

Inactivation of Glyceraldehyde-3-Phosphate Dehydrogenase by Fumarate in Diabetes

Formation of S-(2-Succinyl)Cysteine, a Novel Chemical Modification of Protein and Possible Biomarker of Mitochondrial Stress

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OBJECTIVE—S-(2-succinyl)cysteine (2SC) is formed by a Michael addition reaction of the Krebs cycle intermediate, fumarate, with cysteine residues in protein. We investigated the role of fumarate in chemical modification and inhibition of the sulfhydryl enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in vitro and in tissues of diabetic rats.

RESEARCH DESIGN AND METHODS—GAPDH was incubated with fumarate in PBS to assess effects of fumarate on enzyme activity in vitro. Sites of 2SC formation were determined by analysis of tryptic peptides by high-performance liquid chromatography–quadrupole/time-of-flight mass spectrometry. 2SC and fumarate in gastrocnemius muscle of control and streptozotocin-induced diabetic rats were measured by liquid chromatography/tandem mass spectrometry and by gas chromatography/mass spectrometry, respectively. GAPDH was isolated from muscle by immunoprecipitation, and sites of modification of GAPDH were determined by mass spectrometry analysis.

RESULTS—2SC was found, both in vitro and in vivo, about equally at active-site Cys-149 and nucleophilic Cys-244. Inactivation of GAPDH by fumarate in vitro correlated with formation of 2SC. In diabetic compared with control rats, fumarate and 2SC concentration increased approximately fivefold, accompanied by an ~25% decrease in GAPDH specific activity. The fractional modification of GAPDH by 2SC was significantly increased in diabetic versus control animals, consistent with the decreased specific activity of GAPDH in muscle of diabetic animals.

CONCLUSIONS—Fumarate contributes to inactivation of GAPDH in diabetes. 2SC may be a useful biomarker of mitochondrial stress in diabetes. Modification of GAPDH and other enzymes and proteins by fumarate may contribute to the metabolic changes underlying the development of diabetes complications. *Diabetes* 57:41–49, 2008

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Received for publication 9 August 2007 and accepted in revised form 20 September 2007.

Published ahead of print at <http://diabetes.diabetesjournals.org> on 12 October 2007. DOI: 10.2337/db07-0838.

2SC, S-(2-succinyl)cysteine; AGE, advanced glycation end product; ALE, advanced lipoxidation end product; ESI, electrospray ionization; G3P, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KCl, Krebs cycle intermediate; MALDI, matrix-assisted laser desorption/ionization; MS/MS, tandem mass spectrometry; NEM, N-ethylmaleimide; NFPA, nonafluoropentanoic acid; QTOF, quadrupole time of flight; ROS, reactive oxygen species; TOF, time of flight; UPLC, ultra-performance liquid chromatography.

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Increased chemical modification of proteins as a result of oxidative stress is implicated in the pathogenesis of chronic diseases, including diabetes, atherosclerosis, and Alzheimer's disease (1,2). In diabetes, oxidative damage is amplified by the accumulation of advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs) in tissue proteins (3), which bind to scavenger receptors and the receptor for AGEs and induce inflammatory responses (4). Research on the chemistry of AGE/ALE formation in diabetes has focused primarily on modification of lysine and arginine residues in protein (3,5,6). However, sulfhydryl groups on intracellular proteins are more nucleophilic than the amino or guanidino groups, suggesting that cysteine modifications should also be abundant. We have recently identified S-(2-succinyl)cysteine (2SC) as a chemical modification of plasma and tissue proteins, formed by a Michael addition reaction of fumarate with cysteine (7) (Fig. 1). We also showed that 2SC was significantly increased in muscle protein of diabetic versus control rats and that fumarate irreversibly inhibited several sulfhydryl enzymes in vitro, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Brownlee and colleagues (1,8–10) have proposed that during hyperglycemia in diabetes, inhibition of GAPDH during oxidative stress is a critical step in the development of diabetes complications. According to this hypothesis, inhibition of GAPDH limits the flux of glucose through glycolysis and, combined with the increase in ambient glucose concentration, causes an increase in the cytosolic concentration of three- and six-carbon glycolytic intermediates. These changes in metabolite concentrations then affect pathways, enzymes, and processes that are implicated in the pathogenesis of diabetes complications, including the polyol pathway, the hexosamine pathway, protein kinase C, and formation of AGEs. All of these changes were attributed indirectly to the accumulation of triose phosphates as a result of partial inactivation of GAPDH by poly-ADP-ribosylation. In studies of aortic endothelial cells in vitro, the inhibition of GAPDH could be prevented by increased expression of uncoupling protein 1, indicating that a high redox potential across the inner-mitochondrial membrane (hyperpolarization) and reduction of the electron transport chain contributed to the increase in mitochondrial reactive oxygen species (ROS) production during hyperglycemia.

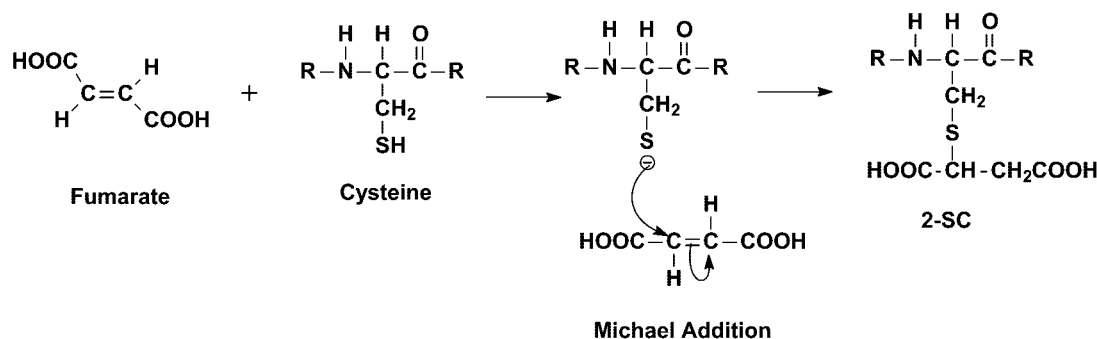


FIG. 1. Mechanism of formation of 2SC. Nucleophilic addition of fumarate to cysteine yields 2SC by a Michael addition reaction. R indicates peptide chain.

Based on the detection of increased levels of 2SC in muscle protein in diabetes, we speculated that oxidative stress and inhibition of electron transport during hyperglycemia might lead to an increase in concentration of Krebs cycle intermediates in mitochondria, and that, among these, fumarate, through formation of 2SC, might contribute to the inactivation of GAPDH in diabetes. We show here that fumarate concentration is significantly increased in muscle of diabetic rats and that the decrease in specific activity of GAPDH in diabetic muscle can be explained, at least in part, by increased chemical modification of cysteine residues by fumarate. We describe this process as succinylation of protein (in order to distinguish it from succinylation in which an amide or ester bond is formed) and propose that 2SC may be a useful biomarker of mitochondrial stress in diabetes.

RESEARCH DESIGN AND METHODS

Unless otherwise indicated, all chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO); $U\text{-}^{13}\text{C}_3$, ^{15}N -cysteine, and d_8 -lysine were from Cambridge Isotope Laboratories (Woburn, MA). 2SC or $U\text{-}^{13}\text{C}_3$, ^{15}N -2SC were synthesized as previously described (7). Polyclonal antibody to GAPDH was from Lab Frontier (Seoul, Korea). Protein A/G agarose beads (product 20421) were obtained from Pierce (Rockford, IL). Organic solvents were of the highest purity available from Acros Chemicals (Atlanta, GA).

Incubation and assay of GAPDH. For *in vitro* studies, GAPDH (1 mg/ml) was incubated in the presence of fumarate (0.5–500 mmol/l), succinate (5 mmol/l), or *N*-ethylmaleimide (NEM) (1 mmol/l), with or without substrate (1 mmol/l D-glyceraldehyde-3-phosphate [G3P]) and/or 1 mmol/l NAD or NADH, in PBS (50 mmol/l, pH 7.4, 37°C). Enzyme activity from *in vitro* studies and in muscle homogenates was measured at room temperature by recording absorbance at 340 nm using a TECAN Safire 2 (Zurich, Switzerland) plate reader.

Quantification of 2SC in GAPDH incubated with fumarate. Aliquots of reaction mixtures were dialyzed against deionized water at 4°C and hydrolyzed in 6M HCl for 1 h at 150°C. The hydrolysates were dried *in vacuo*, then dissolved in deionized water. 2SC was quantified by amino acid analysis using cation exchange chromatography with a Pickering (Mountain View, CA) Na^+ -based buffer system and postcolumn fluorescence detection with o-phthalaldehyde.

Preparation of GAPDH peptides from protein modified *in vitro*. Aliquots or reaction mixtures were dialyzed, lyophilized, and resuspended in 25 μl deionized water. Digestion buffer (50 μl of 6 mol/l urea, 0.1 mol/l morpholinopropanesulfonic acid, buffer, and 1 mmol/l EDTA, pH 7.2) was added and thiols reduced by addition of dithiothreitol (2.5 μmol), followed by incubation for 1 h at 37°C. Cysteine residues were alkylated by addition of 3 μmol 4-vinylpyridine for 1 h. Dithiothreitol (3.5 μmol) was added to consume the remaining 4-vinylpyridine, followed by the addition of 355 μl deionized water (final urea concentration 0.6 mol/l). Trypsin (1 $\mu\text{g}/\mu\text{l}$ in 0.1 mol/l HCl) was added at a ratio of 1:50 (wt/wt to protein), and digestion was carried out at 37°C for 24 h.

Analysis of loss of native cysteine peptides by ultra-performance liquid chromatography/mass spectrometry. A 5- μg sample of peptides was injected onto a Waters Acquity BEH shielded C18 reversed-phase column (2.1 mm \times 50 mm, 1.7 μm), interfaced to a Waters Quattro Premier XE mass

spectrometer (Milford, MA). The mobile phases were solvent A (5 mmol/l nonafluoropentanoic acid [NFPA]) and solvent B (100% acetonitrile). Peptides were eluted with a linear gradient of solvent B (2–60% in 12.6 min) at a flow rate of 0.2 ml/min. Electrospray voltage was 3.2 kV, cone voltage was 30 V, and the source and desolvation temperatures were 120°C and 350°C, respectively. Peptides were detected in positive-ion continuum-mode scanning from 250 to

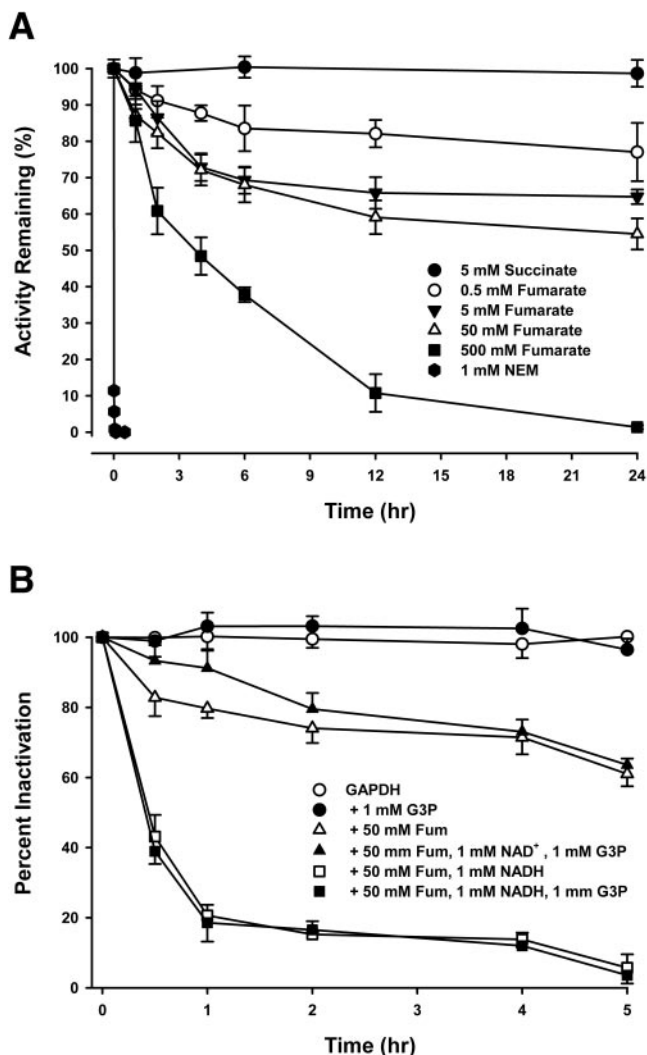


FIG. 2. Kinetics of GAPDH inhibition by fumarate *in vitro*. **A:** GAPDH (1 mg/ml) was incubated in the presence of fumarate (0.5, 5, 50, and 500 mmol/l), succinate (5 mmol/l), or NEM (5 mmol/l) in 50 mmol/l PBS (pH 7.4, 37°C). **B:** GAPDH (1 mg/ml) was incubated in the presence of 50 mmol/l fumarate (Fum) with or without 1 mmol/l coenzyme and/or 1 mmol/l substrate in 50 mmol/l PBS (pH 7.4, 37°C). Data are means \pm SD from three independent experiments.

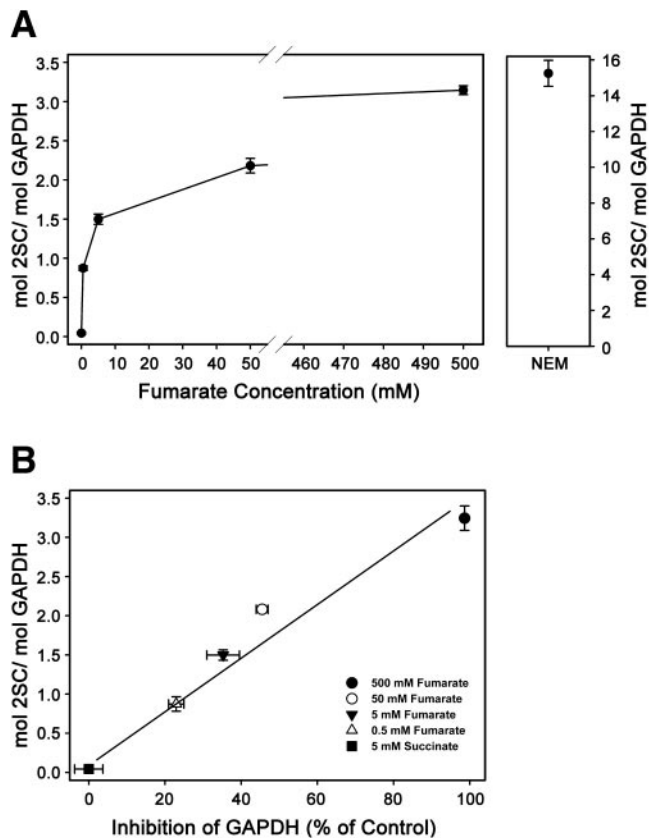


FIG. 3. Kinetics of formation of 2SC during incubation of GAPDH with fumarate. **A:** GAPDH (1 mg/ml) was incubated with various concentrations of fumarate (0.5, 5, 50, and 500 mmol/l) in 50 mmol/l PBS (pH 7.4, 37°C, 24 h), then dialyzed and hydrolyzed in 6 mol/l HCl for quantification of 2SC by amino acid analysis. **B:** Levels of 2SC (Fig. 2A) correlate with the percent inhibition of GAPDH (Fig. 1A). Data are means \pm SD from three independent experiments ($r^2 = 0.78$, $P < 0.01$).

1,000 m/z. Loss of native cysteine-containing peptides was calculated by summation of the +2 and +3 charge states of the peptide divided by the same ions for an internal reference peptide A¹⁹AFNSGK²⁵, which was chosen because of its signal strength and resolution from other peptides.

Peptide sequencing by liquid chromatography/quadrupole time-of-flight tandem mass spectrometry. Peptides (10 μ g) were injected onto an AquaSep C-18 reversed-phase column (2.1 mm \times 150 mm, 5 μ m) (ES industries, West Berlin, NJ), coupled to a quadrupole time-of-flight (QTOF) mass spectrometer (Micromass QTOF; Micromass, Manchester, U.K.). The mobile phases were solvent A (0.1% formic acid) and solvent B (100% acetonitrile). Peptides were eluted with a linear gradient of 2–50% solvent B in 22 min at a flow rate of 0.2 ml/min. Electrospray voltage was 3.5 kV, the cone voltage was 35 V, the collision energy was 40 eV, and source temperature and desolvation temperatures were 100°C and 350°C, respectively. Peptides were detected and fragmented in continuum positive-ion mode.

Preparation of rat muscle samples. Gastrocnemius muscles from six control and six streptozotocin-induced diabetic animals were obtained from a previous study (7). These animals were 34 weeks of age, with 28 weeks' duration of diabetes, and were treated with 3–5 units ultralente insulin (Humulin, Eli Lilly, Indianapolis, IN) three times per week throughout the study. Mean fasting blood glucose for the diabetic animals was 27 mmol/l compared with 5 mmol/l for nondiabetic controls. The animals were only mildly ketotic (twofold increase in acetoacetate) and gained weight during the course of the 6-month study (65% weight gain for diabetic rats vs. 78% for nondiabetic controls). The animals had developed early stages of nephropathy, based on a twofold increase in plasma creatinine concentration and urinary albumin excretion (30 mg/24 h); other complications were not evaluated.

Gastrocnemius muscle was isolated by dissection, scraped free of tendons and connective tissue, frozen at -70°C , and then pulverized in a ceramic mortar on dry ice. The muscle powder was resuspended with brief sonication (10 s, Branson 12; Branson, Danbury, CT) in ice-cold homogenizing buffer (100 mmol/l KCl, 20 mmol/l imidazole, 5 mmol/l diethylenetriaminepentaacetic

acid, and 1% Triton X-100, pH 6.8) at a concentration of 100 mg powder/ml buffer. Protein concentration was measured by the assay used by Lowry et al. (11).

Quantification of fumarate concentration in muscle by gas chromatography/mass spectrometry. Fumarate concentration was measured as the *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (Pierce, Rockford IL) derivative, as described by Tanaka et al. (12). The analyses were conducted on a HP 6890 GC/MS using a Zebron ZB-5w/Guardian column (Phenomenex, Torrance, CA). Nonanoic acid was used as internal standard.

Quantification of 2SC in muscle protein by ultra-performance liquid chromatography/tandem mass spectrometry. An amount of homogenate containing 1 mg protein was precipitated with an equal volume of 20% cold trichloroacetic acid. The supernatant was decanted and the precipitated protein hydrolyzed in 6 mol/l HCl containing ¹³C₃, ¹⁵N-2SC, and *d*₆-lysine internal standards for 1 h at 150°C. Samples were dried under vacuum, then dissolved in 200 μ l of 5 mmol/l NFPA. A 20- μ l (100- μ g) aliquot of protein hydrolysate was injected on a Waters C18 Acquity column (2.1 mm \times 50 mm, 1.7 μ m) interfaced to a Waters Premier UPLC ESI-MS/MS system (ultra-performance liquid chromatography/electrospray ionization/tandem mass spectrometry). The mobile phases were solvent A (5 mmol/l NFPA) and solvent B (100% acetonitrile). 2SC was eluted using a linear gradient from 10 to 40% solvent B over 4.2 min at a flow rate of 0.2 μ l/min and a column temperature of 30°C. The mass spectrometer was used in positive-ion mode, and quantitative analysis was in the multiple-reaction monitoring mode. Collision gas (nitrogen) pressure was 10 Pa, the capillary voltage was 3.4 kV, the cone voltage was 22 V, the collision energy was 20 V, and the source and desolvation temperatures were 100°C and 350°C, respectively. The multiple-reaction monitoring transitions monitored were 2SC, *m/z* 238 > 174; U-¹³C₃, ¹⁵N-2SC, *m/z* 242 > 178; lysine, *m/z* 147 > 84; and *d*₆-lysine, *m/z* 155 > 92. Quantification was performed by isotope-dilution mass spectrometry based on standard curves. The 2SC concentration was normalized to the lysine content of the samples.

Immunoprecipitation and mass spectrometry analysis of GAPDH from rat muscle. Muscle homogenate (1 mg protein) was diluted into 300 μ l radiomunoprecipitation buffer (5 mmol/l Tris-HCl, 150 mmol/l NaCl, 1 mmol/l EDTA, 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate, pH 7.4). Protease inhibitor cocktail (10 μ l; Sigma-Aldrich) was added, and the samples were precleared by incubation with 6 μ l protein A/G agarose beads, with rotation for 30 min at room temperature. After centrifugation at 10,000 rpm for 2 min, the supernatant was transferred to a fresh centrifuge tube, followed by further incubation with 6 μ g anti-GAPDH antibody for 3 h at room temperature. Protein agarose A/G beads (20 μ l) were then added, followed by incubation for 1 h at room temperature. The beads were washed three times by suspension in 0.5 mol/l NaCl in 50 mmol/l PBS, pH 7.4, followed by centrifugation at 10,000 rpm for 2 min.

The agarose beads from the immunoprecipitation were suspended in SDS denaturing buffer (5 μ l, 10% SDS, 20% glycerol, 25 mmol/l TRIS-HCl, and 5% 2-mercaptoethanol), then boiled for 10 min at 95°C. Following centrifugation, the supernatant was loaded onto a 10% precast gel (BioRad, Hercules, CA) and electrophoresed at 200 V for 50 min. The gel was stained with Bio-Safe Coomassie (BioRad), and GAPDH bands were manually excised for digestion. The excised bands were placed into silicon-coated 1.5-ml plastic centrifuge tubes and washed overnight in methanol/acetic acid (1:1, 300 μ l). The protein from the gel was digested and extracted, as described by Kinter and Sherman (13). A 10- μ l aliquot of peptides extracted from a gel piece was analyzed by UPLC ESI-MS/MS, as described above.

Peptide profiling by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis. Mass and product ion mass spectra of peptides were acquired on a Bruker Daltonics (Billerica, MA) Ultraflex 2 MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometer, using a matrix solution containing 7 mg α -cyano-4-hydroxycinnamic acid in 1 ml of 90% acetonitrile. Samples were desalted using a μ C18 Zip Tip (Millipore, Bedford, MA) and eluted onto the target plate with 2 μ l matrix solution. Mass spectra were recorded in the positive-ion reflector-mode scanning from 800 to 4,000 m/z. Ions of interest were selected and subjected to product ion analysis using the LIFT cell to confirm identity. Data were collected until the native peptide mass envelope reached an intensity of 3,000 area units.

RESULTS

Inhibition of rabbit muscle GAPDH by fumarate. To examine the effect of fumarate on GAPDH activity, GAPDH was incubated with fumarate at a range of concentrations or with succinate as a control for 24 h at 37°C (Fig. 2A). GAPDH enzyme activity was inhibited by fuma-

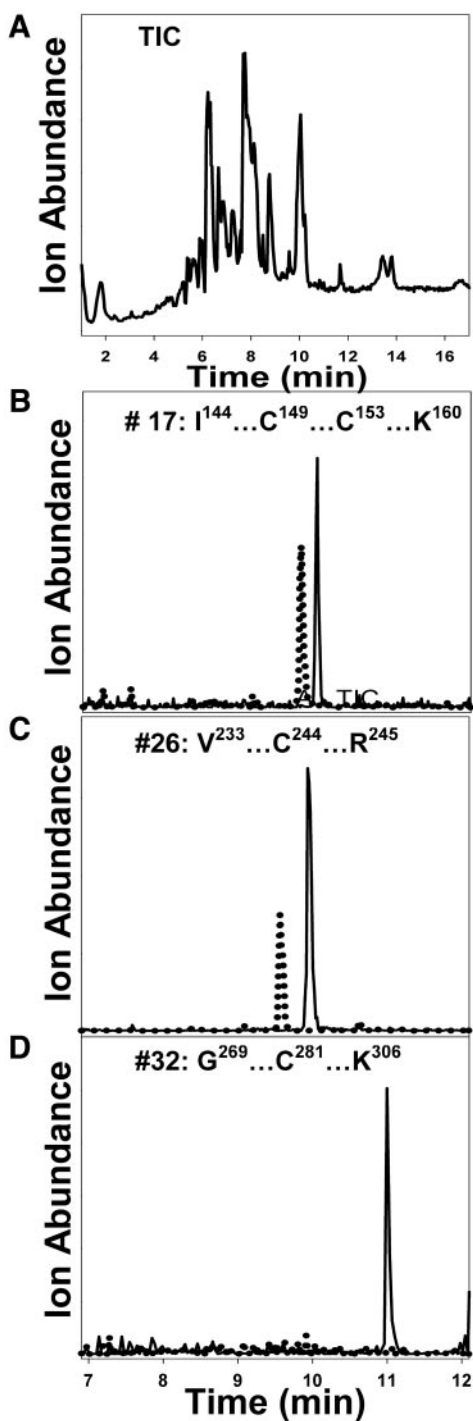


FIG. 4. Total and extracted ion chromatograms of the +2 charge states of the pyridylethyl and 2SC peptides from GAPDH. A 5- μ g sample of tryptic peptides from incubations of GAPDH with 50 mmol/l fumarate for 24 h was analyzed by UPLC-ESI/MS/MS. **A:** Total ion chromatogram (TIC) of GAPDH digested with trypsin. **B:** Extracted ion chromatograms of the +2 charge state of pyridylethyl and 2SC peptide 17. **C:** Extracted ion chromatograms of +2 charge state of pyridylethyl and 2SC peptide 26. **D:** Results of analysis of peptide 32, illustrating absence of 2SC on Cys-281. Solid lines: pyridylethyl peptide; dotted lines: 2SC peptide.

rate in a time- and concentration-dependent manner. Approximately 20% of GAPDH activity was lost by incubation with 0.5 mmol/l fumarate for 24 h, and 100% activity was lost in incubations with 500 mmol/l fumarate at 24 h. Succinate had no effect on enzyme activity, while 1 mmol/l

NEM completely inactivated the enzyme in 10 min (Fig. 2A). As shown in Fig. 2B, the kinetics of inactivation of GAPDH by fumarate were not affected by the presence of G3P, regardless of whether NAD^+ was in the reaction mixture. In contrast, NADH in the presence or absence of G3P significantly accelerated the inactivation of GAPDH by fumarate (Fig. 2B).

Formation of 2SC in incubations of GAPDH with fumarate. To determine the extent of modification of cysteine residues in GAPDH by fumarate, GAPDH was hydrolyzed after incubation with fumarate or succinate and 2SC was measured by amino acid analysis. As shown in Fig. 3A, there was a concentration-dependent increase in 2SC formation at 24 h, approaching 4 mol 2SC/mol protein (1 mol 2SC/mol subunit) in incubations with 500 mmol/l fumarate (Fig. 3A). NEM yielded ~ 16 mol 2SC/mol protein. The increase in 2SC correlated inversely with the loss of enzyme activity (Fig. 3B). Overall, these experiments established that GAPDH was inhibited irreversibly by fumarate in a concentration-dependent manner *in vitro* and that the extent of inhibition of the enzyme by fumarate could be attributed to modification of cysteine residues and formation of 2SC.

UPLC ESI-MS/MS analysis of 2SC-GAPDH. To identify the site(s) of formation of 2SC, GAPDH, incubated for 24 h with 50 mmol/l fumarate, was digested with trypsin and peptides analyzed by UPLC ESI-MS/MS (Fig. 4A). Two cysteine-containing peptides with mass additions equivalent to fumarate were detected: active-site peptide 17 ($^{143}\text{IVSNASCCTTNCLAPLAK}^{159}$, containing Cys-149 and Cys-153) and peptide 26 ($^{232}\text{VPTPNVSVVDLTCR}^{245}$, containing Cys-244) (Fig. 4B and C). There was no evidence of modification of Cys-281 in peptide 32 (Fig. 4D), either in this experiment or at any other concentration of fumarate studied (Fig. 5, *top line*).

The fractional loss of native peptides 17 and 26, compared with a reference peptide ($\text{A}^{19}\text{AFNSGK}^{25}$) from GAPDH, is shown as a function of fumarate concentration in Fig. 5. The total of 55 and 40% losses of peptides 17 and 26, respectively, in incubations with 500 mmol/l fumarate for 24 h (Fig. 5), was consistent with the estimated total extent of modification of the enzyme, ~ 1 mol 2SC/mol subunit (Fig. 3).

Confirmation of peptide identity and specificity of modification by ESI QTOF. A sample of tryptic peptides from incubations with 50 mmol/l fumarate for 24 h was fractionated by reversed-phase high-performance liquid chromatography and analyzed by QTOF mass spectrometry. Based on the fragment ion spectra, the doubly charged ion 963.99 m/z, identified as peptide 17, contained a 2SC adduct on the y_{11} ion but not on the y_7 ion (Fig. 6A), indicating selective modification of Cys-149, the active-site cysteine residue. The doubly charged ion 808.42 m/z, identified as peptide 26, contained a 2SC adduct on the y_2 ion (Fig. 6B), indicating modification at Cys-244.

2SC and fumarate concentrations increase and the specific activity of GAPDH decreases in diabetic skeletal muscle. To assess the role of 2SC in inhibition of GAPDH activity in diabetes, gastrocnemius muscle was obtained from streptozotocin-induced diabetic rats and age-matched control animals. Both 2SC and fumarate were significantly increased in diabetic versus control muscle tissue (Fig. 7A and B), while GAPDH specific activity was decreased $\sim 25\%$ in diabetic versus control animals (Fig. 7C). There was a significant inverse correlation between the 2SC content of total muscle protein and the specific

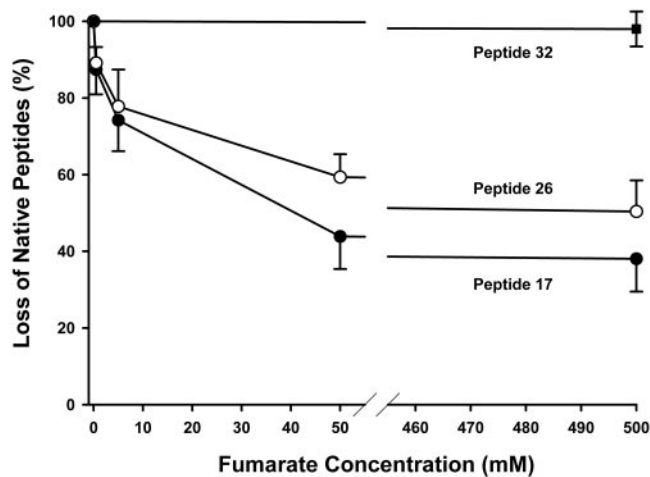


FIG. 5. Loss of native cysteine-containing peptides from GAPDH incubated with fumarate. GAPDH (1 mg/ml) was incubated with fumarate (0.5, 5, 50, and 500 mmol/l) for 24 h, dialyzed, and digested with trypsin. A 5- μ g sample of peptide was analyzed by UPLC ESI-MS/MS and the loss of native peptide calculated as described in RESEARCH DESIGN AND METHODS. Data are means \pm SD ($n = 5$).

activity of GAPDH (Fig. 7D). While GAPDH is only a small fraction of total cellular protein, these experiments suggested that fumarate might have a role in inactivation of GAPDH in diabetes.

Succination of GAPDH in muscle of diabetic rats. To determine the extent of 2SC formation on GAPDH in vivo, GAPDH was immunoprecipitated from control and diabetic rat muscle, electrophoresed under denaturing conditions, stained with Coomassie blue, and then digested in gel with trypsin. Because the quantities of protein available were insufficient for QTOF analysis and sequencing, we applied two different methods to demonstrate that GAPDH was modified in vivo and that the extent of modification was increased in diabetes. First, both of the 2SC-peptides were detectable by UPLC ESI-MS/MS in GAPDH isolated from muscle. As shown in Fig. 8, the ratio of the 2SC peptide to the pyridylethyl peptide was increased for both peptides 17 and 26 from diabetic versus control muscle. The data are presented as a ratio because they are only an approximation of the relative extent of 2SC modification of GAPDH, as the 2SC and pyridylethyl peptides may not have the same ESI-MS/MS response factors. As observed for the relationship between the 2SC content of total muscle protein and the specific activity of GAPDH (Fig. 7D), there was a significant inverse correlation between the peptide ratios in Fig. 8 and the specific activity of GAPDH ($P < 0.01$ for peptide 17 and $P < 0.05$ for peptide 26). As in Fig. 4D, pyridylethyl peptide 32 was present, but there was no detectable signal for 2SC-modified peptide 32 in GAPDH isolated from muscle protein (data not shown).

Analysis of peptides by MALDI-TOF mass spectrometry (Fig. 9) also showed the presence of 2SC on peptides 17 and 26 in GAPDH isolated from control and diabetic animals; peptide 32 was too large for analysis on our MALDI-TOF mass spectrometer. The signal strength for the 2SC-peptides was not strong enough for sequence analysis, but MALDI-TOF provides high-resolution mass spectrometry identification of the peptides. While MALDI-TOF is not considered as quantitative a technique as ESI-MS/MS, the increase in relative signal strength for the 2SC peptides for GAPDH from diabetic versus control

muscle by MALDI-TOF analysis is consistent with the results of the liquid chromatography/mass spectrometry analysis (Fig. 8). The ratio of modified to unmodified peptide was also correlated inversely with the specific activity of GAPDH in the muscle samples ($P < 0.05$), consistent with increased modification of peptides in GAPDH isolated from muscle of diabetic versus control rats.

DISCUSSION

Specificity of modification and inactivation of GAPDH by fumarate. The present work was precipitated by the discovery of 2SC in tissue proteins and the observation that 2SC was increased in muscle proteins of diabetic rats (7). While studying reactions of fumarate with proteins, we learned that fumarate inhibited several sulfhydryl enzymes in vitro, including GAPDH, glucose-6-

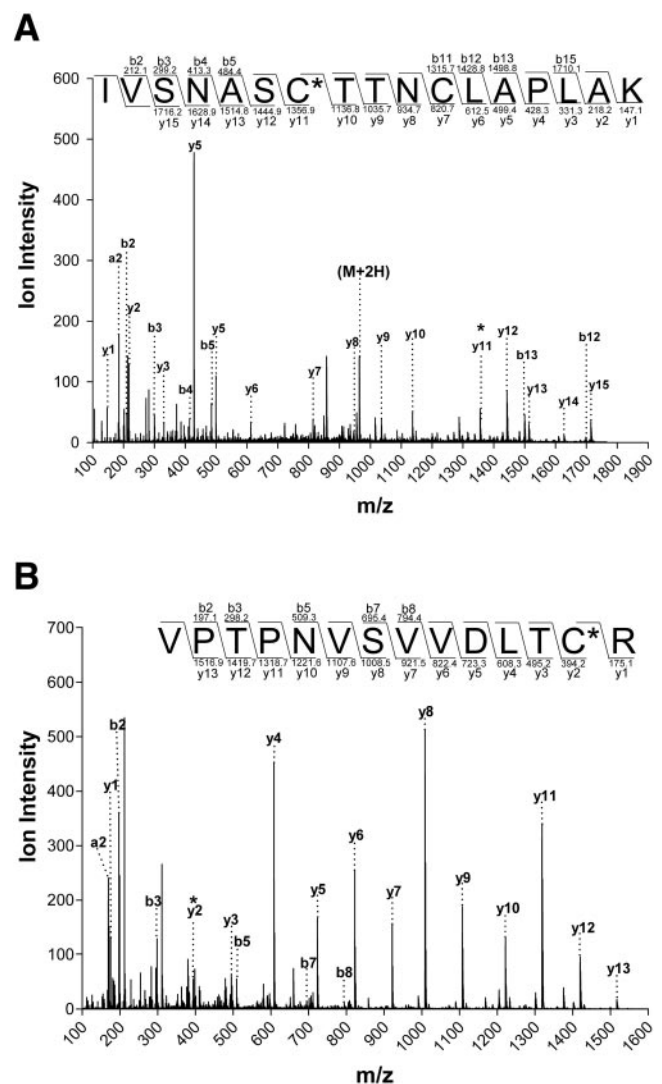


FIG. 6. Mass spectrometric analysis of peptide fragmentation patterns from GAPDH treated with fumarate. GAPDH (1 mg/ml) was incubated with 50 mmol/l fumarate for 24 h, dialyzed, and digested with trypsin. A 20- μ g sample of peptides was analyzed by tandem mass spectrometry, as described in RESEARCH DESIGN AND METHODS. A: Spectrum of the doubly charged ion 963.99 m/z, corresponding to active-site peptide 17. B: Spectrum of the doubly charged ion 808.42 m/z, corresponding to peptide 26. Insets: Amino acid sequence of peptides 17 and 26 indicating the major COOH- and NH₂-terminal fragment ions detected by full-scan MS/MS. *2SC y-ion.

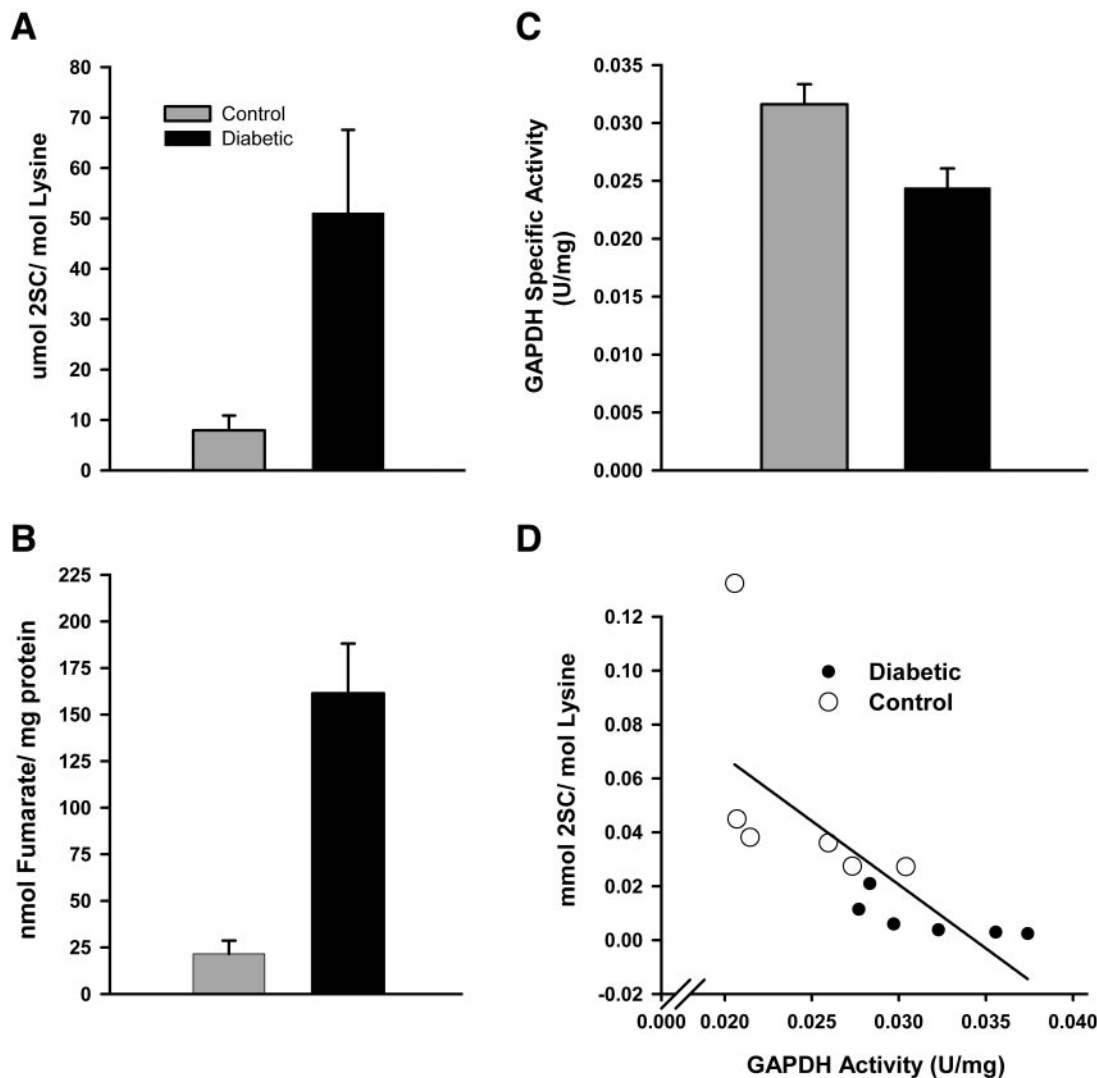


FIG. 7. Measurement of 2SC, fumarate, and GAPDH specific activity in control and diabetic rat gastrocnemius muscle. **A:** Levels of 2SC, measured by isotope dilution UPLC ESI-MS/MS, were 7.83 ± 2.9 and 48.6 ± 17 μmol 2SC/mol lysine in control and diabetic muscle, respectively ($P < 0.01$). **B:** Levels of fumarate, measured by isotope dilution gas chromatography/mass spectrometry, were 21.6 ± 17.2 and 161 ± 65.4 nmol/mg protein, respectively ($P < 0.01$). **C:** GAPDH specific activity was 0.032 ± 0.004 and 0.024 ± 0.004 units/mg protein ($P < 0.02$). **D:** Correlation between 2SC content of muscle protein and specific activity of GAPDH ($r^2 = 0.76$, $P < 0.01$). Data are means \pm SE ($n = 6$).

phosphate dehydrogenase and alcohol dehydrogenase. We focused on GAPDH because of Brownlee's hypothesis that inhibition of this enzyme and the resultant increase in glycolytic intermediates might explain alterations in critical enzymes and pathways implicated in the development of complications in diabetes (1,9): the polyol, protein kinase C, and hexosamine pathways and AGE formation. Although muscle is not considered a critical site for development of diabetes complications, it was chosen for this study because of availability of a considerable mass of homogeneous tissue, for example, compared with microvascular endothelial cells. Muscle metabolism is also substantially altered in diabetes, and there is increasing evidence for defects in mitochondrial metabolism in muscle in diabetes (14,15). Thus, analysis of muscle might serve as a model for studies on mitochondrial stress and dysfunction and development of complications in other tissues.

GAPDH is a tetrameric enzyme with four free sulfhydryl groups per subunit; one of these, Cys-149 in the active site, is a nucleophilic residue and forms a thiohemiacetal

linkage with the substrate G3P. The inactivation of rabbit muscle GAPDH by fumarate was readily demonstrated in vitro (Fig. 2). Complete inactivation of free enzyme by 500 mmol/l fumarate at 24 h was accompanied by formation of ~ 1 mol 2SC/mol subunit (3.5 mol/mol GAPDH; Fig. 3). Analysis of tryptic peptides indicated approximately equal modification of the active-site Cys-149 and of a peripheral cysteine residue, Cys-244 (Fig. 4). Cys-244, like Cys-149, is known to be a nucleophilic residue in GAPDH; it is the primary site of covalent modification of GAPDH by the lipophilic compounds hydroxynonenal (16) and palmitoyl-CoA (17), confirming its reactivity with electrophiles. Modification of this cysteine by palmitate and hydroxynonenal also inhibits enzyme activity, suggesting that modification of Cys-149 or Cys-244 by fumarate is mutually exclusive and that modification at either site inactivates the enzyme. While the other two cysteine residues in GAPDH did not react measurably with fumarate under experimental conditions, all 16 cysteines reacted rapidly with low concentrations of NEM, suggesting that charge-

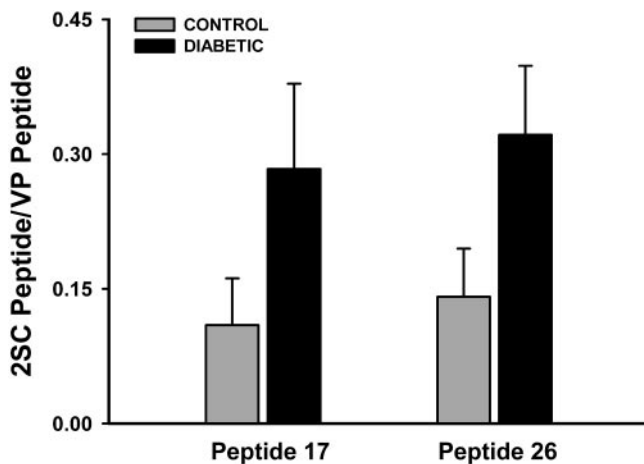


FIG. 8. Increased 2SC modification of GAPDH peptides isolated from gastrocnemius muscle of diabetic versus control rats. GAPDH immunoprecipitates were analyzed by SDS-PAGE, stained with Coomassie blue, digested in gel with trypsin, and then analyzed by UPLC ESI-MS/MS. The ratio of the area units of the +2 ions for the 2SC and pyridylethyl peptides indicated significant increases in 2SC modification of peptides 17 and 26 in diabetic versus control muscle. Data are means \pm SD ($n = 6$; $P < 0.01$ for both peptides). VP, vinylpyridine.

charge interactions restrict modification of the protein by fumarate.

Cys-149 and Cys-244 were also identified as sites of 2SC formation in GAPDH isolated from rat muscle. A significant increase in 2SC modification of these cysteine residues in diabetic muscle was detectable by both UPLC ESI-MS/MS and MALDI-TOF mass spectrometry analysis (Figs. 8 and 9). While neither of these mass spectrometric techniques is rigorously quantitative because of possible difference in mass spectrometer response to the modified and control peptides, comparison of the MALDI-TOF spectra of GAPDH modified by fumarate *in vitro* with those obtained from the tissue enzyme (Fig. 9) is consistent with a 25% modification and decrease in specific activity of the muscle enzyme in diabetes. The loss of activity of GAPDH in diabetic compared with control muscle (25%; Fig. 7B) was less than might be expected from the six- to sevenfold increase in fumarate (Fig. 7C) and 2SC concentration (Fig. 7A). However, some 2SC is detectable in GAPDH of control animals (Figs. 8 and 9). Assuming 4% 2SC content and 96% of the maximal specific activity of GAPDH in control animals, a sixfold increase in 2SC in diabetes would be consistent with a 25% decrease in specific activity of the enzyme. Although this discussion is meant to be illustrative rather than quantitative, it points out the need to apply more rigorous quantitative proteomic techniques to validate the quantitative role of fumarate in protein modification in diabetes.

Endogenous defenses. Glutathione, which is present at millimolar concentrations in cells, could be important in limiting succination of proteins and blunting the effects of increasing cellular fumarate concentration. However, the high pKa of the cysteine residue of glutathione, ~ 9.0 (18), would limit its effectiveness in protecting the nucleophilic cysteine residues of GAPDH. Indeed, the rate of succination of GAPDH is ~ 250 times faster than that of *N*-acetylcysteine at physiological pH (7). Despite protective mechanisms, the inactivation of GAPDH by fumarate is an observable and measurable consequence of fumarate accumulation in tissues in diabetes. Notably, the mild decrease in specific activity of GAPDH (Fig. 7) is consistent

with a chronic, partial inhibition of glycolysis in skeletal muscle in diabetes, which may be sufficient to cause the metabolic and chemical imbalances implicated in the development of diabetes complications (1).

Oxidative stress and mitochondrial dysfunction in diabetes. The mitochondrial electron transport chain is one of the major sources of ROS production in the cell and is implicated in both glucotoxicity to cells in culture and development of complications in diabetes (1,10). Hyperpolarization of the electron transport chain appears to be part of the mechanism of glucotoxicity because inhibitors of the electron transport chain enhance mitochondrial ROS production, while uncouplers and uncoupling proteins have the opposite effect (10,19,20). One consequence of inhibition of the electron transport chain would be an increase in Krebs cycle intermediates (KCI), particularly those oxidized by NAD-dependent dehydrogenases. Under such conditions, the full spectrum of KCIs would probably accumulate in the mitochondrion, as happens in muscle mitochondria during prolonged, strenuous exercise when inhibition of the electron transport chain and oxidative stress are mediated by exercise-induced oxygen debt or hypoxia (21,22). These intermediates, including fumarate, would then migrate into the cytosol through one of several passive mitochondrial carboxylic acid transporters (23,24).

Fumarate may have a unique role as a chemical communicator between the mitochondrion and the cytosol and other subcellular compartments during mitochondrial stress. We use the word stress rather than dysfunction here because the mitochondrial response in diabetes, like that in exercise, may be a perfectly reasonable response to stress, not necessarily an indication of dysfunction. During prolonged exercise, for example, the accumulation of fumarate and other KCIs is the result of lack of oxygen, not mitochondrial dysfunction. In diabetes, the mitochondrial stress and accumulation of fumarate may result from the excess concentration of carbohydrate and lipid substrates.

A number of mechanisms have been described for inactivation of thiol enzymes in diabetes and other diseases, including direct oxidation of cysteine residues, glutathionylation, nitrosation, poly-ADP-ribosylation, reaction with intermediates in formation of AGEs and ALEs, and now reaction with fumarate to form 2SC. The relative contribution of these reversible and irreversible mechanisms to inactivation of various enzymes, including GAPDH, remains to be determined. It seems likely that the accumulation of fumarate might occur at an early stage of stress, in concert with the increase in ROS production, and that succination of protein by fumarate might be one mechanism for modulating the activity of extramitochondrial enzymes, such as GAPDH, limiting the flux of glycolytic intermediates into the Krebs cycle in diabetes. In this context, succination of protein may be one of the early indicators of glucotoxicity, possibly a protective response preceding changes in mitochondrial permeability and swelling and increased generation of ROS—processes that set the stage for apoptosis and cell death. Prolonged oxidative stress could also overwhelm antioxidant defenses, leading to DNA damage and eventually to activation of poly-ADP-ribose polymerase, which may also contribute to inactivation of GAPDH (8).

In this study, we have addressed only a single enzyme, GAPDH, and its response to modification by fumarate. There are obviously a number of other sulfhydryl enzymes and proteins that are likely targets for modification by

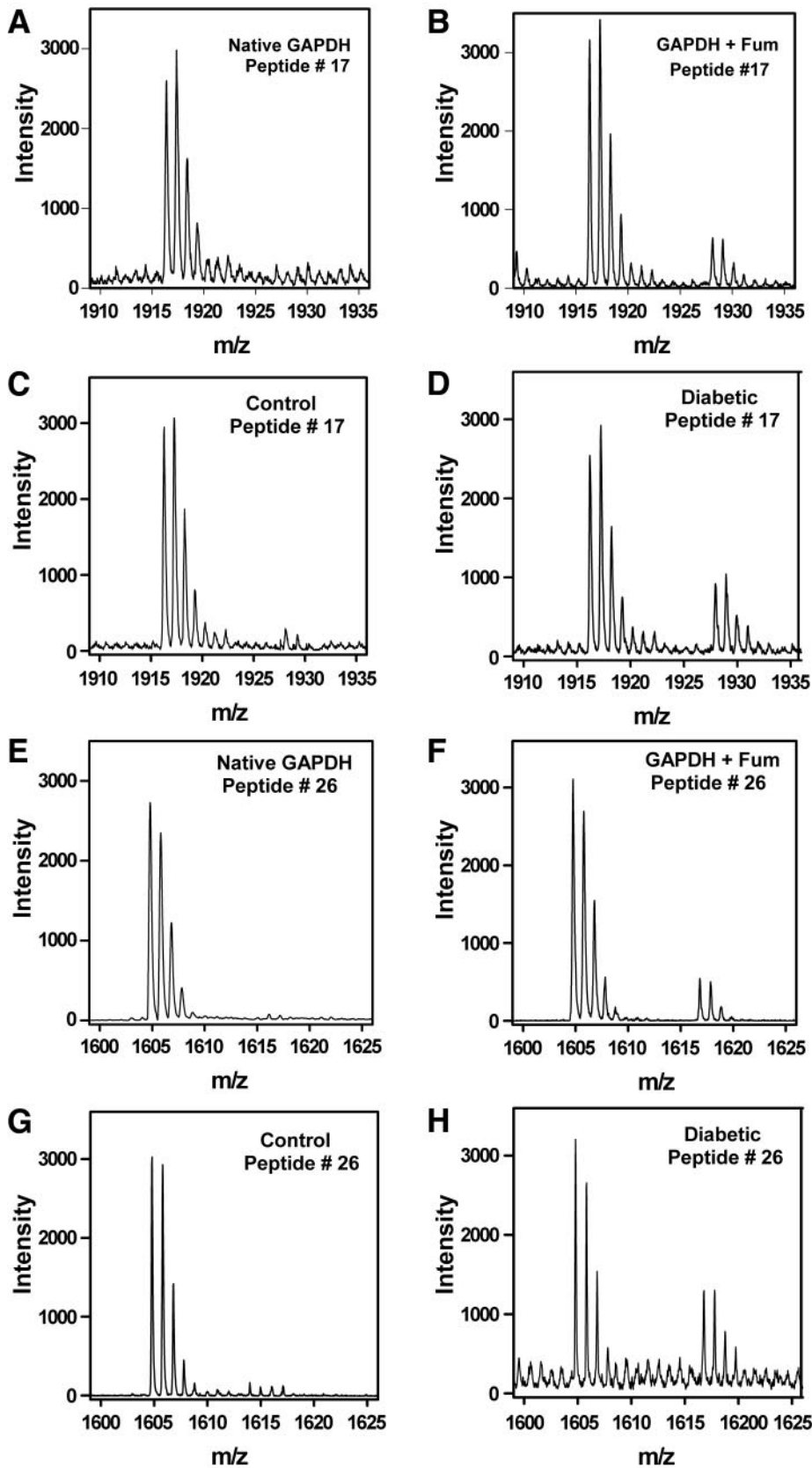


FIG. 9. MALDI-TOF mass spectra of 2SC and pyridylethyl peptides from GAPDH immunoprecipitated from rat muscle. Results are shown for analyses of commercial GAPDH, before and after incubation with fumarate (Fum), and of GAPDH immunoprecipitated from rat muscle and then digested in gel with trypsin. *A*: Tryptic peptide 17 from native GAPDH, showing the pyridylethyl peptide at 1916.3 m/z. *B*: Tryptic peptide 17 from fumarate-modified GAPDH (50 mmol/l, 24 h), showing the 2SC peptide at 1928.1 m/z. *C*: GAPDH from a control rat, showing a strong signal for pyridylethyl peptide 17 at 1916.20 m/z and a weak signal for the 2SC peptide at 1928.08 m/z. *D*: GAPDH from a diabetic rat, demonstrating the presence of 2SC on tryptic peptide 17 at 1928.08 m/z. *E*: Tryptic peptide 26 from native GAPDH showing the pyridylethyl peptide at 1604.79 m/z. *F*: Tryptic peptide 26 from fumarate-modified GAPDH, showing the 2SC peptide at 1616.84 m/z. *G*: GAPDH from a control rat, showing a strong signal for the pyridylethyl peptide 26 at 1604.79 m/z and a weak signal for the 2SC peptide at 1616.78 m/z. *H*: GAPDH from a diabetic rat, demonstrating the presence of 2SC on tryptic peptide 26 at 1616.78 m/z.

fumarate (7). A number of other 2SC-modified proteins have been identified in 3T3 L1 fibroblasts during differentiation to adipocytes in high-glucose medium and have been characterized by 2D-PAGE, Western blotting, and protein mass spectrometry (25). For proteins other than GAPDH, it is possible that 2SC may have a other functions (e.g., trafficking and targeting of protein molecules or acting as a biomarker for protein turnover). Reactive protein thiols are not only important in active and regulatory sites of enzymes but also have a functional role in transporters, the electrophile response element, signaling cascades, and transcription factors, suggesting that fumarate concentration and succination of protein formation may have a broader impact on metabolism in diabetes.

ACKNOWLEDGMENTS

Supported by research grant DK-19971 from the National Institute of Diabetes and Digestive and Kidney Diseases.

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