supernatants and the pellet were electrophoresed and subjected to western blotting using antibody against GAPDH. Western blotting demonstrated that the amount of lipid vesicle-associated GAPDH was about 4-fold higher in the sample containing GAPDH preincubated with palmitoyl-CoA than in the sample containing nontreated GAPDH (Figure 8A, pellet). Consistent with these results, the amount of GAPDH in the supernatant fractions was less in the sample containing GAPDH preincubated with palmitoyl-CoA than in the sample containing nontreated GAPDH (Figure 8A). To test whether similar results could be obtained with naturally occurring biologic membranes, we purified rabbit brain microsomes and incubated them with either nontreated GAPDH or GAPDH preincubated with palmitoyl-CoA. Using the same experimental approaches described above, we demonstrated that the amount of microsome-associated GAPDH was about

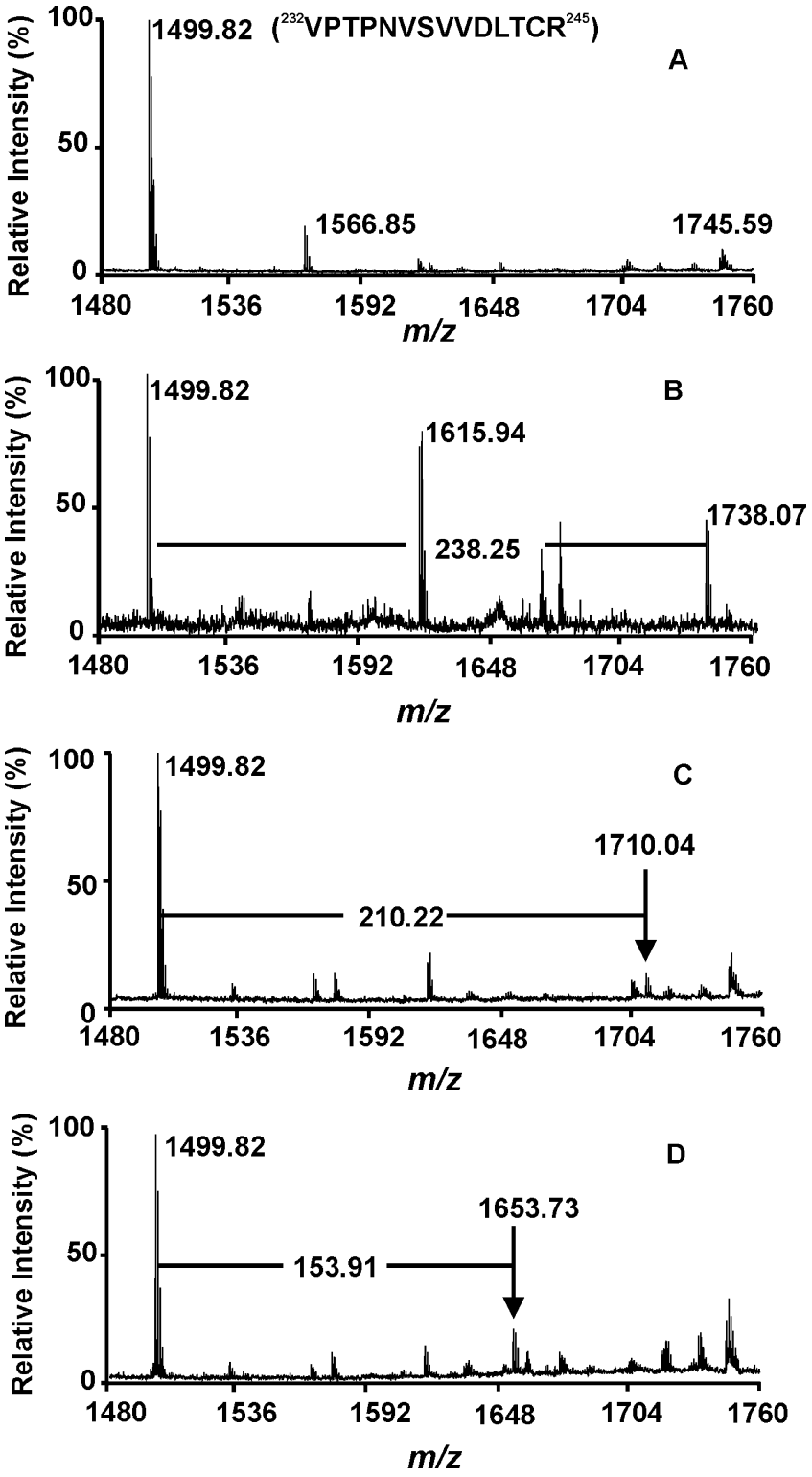

FIGURE 6: MALDI-TOF mass spectra of the GAPDH tryptic peptides. GAPDH (0.4 μM) was modified by 0.8 μM palmitoyl-CoA, myristoyl-CoA, or decanoyl-CoA. Trypsin was added at a ratio of 1:30 (w/w, trypsin/protein), and the mixture was incubated at 37 °C for 24 h. The resultant tryptic peptides were analyzed by MALDI-TOF in the positive ion reflector mode utilizing a 4700 proteomics analyzer. The *m*/*z* of 1499.82 matches the theoretical $[M + H]^+$ value of the GAPDH tryptic peptide ^{232}VPTPNVSVVDLTCR^{245}. Unique peaks at *m*/*z* 1738.07, *m*/*z* 1710.04, and *m*/*z* 1653.73 were present in the mass spectra of the tryptic peptides from the acyl-CoA-treated GAPDH. The mass shifts of these peaks from the unmodified peak at *m*/*z* 1499.82 were 238.25, 210.22, and 153.91, respectively. Key: (A) native GAPDH; (B) palmitoyl-CoA-treated GAPDH; (C) myristoyl-CoA-treated GAPDH; (D) decanoyl-CoA-treated GAPDH.

2-fold higher in membranes incubated with GAPDH that had been preincubated with palmitoyl-CoA (Figure 8B, pellet). The amount of GAPDH in the supernatant fractions was also less in samples containing palmitoylated GAPDH than in the sample containing nonpalmitoylated GAPDH (Figure 8B). The relative difference in partitioning largely reflects the amount of endogenous GAPDH present in rabbit brain microsomes. Collectively, the results obtained using either lipid vesicles or rabbit brain microsomes each demonstrated that palmitoylation of GAPDH facilitated the translocation

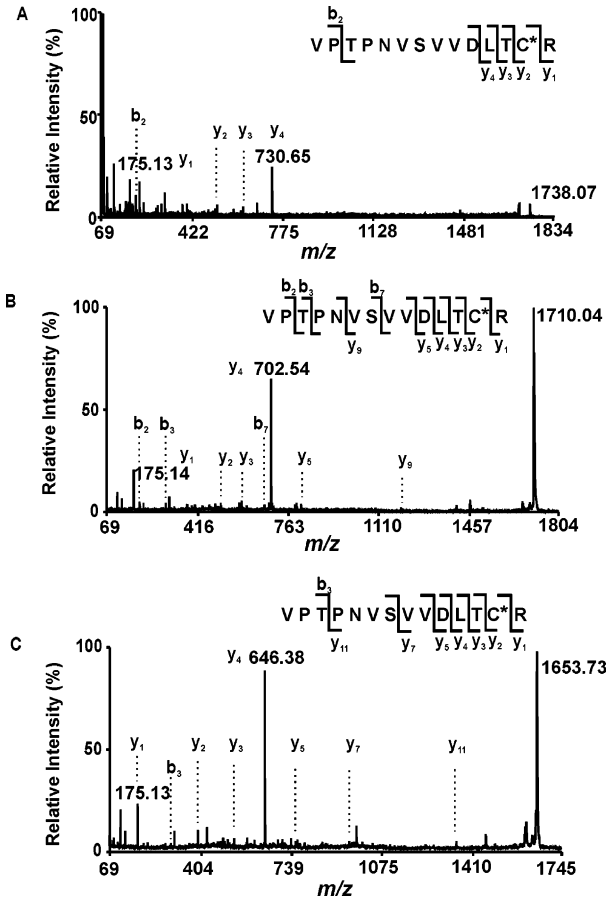

FIGURE 7: MALDI product ion mass spectra of fatty acylated peptides. MALDI-TOF mass spectra of the GAPDH tryptic peptides were acquired (Figure 6), and unique ions were selected for product ion mass spectrometric analyses by MALDI-TOF/TOF with the CID gas off. The series of y ions and b ions as labeled demonstrate that the sequence of the modified peptide is ^{232}VPTPNVS-VVDLTC*R^{245} with Cys-244 modified by the fatty acyl group. Key: (A) product ion mass spectrum of the ion at *m*/*z* 1738.07 (tryptic peptide of palmitoyl-CoA-treated GAPDH); (B) product ion mass spectrum of the ion at *m*/*z* 1710.04 (tryptic peptide of myristoyl-CoA-treated GAPDH); (C) product ion mass spectrum of the ion at *m*/*z* 1653.73 (tryptic peptide of decanoyl-CoA-treated GAPDH).

of a nominally cytosolic enzyme to either synthetic lipid vesicles or naturally occurring membrane fractions.

DISCUSSION

The results of the present study demonstrate the covalent modification of GAPDH by submicromolar concentrations of acyl-CoA that are functionally accompanied both by inhibition of dehydrogenase activity and by increased membrane partitioning. Moreover, they demonstrate that covalent modification of GAPDH by acyl-CoA is specific for acylation at Cys-244 and that this specific modification results in ablation of enzyme activity. Finally, the results demonstrate that acylation of only one unit of the GAPDH tetramer is likely sufficient to result in the translocation of a substantive portion of the tetramer to biologic membranes.

The results of the present study clearly identify one potential mechanism, protein acylation, which can modulate GAPDH enzyme activity. Moreover, we demonstrated that GAPDH acylation could facilitate the translocation of GAPDH to cellular membranes. Protein acylation is a critical

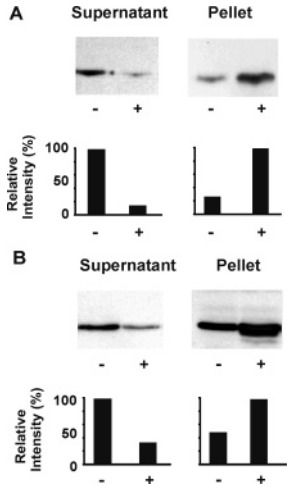

FIGURE 8: Association of palmitoyl-CoA-treated GAPDH to lipid vesicles and rabbit brain microsomes. Lipid vesicles were prepared by sonication, and rabbit brain microsomes were purified by differential centrifugation as described in Materials and Methods. GAPDH (0.4 μM) was incubated with palmitoyl-CoA (1 μM) in 50 mM Tris-HCl, pH 7.0, 2 mM EGTA, and 1 mM DTT at 37 °C for 1 h. A portion of the GAPDH sample was concentrated by centrifugation before incubations with rabbit brain microsomes. GAPDH (−) or palmitoyl-CoA-treated GAPDH (+) was incubated with lipid vesicles (A) or rabbit brain microsomes (B) at 37 °C for 15 min. Following the incubation, the lipid vesicles or rabbit brain microsomes were pelleted by ultracentrifugation. The pellets and the supernatants were electrophoresed on a 12% gel and transferred to a PVDF membrane. The membrane was probed with a GAPDH monoclonal antibody, washed, incubated with HRP-conjugated secondary antibody, and developed by ECL. Quantitative analyses were performed using a Kodak Image Station 440CF.

modification that has been demonstrated to be employed by many proteins to regulate their activities and facilitate their translocation to critical membrane loci interfaces to fulfill their biologic function through modulating their interactions with membrane proteins and determining their residence times after removal of the acyl group (*24*, *25*). We also recognize the possibility that, in addition to acylation of GAPDH in vitro, there may be additional mechanisms which accelerate and regulate the net membrane concentration of acylated GAPDH in vivo (e.g., acyl protein thioesterase 1, acyl protein thioesterase 2) or even potential proteins that can multiply acylate the monoacylated GAPDH moiety described herein. Since submicromolar concentrations of acyl-CoA result in the specific acylation of a single cysteine, substantially inhibit GAPDH enzyme activity, and alter the localization of acylated GAPDH, the possibility that cellular lipid loading (such as occurs in the lipotoxic state) contributes to insulin resistance merits consideration. The presence of an increased intramuscular acyl-CoA content either during acute lipid loading or in chronic lipotoxic states is well-known (*7*). Even minor excursions from normal acyl-CoA levels over an extended duration [e.g., submicromolar concentrations of acyl-CoA over days, anticipated lifetime of GAPDH in cytosol (*26*)] could result in abnormalities of intermediary metabolism either through attenuation of the enzyme activity of GAPDH or through the partitioning of the acylated GAPDH molecule to alternative subcellular locations. The levels of long-chain acylcarnitines increase over 4-fold in diabetic myocardium, demonstrating that mitochondrial utilization is insufficient to keep up with acyl-CoA production during lipotoxicity (*27*). Moreover, acyl-

carnitines that accumulate in diabetic myocardium inhibit the activity of a cytosolic acyl protein thioesterase, which is thought to be a key mediator in the removal of the palmitate moiety from palmitoylated proteins (*28*, *29*). In this study, we have identified that palmitoylation of GAPDH can occur as a result of only minor increases in acyl-CoA levels that would have profound effects on the endogenous enzyme activity of a key enzyme in glycolysis as well as initiate the potentially pathologic membrane partitioning of this essential regulatory enzyme to critical membrane compartments. It seems clear that palmitoylation of GAPDH would lead to attenuation of glycolytic flux resulting in decreased rates of glycolysis and decreased clearance of serum glucose after glucose loading, the hallmark of insulin resistance.

By taking advantage of the mass resolving power of modern mass spectrometers, we have demonstrated that GAPDH is covalently acylated after incubation with various fatty acyl-CoAs. We identified the modification site as cysteine 244 by MALDI-TOF/TOF. GAPDH is a tetrameric enzyme (a dimer of dimers) consisting of four 35 kDa subunits. Each subunit has four cysteines (Cys-149, Cys-153, Cys-244, and Cys-281) with Cys-149 being typically considered as the most nucleophilic. Due to its critical role in GAPDH activity, Cys-149 is the target for various GAPDH nucleophilic inhibitors and regulators, such as iodoacetate. However, in this study, we found that Cys-244 was the primary target for fatty acyl-CoA modification and this modification was correlated with a loss of GAPDH enzyme activity. Interestingly, GAPDH activity was ablated when only about 25% of the GAPDH was modified. These results implied that GAPDH activity was allosterically regulated by fatty acyl-CoAs. Fatty acyl-CoAs are not the only GAPDH inhibitor which targets Cys-244 primarily. Indeed, Ishii et al. reported that Cys-244 and Cys-281 are covalently modified in rabbit muscle GAPDH by 4-hydroxy-2-nonenal, resulting in the inhibition of enzyme activity (*30*). Moreover, the two most nucleophilic cysteines at the active site (Cys-149 and Cys-153) remained unmodified by 4-hydroxy-2-nonenal in their study and ours. These results collectively identify the intrinsic nucleophilicity of Cys-244. Comparison of the sequence database revealed that Cys-244 is conserved in multiple mammalian GAPDH sequences, including humans.

Previous work has largely relegated the role of GAPDH to that of a classical glycolytic enzyme, freely soluble in the cytosol and working in conjunction with other glycolytic enzymes to produce ATP and NADH. Recent studies have identified the emerging theme that GAPDH undergoes a signal-mediated translocation to critical subcellular membrane compartments where it regulates a multiple of cellular processes including membrane trafficking, synaptic vesicle function, and neurotransmitter dynamics (*19*, *20*, *31*−*33*). The possibility that acylation of GAPDH contributes to membrane trafficking or membrane fusion merits consideration. Furthermore, GAPDH is also part of a complex regulatory network in the sarcoplasmic reticulum where it is phosphorylated by a muscle-specific calmodulin-dependent protein kinase (CaMKIIβ_M) leading to the regulation of sarcoplasmic reticular calcium uptake and homeostasis through the effects of local ATP and NADH concentration near the ryandodine receptor and the calcium pump (*34*). The dynamic behavior of GAPDH during signal-induced translocation and phosphorylation and its extensive use of microtubule networks make it an attractive candidate to integrate glycolytic flux with membrane trafficking. However, there is still substantial controversy over the mechanisms through which these effects are mediated and the chemical and physical sequelae of GAPDH membrane association. It is hoped that identification of GAPDH palmitoylation in conjunction with the known requirement for acyl-CoA in membrane trafficking and fusion will provide further insight into the mechanisms underlying the multiple roles of GAPDH in biologic cellular function and as a mediator contributing to the production of the lipotoxic state.

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