O-GlcNAcomic Profiling Identifies Widespread O-Linked β-N-Acetylgulosamine Modification (O-GlcNAcylation) in Oxidative Phosphorylation System Regulating Cardiac Mitochondrial Function*§*§

Received for publication, September 14, 2015  Published, JBC Papers in Press, October 7, 2015, DOI 10.1074/jbc.M115.691741

Junfeng Ma1, Ting Liu1, An-Chi Wei1, Partha Banerjee1, Brian O’Rourke1, and Gerald W. Hart†1

From the 4Department of Biological Chemistry and 6Division of Cardiology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Background: Mitochondrial protein O-GlcNAcylation is not well understood.

Results: Eighty eight mitochondrial proteins, involved in diverse pathways, are O-GlcNAcylated, and an overall increased O-GlcNAcylation leads to altered mitochondrial function.

Conclusion: O-GlcNAcylation is on many mitochondrial proteins within the oxidative phosphorylation system, modulating cardiac mitochondrial function.

Significance: O-GlcNAc cycles on many proteins within mitochondria, leading to altered function.

Dynamic cycling of O-linked β-N-acetylgulosamine (O-GlcNAc) on nucleocytoplasmic proteins serves as a nutrient sensor to regulate numerous biological processes. However, mitochondrial protein O-GlcNAcylation and its effects on function are largely unexplored. In this study, we performed a comparative analysis of the proteome and O-GlcNAcome of cardiac mitochondria from rats acutely (12 h) treated without or with thiamet-G (TMG), a potent and specific inhibitor of O-GlcNAcase. We then determined the functional consequences in mitochondria isolated from the two groups. O-GlcNAcomic profiling finds that over 88 mitochondrial proteins are O-GlcNAcylated, with the oxidative phosphorylation system as a major target. Moreover, in comparison with controls, cardiac mitochondria from TMG-treated rats did not exhibit altered protein abundance but showed overall elevated O-GlcNAcylation of many proteins. However, O-GlcNAc was unexpectedly down-regulated at certain sites of specific proteins. Concomitantly, TMG treatment resulted in significantly increased mitochondrial oxygen consumption rates, ATP production rates, and enhanced threshold for permeability transition pore opening by Ca2+.

Our data reveal widespread and dynamic mitochondrial protein O-GlcNAcylation, serving as a regulator to their function.

Three decades after its discovery (1, 2), O-linked β-N-acetylgulosamine (O-GlcNAc) modification has been found to be a highly dynamic post-translational modification of serine and threonine residues of nuclear and cytosolic proteins in all metazoans, some protzoans, and some bacteria (3). O-GlcNAc cycling is regulated by two enzymes, O-GlcNAc transferase (OGT, which adds O-GlcNAc onto proteins) and O-GlcNAcase (which removes O-GlcNAc from target proteins). The OGT knock-out is embryonically lethal (4), and loss of O-GlcNAcase leads to neonatal lethality in mammals (5). Dynamic O-GlcNAcylation is directly involved in the regulation of multiple physiological and pathological processes (6–10).

In contrast to many studies on nucleocytoplasmic O-GlcNAcylation, mitochondrial O-GlcNAcylation and its roles in the regulation of mitochondrial functions are poorly understood. Adenovirus-mediated overexpression of the nucleocytoplasmic form of OGT initiated changes in mitochondrial protein expression and mitochondrial functions (11, 12). The identification of a splice variant of OGT, which is targeted to mitochondria (mOGT), suggested that O-GlcNAcylation might also occur on mitochondrial proteins (13). Moreover, the induction of mOGT expression triggered programmed cell death (14). In addition, an increase in O-GlcNAcylation on several mitochondrial proteins has been observed in animals selected for low running capacity (15). Although functionally important, only a few mitochondrial proteins were identified to be O-GlcNAcylated (11, 15). The identification of O-GlcNAc targets is important for further

* This work was supported by National Institutes of Health Grants N01-HV-00240, P01HL107153, and R01DK61671 and Award S10RR26474 from the NCRR (for sharing the Seahorse instrument). Dr. Hart receives a share of profits of O-GlcNAc from Apeer Bioanalytical, Inc. Dr. Banerjee receives support from a grant from the National Institutes of Health with Dr. Hart. The authors declare no conflict of interest.

†To whom correspondence should be addressed: Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205–2185. Tel.: 410-614-8804; E-mail: gwhart@jhmi.edu.

‡1 This article was selected as a Paper of the Week.

§2 The abbreviations used are: O-GlcNAc, O-linked β-N-acetylgulosamine; O-GlcNAcylation, O-linked β-N-acetylgulosamine modification; BN-PAGE, blue native-PAGE; BEAMID, β-elimination/Michael addition with DTT; G/M, glutamate/malate; ΔΨm, mitochondrial inner membrane potential; mPTP, permeability transition pore opening; ROR, relative site occupancy ratio; TCA, tricarboxylic acid cycle; TMG, thiamet-G; PNGase F, peptide N-glycosidase F; OGT, O-GlcNAc transferase; PUGNac, O-(2-acetamido-2-deoxy-o-glucopyranosylidene)aminopyridoxylcarbamate.
Cardiac Mitochondrial Protein O-GlcNAcylation

exploring the sugar’s important roles in mitochondrial physiopathology.

To this end, by using an improved approach (16–18), we performed comparative O-GlcNAcosmic profiling on purified cardiac mitochondria from rats treated with or without TMG, a potent and specific inhibitor of O-GlcNCase which globally increases O-GlcNAcylation. We then investigated the regulatory roles of O-GlcNac in cardiac mitochondrial function. We not only found that many mitochondrial proteins are O-GlcNAcylated, particularly in the oxidative phosphorylation system, but also show that protein O-GlcNAcylation regulates cardiac mitochondrial function.

Experimental Procedures

Animal Models—Male Sprague-Dawley rats (200–250 g) were housed at 22 ± 1 °C in a 12-h light/dark cycle, with tap water and standard chow diet ad libitum. Control rats were received with vehicle buffer. For thiamet-G treatment, rats were injected intraperitoneally with a single dose of thiamet-G (50 mg/kg). After 12 h of treatment, rats from both groups were subjected to anesthesia and decapitation. All experimental protocols were approved by the Institutional Animal Care and Use Committee of The Johns Hopkins University.

Mitochondrial Isolation—Mitochondrial fractions were isolated using differential centrifugation, according to the method previously described (19). In brief, the heart was quickly removed from the rat, finely minced, and homogenized on ice in ice-cold isolation buffer A (75 mM sucrose, 225 mM mannitol, 1 mM EGTA, and 0.2% fatty acid-free BSA (pH 7.4), supplemented with Complete EDTA-free protease inhibitor mixture (Roche Applied Science)) using a Potter-Elvehjem glass homogenizer. The resulting homogenates were centrifuged for 15 min at 500 × g, and the resulting supernatants were centrifuged for 10 min at 7,700 × g. The mitochondrial pellets were washed twice thereafter by centrifugation at 7,700 × g for 5 min each. All centrifugation steps were carried out at 4 °C. Mitochondrial pellets were resuspended in a small amount of isolation buffer, with the protein concentration determined by using the BCA Assay kit (Thermo Fisher Scientific).

Mitochondrial Oxygen Consumption Rate Assay—Respiration was assayed with a high throughput automated 96-well extracellular flux analyzer (XF96; Seahorse Bioscience), as described previously (20). Briefly, freshly isolated mitochondria were resuspended in the assay buffer (buffer B) containing 137 mM KCl, 2 mM KH₂PO₄, 0.5 mM EGTA, 2.5 mM MgCl₂, and 20 mM HEPES at pH 7.2, with the presence of 0.2% fatty acid-free BSA. Appropriate amounts of mitochondrial suspension were transferred into a 96-well XF96 plate (5–15 μg of mitochondrial protein/well) precoated with polyethyleneimine. After centrifugation at 3,000 × g for 7 min at 4 °C, the plate was incubated at 37 °C for 20 min before starting the assay in the Seahorse Bioscience equipment, and respiration was evaluated with substrates of complex I (glutamate/malate (G/M), 5 mM each) and complex II (succinate, 5 mM each) without (state 4) or with (state 3) 1 mM ADP. Oxygen consumption was measured following a 2-min mix three times in each step (baseline, state 4, and state 3), and the first three points were averaged for the consumption rate of that measurement. The average of three measurements is the oxygen consumption rate of the mitochondria from each heart.

Mitochondrial ATP Production Rate Assay—ATP synthesis of fresh mitochondria was measured by using a luciferase luminescence-based kinetic assay approach. Specifically, 20 μg of mitochondria in buffer B were supplemented with 5 mM glutamate/malate or 5 mM succinate, 1 μM rotenone, with the addition of luciferin-luciferase (Life Technologies, Inc.). The luminescence was recorded with a FlexStation Plate Reader (Molecular Devices) before and after the addition of 1 mM ADP. The produced ATP amount was calculated from a standard curve for a series of ATP concentrations.

Mitochondrial Ca²⁺ Uptake and Membrane Potential Assay—Mitochondrial Ca²⁺ uptake capacity and membrane potential were measured as reported previously (21). In brief, fresh mitochondria (0.5 mg) were suspended in a potassium-based buffer solution consisting of 137 mM KCl, 2 mM KH₂PO₄, 20 μM EGTA, 20 mM HEPES, and 5 mM glutamate/malate at pH 7.15. Calcium green-5N (0.1 μM) fluorescence was recorded at excitation and emission wavelengths of 505 and 535 nm with a fluorometer (QuantaMaster, Photon Technologies International) at 37 °C. Doses of CaCl₂ were added until the opening of mPTP (the first calcium pulse is 15 μM free Ca²⁺ and each pulse afterward was 25 μM). Mitochondrial 90° light scattering was monitored at 540 nm with a second detector. With the presence of either 5 mM glutamate/malate or 5 mM succinate, NADH fluorescence was recorded at excitation at 350 nm and emission at 450 nm. The mitochondrial membrane potential (ΔΨm) was monitored with tetramethylrhodamine methyl ester as the dye at excitations of 546 and 573 nm and emission at 590 nm.

BN-PAGE/SDS Separation of Mitochondrial Protein Complexes—For BN-PAGE analysis, mitochondrial pellets were solubilized as described previously (22). Briefly, mitochondria were resuspended in buffer C (50 mM Tris-HCl, 50 mM NaCl, 10% glycerol w/v, 0.001% Ponceau S, pH 7.2, plus protease inhibitor mixture) with the presence of 1% (v/v) n-dodecyl-β-d-maltoside on ice for 30 min. Samples were then centrifuged for 30 min at 12,000 × g at 4 °C. The supernatants containing 50 μg of protein were supplemented with 0.5% (w/v) Coomassie G-250, loaded on a 4–12% native PAGE Novex gel (Invitrogen), and run according to the manufacturer’s protocols. For the second dimension, individual sample lanes from the first dimension were excised, placed on the top of a 10–20% SDS-polyacrylamide gel (Bio-Rad), and overlaid with 0.5% agarose. The gels were run using SDS-PAGE running buffer (25 mM Tris, 125 mM glycine, 0.1% w/v SDS, pH 7.4) at 110 V for 2 h. Gels were either stained with Coomassie Brilliant Blue G-250 overnight or transferred to PVDF membrane for immunoblotting as described below.

Immunoblotting of Mitochondrial Proteins—PVDF membrane was blocked using 5% BSA in Tris-buffered saline with Tween 20 (TBS-T: 100 mM Tris-HCl, 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20), incubated with O-GlcNAc antibody CTD 110.6 in TBS-T under gentle agitation overnight, and then incubated with HRP-conjugated IgM (Santa Cruz Biotechnology) in TBST under gentle agitation for 1 h. Chemiluminescence was performed under manufacturer’s protocols, by using ECL films
Cardiac Mitochondrial Protein O-GlcNAcylation

(GE Healthcare). Between blocking, antibody incubations, and chemiluminescence detection, blots were washed five times for 10 min in TBS-T. For the competitive assay, CTD 110.6 was incubated with 0.5 mM GlcNAc for at least 2 h at 4 °C before blotting. For the immunoblotting of specific mitochondrial proteins, protein samples were immunoprecipitated with NDUF51 (Abcam) and ATP5A1 (Abcam) by gentle shaking at 4 °C overnight. After extensive washes, the immunoprecipitates were added with Laemmli buffer followed by SDS-PAGE and Western blotting with specific antibodies.

Mitochondrial Protein Sample Preparation—Highly purified mitochondria samples were prepared by a discontinuous gradient-based ultracentrifugation for the tandem mass spectrometry-based proteomics, according to a previous protocol (23). In brief, cardiac mitochondria were isolated from six rats treated with or without thiamet-G as described above, with the presence of 2 mM O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino N-phenylcarbamate (PUGNAc). Mitochondrial pellet was then resuspended in sucrose buffer (10 mM Tris-HCl and 250 mM sucrose, pH 7.5) and layered onto a discontinuous gradient consisting of (from top to bottom) 6% Percoll, 17 and 35% Histodenz (Sigma), followed by centrifugation with a Ti41 rotor (Backman) at 17,500 rpm for 30 min at 4 °C. The layer at the 17/35% interface was collected and washed with the isolation buffer supplemented with 2 mM PUGNAc twice to remove trace Histodenz, yielding a highly mitochondrial-enriched fraction. After determining the protein concentration with the BCA assay, an equal amount of mitochondrial pellet from six preparations was pooled and then resuspended in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM NaF, 1 mM β-glycerophosphate, and 2 mM PUGNAc, and 1X Protease Inhibitor Mixture 1. The suspension was sonicated two times for 10 s and spun down at 12,000 × g for 15 min. The supernatant was collected, and the mitochondrial proteins were extracted by acetone precipitation. The precipitated proteins were vacuum dried and then dissolved in 8 M urea and 50 mM triethylammonium bicarbonate, pH 8, with the protein concentration determined by the BCA assay. Nine mg of mitochondrial proteins from control hearts and TMG-treated hearts were reduced with 10 mM DTT for 30 min at 37 °C and alkylated with 30 mM iodoacetamide for 30 min at room temperature. After decreasing the urea concentration with 50 mM triethylammonium bicarbonate, pH 7.5, and 250 mM sucrose, pH 7.5) and layered onto a discontinuous gradient consisting of (from top to bottom) 6% Percoll, 17 and 35% Histodenz (Sigma), followed by centrifugation with a Ti41 rotor (Backman) at 17,500 rpm for 30 min at 4 °C. The layer at the 17/35% interface was collected and washed with the isolation buffer supplemented with 2 mM PUGNAc twice to remove trace Histodenz, yielding a highly mitochondrial-enriched fraction. After determining the protein concentration with the BCA assay, an equal amount of mitochondrial pellet from six preparations was pooled and then resuspended in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM NaF, 1 mM β-glycerophosphate, and 2 mM PUGNAc, and 1X Protease Inhibitor Mixture 1. The suspension was sonicated two times for 10 s and spun down at 12,000 × g for 15 min. The supernatant was collected, and the mitochondrial proteins were extracted by acetone precipitation. The precipitated proteins were vacuum dried and then dissolved in 8 M urea and 50 mM triethylammonium bicarbonate, pH 8, with the protein concentration determined by the BCA assay. Nine mg of mitochondrial proteins from control hearts and TMG-treated hearts were reduced with 10 mM DTT for 30 min at 37 °C and alkylated with 30 mM iodoacetamide for 30 min at room temperature. After decreasing the urea concentration with 50 mM triethylammonium bicarbonate, pH 7.5, and 250 mM sucrose, pH 7.5) and layered onto a discontinuous gradient consisting of (from top to bottom) 6% Percoll, 17 and 35% Histodenz (Sigma), followed by centrifugation with a Ti41 rotor (Backman) at 17,500 rpm for 30 min at 4 °C. The layer at the 17/35% interface was collected and washed with the isolation buffer supplemented with 2 mM PUGNAc twice to remove trace Histodenz, yielding a highly mitochondrial-enriched fraction. After determining the protein concentration with the BCA assay, an equal amount of mitochondrial pellet from six preparations was pooled and then resuspended in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM NaF, 1 mM β-glycerophosphate, and 2 mM PUGNAc, and 1X Protease Inhibitor Mixture 1. The suspension was sonicated two times for 10 s and spun down at 12,000 × g for 15 min. The supernatant was collected, and the mitochondrial proteins were extracted by acetone precipitation. The precipitated proteins were vacuum dried and then dissolved in 8 M urea and 50 mM triethylammonium bicarbonate, pH 8, with the protein concentration determined by the BCA assay. Nine mg of mitochondrial proteins from control hearts and TMG-treated hearts were reduced with 10 mM DTT for 30 min at 37 °C and alkylated with 30 mM iodoacetamide for 30 min at room temperature. After decreasing the urea concentration with 50 mM triethylammonium bicarbonate to <1 M, 200 μg of trypsin was added and incubated overnight at 37 °C with gentle shaking. The resulting tryptic peptides were then treated with calf alkaline phosphatase (New England Biolabs) and PNGase F (New England Biolabs) for another 12 h. The peptides were desalted with C18 Sep-Pak columns (Waters) and dried with lyophilization. A process replicate was performed with another six control rats and six TMG-treated rats.

Isotopic Labeling and O-GlcNAc Enrichment—Equal amounts of aliquots of peptides were isotopically labeled by a 6-plex TMT kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Specifically, 100 μg of peptides from control rats were labeled with TMT-126, and 100 μg of peptides from TMG-treated rats were labeled with TMT-128. For peptides from the process replicate, TMT-129 and TMT-131 were used, respectively. All the labeled peptides were pooled and then separated by a basic pH reversed-phase liquid chromatography (as described previously (24)) into 12 fractions. Each fraction was dried down via SpeedVac and analyzed by LC-MS/MS as described below for protein quantification. The remaining peptides from control and TMG-treated hearts were subjected to β-elimination and Michael-addition with d6-N- and d6,E′-DTT (BEMAD), respectively, according to our previous protocols (16–18). Briefly, peptides were dissolved in BEMAD buffer (1.5% (v/v) triethylamine, 20 mM d6,N-DTT or 20% (v/v) ethanol, pH 12.5, by NaOH) and incubated at 50 °C for 4 h with gentle shaking. The reaction was quenched by neutralizing to 2% trifluoroacetic acid. The peptides were pooled and desalted as described above. To enrich DTT-substituted peptides, thiol-Sepharose resin (Sigma) was swelled in degassed PBS containing 1 mM EDTA (PBS/EDTA, pH 7.4). Dried peptides were then suspended in the same buffer and incubated with the activated resin for 4 h at room temperature. After extensive washing with PBS/EDTA and 40% acetonitrile, the resin was incubated with PBS/EDTA containing 20 mM free DTT for 30 min at 37 °C. Peptides eluted were acidified and desalted with C18 spin columns (Nest Group) for subsequent analysis.

LC-MS/MS and Data Analysis—Liquid chromatography coupled with tandem mass spectrometry analysis of the peptides was carried out on an LTQ-Orbitrap Velos (Thermo Fisher Scientific) attached to a Waters UPLC system with the auto sampler. Peptides were enriched on a 2-cm trap column (YMC gel ODS-A S-10 μm), fractionated on Magic C18 AQ5, 5 μm, 100 Å (Michrom Bioresources), 75 μm × 15-cm column, and electrosprayed through a 15-μm emitter (New Objective). Reversed-phase solvent gradient consisted of solvent A (0.1% formic acid) with increasing levels of solvent B (0.1% formic acid, 90% acetonitrile) over a period of 90 min. LTQ Orbitrap Velos was set at 2.0-kV spray voltage, full MS survey scan range of 350–1,800 m/z, data-dependent HCD MS/MS analysis of top eight precursors with minimum signal of 2,000, isolation width of 1.2, 30-s dynamic exclusion limit, and normalized collision energy of 40. Precursor and the fragment ions were analyzed at 30,000 and 15,000 resolutions, respectively. Peptide sequences were identified from isotopically resolved masses in MS and MS/MS spectra. Tandem MS2 mass spectra were processed by Proteome Discoverer (version 2.0 Thermo Fisher Scientific) in three ways, using three nodes as follows: common, Xtract (spectra were extracted, charge state deconvoluted, and deisotoped using Xtract option); and MS2 Processor. MS/MS spectra from three nodes were analyzed with Mascot version 2.2.2 (MatrixScience) using the RefSeq rat database (2012), with concatenated decoy database, specifying Rattus as species, trypsin/Pro as enzyme, missed cleavage 2, precursor mass tolerance 10 ppm, and fragment mass tolerance 0.03 Da. For the TMT labeling, carboxymethylation (Cys) and TMT (Lys and N-terminal) were set as fixed modification, with deamidation (Asn/Gln) as variable modifications. Reporter Ions Quantifier node was used for peptide quantification by determining reporter ion abundances. For the enriched DTT peptides, oxidation (Met), d6,N-DTT/Ser/Thr/Cys, and carboxamidomethylation (Cys), and deamidation (Asn/Gln) were set as variable modifications.
Cardiac Mitochondrial Protein O-GlcNAcylation

The false discovery rate was set to 0.01 for peptides and proteins. Precursor Ions Area Detector node was used to provide O-GlcNAc quantitation by averaging peak areas over the time of elution of given ion pairs within 3 ppm mass accuracy. All MS/MS spectra identifying proteins, peptides, or sites were further inspected manually for accuracy.

Bioinformatics—Proteins with mitochondrial location annotations from at least two prediction software, including Target P 1.1 (25), Mitoprot (26), and Predator (27), were accepted as mitochondrial proteins. To avoid possibly missed assignment of mitochondrial proteins, we also compared our results with previous dataset on the rat heart mitochondrial proteome to confirm its subcellular location. Pathway analysis of proteins was performed with Panther annotation. Topological prediction was obtained by the TOPO2 software.

Results

Widespread O-GlcNAcylation of the Mitochondrial Oxidative Phosphorylation System—To probe O-GlcNAcylation of proteins within the oxidative phosphorylation system, Percoll gradient-purified cardiac mitochondria from normal rats were subjected to two-dimensional BN-PAGE and SDS-PAGE separation. The protein subunits from the five respiratory complexes (i.e. complex I–V) were readily separated in the first dimension, and their individual subunits were well resolved after the second dimension. When blotted with a pan-specific O-GlcNAc antibody CTD110.6, there was an extensive signal on a number of proteins in control cardiac mitochondria (left panel in Fig. 1A). As a specificity control, immunoblots probed with the same antibody, but in the presence of 0.5 m GlcNAc, showed very few reactive spots (Fig. 1B). These results suggest that many proteins within the oxidative phosphorylation system are O-GlcNAcylated. For comparison, TMG, a potent and specific inhibitor of O-GlcNAcase, which has been applied to increase global nucleocytoplasmic O-GlcNAc levels in rodents (28–30), was used to induce potential changes in cardiac mitochondrial O-GlcNAcylation. After an acute 12-h treatment, the blood glucose concentration of rats remained unchanged (135.67 ± 7.99 mg/dl for the untreated group versus 130.33 ± 12.34 mg/dl for the treated group, n = 6). Immunoblotting shows that the oxidative phosphorylation system of TMG-treated rats is more heavily O-GlcNAcylated, with a substantial increase compared with the controls (Fig. 1A; with the loading controls shown in Fig. 1C).

To investigate possible changes in protein abundance and to identify the mitochondrial O-GlcNAc targets, a modified glycomic/proteomic approach was adopted for global profiling of the mitochondrial O-GlcNAcome (Fig. 2). A small portion (1%) of peptides from an equal amount of cardiac mitochondrial proteins from control and TMG-treated rats was isotopically labeled with 6-plex TMT reagents (Thermo Fisher Scientific) for protein level quantification. Among the identified 524 mitochondrial proteins, 353 were quantified with ≥2 unique peptides (supplemental Table 1). The quantification results revealed that the abundance of almost all proteins did not differ substantially between control and TMG rats (with a fold change within the range of 0.8–1.2; supplemental Table 1 and Fig. 3), with only a few proteins (<0.01%) decreased by ~30% (supplemental Table 1), suggesting that acute TMG treatment did not affect the abundance of mitochondrial proteins. This finding is consistent with our previous observations from cell lines showing that short term treatment with O-GlcNAcase inhibitors does not change the overall abundance of intracellular proteins dramatically (31).

To map the O-GlcNAc sites and quantify their changes on mitochondrial proteins, tryptic peptides were treated exhaustively (12 h) with alkaline phosphatase to remove phosphates and with PNGase F to remove any potential N-glycans. The resulting peptides were subjected to BEMAD using either d_4O or d_6O-DTT under very mild conditions, as described previously (16–18). The peptides were pooled, fractionated, enriched with thio-affinity columns, and detected by LC-ESI-HCD-MS/MS (Fig. 2). Although previous reports have suggested that only the O-GlcNAc moiety could be substituted with d_4O/d_6O-DTT under mild BEMAD conditions (16–18), the specificity of the refined approach was further demonstrated by samples supplemented with a synthetic O-GlcNAc peptide FSTVAGESGSADTVR (where underlined S denotes the modified Ser) and the digest of a standard phosphoprotein β-casein (with three known phosphorylated Ser/Thr sites). The O-GlcNAc peptide was positively identified, whereas none of the β-casein peptides were detected, demonstrating the method’s selectivity toward the O-GlcNAc moiety on peptides. With this approach, 184 putative O-GlcNAc peptides were assigned to 88 mitochondrial proteins, which are involved in multiple pathways including the TCA cycle, fatty acid utilization, and others (Fig. 4A; supplemental Table 2). Nearly half of the O-GlcNAcylated proteins are components of the oxidative phosphorylation system (i.e. the respiratory chain and ATP synthase/complex V), suggesting that it is indeed enriched for protein O-GlcNAcylation (Fig. 4B).

To further validate the identification of mitochondrial O-GlcNAcylated proteins, we immunoprecipitated NDUF51 and ATP5A1, two important proteins of the oxidative phosphorylation system. NDUF51, the largest and core subunit of complex I, transfers electrons from NADH to the respiratory chain. It may form part of the active site crevice where NADH is oxidized (32). By probing with CTD110.6, a pan-specific antibody, NDUF51 is indeed O-GlcNAcylated in cardiac mitochondria (Fig. 5A, with the representative mass spectrum of one O-GlcNAc peptide shown in Fig. 5B). Moreover, we observed an ~2-fold increase in O-GlcNAcylation of NDUF51 in TMG-treated rats (Fig. 5A). ATP5A1, one major component of the catalytic portion of ATP synthase, was also O-GlcNAcylated (Fig. 5D, with the representative spectrum of one O-GlcNAc peptide shown in Fig. 5E). Although it was previously found O-GlcNAcylated (with two modification sites) in mouse brain synaptosomes (33) and rat liver (34), herein we show that about 10 other sites are also O-GlcNAcylated in cardiac mitochondria. Intriguingly, the overall O-GlcNAc level was even slightly decreased upon TMG treatment (Fig. 5D).

It is noteworthy, among the O-GlcNAc proteins identified herein, that nine were previously identified as O-GlcNAc-modified in mitochondria from other tissues (i.e. brain synaptosomes and liver) using different approaches (11, 33, 34). Moreover, three O-GlcNAc sites on these proteins have also been unambiguously detected (supplemental Table 2) 11, 33,
These data suggest that some mitochondrial O-GlcNAcylation sites might be conserved between different tissues. Interestingly, NADH dehydrogenase subunit 4 (mt-ND4), a mitochondrial DNA-encoded protein, was also O-GlcNAcylated (supplemental Table 2), suggesting that protein O-GlcNAcylation can occur within the mitochondrial matrix.

Site-specific O-GlcNAcylation of Proteins within the Mitochondrial Oxidative Phosphorylation System—To quantitatively analyze the O-GlcNAc response, we investigated site-specific O-GlcNAcylation in cardiac mitochondria from rats treated with or without TMG. We calculated the O-GlcNAc site relative occupancy ratio (ROR) of mitochondrial proteins according to Equation 1.

$$ROR_{\text{TMG/Ctrl}} = \frac{[\text{O-GlcNAc}]_{\text{Rabbit}}/[\text{protein}]_{\text{Rabbit}}}{[\text{O-GlcNAc}]_{\text{Ctrl}}/[\text{protein}]_{\text{Ctrl}}}$$

(Eq. 1)

**FIGURE 1.** Cardiac mitochondrial electron transport chain proteins are O-GlcNAcylated. Percoll gradient-purified mitochondria from rats treated with vehicle (left panel) and TMG (right panel) were first separated by BN-PAGE. The resulting two one-dimensional gels were laid onto the top of one SDS-polyacrylamide gel for a second dimension separation. After electrotransferring onto a PVDF membrane, proteins were probed with CTD 110.6 without (A) or in the presence (B) of 0.5 M GlcNAc for specificity confirmation (CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CV, complex V). WB, Western blot. C, BN-PAGE/SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue (CBB) G-250 as a loading control (Ctrl.).
As expected, upon TMG treatment, many of the sites (62 sites, 33%) on the proteins in the oxidative phosphorylation system showed >1.2-fold increase in the O-GlcNAYlation ROR (supplemental Table 2), suggesting that the cycling of O-GlcNAYlation on these sites was effectively blocked due to the inhibition of O-GlcNAYlation. Interestingly, a number of other sites showed an ROR of <0.8 after TMG treatment (supplemental Table 2). This seemingly paradoxical observation is consistent with prior studies in which decreased O-GlcNAYlation occupancy at some sites was seen despite a globally increased O-GlcNAYlation (35). A further examination of the sites with decreased ROR reveals that many of these sites are adjacent to proximal sites with increased ROR on the same polypeptide. Notably, among the sites with decreased ROR, quite a few occur on proteins with multiple O-GlcNAYlation sites (e.g. ATP5A1, see Fig. 5F). Indeed, we found multiple cases in which decreased O-GlcNAYlation was accompanied by an increased ROR on proximal O-GlcNAYlation sites (within 10 amino acids), such as Ser-315 and Ser-325 on ATP5A1 and Ser-517 and Ser-513 on ATP5A1 (Fig. 5F). The apparent competitive inhibitory interaction between different O-GlcNAYlation sites is akin to what occurs with protein phosphor-

ylation, where reciprocal interactions between proximal phosphorylation sites are a common signaling motif (36). Besides proximal site inhibition, conformational inhibition between O-GlcNAYlation sites within proteins could also be a contributor to the decreased ROR observed at certain sites. In addition, the decreased ROR could also result from the altered OGT activity, considering that OGT itself is O-GlcNAYlated (37). It is possible that TMG-induced O-GlcNAYlation changes of OGT might alter its activity on certain O-GlcNAYlation sites. Another possibility is that because many kinases/phosphatases are O-GlcNAYlated, TMG treatment may change the phosphorylation of proteins, which could then lead to reciprocal changes in O-GlcNAYlation at certain sites, as has been observed on many nuclear and cytoplasmic proteins (31, 38).

The site-specific O-GlcNAYlation changes provide direct insights to the observed alteration in protein O-GlcNAYlation levels between control and TMG treatment. The increased O-GlcNAYlation on Ser-177 could be the major site contributing to elevated O-GlcNAYlation of NDUF51 after TMG treatment, because the other three sites did not show obvious changes (Fig. 5, A and C). In terms of ATP5A1, although the O-GlcNAYlation ROR on several sites was increased (e.g. Ser-315 and Ser-517), the decreased ROR on many other sites (e.g. Ser-325, Ser-502, and Ser-513) might mask this effect, leading to an overall decreased O-GlcNAYlation level upon TMG treatment (Fig. 5, D and F).

Taken together, these results suggest that mitochondrial protein O-GlcNAYlation is a dynamic process, which is regulated in a site-specific manner. Although the O-GlcNAYlation at specific sites on certain proteins might undergo down-regulation, an overall increase in O-GlcNAYlation has been achieved after acute TMG treatment (Fig. 1).

Mitochondrial O-GlcNAYlation Modulates Oxygen Consumption Rates—Because acute TMG treatment did not change blood glucose levels, we determined whether an overall increased O-GlcNAYlation altered cardiac mitochondrial function. The respiratory activity of freshly isolated mitochondria at different metabolic states was measured with an extracellular flux analyzer (Seahorse XF 96; Fig. 6, A and B). State 4 respiration (i.e. resting respiration) was determined with G/M or succinate, electron donors to NADH-dependent complex I and FADH-dependent complex II, respectively. State 3 respiration (oxidative phosphorylation-coupled respiration) was stimulated with the addition of ADP, and maximal uncoupled respiration was induced with the addition of 2,4-dinitrophenol. Respiratory control ratios of mitochondria, calculated as the ratio of state 3 to state 4 oxygen consumption rates, were similar in the absence or presence of TMG treatment, suggesting that the quality of the mitochondrial preparation was equivalent between groups. However, the absolute respiration rates (normalized to mitochondrial protein level) of state 3 and state 4 of mitochondria from TMG-treated rats were significantly higher compared with the control mitochondria, with either G/M or succinate as the substrate. Moreover, there was a trend (not statistically significant) toward higher uncoupled respiration in the TMG group compared with the controls (data not shown), suggesting an increase in the maximal respiratory

**FIGURE 2.** Scheme for the cardiac mitochondrial O-GlcNAYlation site mapping and quantification. The equal amounts of mitochondrial proteins were digested by trypsin. Aliquots of the digest were labeled with a 6-plexTMT for protein level quantification. The remaining peptides were enriched with thio-affinity columns and analyzed by LC-MS/MS for O-GlcNAYlation site mapping and quantification. Ctrl, control. (See “Experimental Procedures” for more details.)
Thus, acute O-GlcNAcylation leads to an increase in G/M- or succinate-dependent mitochondrial oxygen consumption rate.

Mitochondrial O-GlcNAcylation Modulates Oxidative Phosphorylation—We next assessed the key bioenergetic parameters of mitochondria from control or TMG-treated animals. Mitochondrial inner membrane potential ($\Delta \Psi_m$) under different conditions of energy demand was determined. When mitochondria were energized with G/M, a rapid increase in $\Delta \Psi_m$ was detected, and adding a limited amount of ADP resulted in transient depolarization followed by a return to the initial level as the ADP was converted to ATP (Fig. 7A). Of note, although the initial $\Delta \Psi_m$ level was similar, a significantly higher maximum $\Delta \Psi_m$ in the presence of G/M was achieved for mitochondria from TMG-treated rats. However, we did not observe a significant increase in $\Delta \Psi_m$ when succinate was used as the electron donor (Fig. 7B). A possible explanation for this differential effect could be that TMG treatment selectively enhances proton pumping at complex I, which would be bypassed by succinate (complex II). Nevertheless, an increase in the overall rate of ADP phosphorylation downstream of complexes I and II for both G/M and succinate was indicated by the more rapid recovery of $\Delta \Psi_m$ (transition from state 3 to state 4) during the ADP challenge in mitochondria from TMG-treated animals (Fig. 7B).

Next, we directly measured the ATP production rate, under the presence of excessive amounts of ADP, with isolated cardiac mitochondria from control and TMG-treated rats. In comparison with the control, the maximum ATP production rate was significantly higher in the TMG treatment group in the presence of G/M (Fig. 7C). However, the succinate-dependent maximum ATP production rate was quite similar in both conditions (Fig. 7D), which is somehow divergent from the increased ADP
oxidation rate with a low-dose ADP. The dose-dependent effects might be ascribed to the potentially shifted ADP affinity of adenine nucleotide translocase 1 (ANT1/SLC25A4), which is O-GlcNAcylated differentially after TMG treatment (supplemental Table 2).

Taken together, these data suggest that mitochondrial O-GlcNAcylation regulates oxidative phosphorylation at multiple sites in the respiratory chain, contributing to the increased ΔΨm and ATP production rates.

Mitochondrial O-GlcNAcylation Modulates Ca2+ Uptake Capacity before Opening of the Permeability Transition Pore—Ca2+ handling capacity is another important factor reflecting the functional status of mitochondria that is also relevant to cell injury and death. The capacity of cardiac mitochon-
Cardiac Mitochondrial Protein O-GlcNAcylation

FIGURE 5. O-GlcNAcylation of NDUFS1 (A–C) and ATP5A1 (D–F). NDUFS1 (A) and ATP5A1 (D) were immunopurified from lysates from cardiac mitochondria extracted from rats treated without or with TMG. Precipitates were washed, separated on SDS-PAGE, and transferred to PVDF membranes for blotting. Membranes were probed for O-GlcNAc. Representative mass spectrum for the modified peptides, IASQAVALDLGYKPGVEAIR (from NDUFS1) and ILGADTSVDEETGR (from ATP5A1), with the modified sites underlined (B and E). Site-specific O-GlcNAc ROR of NDUFS1 (C) and ATP5A1 (F) is shown. For NDUFS1, the increase in O-GlcNAc in the mitochondria with the treatment of TMG is evident, which could be ascribed to the increased O-GlcNAc ROR on Ser-177 because other sites did not show obvious changes. For ATP5A1, the O-GlcNAcylation is even slightly decreased after TMG treatment, which may be due to the presence of several O-GlcNAcylation sites exhibiting decreased ROR values.

dria to accumulate Ca$^{2+}$ before triggering permeability transition pore (mPTP) opening was measured for multiple additions of Ca$^{2+}$ while simultaneously monitoring $\Delta \Psi_m$ and light scattering (Fig. 8A). The first addition of Ca$^{2+}$ evoked a rapid Ca$^{2+}$ uptake from the media. After the ninth addition, however, corresponding to a total mitochondrial Ca$^{2+}$ load of $\sim 600$ nmol of Ca$^{2+}$/mg of mitochondrial protein, the $\Delta \Psi_m$ collapsed and additional swelling (indicated by a decrease in light scattering) occurred, with Ca$^{2+}$ released into the media (Fig. 8, A and B). In contrast, the cardiac mitochondria isolated from TMG-treated rats readily took up $\geq 10$ additions of Ca$^{2+}$ while maintaining the $\Delta \Psi_m$ and mitochondrial volume, with the maximum mitochondrial Ca$^{2+}$ uptake capacity reaching $\sim 750$ nmol of Ca$^{2+}$/mg of mitochondrial protein. These data indicate that acute TMG treatment increases the Ca$^{2+}$ threshold for mPTP opening,
suggesting a beneficial role that could contribute to cardio-
protection and prevention of cell death (40).

**Discussion**

Mitochondria were once regarded as organelles with very few O-GlcNAcylated proteins (2, 13). However, growing evi-
dence suggests that protein O-GlcNAcylation exists in mito-
chondria. Very recently, several mitochondrial proteins have
been identified from different tissues of rodents (i.e. heart
(11), liver (34), and brain synaptosomes (33)) by advanced
mass spectrometry techniques.

By using two-dimensional BN-PAGE/SDS-PAGE separa-
tion, followed by probing with a pan-specific O-GlcNAc anti-
FIGURE 6. Mitochondrial O-GlcNAcylation promotes oxygen consumption. G/M (A) and succinate (Succ.) (B)-dependent oxygen consumption rates of mitochondria isolated from control and TMG rats, without (i.e. state 4, left panel) or with (i.e. state 3, right panel) the presence of ADP. n = 4, *p < 0.05; **, p < 0.01, and the error bar shows the standard deviation of the mean.

FIGURE 7. Mitochondrial O-GlcNAcylation modulates oxidative phosphorylation. Mitochondrial membrane potential changes with the addition of G/M (A) or succinate (Succ.) (B), followed by the addition of a limited amount of ADP (0.2 mM) are shown. G/M-linked (C) or succinate-linked (D) mitochondrial ATP production rates in control and TMG-treated rats are shown. n = 3, *p < 0.05, and the error bar shows the standard deviation of the mean.
body, we have found that a number of cardiac mitochondrial proteins are O-GlcNAc-modified. To further elucidate the O-GlcNAc targets, a modified BEMAD-based O-GlcNAc mimetic approach has been applied for the profiling of mitochondrial proteins. Our data reveal that among the 88 O-GlcNAcylated mitochondrial proteins identified, nearly half of them are located within the oxidative phosphorylation system, further confirming that it is indeed a major target for mitochondrial protein O-GlcNAcylation. The localization of mOGT, which has been identified to be tightly associated with mitochondrial inner membrane (13), could be one reason for such a preference. Interestingly, the O-GlcNAcylation of NADH dehydrogenase subunit 4, one of the 13 mitochondrial DNA-encoded proteins, provides another line of evidence that protein O-GlcNAcylation can occur inside the mitochondria. Indeed, our recent study suggests that there is a complete set of mitochondrial O-GlcNAc cycling components (i.e. mOGT, O-GlcNAcase, and a UDP-GlcNAc transporter (41)), which might leads to mitochondrial O-GlcNAcylation on proteins. However, we cannot rule out the possibility that some other proteins may be O-GlcNAcylated in cytosol and subsequently transported into the mitochondria.

A single overnight TMG treatment of animals increased the overall O-GlcNAcylation levels on cardiac mitochondrial proteins without perturbing the protein abundance, suggesting that mitochondrial protein O-GlcNAcylation is a highly dynamic process, similar to nucleocytoplasmic O-GlcNAcylation, which also rapidly responds to acute inhibition of O-GlcNAcase. These rapid changes in O-GlcNAcylation of mitochondrial proteins occur in a site-specific manner. Although the ROR on many sites of cardiac mitochondrial proteins is increased as a result of TMG treatment, other sites show decreased ROR with TMG treatment. Whether it is a common phenomenon (akin to the interactions between multiple phosphorylation sites) or there is some compensatory mechanism(s)

within cardiac mitochondria is still unclear. But likely, considering that many proteins (including kinases, phosphatases, and OGT) are O-GlcNAc targets, intricate cross-talk between multiple O-GlcNAc sites or between O-GlcNAc and other PTMs (e.g. phosphorylation) is likely one underlying factor to the altered site-specific O-GlcNAcylation that impacts mitochondrial function.

Adenovirus-mediated overexpression of nucleocytoplasmic form of OGT and/or O-GlcNAcase provides some insights into the protein O-GlcNAcylation-related mitochondrial functions (11, 12). However, the functional changes can potentially be attributed to alterations of mitochondrial protein abundance (12). In our study, acute TMG treatment of rats caused an overall increase in O-GlcNAcylation without changing the protein abundance, providing an excellent model for a systematic investigation of O-GlcNAcylation modulation of cardiac mitochondrial function. In comparison with controls, cardiac mitochondria from TMG-treated animals had higher oxygen consumption and ATP production rates. Effects on complex I supported respiration and ΔΨm indicate a special role for O-GlcNAcylation of the NADH dehydrogenase complex, consistent with the extensive number (12 of 45) of its subunits modified.

Moreover, acute increases in the O-GlcNAc level were correlated with enhanced cardiac mitochondrial Ca2+ uptake capacity and attenuation of mPTP opening, a critical step in the initiation of necrotic and programmed cell death. Two proteins in the regulation of the mPTP, voltage-dependent anion channel and ANT1, were also found O-GlcNAcylated in this study, which could potentially contribute to the shift in Ca2+ sensitivity of mPTP opening. In addition, the identified O-GlcNAc modifications on adenine nucleotide translocase, voltage-dependent anion channel, and the ATP synthase, which coordinately mediate mitochondrial ADP/ATP transport and phosphorylation, could explain the observed functional effects. Collectively, our data offer direct evidence that an acute overall

---

**FIGURE 8. Mitochondrial O-GlcNAcylation modulates Ca2+ capacity before permeability transition pore opening.** A, representative traces of the uptake capacity of isolated cardiac mitochondria (green lines) by monitoring changes of light scattering (gray lines) and membrane potential (red lines). B, calculated Ca2+ capacity in mitochondria isolated from control and TMG-treated rats. n = 5, *p < 0.05, and the error bar shows the standard deviation of the mean.
increase of mitochondrial O-GlcNAcylation is likely beneficial to cardiac mitochondrial function, providing novel molecular targets that may explain previously observed cardioprotective effects when perfused heart or myocytes were subjected to glucosamine treatment, OGt overexpression, or O-GlcNAcase inhibition (42–45). Importantly, our data also suggest that the acutely increased O-GlcNAcylation within the mitochondria could be one mechanism that serves to increase ADP oxidation. The category of mitochondrial O-GlcNAc targets and their specific sites would largely facilitate the elucidation of the detailed molecular mechanisms and the roles of individual O-GlcNAcylated proteins in mitochondrial (dys)function even in other biological contexts.

Of note, it appears that acute activation of O-GlcNAcylation of mitochondrial proteins exerts a protective role. However, prolonged elevated protein O-GlcNAcylated, as occurs in diabetes, underlies the progression of diabetic complications (46–49). Considering that mitochondrial dysfunction is etiological in diabetic cardiomyopathy (50, 51), alteration of the O-GlcNAcylation on key mitochondrial proteins may also contribute to nutrient toxicity in diabetic tissues, which needs to be further investigated.

Author Contributions—J. M., T. L., A. W., P. B., B. O., and G. W. H. designed research; J. M., T. L., A. W., P. B. performed research; J. M., T. L., A. W., P. B., B. O., and G. W. H. analyzed data; J. M., B. O., and G. W. H. wrote the manuscript; all authors edited the paper.

Acknowledgments—We thank the Jennifer Van Eyk laboratory (Drs. Shengbing Wang, Haodong Li, and Irina Chernysheva), the Hui Zhang laboratory (Dr. Shuang Yang), The Johns Hopkins University Mass Spectrometry Core Facility (Drs. Bob Cole, Raghothama Chae-rkady, Tatiana Boronina, Robert O’Meally, and Lauren Devine), and Dr. Lance Wells at the University of Georgia for their great help on two-dimensional BN/SDS-PAGE separation and mass spectrometry as well as data analysis. We appreciate the Brian Foster laboratory, the Anne Murphy laboratory (Dr. Genaro Ramirez-Correa), the David Kass laboratory (Djahida Bedja), and the Roselle Abraham laboratory (Junaid Afzal) for insightful discussions. We also thank the Brian O’Rourke laboratory (Drs. Alice Ho, Miguel Aon, and Sonia Cortassa), the Natasha Zachara laboratory, and the current Gerald Hart laboratory and its former members, especially Drs. Chad Slawson, Kaoru Sakabe, Quira Zeidain, Ron Copeland, and John Bullen, for their kind help at the beginning of the project.

References
1. Torres, C. R., and Hart, G. W. (1984) Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes: evidence for O-linked GlcNAc. J. Biol. Chem. 259, 3308–3317
2. Holt, G. D., and Hart, G. W. (1986) The subcellular distribution of terminal N-acetylglucosamine moieties: localization of a novel protein–saccharide linkage, O-linked GlcNAc. J. Biol. Chem. 261, 8049–8057
3. Hart, G. W., Housley, M. P., and Slawson, C. (2007) Cycling of O-linked β-N-acetyl-glucosamine on nucleo-cytoplasmic proteins. Nature 446, 1017–1022
4. Shafi, R., Iyer, S. P., Ellies, L. G., O’Donnell, N., Marek, K. W., Chui, D., Hart, G. W., and Martin, J. D. (2000) The O-GlcNAc transferase gene resides on the X chromosome and is essential for embryonic stem cell viability and mouse ontology. Proc. Natl. Acad. Sci. U.S.A. 97, 5735–5739
5. Yang, Y. R., Song, M., Lee, H., Jeon, Y., Choi, E. J., Jang, H. J., Moon, H. Y., Byun, H. Y., Kim, E. K., Kim, D. H., Lee, M. N., Koh, A., Ghim, J., Choi, J. H., and Lee-Kwon, W., et al. (2012) O-GlcNAcase is essential for embryonic development and maintenance of genomic stability. Aging Cell 11, 439–448
6. Hardiville, S., and Hart, G. W. (2014) Nutrient regulation of signaling, transcription, and cell physiology by O-GlcNAcylated. Cell Metab. 20, 208–213
7. Yuzwa, S. A., and Vocadlo, D. J. (2014) O-GlcNAc and neurodegeneration: biochemical mechanisms and potential roles in Alzheimer’s disease and beyond. Chem. Soc. Rev. 43, 6839–6858
8. Vaidyanathan, K., and Wells, L. (2014) Multiple tissue-specific roles for the O-GlcNAc post-translational modification in the induction of and complications arising from type II diabetes. J. Biol. Chem. 289, 34446–34451
9. Slawson, C., and Hart, G. W. (2011) O-GlcNAc signaling: implications for cancer cell biology. Nat Rev. Cancer 11, 678–684
10. Marsh, S. A., Collins, H. E., and Chatham, J. C. (2014) Protein O-GlcNAcylation and cardiovascular (patho)physiology. J. Biol. Chem. 289, 34449–34456
11. Hu, Y., Suarez, J., Fricovsky, E., Wang, H., Scott, B. T., Trauger, S. A., Han, W., Hu, Y., Oyeleye, M. O., and Dillmann, W. H. (2009) Increased enzymatic O-GlcNAcylation of mitochondrial proteins impairs mitochondrial function in cardiac myocytes exposed to high glucose. J. Biol. Chem. 284, 547–555
12. Tan, E. P., Villar, M. T., E. I., Lu, J., Selfridge, J. E., Artigues, A., Swerdlov, R. H., and Slawson, C. (2014) Altering O-linked β-N-acetylglucosamine cycling disrupts mitochondrial function. J. Biol. Chem. 289, 14719–14730
13. Love, D. C., Kochan, J., Cathey, R. L., Shin, S. H., Hanover, J. A., and Kochran, J. (2003) Mitochondrial nucleo-cytoplasmatic targeting of O-linked GlcNAc transferase. J. Cell Sci. 116, 647–654
14. Shin, S. H., Love, D. C., and Hanover, J. A. (2011) Elevated O-GlcNAc-dependent signaling through inducible mOGT expression selectively triggers apoptosis. Amino Acids 40, 885–893
15. Johnsen, V. L., Belke, D. D., Hughey, C. C., Hittel, D. S., Hepple, R. T., Koch, L. G., Britton, S. L., and Shearer, J. (2013) Enhanced cardiac protein glycosylation (O-GlcNAc) of selected mitochondrial proteins in rats artificially selected for low running capacity. Physiol. Genomics 45, 17–25
16. Wells, L., Vosseller, K., Cole, R. N., Cronshaw, J. M., Matunis, M. J., and Hart, G. W. (2002) Mapping sites of O-GlcNAc modification using affinity tags for serine and threonine post-translational modifications. Mol. Cell. Proteomics 1, 791–804
17. Vosseller, K., Hansen, K. C., Chalkley, R. J., Trinidad, J. C., Wells, L., Hart, G. W., and Burlingame, A. L. (2005) Quantitative analysis of both protein expression and serine/threonine post-translational modifications through stable isotope labeling with dithiothreitol. Proteomics 5, 388–398
18. Wang, Z., Park, K., Comer, F., Hsieh-Wilson, L. C., Saudek, C. D., and Hart, G. W. (2009) Site-specific GlcNAcylation of human erythrocyte proteins: potential biomarker(s) for diabetes. Diabetes 58, 309–317
19. Aon, M. A., Cortassa, S., Wei, A. C., Grunnet, M., and O’Rourke, B. (2010) Energetic performance is improved by specific activation of K+ fluxes through K(Ca) channels in heart mitochondria. Biochim. Biophys. Acta 1797, 71–80
20. Aon, M. A., Stanley, B. A., Sivakumaran, V., Kembro, J. M., O’Rourke, B., Paolocci, N., and Cortassa, S. (2012) Glutathione/thioredoxin systems modulate mitochondrial H2O2 emission: an experimental-computational study. J. Gen. Physiol. 139, 479–491
21. Wei, A. C., Liu, T., Cortassa, S., Winslow, R. L., and O’Rourke, B. (2011) Mitochondrial Ca2+ influx and efflux rates in guinea pig cardiac mitochondria: low and high affinity effecytosines of cyclosporine A. Biochim. Biophys. Acta 1813, 1373–1381
22. Wang, S. B., Foster, D. B., Rucker, J., O’Rourke, B., Kass, D. A., and Van Eyk, J. E. (2011) Redox regulation of mitochondrial ATP synthase: implications for cardiac resynchronization therapy. Circ. Res. 109, 750–757
23. Foster, D. B., Rucker, J. J., and Marban, E. (2008) Is Kir6.1 a subunit of mitoKATP? Biochem. Biophys. Res. Commun. 366, 649–656
24. Baycin-Hizal, D., Tabb, D. L., Chaerkady, R., Chen, L., Lewis, N. E., Naga-rajan, H., Sarkaria, V., Kumar, A., Wolozny, D., Colao, J., Jacobson, E., Tian, Y., O’Meally, R. N., Krug, S. S., Cole, R. N., et al. (2012) Proteomic
analysis of Chinese hamster ovary cells. J. Proteome Res. 11, 5265–5276
25. Emanuelsson, O., Nielsen, H., Brunak, S., and von Heijne, G. (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. 300, 1005–1016
26. Claros, M. G., and Vincens, P. (1996) Computational method to predict mitochondrial imported proteins and their targeting sequences. Eur. J. Biochem. 241, 779–786
27. Small, I., Peeters, N., Legeai, F., and Lurin, C. (2004) Predotar: a tool for rapidly screening proteomes for N-terminal targeting sequences. Proteomics 4, 1581–1590
28. Yuzwa, S. A., Macauley, M. S., Heinenon, J. E., Shan, X., Dennis, R. J., He, Y., Whitworth, G. E., Stubb, K. A., McEachern, E. J., Davies, G. J., and Vocadlo, D. J. (2008) A potent mechanism-inspired O-GlcNacase inhibitor that blocks phosphorylation of tau in vitro. Nat. Chem. Biol. 4, 483–490
29. Yuzwa, S. A., Shan, X., Macauley, M. S., Clark, T., Skorobogatko, Y., Vosseller, K., and Vocadlo, D. J. (2012) O-GlcNac slows neurodegeneration and stabilizes tau against aggregation. Nat. Chem. Biol. 8, 393–399
30. Andrés-Bergoñs, J., Tardío, L., Larranaga-Vera, A., Gómez, R., Herrero-Beaumont, G., and Largo, R. (2012) The increase in O-linked N-acetylglucosamine protein modification stimulates chondrogenic differentiation both in vitro and in vivo. J. Biol. Chem. 287, 33615–33628
31. Wang, Z., Gueck, M., and Hart, G. W. (2008) Cross-talk between GlcNAcylation and phosphorylation: site-specific phosphorylation dynamics in response to globally elevated O-GlcNac. Proc. Natl. Acad. Sci. U.S.A. 105, 13793–13798
32. Sumegi, B., and Sere, P. A. (1984) Complex I binds several mitochondrial NAD-coupled dehydrogenases. J. Biol. Chem. 259, 15040–15045
33. Trinidad, J. C., Barkan, D. T., Gulledge, B. F., Thalhammer, A., Sali, A., Schoepfer, R., and Burlingame, A. L. (2012) Global identification and characterization of both O-GlcNAcylation and phosphorylation at the murine synapse. Mol. Cell. Proteomics 11, 215–229
34. Cao, W., Cao, J., Huang, J., Yao, J., Yan, G., Xu, H., and Yang, P. (2013) Discovery and confirmation of O-GlcNAcylated proteins in rat liver mitochondria by combination of mass spectrometry and immunological methods. PLoS ONE 8, e76399
35. Wang, Z., Udeshi, N. D., Slawson, C., Compton, P. D., Sakabe, K., Cheung, W. D., Shabanowitz, J., Hunt, D. F., and Hart, G. W. (2010) Extensive crosstalk between GlcNAc and phosphorylation regulates cytoskeleton. Sci. Signal. 3, r2a
36. Ubersax, J. A., and Ferrell, J. E., Jr. (2007) Mechanisms of specificity in protein phosphorylation. Nat. Rev. Mol. Cell Biol. 8, 530–541
37. Kreppel, L. K., and Hart, G. W. (1999) Regulation of a cytosolic and nuclear O-GlcNAc transferase. J. Biol. Chem. 274, 32015–32022
38. Hu, P., Shimoji, S., and Hart, G. W. (2010) Site-specific interplay between O-GlcNAcylation and phosphorylation in cellular regulation. FEBS Lett. 584, 2526–2538
39. Nicholls, D. G., and Ferguson, S. (2013) Bioenergetics, 4th Ed., pp. 89–136, Elsevier, Amsterdam
40. Halestrap, A. P., Clarke, S. I., and Javadov, S. A. (2004) Mitochondrial permeability transition pore opening during myocardial reperfusion: a target for cardioprotection. Cardiovasc. Res. 61, 372–385
41. Banerjee, P. S., Ma, J., and Hart, G. W. (2015) Diabetes-associated dysregulation of O-GlcNac in rat cardiac mitochondria. Proc. Natl. Acad. Sci. U.S.A. 112, 6050–6055
42. Liu, J., Pang, Y., Chang, T., Bounelis, P., Chatham, J. C., and Marchase, R. B. (2006) Increased hexose biosynthesis and protein O-GlcNAc levels associated with myocardial protection against calcium paradox and ischemia. J. Mol. Cell. Cardiol. 40, 303–312
43. Jones, S. P., Zachara, N. E., Ngoh, A. G., Hill, B. G., Teshima, Y., Bhatnagar, A., Hart, G. W., and Marbán, E. (2008) Cardioprotection by N-acetylglucosamine linkage to cellular proteins. Circulation 117, 1172–1182
44. Wu, T., Zhou, H., Jin, Z., Bi, S., Yang, X., Yi, D., and Liu, W. (2009) Cardioprotection of salidroside from ischemia/reperfusion injury by increasing N-acetylglucosamine linkage to cellular proteins. Eur. J. Pharmacol. 613, 93–99
45. Laczy, B., Fülöp, N., Onay-Besikci, A., Des Rosiers, C., and Chatham, J. C. (2011) Acute regulation of cardiac metabolism by the hexosamine biosynthesis pathway and protein O-GlcNAcylation. PLoS ONE 6, e18417
46. Clark, R. J., McDonough, P. M., Swanson, E., Trost, S. U., Suzuki, M., Fukuda, M., and Dillmann, W. H. (2003) Diabetes and the accompanying hyperglycemia impairs cardiomyocyte cycling through increased nuclear O-GlcNAcylation. J. Biol. Chem. 278, 44230–44237
47. Ramirez-Corream, G. A., Ma, J., Slawson, C., Zeidan, Q., Lugo-Fagundo, N. S., Xu, M., Shen, X., Gao, W. D., Caceres, V., Chakir, K., DeVine, L., Cole, R., Marchionni, L., Paolocci, N., Hart, G. W., et al. (2015) Removal of abnormal myofilament O-GlcNAcylation restores Ca2+ sensitivity in diabetic cardiac muscle. Diabetes 10.2337/db14–1107
48. Erickson, J. R., Pereira, L., Wang, L., Han, G., Ferguson, A., Dao, K., Cope, R., Despa, F., Hart, G. W., Ripplinger, C. M., and Bers, D. M. (2013) Diabetic hyperglycaemia activates CaMKII and arrhythmias by O-linked glycosylation. Nature 502, 372–376
49. Heath, J. M., Sun, Y., Yuan, K., Bradley, W. E., Litovskaya, S., Dell’Italia, L. J., Chatham, J. C., Wu, H., and Chen, Y. (2014) Activation of AKT by O-linked N-acetylglucosamine induces vascular calcification in diabetes mellitus. Circ. Res. 114, 1094–1102
50. Buggler, H., and Abel, E. D. (2010) Mitochondria in the diabetic heart. Cardiovasc. Res. 88, 229–240
51. Duncan, J. G. (2011) Mitochondrial dysfunction in diabetic cardiomyopathy. Biochim. Biophys. Acta 1813, 1351–1359