Diabetes Induces Lysine Acetylation of Intermediary Metabolism Enzymes in the Kidney

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Cells in which insulin is not required for glucose uptake are susceptible to the long-term complications of diabetes. Even in these tissues, however, the major perturbations that would otherwise be engendered by the greatly increased intracellular glucose concentration are mollified by adaptive changes in the enzymes of intermediary metabolism. These include allosteric regulation, product inhibition, and covalent modification as well as alterations in gene transcription. More recently, advances in proteomic technology have shown that reversible acetylation of the ε-amino group of lysine provides an additional means of modulating protein function and, in particular, enzyme activity. Here, we explored the extent of protein acetylation in an organ susceptible to the long-term complications of diabetes, examining the kidneys of rats with streptozotocin-induced diabetes and kidney cells exposed to high glucose. Using high-resolution mass spectrometry coupled with immunoaffinity enrichment, we identified 47 lysine-acetylated proteins in the kidneys of diabetic rats compared with 11 in control kidneys. Bioinformatic interrogation of the acetylome from diabetic animals showed a predominance of metabolic pathway involvement including the citrate acid cycle, glycolysis/gluconeogenesis, and metabolism of branched chain amino acids. Increased lysine acetylation was also noted in mesangial and tubular cells exposed to 25 mmol/L compared with 5.6 mmol/L glucose. These findings highlight acetylation as a posttranslational modification affecting numerous proteins. Current drug discovery efforts to develop small molecule inhibitors and activators of various lysine acetylases and deacetylases offer a new potential strategy to reduce the likelihood of diabetes complications.

As a consequence of their direct contact with the external environment, unicellular organisms need to rapidly regulate intermediary metabolism in response to wide fluctuations in ambient nutrient concentration. Complex metazoa, on the other hand, maintain a relatively constant milieu intérieur, despite changes in nutrient availability. This stability is dramatically eroded in diabetes where inappropriate hyperglycemia leads to profoundly disordered intermediary metabolism. In cells that require insulin for glucose uptake, such as the liver, muscle, or adipose tissue, insulin deficiency or resistance leads to a diminution in intracellular glucose that accelerates gluconeogenesis, lipolysis, and ketogenesis. These tissues, pivotal to the development of the acute complications of diabetes, are, however, not subject to the chronic complications of the disease. Instead, it is the cells that continue to transport glucose in the face of hyperglycemia, such as those of the eye, kidney, nervous system, and endothelium, that face long-term damage.

Intermediary metabolism, with its central role in providing cells with energy and the building blocks of macromolecules, is regulated at multiple levels. Its enzymes, for instance, may be modulated by allosteric regulation, product inhibition, and covalent modification. For the last of these, recent technological advances in

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mass spectrometry and immunoenrichment have indicated that the activity of metabolic enzymes can be modulated by the reversible acetylation of the ε-amino group of lysine through the transfer of an acetyl group from acetyl-CoA (1).

The acetylation and deacetylation of proteins, identified in the regulation of histone function some 40 years ago, is now appreciated to be far more widespread, rivaling phosphorylation/dephosphorylation, both in breadth and specificity, as a key regulator of protein function (2,3). Like phosphorylation, where ATP functions as both a marker of energy stores and the major phosphate donor, acetyl-CoA is similarly both a central component of intermediary metabolism and a major donor for acetyl groups in acetylation (4). However, unlike phosphorylation where a diverse array of proteins is modified, lysine acetylation appears to affect functional networks, which suggests a role in the regulation of multiprotein complexes (5). This is particularly evident in intermediary metabolism, where lysine acetylation coordinately modulates glycolysis, the tricarboxylic acid (TCA) cycle, fatty acid oxidation, amino acid metabolism, and ketogenesis, according to nutrient availability (6).

Given the central role of intracellular nutrient (glucose) excess and consequent metabolic stress in the pathogenesis of diabetes complications, we sought to examine changes in the acetylome in the kidneys of animals with long-term diabetes. Here we show that multiple enzymes of the glycolytic and TCA cycle are differentially acetylated in diabetes.

RESEARCH DESIGN AND METHODS

Animal Studies

Study 1
Eight-week-old male, heterozygous (mRen-2)27 rats were assigned to receive either 55 mg/kg streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO) diluted in 0.1 mol/L citrate buffer, pH 4.5, or citrate buffer alone (nondiabetic) by tail vein injection after an overnight fast. Only STZ-treated animals with blood glucose >15 mmol/L were considered to have diabetes. Both diabetic and nondiabetic rats received regular chow (Certi-Sure Rodent Diet #5002; LabDiet, St. Louis, MO) and drinking water ad libitum. Diabetic rats received a daily injection of insulin (2–4 units subcutaneously; Humulin NPH; Eli Lilly and Company, Indianapolis, IN) to reduce mortality and to promote weight gain. Animals were housed in a stable environment maintained at 22 ± 1°C with a 12-h light/dark cycle commencing at 6:00 A.M. Eight weeks after receiving STZ (or buffer), rats were terminated by lethal anesthesia with Nembutal. Kidneys were excised, decapsulated, and snap frozen in liquid nitrogen prior to storage at −80°C.

Study 2
To determine whether normalization of plasma glucose would alter protein acetylation in the kidneys of diabetic animals, we induced diabetes in six Ren-2 rats, as described in Study 1. After confirmation of diabetes, rats were randomized to receive either vehicle or subcutaneous slow-release insulin implants containing bovine insulin and microrecrystallized palmitic acid (0.5–1 pellet; 1–2 units insulin/day; Linshin Canada, Inc., Toronto, ON, Canada) for 7 days, prior to killing. Animals were housed as in Study 1, with all experimental procedures in both studies adhering to the guidelines of the Canadian Council on Animal Care and were approved by St. Michael’s Hospital Animal Care Committee.

Cell Culture Studies

The effects of high glucose (HG) were also examined in rat mesangial (IRMC; American Type Culture Collection [ATCC CRL-2573]) and the proximal tubular epithelial cell line, NRK-52E, cells (ATCC CRL-1571). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% FBS (Life Technologies, Burlington, Ontario, Canada), streptomycin (100 g/mL), penicillin (100 units/mL), and 2 mmol/L glutamine at 37°C in 95% air/5% CO2 as previously reported (7). Cells were cultured in DMEM containing either 5.6 mmol/L or supplemented with 19.4 mmol/L glucose (final concentration 25 mmol/L) or 19.4 mmol/L mannitol (final concentration 25 mmol/L). To assess the impact of lysine deacetylase inhibition, NRK-52E cells were exposed to 5.6, 25, or 5.6 mmol/L glucose in the presence of the Sirt1 deacetylase inhibitor EX527 2 μmol/L (Cayman Chemical, Ann Arbor, MI) for 24 h.

Immunoblot

An aliquot of lysate from cell culture and kidney tissue was assayed in a Bio-Rad (Bradford) protein assay. Fifty micrograms of protein was then examined by Western blot analysis as previously described (8) using an anti-acetyl-lysine antibody that detects acetylation of the ε-amine groups of lysine residues (Cell Signaling #9441; Cell Signaling Technology, Beverly, MA). Equivalent protein loading was determined by β-actin immunolabeling. The ability of the antibody to detect lysine-acetylated proteins was confirmed in cell lysates from rat mesangial IRMC cells exposed to a histone deacetylase inhibitor, vorinostat (SAHA; 5 μmol/L for 24 h) (Supplementary Fig. 1).

Protein Extraction and Liquid Chromatography–Tandem Mass Spectrometry Analysis

To extract proteins for mass spectrometry analysis, frozen tissues (50 mg) and cells (NRK-52E and IRMC, ∼20 million) were homogenized or lysed in 2 mL 0.5% RapiGest (Waters Corporation, Milford, MA) containing 0.5 μmol/L trichostatin A and protease inhibitors (Protease Inhibitor Cocktail; Sigma-Aldrich). Proteins were reduced and alkylated with dithiothreitol prior to digestion with sequencing-grade trypsin (Promega, Madison, WI). Tryptic peptides were desalted and immunopurified using anti-acetyl-lysine antibodies immobilized on agarose beads (ImmuneChem Pharmaceuticals, Burnaby, BC, Canada) according to the
Histones were not differentially acetylated in the diabetic kidney (Supplementary Table 3). The acetylome of cells incubated with 19.4 mmol/L mannitol in addition to 5.6 mmol/L glucose was substantially different, although some overlap between the two conditions in both cell types was noted (Supplementary Fig. 2 and Supplementary Tables 7 and 8).

Administration of insulin to diabetic rats in Study 2 led to reversal of hyperglycemia (glucose [mean ± 5D], 5.4 ± 3.4 mmol/L). Despite attaining near-normal blood glucose concentrations for 7 days, the extent of protein acetylation was only modestly altered. Of the 109 acetylated proteins identified in the kidneys of untreated diabetic animals, 66 were also detected in the kidneys of animals that achieved 7 days of near normoglycemia with insulin implants. Twenty-six acetylated proteins were identified exclusively in the kidneys of animals that had received insulin (Supplementary Tables 1–6).

Cell Culture
In IRMC and NRK-52E cells exposed to HG, 50 and 99 proteins were identified, respectively. In the setting of normal glucose (NG), 31 and 39 acetylated proteins were identified in IRMC and NRK-52E cells, respectively (Fig. 2B and C and Supplementary Tables 1 and 3). Although increased protein acetylation was substantially increased in cells exposed to HG, and some overlap was noted, the patterns of acetylation differed substantially between cell types and between cultured cells and kidney tissue (Fig. 2D). A minority (~10%) of the acetylated proteins found in IRMC and NRK-52E cells exposed were also found when these same cell types were exposed to isosmotic mannitol. No enzymes of intermediary metabolism were commonly acetylated in cells exposed to both mannitol and HG (Supplementary Fig. 2 and Supplementary Tables 7 and 8).

To gather insight into the enzymes that might be contributing to the lysine acetylation induced by HG, NRK-52E cells incubated in 25 mmol/L glucose were compared with cells exposed to 5 mmol/L glucose in the presence of the Sirt1 deacetylase inhibitor EX527. Although differences between the acetylomes were noted, substantial overlap was also evident (Fig. 3 and Supplementary Tables 1–15).

Pathway Analysis
Gene ontology analysis revealed that lysine-acetylated proteins in the kidney are localized within the nucleus (35%), mitochondria (32%), and cytoplasm (30%). Some of the 168 proteins were classified in more than one cellular compartment. We used Protein Center to identify overrepresented pathways (KEGG database) in diabetes. The top five statistically significant pathways were citrate acid cycle (P = 2.24 × 10⁻⁹), metabolic pathways (P = 4.91 × 10⁻⁷), glycolysis/gluconeogenesis (P = 4.87 × 10⁻⁶), and metabolic pathways for valine, leucine, and isoleucine (P = 1.02 × 10⁻⁷) as well as glycine, serine, and threonine (P = 5.41 × 10⁻⁵) (Table 1).
From 37 proteins that were differentially acetylated in the diabetic kidney, a substantial proportion of these were associated with enzymes that catalyze intermediary metabolism (Fig. 4) with six of the eight enzymes of the TCA cycle acetylated: citrate synthase, malate dehydrogenase (MDH), isocitrate dehydrogenase, fumarate hydratase, cis-aconitase, and succinate dehydrogenase. For glycolysis, three enzymes (pyruvate carboxylase, glyceraldehyde-3-phosphate dehydrogenase [GAPDH], and triose phosphate isomerase) were acetylated, as was the ketogenic enzyme acetyl-CoA acetyltransferase. For fatty acid metabolism, enoyl-CoA isomerase and hydroxycyacyl-CoA dehydrogenase were both acetylated in the diabetic kidney along with enzymes of amino acid metabolism/urea cycle, glutamate dehydrogenase, aspartate aminotransferase, aminoadipic semialdehyde synthase, and ornithine carbamoyltransferase. Other enzymes acetylated in the diabetic kidney included those involved with ATP synthesis, such as ATP synthase α and β and electron transfer flavoprotein ADP/ATP translocase (Supplementary Tables 1 and 2).

Although increased enzyme acetylation was also noted in IRMC and NRK-52E cells exposed to 25 mmol/L glucose, differences were noted between the cell types and between the cells and whole kidney. For instance, of the 11 enzymes of intermediary metabolism that were identified in the kidneys of diabetic animals, 5 and 4 of these were also detected in the acetylomes of IRMC and NRK-52E cells, respectively, when exposed to HG with only a single enzyme, glutamate dehydrogenase, common to both cell types (Supplementary Table 16).

We used the Motif analysis tool embedded within the Scaffold PTM software to identify a potential consensus motif for lysine acetylation. The analysis determined GK* (glycine-lysine) as the preferential motif for lysine acetylation in our dataset. Nineteen percent of our lysine-acetylome proteins (36 peptides) matched with this motif.
preferential motif for the −1 position (Fig. 5). Scaffold PTM software was similarly used to identify the subcellular localization of acetylated proteins in the kidneys of diabetic rats (Fig. 6).

**DISCUSSION**

Although numerous studies have examined the urinary proteome of patients with diabetic nephropathy and some have examined the tissue proteome in animals and humans with diabetic nephropathy (9,10), to our knowledge, the current study is the first to examine the spectrum of lysine acetylation (the acetylome) in the diabetic kidney. In the current study, we identified 39 proteins that were differentially acetylated in the diabetic kidney, the majority of which were enzymes of intermediary metabolism where acetylation has been shown not only to be widespread but also to modulate activity (1,11). How lysine acetylation affects protein function is only partially understood but pivots around the neutralization of lysine’s positive charge and consequent modification of protein-protein interactions, subcellular distribution, and stability (12). Mostly, however, the impact of lysine acetylation on enzyme activity is unknown, and in the few cases studied in animals and humans, we found very little acetylation in the kidneys of nondiabetic animals and a marked increase in the acetylation of intermediary metabolism enzymes in the setting of diabetes.

In general terms, acetylation is known to affect three key determinants of enzyme activity: abundance, catalytic activity, and accessibility to substrate (14). Accordingly, acetylation can now be added to an already long list of HG-mediated post-translational modifications that reduce GAPDH activity, including ADP ribosylation, succinylation, and oxidation (15,16). Because a diminution in GAPDH activity leads to an increase in the abundance of glycolytic intermediates and thus augmentation of the polyol, hexosamine, protein kinase C, and advanced glycation end product pathways, GAPDH is viewed as having a central role in the pathogenesis of diabetes complications (17). Moreover, in addition to its role as a regulator of glycolytic flux, GAPDH also modulates cell signaling, chromatin structure, DNA integrity, transport, autophagy, and apoptosis (18) such that changes in its activity may have diverse consequences. However, given the enormity of the acetylic changes that we found in diabetes, the diversity of functional changes induced by acetylation and the cell-to-cell variations in their acetylomes, the sum of the metabolic effects of this posttranslational modification in the diabetic context remain to be determined.

Acetyl-CoA, the principal acetyl group donor in lysine acetylation, is a product of intermediary metabolism that is generated in both mitochondrial and cytosolic compartments by pyruvate dehydrogenase and ATP-citrate lyase, respectively in response to nutrient availability (19). As such, lysine acetylation is ideally placed for its role in coordinately modulating glycolysis, the TCA cycle, fatty acid oxidation, amino acid metabolism, and ketogenesis (6). Previous studies have examined changes in the acetylome of prokaryotes and in the livers of humans and mice, showing major changes that suggest a key role in metabolic regulation (6,11,20). Indeed, in the liver, virtually all enzymes of glycolysis, gluconeogenesis, TCA cycle, urea cycle, fatty acid metabolism, and glycogen metabolism are preferentially acetylated (11). In contrast to the descriptions of marked acetylation in the liver, we found very little acetylation in the kidneys of nondiabetic animals and a marked increase in the acetylation of intermediary metabolism enzymes in the setting of diabetes.

**Table 1**—Overrepresented pathways (determined by Protein Center) associated with differentially expressed lysine acetylome data

<table>
<thead>
<tr>
<th>Description</th>
<th>Count*</th>
<th>Ref. count#</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Citrate cycle (TCA cycle) (mo00020)</td>
<td>8</td>
<td>29</td>
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<tr>
<td>Metabolic pathways (mo01100)</td>
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<td>1,061</td>
<td>4.91E−07</td>
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<tr>
<td>Glycolysis/gluconeogenesis (mo00010)</td>
<td>8</td>
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<td>Valine, leucine, and isoleucine degradation (mo00280)</td>
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<td>39</td>
<td>1.02E−05</td>
</tr>
<tr>
<td>Glycine, serine, and threonine metabolism (mo00260)</td>
<td>5</td>
<td>32</td>
<td>5.41E−05</td>
</tr>
<tr>
<td>Pyruvate metabolism (mo00620)</td>
<td>5</td>
<td>37</td>
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</tr>
<tr>
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<tr>
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<td>3</td>
<td>22</td>
<td>2.86E−03</td>
</tr>
<tr>
<td>Propanoate metabolism (mo00640)</td>
<td>3</td>
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</tr>
<tr>
<td>Butanoate metabolism (mo00650)</td>
<td>3</td>
<td>26</td>
<td>4.64E−03</td>
</tr>
</tbody>
</table>

*Number of proteins identified in this study associated with corresponding pathway. #Total number of proteins reported to be associated with corresponding pathway.
These findings are consistent not only with increased glucose entry into cells but also with the additional energy requirements entailed by hyperfiltration and the augmented tubular transport of glucose from the tubular lumen to the basolateral interstitium in the presence of diabetes.

Although acetylation of lysine residues on proteins was described 45 years ago (21), it was viewed, until recently, as mostly confined to histones. However, the ability to enrich for acetylated proteins with anti–acetyl-lysine antibodies in combination with mass spectrometry has shown the breadth of this posttranslational modification (5). Consistent with this realization that nonhistone proteins are also subject to reversible acetylation and deacetylation, the enzymes responsible for catalyzing these reactions are now often referred to as lysine (K), rather than histone acetylases and deacetylases (22). Indeed, in the current study, although many proteins were differentially acetylated in the diabetic kidney, histone proteins were not, contrasting it to recent reports of histone acetylation in the retinae of diabetic rats (23). Although in theory, the ε-amino group of any lysine may be subject to enzymatic acetylation, organ-wide mapping of the sites involved indicate site-specific sequence motifs (24). In our study, where ~20% of the acetylome displayed the consensus motif G-AcK, the acetylated lysine was preceded by glycine.

Adding to the complexity of examining the acetylome is the knowledge that most organs are comprised of numerous different cell types. The kidney, for instance, consists of ~20 different cell types with various functions, energy requirements, and metabolic activity, such that the contribution of each of these is difficult to ascertain in the in vivo setting. Accordingly, we focused on the
cell types that have most often been implicated in the pathogenesis of declining GFR in diabetic nephropathy, the mesangial cell and the proximal tubular epithelium. Although exposure to HG led to increased lysine acetylation, its pattern varied substantially between cell types and kidney tissue. These findings suggest that although exposure to HG stimulates lysine acetylation, the acetylome of a tissue exposed to hyperglycemia in the in vivo setting cannot be directly inferred from in vitro findings. Such variations presumably reflect various factors that include cell-specific acetylome “signatures,” the major differences between the in vivo multicellular context and a two-dimensional single cell culture dish, and the incompleteness of 25 mmol/L glucose as a surrogate for the diabetic milieu that in addition includes alterations in the concentrations of circulating free fatty acids, amino acids, and ketones.

Numerous studies have examined the urinary proteomes of patients with diabetic nephropathy that reflect mostly those plasma proteins that have passed through the glomerulus. In contrast, our study focused on kidney tissue and cells, where to the best of our knowledge, only two studies have reported the diabetic kidney tissue proteome (9,10). The majority of proteins that were identified in those studies were derived from the extracellular space. Indeed, when we compared the acetylated proteins found in our study with the published total proteome in human diabetic nephropathy, we found only a single protein, GAPDH, in common. These findings suggest that changes in protein expression and posttranslational modifications, such as lysine acetylation, are regulated by different mechanisms.

That the downstream consequences of even brief exposure to HG lead to enduring changes in cell function has long been known (25), with prolonged follow-up of glycemic intervention studies providing evidence of a clinical correlate (26,27). Consistent with this notion, we found that the majority of proteins that were differentially acetylated after the induction of experimental diabetes remained so, despite achieving normoglycemia for 7 days. As such, it seems plausible that transiently elevated glucose concentrations may lead to persistent changes in the acetylome of individuals with diabetes, resulting in long-term changes to protein function. Accordingly, the relevance of lysine acetylation in diabetes lies not only in its biology but also in its potential as a target for drug discovery. Notably, given the role of histone acetylation in modifying gene expression and the rediscovery of the Warburg effect, attempts to modulate lysine acetylation as a therapeutic strategy in cancer have led to the development of a number of small-molecule inhibitors and activators of various lysine acetylases and deacetylases (28). To date, these compounds are relatively nonspecific, mostly targeting a number of acetylases and deacetylases. Moreover, the precise targets for these agents are uncertain, although they seem likely to include histones and thereby modify gene expression (23). Notably, compounds that modify lysine acetylation have been shown to attenuate retinal inflammation in diabetes (23) and to diminish the structural and functional attributes of diabetic nephropathy in rodent models (29–32). These considerations, along with the development of some of them for human use (33), raise the possibility of a new therapeutic strategy in the prevention and treatment of diabetes complications. In the current study, we noted substantial overlap between the acetylomes of mesangial cells exposed to HG and those incubated in 5 mmol/L glucose in the presence of an inhibitor of Sirt1, a class III lysine deacetylase. These studies raise the possibility that increasing sirtuin deacetylase activity may provide a new strategy to reverse the cell dysfunction associated with diabetes (34).

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