Quantitative \(N\)-linked Glycoproteomics of Myocardial Ischemia and Reperfusion Injury Reveals Early Remodeling in the Extracellular Environment* □

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Extracellular and cell surface proteins are generally modified with \(N\)-linked glycans and glycopeptide enrichment is an attractive tool to analyze these proteins. The role of \(N\)-linked glycoproteins in cardiovascular disease, particularly ischemia and reperfusion injury, is poorly understood. Observation of glycopeptides by mass spectrometry is challenging due to the presence of abundant, nonglycosylated analytes, and robust methods for purification are essential. We employed digestion with multiple proteases to increase glycoproteome coverage coupled with parallel glycopeptide enrichments using hydrazide capture, titanium dioxide, and hydrophilic interaction liquid chromatography with and without an ion-pairing agent. Glycosylated peptides were treated with PNGase F and analyzed by liquid chromatography-MS/MS. This allowed the identification of 1556 nonredundant \(N\)-linked glycosylation sites, representing 972 protein groups from ex vivo rat left ventricular myocardium. False positive “glycosylations” were observed on 44 peptides containing a deamidated Asn-Asp in the \(N\)-linked sequon by analysis of samples without PNGase F treatment. We used quantitation via isobaric tags for relative and absolute quantitation (iTRAQ) and validation with dimethyl labeling to analyze changes in glycoproteins from tissue following prolonged ischemia and reperfusion (40 mins ischemia and 20 mins reperfusion) indicative of myocardial infarction. The iTRAQ approach revealed 80 of 437 glycopeptides with altered abundance, while dimethyl labeling confirmed 46 of these and revealed an additional 62 significant changes. These were mainly from predicted extracellular matrix and basement membrane proteins that are implicated in cardiac remodeling. Analysis of \(N\)-glycans released from myocardial proteins suggest that the observed changes were not due to significant alterations in \(N\)-glycan structures. Altered proteins included the collagen-laminin-integrin complexes and collagen assembly enzymes, cadherins, mast cell proteases, proliferation-associated secreted protein acidic and rich in cysteine, and microfibril-associated proteins. The data suggest that cardiac remodeling is initiated earlier during reperfusion than previously hypothesized. Molecular & Cellular Proteomics 10: 10.1074/mcp. M110.006833, 1–13, 2011.

\(N\)-linked glycosylation is a common post-translational modification linked to asparagine (Asn) residues at the consensus motif N-X-S/T/C in cell surface and extracellular proteins. The attachment of usually large carbohydrates is likely to have significant conformational consequences and thus be a strong influence on protein structure and function. Glycosylation has been implicated in many biological processes including adhesion and cell-cell communication, protein folding, stabilization and translocation, differentiation and development (1–4), and may be associated with diseases such as diabetes (5) and cancer (6). The glycoproteome represents an attractive subproteome for investigation because cell surface proteins are generally glycosylated and these proteins are at the interface that mediates the relationship between the cell and environment. Glycoproteomics tools offer an effective means for enriching this typically difficult set of proteins that are predominantly hydrophilic and often of low abundance (7–11).

Large-scale glycoproteomic profiling has proven challenging, and only a handful of methods have been developed to investigate specific glycan-containing glycopeptides, for example lectin affinity for specific sugar subunits (12) or titanium
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dioxide (TiO₂) for sialic acids (SA)¹ (13). A comprehensive glycoproteomic profile most likely requires multiple fractionation, enrichment and mass spectrometric procedures applied in parallel. Generally, the study of protein glycosylation has been divided into three broad fields: 1) glycoproteomics, where modified proteins are identified and the sites of glycan attachment characterized from complex mixtures; 2) glycomics, where glycans are chemically or enzymatically released and structurally characterized; and 3) a more targeted approach where individual glycoproteins are subjected to rigorous characterization of glycan heterogeneity at one, or multiple, attachment sites. The development of advanced fragmentation techniques, such as electron transfer dissociation, applied in combination with conventional (collision-induced dissociation; CID) or higher energy (high energy collisional dissociation) fragmentation has alleviated the problem of in situ glycan-peptide characterization to a degree, allowing information regarding both to be recovered from the same experiment (14). Very large or complex glycans however, remain problematic and currently limit this approach to only a very specific set of glycoproteins. Glycoproteins and peptides also require enrichment as their heterogeneity and low abundance make them difficult to analyze from among a complex mixture. Lectin affinity (12, 15, 16) is often employed but suffers from low binding affinities, glycoprotein solubility problems and extensive copurification of false positives. Alternative methods include hydrazide capture (17, 18), hydrophilic interaction liquid chromatography (HILIC; (19, 20)) or TiO₂ chromatography (13), which each target a different oligosaccharide interaction set. A combination of different glycopeptide enrichment approaches is thus required to achieve a more comprehensive glycoproteome coverage.

There is growing evidence that glycosylation plays an important role in mediating cardiomyocyte function and survival. The myocardial sarclemma and interstitium is abundantly glycosylated though the precise physiological function of N-linked glycans and their role in cardiovascular disease are not clear. Removal of SA from the sarclemma results in decreased contractile function and the development of arrhythmias (21–22), demonstrating the importance of SA in mediating contraction. Despite this, only a few confirmed molecular targets of glycosylation are currently known. The voltage-gated ion channels appear to be extensively glycosylated.

¹ The abbreviations used are: SA, sialic acids; AMI, acute myocardial infarction; ECM, extracellular matrix; HILIC, hydrophilic interaction liquid chromatography; I/R, ischemia/reperfusion; LV, left ventricle; MMP, matrix metalloproteinases; NITC, non-ischemic time control; SPARC, secreted protein acidic and rich in cysteine; TTC, triphenyltetrazolium chloride; CID, collision induced dissociation; RPP, rate pressure product; TEAB, triethylammonium bicarbonate; iTRAQ, isobaric tag for relative and absolute quantitation; TFA, trifluoroacetic acid; ZIC, Zwitterionic; HPLC, high performance liquid chromatography; MS/MS, tandem MS; PVDF, polyvinylidene difluoride; LV, left ventricle; IP, ion pairing.

Modulation of ion channel glycosylation is implicated in altering electrical signaling in the heart (23) and has been linked with cardiac conduction disorders and heart failure (22, 24). There is also growing evidence that extracellular glycosidase activity modifies surface glycans and alters protein function. An example of this is the extracellular glycosidase, Klotho, whose regulation has been associated with hypertension and myocardial infarction (25).

Reduction or cessation of blood flow (ischemia) generally results from the growth and eventual rupture of atherosclerotic lesions within the coronary arteries. The re-establishment of flow (reperfusion) by thrombolysis and angioplasty remains the best strategy for resolving ischemia and preventing cell death, necrosis, and thus permanent cardiac dysfunction (infarction). Ischemia induces fundamental changes within the myocardium, including a switch to anaerobiosis, a mismatch in energy supply and demand to the contractile apparatus, and a reduction in intracellular pH causing Ca²⁺ levels to rise within myocytes (26). Reperfusion itself is also associated with injury, mainly because of a dramatic surge in reactive oxygen species in the first 2 to 3 min of reflow. The myocardial extracellular matrix (ECM) is a dynamic environment that is rich in glycosylated and secreted proteins, growth factors, signaling molecules, and proteases. The ECM provides an organized framework that allows for strict myocyte orientation and structural integrity allowing myocardial contraction and myofibril alignment (27). Following myocardial infarction, the ECM is involved in the process of cardiac remodeling (28). Remodeling is typically described as a process involving three main, overlapping phases: inflammatory, proliferative, and maturation (29). The entire process may take up to 2 months in humans, although in rodents may be completed in as few as 5 days in surviving animals (30). The inflammatory, or acute, phase begins sometime after the onset of ischemia and is typified by the degradation of the ECM, increased proteolytic (matrix metalloproteinases [MMP]) (31) activity, increased expression of adhesion molecules, and by an influx of inflammatory cells. ECM degradation is essential as it allows for inflammatory cell infiltration, fibroblast proliferation and becomes the scaffold for the deposition of the eventual scar tissue. Fibroblast proliferation and differentiation into ‘contractile’ myofibroblasts leads to the synthesis and secretion of new ECM proteins ~48 h post-infarct in rodents (29), however it has also been suggested that synthesis of ECM proteins may begin during the inflammatory phase, even during ECM turnover. At the protein level, several ECM proteins are induced during proliferation including fibronectin, thrombospondins, secreted protein acidic and rich in cysteine (SPARC) and tenascin-C, and anti-inflammatory signals, including elevation of tissue inhibitor of metalloproteinase expression (29). Eventually the mature scar is formed by the deposition of collagen from myofibroblasts and maturation is further characterized by matrix cross-linking and myofibroblast apoptosis. Since many proteins involved in the
ECM scaffold and remodeling are glycosylated, an understanding of these processes at the molecular level may be possible through a glycoproteomic approach. We therefore used complementary quantitative glycoproteomics and glycomics to analyze animal model (rat) myocardium subjected to I/R injury and have identified ECM and cell surface proteins involved in disease-induced cardiac remodeling.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. Hydrazide support was obtained from Bio-Rad (Hercules, CA). Titanium dioxide was obtained from GL Sciences (Japan), ZIC-HILIC was obtained from Sequest (Umea, Sweden). Sequencing-grade trypsin was from Promega (Madison, WI). Calf bovine intestinal phosphatase, PNGase F, and endoprotease Asp-N were obtained from New England Biolabs (Ipswich, MA). All solutions were made with ultrapure Milli-Q water (Millipore, Bedford, MA).

**Rat Heart Isolation**—Male Lewis rats (~200 g) were euthanized with an intraperitoneal injection of sodium pentobarbital (0.5 mg/g) using the guidelines set out by the University of Sydney Animal Ethics Committee (approval no. K20/6–2009/3/5078). Immediately after the loss of corneal and hind-limb reflexes, a median sternotomy incision was made and the heart exposed. The aorta was cut ~1 cm above the aortic valve and the heart removed from the thoracic cavity and placed into ice-cold saline solution (0.9% (w/v) NaCl, pH 7.4). Hearts were attached to a Langendorff perfusion system (ADInstruments, Castle Hill, Australia) via insertion of a stainless steel cannula into the aorta, which was then firmly tied in position with a 3.0 silk suture. Oxygenated Krebs-Henseleit buffer (118 mM NaCl, 25 mM NaHCO3, 1.2 mM KH2PO4, 4.8 mM KCl, 1.2 mM MgSO4, 11 mM glucose, 2.0 mM CaCl2, pH 7.4) at 37 °C was pumped into the aorta at a pressure of 80 mmHg, which upon entry into the heart forces the aortic valve shut and perfusate is thus diverted into the coronary ostia and coronary circulation. The performance of the heart was monitored using a saline-filled latex balloon that was inserted into the left ventricle (LV) through an incision in the left auricle and passing through the mitral valve. The balloon was attached to a calibrated pressure transducer and the heart rates and LV-developed pressure recorded (LabChart, ADInstruments). Hearts were equilibrated for a period of 15 mins and then assigned into two groups: 1) 60 mins of normoxic perfusion (n = 6); non-ischemic time control (NITC); or 2) 40 mins of no-flow ischemia followed by 20 mins of full reperfusion (40I/20R; n = 6). Hearts that failed to attain a rate pressure product (RPP) of 20,000 mmHg/min or a heart rate of 200 beats/min at the end of the 15 min equilibration period were excluded. Following perfusion, atria were removed and ventricles were immediately snap-frozen in liquid nitrogen. Samples were stored at –80 °C until analysis.

**Assessment of Myocardial Necrosis**—Myocardial necrosis was determined by staining hearts with triphenyltetrazolium chloride (TTC). Hearts were removed from the Langendorff apparatus, sealed in an airtight bag, and incubated at ~20 °C overnight. Frozen hearts were sectioned perpendicular to the aortic root-apex axis into 2 mm slices. Slices were incubated in 50 mM sodium phosphate buffer, pH 7.4 containing 1% (w/v) TTC for 15 mins at 37 °C with gentle agitation. Slices were then counter-stained for 10 mins in 10% (v/v) formalin. Viable tissue appears red while areas of necrosis appear tan/beige in color.

**Membrane Protein Preparation**—Myocardial tissue (500–600 mg) was homogenized in 1.5 ml of lysis buffer containing 10 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM phenylmethylsulfonylfluoride, 0.2% (w/v) pepstatin, 0.2% (w/v) aprotonin, 0.2% (w/v) leupeptin, 1 mM dithiothreitol, pH 8.0 using an Omni homogenizer (Omni International, Keneshaw, Ga). The homogenate was centrifuged at 10,000 × g for 15 mins at 4 °C. The supernatant was removed and the pellet resuspended in 1 ml of 100 mM Na2CO3 and rotated at 4 °C for 2 h followed by centrifugation at 200,000 × g for 1.5 h to collect membranes. The supernatant was removed and the pellet resuspended in 6 M urea, 2 M thiourea, 1% (w/v) SDS, 50 mM triethylammonium bicarbonate (TEAB) pH 8.0, to solubilize membrane proteins.

**Reduction, Alkylation and Proteolytic Digestion**—Proteins were reduced in 10 mM DTT for 1 h at 25 °C and alkylated in 50 mM iodoacetamide for 1 h at 25 °C in the dark. The reaction was diluted 1:10 with 50 mM TEAB pH 8.0 and digested with 1% (w/v) trypsin or endoprotease Asp-N for 16 h at 25 °C; or thermolysin for 6 h at 25 °C. Fifty units of calf intestinal phosphatase was added to each digestion and incubated for a further 2 h at 25 °C. The dephosphorylated and digested samples were acidified to below pH 3.0 with 100% formic acid, centrifuged at 20,000 × g for 10 mins and the supernatant desalted using Hydrophilic Lipophilic Balance solid phase extraction cartridges (Waters Corp, Milford, MA), according to the instructions and then dried by vacuum centrifugation.

**Isotopic Labeling**—Digested proteins were resuspended in 100 mM TEAB, pH 8.0 and quantified in triplicate with Qubit (Invitrogen, Carlsbad CA) according to the manufacturer’s instructions. Four-plex isotopic barbs for relative and absolute quantitation (TIFQA) (Applied Biosystems, Foster City CA) labeling was carried out in duplicate with 100 µg digested proteins from NITC hearts labeled with 114 and 115 mass tags and 100 µg digested proteins from hearts subjected to 40I/20R labeled with 116 and 117 mass tags, according to the manufacturer’s instructions. Dimethyl labeling was carried out essentially as described previously (32). 2 mg of digested proteins from NITC and 40I/20R hearts were loaded onto separate HLB columns and washed with 5 ml of 50 mM sodium phosphate buffer, pH 7.5 containing 0.2% formaldehyde (CH2O or CD2O) and 30 mM cyano-borohydride.

**N-linked Glycopeptide Capture onto Hydrazide Support**—Glycopeptide capture was performed as described previously (17, 18). Peptides were resuspended in coupling buffer containing 100 mM NaAc, 150 mM NaCl, pH 5.0 and oxidized with 15 mM NaIO4 in the dark for 1 h at 25 °C. The reaction was quenched with 50 mM Na2S2O3 for 10 mins at 25 °C and coupled to hydrazide resin overnight at 25 °C with rotation. The resin was washed three times with 1 ml of coupling buffer; 3 M urea, 1 M thiourea; 4 M NaCl; methanol; acetonitrile (MeCN); 50 mM TEAB, sequentially to remove nonglycosylated peptides. Formerly N-linked glycopeptides were released from the resin using 250 U of PNGase F in 200 µl of 50 mM TEAB overnight at 37 °C with rotation. Peptides were collected by washing the resin 3 times with 50% MeCN and were then dried by vacuum centrifugation. Peptides were purified using a Poros R2 reversed phase micro-column and stored at –20 °C.

**Enrichment of Sialic Acid-containing N-Linked Glycopeptides**—SA-containing N-linked glycopeptides were purified essentially as described (13, 33). Digested proteins were resuspended in loading buffer containing 1 M glycyl acid, 80% MeCN, 5% trifluoroacetic acid (TFA) and loaded onto TiO2 using a batch method (5 µM; 1.5 peptide: TiO2 beads) and shaken gently at 25 °C for 20 mins. The beads were washed with loading buffer followed by 80% MeCN, 2% TFA, and finally 20% MeCN, 0.1% TFA. SA-containing peptides were eluted using 0.5% (v/v) ammonia solution, pH 11 and dried by vacuum centrifugation. Peptides were resuspended in 50 mM TEAB, pH 8.0 and de-glycosylated with 250 U of PNGase F overnight at 37 °C. Former SA-containing glycopeptides were purified using a Poros R2 reversed phase micro-column and stored at –20 °C.

**Enrichment of N-Linked Glycopeptides Using Zwitterionic Hydrophilic Interaction Liquid Chromatography (ZIC-HILIC)**—Glycopeptides were purified as previously described (19). Briefly, peptide digests were resuspended in 80% MeCN containing either 5% formic acid or
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0.1% TFA and loaded onto a ZIC-HILIC prototype micro-column. The column was washed with 80% MeCN containing either 5% formic acid or 0.1% TFA (34) and glycopeptides eluted with water. Eluted peptides were dried by vacuum centrifugation, de-glycosylated using PNGase F and purified using Poros R2 as described above.

Offline TSK Amide-80 HILIC Peptide Fractionation—Peptide fractionation was performed using a modified method of (35, 36) where the stationary phase was replaced with neutral Amide-80 HILIC and mobile phase contained TFA as an ion-pairing reagent (34). Purified peptides were re-suspended in 90% MeCN, 0.1% TFA and loaded onto a 450 μm OD × 320 μm ID × 17 cm micro-capillary column packed with TSK Amide-80 (β µm; Tosoh Bioscience, San Francisco CA) using an Agilent 1200 Series HPLC (Agilent, Santa Clara CA). The detection was measured at 210.8 nm. Fractions were dried by vacuum combined into 8–12 fractions depending on the intensity of UV detection at 250 nL/min. Fractions were collected every 1 min and combined into 8–12 fractions depending on the intensity of UV detection measured at 210.8 nm. Fractions were dried by vacuum centrifugation.

LC-ESI-MS/MS—Fractionated peptides were re-suspended in 0.1% formic acid and loaded onto a 320 μm OD × 100 μm ID × 17 cm Reprosil-Pur C18-AQ (3 µm; Dr. Maisch GmbH, Germany) nanocolumn using an EASY-nLC nanoHPLC (Proxeon, Odense, Denmark). The HPLC gradient was 0–40% solvent B (A = 0.1% formic acid; B = 95% MeCN, 0.1% formic acid) over 55 mins at a flow-rate of 250 nL/min. Mass spectrometric detection was achieved using an LTQ Orbitrap XL (Thermo Scientific, San Jose CA). An MS scan (400–2000 m/z) was recorded in the Orbitrap set at a resolution of 60,000 at 400 m/z followed by data-dependent CID MS/MS on the four most intense ions in the LTQ. For iTRAQ analysis, data-dependent CID MS/MS analysis of the two most intense ions was performed in the LTQ followed by high energy collisional dissociation MS/MS analysis of the corresponding ions with detection in the Orbitrap. Parameters for acquiring CID were: activation time = 15 ms, normalized energy = 35, Q-activation = 0.25, dynamic exclusion = enabled with repeat count 1, exclusion duration = 30 s and intensity threshold = 30,000. Parameters for acquiring high energy collisional dissociation were: activation time = 5 ms, normalized energy = 55, resolution = 7500, dynamic exclusion = enabled with repeat count 1, and exclusion duration = 30 s.

Analysis of Mass Spectrometry Data—Raw data generated on the LTQ Orbitrap XL were processed using Proteome Discoverer v1.1 (Thermo Scientific) into .mgf files and searched against the IPI rat database version 3.53 (39,924 entries) and filtered to a 1% false discovery rate. All database assignments were confirmed by manual interpretation of the MS/MS spectra and deamidation site-assignment assessed using the PTM Scoring algorithm in MSQuant. All glycosylation sites were confirmed by manual inspection of tandem MS (MS/MS) spectra to determine correct deamidation sites within the consensus sequence (N-X-S/T/C). Protein annotation was obtained using ProteinCenter (Proxeon, Odense, Denmark). All accessions codes with peptide sequences containing 100% sequence identity against an individual peptide characterized by MS/MS in the database search were grouped together as a single protein description to remove protein name redundancy.

Release and Purification of N-Linked Glycans—Membrane proteins from NITC and 40I/20R hearts were dot-blotted onto polyvinylidene difluoride (PVDF) membrane (0.45 μm, Millipore) in replicates and allowed to dry overnight at room temperature. The PVDF membrane was washed with methanol followed by water and stained with 0.0085% direct blue 71 in 40% ethanol, 10% acetic acid for 10 mins. The membrane was washed with water and allowed to dry overnight. Immobilized proteins were excised and placed into a 96-well plate containing 1% polyvinyl pyrrolidone 40000 (PVP) for 5 mins. PVDF membrane was then washed 3 times with water. 2.5 μL of PNGase F was added in 15 μL of water and incubated at 37 °C for 16 h. Released glycans were collected and incubated in 100 mM ammonium acetate, pH 5.0 for 1 h at room temperature and dried by vacuum centrifugation. Glycans were then reduced with 1 mM NaBH4 in 50 mM KOH for 3 h at 50 °C and desalted using cation-exchange micro-columns (Dowex, Midland, MI) according to the manufacturer’s procedures and dried. Residual borate was removed by washing 3 times with 1% acetic acid (v/v) in methanol and released glycans dried by vacuum centrifugation.

Liquid Chromatography-Electrospray Ionization Tandem MS (LC-ESI-MS/MS) of Released Glycans—Mass spectrometry of released glycans was performed as described in (39). Briefly, glycans were re-suspended in 10 mM NH4HCO3 and loaded onto a 320 μm ID × 10 cm micro-capillary column packed with porous graphitized carbon (5 μm Hypercarb, Thermo Hypersil, Runcorn UK). Oligosaccharides were separated on an Agilent 1100 Series HPLC using a linear gradient from 2–16% (v/v) MeCN/10 mM NH4HCO3 for 45 mins, followed by a gradient from 16–45% over 20 mins before washing the column with 45% MeCN/10 mM NH4HCO3 at a flow-rate of 5 μL/min for 6 mins. Mass spectrometry was performed using an Agilent MSD ion trap XCT Plus instrument in negative ion mode as described (39). Raw data were viewed using Data Analysis software version 4.0 (Bruker) and a list of precursors generated manually. Potential glycan compositions were determined by importing the list of precursors into the GlycoMod tool (http://www.exasy.ch/tools/glycomod). Compositions were then imported into GlycoSuiteDB (http://glycosuitedb.expasy.ch) to compare with previously experimentally observed N-linked glycan structures. Predicted N-linked structures were matched using GlycoWorkbench (http://www.eurocarbdb.org/applications/ms-tools) and in silico MS/MS was validated to experimental MS/MS. XICs of all multiply charged precursors were performed to provide a semiquantitative glycan comparison of NITC versus 40I/20R hearts.

RESULTS

Left Ventricle (LV) Function and Necrosis—Rat hearts (n = 6, nonischemic time control [NITC] and n = 6, 40I/20R) were evaluated based on hemodynamic data and TTC staining (Fig. 1). LV function was determined by the RPP, an indicator of myocardial viability based on the LV developed pressure and heart rate, and expressed as a percentage of the post-15 min equilibration value. RPP was significantly reduced in the 40I/20R group compared with control hearts (p < 0.001) with ~20% functional recovery after 20 mins reperfusion, consis-
tent with myocardial infarction. TTC staining confirmed large regions of necrosis in 40I/20R hearts (negative staining; 41.8 ± 4.2% of LV mass compared with 0.4 ± 0.1% for NITC hearts).

Profiling Myocardial N-Linked Glycoproteins Using Multiple Proteases and Complementary Enrichment Methods—Membrane-associated proteins from NITC heart tissue were digested in parallel with trypsin, Asp-N and thermolysin. Digests were subjected to different glycopeptide enrichment strategies including hydrazide capture, TiO₂ purification, and HILIC with and without an ion-pairing agent. A total of 1556 non-redundant N-linked glycosylation sites representing 972 protein groups were identified, with concatenated reversed database searching resulting in a 0.8% false discovery rate. Detailed information regarding all identified sites can be found in supporting information (supplementary Table S1). Previously N-glycosylated sites were highlighted by the presence of the PNGase F-mediated deamidation of Asn to Asp (+0.986 Da) that was located within the motif for N-linked glycosylation (N-X-S/T/C, where X can be any amino acid except Pro). Of the 972 protein groups, more than 650 have a predicted transmembrane domain. ProteinCenter analysis of the identified proteins showed that 76.9% (n = 732/972) were predicted to localize to the membrane (Fig. 2A, supplementary Table S2). A further substantial fraction of proteins (37.8%) were predicted to be extracellular. Other predominant localization groups were those from copurifying membranous components such as mitochondrial membrane, endoplasmic and sarcoplasmic reticulum, Golgi and lysosomes.

Previous studies have demonstrated that the use of multiple proteases increases coverage of the observed glycoproteome (40). The enzymes used in this study were: trypsin (cleaves C-terminal to K/R), thermolysin (cleaves on the N-terminal side of W/Y/F/I/L/V/A/M), and Asp-N (cleaves N-terminal to D). These proteases resulted in the identification of 1299, 175, and 372 nonredundant glycosylation sites, respectively (Fig. 2B). As anticipated, the number of identified glycosylation sites was significantly increased using a multiple protease approach and there was little overlap, with only 22 (1.4%) non-redundant sites identified by all three proteases. We also used four enrichment methods based on different chemical mechanisms: 1) hydrazide capture, which binds cis-diol-containing glycopeptides; 2) TiO₂, which forms multivalent complexes with SA-containing glycopeptides; 3) ZIC-HILIC with

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**Fig. 1. Hemodynamics and TTC staining of perfused rat hearts.**
Left ventricular function (RPP) is expressed as a percentage of the post 15 min equilibration value (p < 0.001, paired t test). A, Nonischemic time control (NITC), B, 40 min ischemia/20 min reperfusion (40I/20R).

**Fig. 2. Comparison of subcellular localization of identified glycopeptides, proteases, and enrichment strategies used for glycopeptide preparation.** A, Gene Ontology cellular compartmentalization of the 972 identified N-linked glycoproteins. B, Number of unique and overlapping nonredundant N-linked glycosylation sites identified by the three proteases; trypsin, endoproteinase Asp-N, and thermolysin. (C–E) Number of unique and overlapping nonredundant N-linked glycosylation sites identified by the 4 glycopeptide enrichment strategies for trypsin (C), Asp-N (D), and thermolysin (E).
formic acid, which binds glycopeptides by electrostatic interactions and hydrophilic partitioning and; 4) ZIC-HILIC with TFA (ion-pairing (IP)-ZIC-HILIC), which also binds glycopeptides by electrostatic interactions and hydrophilic partitioning however, the presence of an ion-pairing agent most likely reduces nonspecific binding of non-glycosylated peptides to the stationary phase. IP-ZIC-HILIC resulted in the largest number of identified glycopeptides for both trypsin and Asp-N digested proteins, and combined with non-IP ZIC-HILIC, contributed more glycosylation site information than either hydrazide capture or TiO2 for all protease treatments. Each method however, contributed a substantial number of unique identifications (Figs. 2C, 2D, 2E), most likely due to their differing specificities (e.g. TiO2 selectivity for SA-containing glycopeptides, rather than all N-linked sugars).

False Positive N-Linked Glycosylation Sites Arising from Asn Deamidation at N-G or N-S Motifs—The limitation of N-linked glycoproteomic experiments that use PNGase F is the reliance on the presence of Asn deamidation as a diagnostic for N-linked glycan site assignment. Deamidation of Asn to Asp is a well-characterized in vivo modification, particularly if the following amino acid is glycine or serine (41), and previous work has shown Asn deamidation plays a role in myocardial I/R (42). We therefore performed an additional control to determine whether deamidation of Asn in myocardial tissue may be contributing to false positive assignment of N-linked glycosylation sites. Aliquots of the IP-ZIC-HILIC and TiO2-enriched glycopeptides were analyzed directly by LC-ESI-MS/MS without PNGase F treatment. A total of 44 peptides containing deamidated Asn residues and satisfying the N-X-S/T/C sequon rule were identified (supplementary Table S3), giving rise to a 2.8% false discovery rate. Twenty-five of these deamidations (56.8%) were in N-G-S/T/C or N-S-S/T/C sequences. A substantial number of deamidated asparagines (>100 peptides) not satisfying the N-linked sequon rule were also identified with the majority characterized by Gly or Ser at the +1 position (data not shown).

Quantification of Previously N-Linked Glycopeptides Associated with Myocardial I/R Injury—We investigated the response of the rat model myocardial N-glycoproteome to I/R injury. In the first set of quantitative experiments, membrane proteins from NITC hearts and those subjected to 40I/20R were digested and labeled in duplicate with iTRAQ. The glycopeptides were purified using sequential enrichment with TiO2 (to purify SA-containing glycopeptides) followed by solid phase extraction desalting of the unbound fraction and IP-ZIC-HILIC (to purify neutral glycopeptides) (Fig. 3A). After removal of the N-glycan moiety by PNGase F, a total of 437 non-redundant N-linked glycopeptides were statistically quantified with a standard deviation ratio of 0.17 at the 95% confidence level. Eighty glycosylation sites were altered in abundance with 72 present at increased abundance of greater than two standard deviations (1.3- to 3.0-fold change; p < 0.05) in tissue subjected to 40I/20R and 8 at decreased abundance of less than 2 standard deviations (0.75- to 0.46-fold change; p < 0.05). Detailed information, raw quantitation values and annotated MS/MS spectra can be found in Supplementary Data (supplementary Table S4 and supplementary Fig. S1).

To increase the number of quantified glycopeptides and provide validation for the data derived from iTRAQ quantitation, a second set of experiments was performed using light (NITC) and heavy (40I/20R) dimethyl labeling. This enabled increased amounts of starting material and therefore the use of complementary glycopeptide enrichment methods (Fig. 3B). A total of 590 nonredundant glycosylation sites were quantified by calculating the monoisotopic XIC of the “light” and “heavy” forms of the peptide. The final XIC was calcu-
lated by summing consecutive Fourier transform MS scans for the duration of the chromatographic peak profile. Detailed information regarding all changes, as well as XICs, can be found in the supplementary data (supplementary Table S5 and supplementary Fig. S2). We quantified 113 glycopeptides with altered abundance in hearts subjected to 40I/20R compared with NITC (103 with increased abundance [>1.3-fold] and 10 with decreased abundance [<0.7-fold]; supplementary Table S5). Of these, 46 glycopeptides (40.7%) were in agreement with the data observed in the iTRAQ dataset (23 identical peptides with replicated statistical changes and a further 23 glycopeptides representing additional sites altered similarly to others from the same protein observed by iTRAQ). The dimethyl labeling datasets also contained 62 glycopeptides that were not observed in the iTRAQ comparisons. We did observe five glycopeptides that were statistically significantly altered in the dimethyl labeled data that were classified as “not changing” in the iTRAQ data. Three of these peptides represented three different glycosylation sites from a single protein, integrin β-1 precursor (ITB1_RAT). The identified and quantified glycopeptides from the iTRAQ and dimethyl labeling experiments were clustered according to their corresponding glycoprotein Gene Ontology localization. GO revealed that the majority with altered abundance were predicted extracellular/secreted proteins or cell surface glycoproteins (Fig. 4).

Functional assessment of the molecular targets altered by I/R injury suggests that ECM remodeling is the major process in which changes to glycoproteins occurred (Fig. 5; supplementary Tables S4 and S5). We identified proteins with altered glycopeptide abundance from all three stages of the remodeling process: 1) inflammatory (down-regulated glycopeptides were predominantly associated with cell adhesion [e.g. β-sarcoglycan, nidogen-2, basal cell adhesion molecule (BCAM), desmoglein-2, cadherin-13], while changes were also observed in glycopeptides from proteoglycans [e.g. lumican, basement membrane-specific heparan sulfate proteoglycan, mimecan and Cspg4], and several proteases [e.g. chymase, mast cell carboxypeptidase A, dipeptidyl peptidase 2, carboxypeptidase D, and various cathepsins); 2) proliferative (e.g. isoforms of fibronectin, integrins, laminins, and SPARC all contained glycopeptides present at increased levels); and 3) maturation (predominantly elevated glycopeptides within collagen isoforms). A second group of functionally-related proteins with glycopeptides changing in abundance during I/R were those associated with the sarcolemma and electrochemical signaling and solute transport (e.g. Na+/K+-transporting ATPase β, voltage-dependent anion channel 1 (VDAC-1), voltage-dependent Ca2+-channel α-1 and several solute transporters).

Changes in glycopeptide abundance could reflect: 1) a change in gene/protein expression and commensurate glycosylation; 2) changes in glycosite occupancy (increase or decrease in modification at a particular site with no change in protein expression); 3) a change in protein stability turnover degradation or subcellular location (e.g. cycling); or 4) elements of 1 to 3) combined. The time frame for I/R injury is unlikely to be associated with large changes in protein expression and glycans biosynthesis. It is likely that the changes we have observed are due to protein degradation (for the down-regulated cell adhesion-associated proteins) and deposition for those elevated and associated with pro-
liferation and maturation. We did however, identify an increase in abundance of a glycosyltransferase (STT3B) that is part of the oligosaccharyltransferase (OST) complex (supplementary Table S5). Because N-linked glycosylation occurs co-translationally, it suggests that STT3B expression is elevated during 40I/20R and that increased levels of this protein may be responsible for elevated abundance of glycopeptides.

Analysis of Released N-Linked Glycans During Myocardial I/R Injury—To determine whether changes in the N-glycoproteome were the result of, or concurrent with, alterations to the attached N-linked glycan structures, membrane proteins from both NITC hearts and those subjected to 40I/20R were dot blotted to PVDF, treated with PNGase F and the released glycan structures characterized by graphitized carbon LC-MS/MS. Forty distinct N-glycan structures and their isomers
Structures assigned to some peaks are highlighted (see supplementary Table S6).

were identified as being attached to myocardial glycoproteins (Fig. 6). Detailed information regarding all structures, XIC and annotated MS/MS spectra can be found in the supplementary data (supplementary Table S6, supplementary Figs. S3 and S4). An overlay chromatogram of replicate graphitized carbon LC runs of released N-linked glycans from NITC and 40I/20R tissue however, showed no significant differences in either sugar composition or linkage isoforms of these 40 most abundant glycan structures (Fig. 6). It should be noted, however, that such a comparison is at best semi-quantitative and it remains possible that more subtle changes in glycan composition or abundance may be present on a specific protein or subfraction of myocardial glycoproteins.

DISCUSSION

The analysis of N-linked glycoproteins from isolated animal model (rat) myocardium using complementary glycopeptide enrichment strategies is a powerful tool to identify pathological changes occurring on the cell surface and extracellular environment. As with other studies (40), the combined use of parallel digestions using proteases with differing specificities increases (glyco)proteome coverage. These digestions in combination with enrichment techniques based on different chemical properties of the attached glycans, further increased coverage. For the myocardial N-linked glycoproteome, we found that ZIC-HILIC (both in the presence and absence of an ion-pairing agent) contributed the majority of the unique glycopeptide identifications, when compared with hydrazide capture and TiO2 chromatography, however all four methods contributed a significant number of unique peptides. Our data also confirm that taking a glycoproteomics approach may be the most specific means for enriching plasma membrane/cell surface proteins (7), as 76.9% of the identified N-glycoproteins were predicted to be membrane proteins. The techniques utilized here are therefore specific for this most troublesome subproteome and compare favorably with approaches such as biotinylation, ultracentrifugation and detergent extraction for membrane protein enrichment.

An important aspect of our work was the inclusion of a control to identify the extent of false positive “glycosylations” occurring due to naturally (or experimentally) occurring deamidation of Asn-Asp within the N-linked sequon, rather than the Asn-Asp conversion that results from the PNGase F reaction. We identified 44 nonglycosylated Asn-Asp deamidations within an N-linked sequon, and over 100 more that were not within a sequon. The majority of these were located with either Gly (N-G) or Ser (N-S) at the +1 position. N-G and N-S are common in vivo deamidation motifs (41, 43). Furthermore, since deglycosylation most likely increases the efficiency of proteolytic digestion (particularly where the glycan attachment site is close to the protease cleavage site), it is probable that false positives have been under-estimated in the current study and must contribute very significantly in other studies that have not employed such controls. Our data therefore warn against assigning glycosylation sites with absolute confidence based on Asn-Asp conversion following PNGase F treatment without consideration of false positives that may be due to in vivo, or chemically induced in vitro, deamidation.

Our data support the rationale that the three phases of cardiac remodeling (inflammatory, proliferative and maturation) are largely overlapping (29), as changes to proteins consistent with each were detected. Remodeling is a complex event defined as “genomic expression resulting in molecular, cellular and interstitial changes that are manifested clinically as changes in size, shape and function of the heart after cardiac injury” (44). The balance between ECM deposition and degradation is critical in the clinical outcome - where deposition is excessive, fibrosis leading to LV heart failure may occur; where degradation is predominant, LV wall thinning leading to rupture is possible (28). Anatomical imaging, such as magnetic resonance imaging, is the reference standard for determining prognosis and therapy following acute myocardial infarction (AMI). MRI is however, unable to reveal precise molecular events and therefore the development of molecular imaging (45) based on the quantitative glycoproteomic data seen here (Fig. 5) could provide novel biomarkers. While our data are consistent with the mechanisms of ECM remodeling, the time frame may be considerably more rapid than previously thought. While it is known that rodent models undergo an “accelerated’ inflammatory and repair response (29, 46), the accepted paradigm is that the proliferative phase begins approx. 48 h post-injury, with the maturation phase beginning up to 5 days later.

The inflammatory phase of remodeling is characterized by protease activation (predominantly MMPs and serine proteases (31)) causing degradation of the ECM. MMPs (collagenases) may be activated as early as 10 min post-ischemic onset leading to breakdown of collagen and leakage of collagen peptides into the circulation (47). We identified elevated levels of glycopeptides derived from proteins either involved in the regulation of MMP activity or in proteolytic degradation.

Fig. 6. Overlay graphitized carbon chromatogram of released N-linked glycans from NITC (red) and 40I/20R (purple and blue). Structures assigned to some peaks are highlighted (see supplementary Table S6).
We observed significantly elevated levels of many glycopeptides from laminin type II, IV, as well as nidogen-1 and 2, which are known to form high affinity complexes with collagen. Increased levels of laminins and collagen type VI have been proposed to reflect early remodeling in patients with AMI (61). The picture is further complicated by changes detected in the secretory granules of resident mast cells. It has been proposed that sufficient resident mast cells are normally present in the heart to increase MMP activity and account for early remodeling in volume overload (49). Of the glycoproteins identified as regulated by I/R in our study, cathepsins have been shown to activate mast cell chymases (50) and carboxypeptidase A (51).

Many differentially regulated glycopeptides were from the complex network of the collagen-laminin-integrin matrix (Fig. 5). Collagen changes are indicative of both the inflammatory stage (collagen degradation) and the final (maturation) phase (collagen deposition). Collagen remodeling involves changes in hydroxyproline content (52), ratios of collagen I/III (53) and cross-linking through advanced-glycation end (AGE) products (54). We observed significantly elevated levels of many glycopeptides derived from various collagen isoforms including collagen type I/III1, IVα2, IVα3, IVα4, VIα1, VIα2, VIα3 and, XIVα1 in tissue subjected to 40I/20R. We also identified a number of matricellular proteins that influence collagen fibril morphology and assembly post-infarction. Glycopeptides derived from secreted protein acidic and rich in cysteine (SPARC), peristin and nidogen-1 were detected at increased levels in 40I/20R tissue. SPARC is regarded as a marker of the proliferative phase of remodeling (55) and mRNA levels increase post-AMI and peak at ~3.7-fold by day 7 (56). SPARC-null mice also show a significant increase in myocardial rupture and dysfunction following AMI (57). Peristin is another profibrogenic protein that promotes proliferation of cardiomyocytes in the infarct area and differentiation of progenitor cells into fibroblasts (58). This protein is also highly up-regulated in cardiac injury (59) and this may explain the elevated glycopeptide abundance observed here. Peristin-null mice display increased susceptibility to myocardial rupture (60) however; those that survive show decreased fibrotic scarring and better LV function post-AMI.

We detected elevated levels of glycopeptides from laminin α2, α4, α5, β1, β2 and γ1, as well as nidogen-1 and 2, which are known to form high affinity complexes with collagen. Increased levels of laminins and collagen type VI have been proposed to reflect early remodeling in patients with AMI (61). The picture is further complicated by changes detected in proteins that localize to the cell surface, such as integrins αV, α6, α7, and β1. Integrins link the ECM to the cytoskeleton and cardiomyocyte targeted gene deletion of the major integrin β1 results in cardiac failure (62). Angiogenesis plays a vital role in infarct-induced remodeling. Endothelial cell adhesion is a vital step in angiogenesis and integrin αV/β3 has been identified as a critical component in this process (63). Further proteins involved in cell-cell adhesion between cardiomyocytes in the intercalated discs (caderhins T, N and VE, as well as desmocolin-2 and desmoglein-2) were significantly altered post-I/R. The final ECM remodeling-associated component that was identified in this study included proteins from the microfibrils. Glycopeptides derived from fibrillin-1 and 2, fibulin-5, LTBP-4 and EMILIN-1 were quantified with increased abundance in 40I/20R tissue. The detection of changes in fibulin-5 and EMILIN-1 suggests that microfibril assembly is initiated after only 20 mins reperfusion post-40I in the rodent model. Fibulin-5 expression can also inhibit the expression of MMP-7 (64).

Altered proteins could also be clustered into non-ECM functional pathways. Glycopeptides from voltage-gated and other ion transporters were up-regulated following 40I/20R. Changes in ion channel glycosylation have been associated with altered electrical signaling (23) and changes in sialic acids of voltage-gated sodium channel (Nav) have been linked with cardiac conduction disorders and heart failure (22, 24). A number of α and β subunits are expressed: Navα1.1, 1.3, 1.4, 1.5 and Navα1.6; however, Navα1.5 is the major subunit in the sarcolemma and is primarily responsible for the action potential in ventricular systole (65). Navα1.5 is heavily glycosylated (66) and several glycosylation sites were observed in this study, however none were quantified with statistical confidence post-I/R.

We identified several glycoproteins involved in the initiation and propagation of action potentials, and glycans on these may be involved in altering sarcosomal electrochemical gradients contributing to contrac tile dysfunction, as well as variants of voltage-dependent anion channels (VDAC) that are core components of the mitochondrial permeability transition pore (mPTP) involved in cytochrome c release during cell death. Elevated O-linked GlcNAc glycosylation of VDAC is cardioprotective, most likely via inhibition of mPTP formation (67), however no role has been suggested for complex N-linked glycans in VDAC function.

There is prior evidence that glycan structures may be altered during I/R. Removal of SA by neuraminidase from cultured myocytes increases Ca2+ permeability and thus intracellular Ca2+ (68–69), correlating with the well-documented Ca2+-overload that is associated with I/R (26), and leads to decreased contractility (70). Since we observed changes in abundance of formerly glycosylated peptides, we attempted to determine whether gross changes in glycan structures or abundance could be observed by performing glycan profiling of non-ischemic time control and 40I/20R tissues. Despite characterizing ~40 glycan structures we were unable to observe large changes, nor the appearance or disappearance of major glycans. The method however, is semi-quantitative and...
thus we are unable to rule out subtle changes that may occur on specific proteins and that may reflect previous work regarding a role for SA in I/R.

The proteomic analysis of intact mammalian tissue has the advantage over cell culture models as a better representation of the biological system. The myocardium however, is a mixed-cell population predominantly consisting of cardiomyocytes, but also including fibroblasts, endothelial cells, vascular smooth muscle cells and mast cells that provide the structures necessary to model the ECM. Furthermore, remodeling following infarction occurs in a time- and region-specific manner, giving rise to heterogeneity across the myocardium, which will contain the area of infarct, border zone and remote (normal) region. Examining these in combination may thus dampen signals associated with abundance changes and those we have observed are likely to be significantly more pronounced in the immediate infarct and border zones. Finally, a consequence of myocardial infarction is the generation of areas of necrosis (measured here as ~40% of the LV mass following 40I/20R) that result in a loss of plasma membrane integrity. This means that we cannot unequivocally state that predicted cell surface proteins were identified exclusively in that location. Furthermore, abundance changes in such proteins may reflect this loss of integrity in ischemic, but not control, hearts. Despite these limitations, we have shown that targeting glycosylated proteins is a powerful approach to understand predicted membrane and extracellular proteins in disease-induced tissue remodeling and should provide valuable insights into other remodeling-associated diseases.

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