Proteomic changes associated with diabetes in the BB-DP rat

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Although extensively studied, type 1 diabetes remains a major health concern, and much of the pathogenesis still eludes understanding. In the Diabetes Control and Complications Trial, the most strongly regulated subjects still developed complications despite aggressive intervention (35). The fundamental pathology of type 1 diabetes is the loss of pancreatic β-cell function and the related release of insulin in the regulation of overall metabolic functions of the body. The complications of type 1 diabetes include dramatically altered energy metabolism and glucose handling, protein oxidation, vascular disease, heart disease, retinopathy, neuropathies, nephropathy, and susceptibility to infections. These results showed that the pathology of diabetes exceeds the simple maintenance of plasma glucose and insulin levels. A more global assessment of the diabetic state may provide useful insight. These studies sought to perform a comprehensive screen of the proteomic changes associated with the type 1 diabetic state, utilizing emerging technology in high-throughput semiquantitative proteomics and two-dimensional (2D) gel electrophoresis to detect protein expression differences as well as possible covalent modifications inherent to the diabetic state.

Previous studies have revealed that many enzymes and proteins are under transitional control by insulin (45) that would predictably alter the content of these proteins with the reduction of insulin in type 1 diabetes. The major transcription factors involved in insulin action are adipocyte differentiation and determination factor (ADD)-1/sterol regulatory element binding protein (SREBP)-1/peroxisome proliferator-activated receptor (PPAR)γ, involved in fatty acid and triglyceride metabolism as well as other aspects of intermediary metabolism (61). Several recent screens of gene expression are available in various models of diabetes consistent with a key role of insulin in the gene expression profiles associated with energy metabolism as well as many other metabolic as well as signaling pathways (15, 64). We reasoned that a proteomic screen might provide insight into the complex metabolic and regulatory pathways associated with type 1 diabetes by directly monitoring the protein levels with recently developed differential quantitative proteomic approaches.

Since we chose to examine multiple organs in these studies, we selected an animal model with a matched control. The most common model used in diabetes research is streptozotocin or alloxan treatment, which results in β-cell death, insulin insufficiency, and hence diabetes (5, 53). Although these methods produce animals that model diabetes, streptozotocin and alloxan are oxidative stressors and the secondary oxidative stress effects can confound diabetes in these animals. Because mitochondrial proteins were of interest and these are particularly susceptible to oxidative stress, we decided not to use alloxan and streptozotocin. The use of knockout mouse models is problematic because the mutations that are introduced may induce hyperglycemia by mechanisms that do not normally occur in diabetes. Mice also yield so little tissue that analysis via 2D gel and mass spectrometry without pooling samples becomes problematic. The two rat models considered were the Zucker diabetic fatty/type 2 diabetes (ZDF) model (genetic models) and the Bio-Breeding diabetes-prone (BB-DP) spontaneous type 1 diabetes model (biomedical research models). We chose to avoid the confounding variables associated with obesity in conjunction with diabetes in the ZDF rat. On the basis of these numerous considerations, we selected the BB-DP model. The BB-DP strain is maintained commercially along with a matched-background diabetes-resistant strain (BB-DR) and follows a course similar to human type 1 diabetes, with 96% of the rats becoming diabetic by 120 days. The course is predictable and is autoimmune, similar to human diabetes. The lipid profiles of the rats are similar to the human condition (39, 49).

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PROTEOMICS OF DIABETES IN BB-DP RATS

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We used 2D gel electrophoresis to qualitatively screen for protein content, hydrolysis (decreases in molecular weight), and posttranslational modifications [as demonstrated by isoelectric focusing variants (IEVs)]. IEV can reflect several different posttranslational modifications from phosphorylation, oxidation, ADP-ribosylation, or even selective protein cleavage. Any modification of the protein that would change its surface charge will result in an IEV. Thus this measure must be interpreted with caution. Liquid chromatography-mass spectrometry (LC-MS) was used for more extensive determination of protein content differences associated with the diabetic state because LC-MS has a broader range than standard 2D gel electrophoresis. 2D gel analysis was performed on diabetic and control heart, liver, and muscle. LC-MS analysis was performed on liver and heart, where the initial 2D gel studies demonstrated the largest protein content changes with diabetes. These data were then compiled into a format that permitted functional evaluation of the protein alterations associated with defined metabolic pathways. In addition to confirming some of the known changes associated with diabetes, this approach provided evidence for novel alterations that merit further studies by conventional experimental approaches.

MATERIALS AND METHODS

Tissue harvest. Urine glucose levels were measured daily after the BB-DP rats were ~7 wk old. Urine glucose-positive animals were subjected to blood glucose measurements via tail blood and glucose analyzer determination. To prevent the development of insulin antibodies and potentially confounding data, rats were not treated with insulin. Animals were allowed ad libitum standard chow until the day of tissue harvest. Blood glucose was measured twice daily until blood glucose was ≥500 mg/dl and/or the rats demonstrated significant ketonuria. Fluid therapy was administered if necessary to maintain health. Within 30 h of ≥500 mg/dl blood glucose and/or significant ketonuria, rats were euthanized. This usually occurred when the rats were ~17 wk old. All necropsies were performed at noon to minimize protein expression differences related to circadian rhythm. Acutely diabetic BB-DP and age-matched (±3 days) BB-DR rats were fasted from 7:00 AM until euthanasia on the day of collection. Euthanasia was by decapitation. The guillotine was thoroughly washed and the area of decapitation scrupulously cleaned to prevent stressing the rats en route to euthanasia. Animals were kept in a room separate from the site of decapitation and transported to the guillotine immediately before euthanasia. The whole heart was removed and washed with ice-cold saline in three consecutive dishes. Two pieces from the left side of the liver were removed (~5 g each) and placed briefly into 4°C for 35 min. Lipid phase and protein content of liver and heart were determined with differential in-gel 2D electrophoresis (DIGE) Cy3 and Cy5 dyes, respectively. A standard containing 25 µg of each sample was labeled with Cy2 per the Amersham DIGE protocol (200 pmol/µl labeling). The reaction was then quenched with 10 mM lysine for 10 min. Labeled Cy2-, Cy3- and Cy5-treated samples were combined with 8 µl of DeStreak (GE Biosciences-Amersham) and 4 µl of pH 3–10 ampholytes (GE Biosciences-Amersham). Solution volume was adjusted to 440 µl with the necessary volume of rehydration solution. Samples were loaded onto 24-cm Immobiline DryStrip pH 3–10 (GE Biosciences-Amersham). Isoelectric focusing was achieved by active rehydration for 11 h at 30 V followed by stepwise application of 500 V, 1,000 V, and 8,000 V for a total of ~70,000 Vh (Etan IPG Phor, GE Biosciences-Amersham). Strips were equilibrated in equilibration solution (50 mM Tris-Cl, 6 M urea, 30% glycerol, 2% SDS) with 0.5% DTT for 12 min with agitation and then in equilibration solution with 4.5% iodoacetamide for 12 min with agitation. Strips were then washed on 10–15% SDS-PAGE acrylamide gradient gels (Jule) and sealed with 0.5% agarose. The second-dimension gels were run at 13°C for 30 min at a constant 50 V and then for ~18–21 h at 105 V constant voltage. Gels were rinsed with purified water and then scanned on a Typhoon 9400 scanner (GE Biosciences-Amersham) at 100-µm resolution. Cy2 gels were scanned at 540 Bp40 on a blue2 (488 nm) laser, Cy3 gels were scanned at 580 Bp30 with a green (532 nm) laser, and Cy5 gels were scanned at 670 Bp30 with a red (633 nm) laser. Gels utilized for MS analysis were run with 500 µg of protein. Plates were separated, and gels were fixed overnight in 30% methanol and 10% glacial acetic acid. Gels were then stained overnight in Sypro Ruby dye (Molecular Probes).

Differential gel presentation was created within a custom-written image processing program (IDL, RSI) to correct for scanning gain/detector alterations for the different wavelengths. Since large differences in protein contents were observed in some of these studies, global corrections were found to be inadequate. For this procedure, a nonscaled color overlay of the Cy3 and Cy5 images was used to select a region of interest (ROI) for use in the scaling function. The ROI was selected based on an area of the gel in which there was high signal-to-noise difference in Cy3 and Cy5 with minimal background noise; this optimized the linear least squares approach using the scaling function. In general, the ROI represented <20% of the total image. A linear least squares analysis between the three channels collected was performed with the following equation: I_{Cy5}[Cy3(ROI)] + I_{Cy3}[Cy5(ROI)] = Cy2(ROI) (1).

The ICy3 and ICy5 coefficients were determined with a resident least squares fitting routine within the IDL software environment, minimizing the residuals. To create the final DIGE image, the entire Cy3 and Cy5 images were overlaid after multiplying by the appropriate coefficients. This approach was accurate over a fivefold range in signal amplitude in protein standard experiments. In general, ICy3 and ICy5 ranged from 0.75 to 1.25 in the experimental samples.

Spot picking and protein identification. Spot identification was carried out with an Etan Spot Handling Workstation (GE Biosciences-Amersham) that performs automated extraction and in-gel trypsin digestion of selected protein spots according to an Amersham protocol. Peptides derived from protein spots of the gel were analyzed by conventional experimental approaches.

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with a mass spectrometer (4700 Proteomics Discovery System, Applied Biosystems) using MALDI-TOF and tandem mass spectrometry (MS/MS). At least two peptides were obtained for each protein with MS/MS. Proteins were identified from the acquired spectra with the MASCOT database search function.

Sample preparation for mass spectrometry. Liver or heart tissue was powdered on dry ice as described above. LC-MS was performed and statistical analysis was done as described previously (27). Protein extraction and MS were performed as described previously (27, 33).

There was some concern that the magnitude of protein level changes might be underestimated. In previous studies (32, 33) the ratio of proteins was underestimated as the difference in protein content increased because of the effect of weak background signals preventing the denominator from reaching true zero. To evaluate this, we conducted a trial using five known proteins [APE-1, serine esterase, pyruvate dehydrogenase (PDH), arylamine-N-acetyltransferase, and alcohol dehydrogenase (ADH)] at three different concentrations within a mitochondrial protein background. These standards demonstrated consistent agreement in direction, but the method consistently underestimated the magnitude of difference. That is, the standards revealed a proper qualitative increase in measured amplitude with concentration, but all underestimated the relative increase in protein content. When measured protein ratio between samples was plotted versus the known amount of protein, the slope of the line was 0.5 (y = 0.38 + 0.5x; R = 0.86) rather than 1 for a perfect quantitative analysis. These data are presented in Supplemental Fig. S1.1 These data demonstrate that the relative concentrations of proteins are qualitatively correct; however, the precise ratio of proteins between samples is underestimated by roughly a factor of 2 in the mitochondrial protein environment.

Fig. 1. Two-dimensional differential gel electrophoresis of diabetic tissues. Overlay of diabetic vs. control tissue. A: liver. B: heart. C: skeletal muscle. Protein identifications follow. Liver: 1) aldehyde dehydrogenase 1; 2) sarcosine dehydrogenase; 3) dimethylglycine dehydrogenase; 4) serotransferrin precursor; 5) mitochondrial aconitase; 6) long-chain-fatty acid-CoA ligase; 7) urocanase; 8) glucokinase regulatory protein; 9) phosphoenolpyruvate carboxykinase; 10) succinate dehydrogenase flavoprotein; 11) stress 70 protein; 12) protein disulfide-isomerase; 13) 78-kDa glucose-regulated protein; 14) protein disulfide isomerase A3; 15) pyruvate kinase isozymes R/L; 16) phosphoglucomutase 1; 17) carbamyl-phosphate synthase II; 18) liver carboxylesterase 10; 19) serine protease inhibitor A3k; 20) glycerol kinase; 21) serine protease inhibitor A3L; 22) S-adenosylmethionine synthetase; 23) argininosuccinate lyase; 24) β-ureidopropionase; 25) cysteine sulfenic acid decarboxylase; 26) hydroxymethylglutaryl-CoA synthase; 27) fumarate hydratase; 28) argininosuccinate synthase; 29) glutamate oxaloacetate transaminase 1; 30) apolipoprotein A-IV; 31) regucalcin; 32) 3-oxo-5-β-steroid 4-dehydrogenase; 33) arginase I; 34) N-hydroxyarylamine sulfotransferase; 35) cystosolicmalate dehydrogenase; 36) estrogen sulfotransferase isofrom 2; 37) glutamate-cysteine ligase modifier subunit; 38) prohibitin; 39) proteasome activator complex subunit 2; 40) phenazine biosynthesis-like domain-containing protein; 41) Δ3,5-Δ2,4-dienoyl-CoA isomerase; 42) homogentisate oxidase; 43) glycoprotein gC1qBP; 44) catechol O-methyltransferase, membrane-bound form; 45) major urinary protein; 46) perin-4-carbinolamine dehydratase; 47) D-dopachrome tautomerase; 48) fatty acid-binding protein. Skeletal muscle: 1) glycogen phosphorylase, muscle form; 2) serotransferrin, splice isoform 2; 3) serum albumin; 4) tripartite motif-containing protein 72; 5) enolase 1a; 6) fumerase; 8) apolipoprotein A-I; 9) apolipoprotein A-IV; 10) atrial natriuretic factor; 11) ATP synthase delta chain. Heart: 1) LIM domain-binding protein 3; 2) creatine kinase; 3) ATP synthase subunit β; 4) tripartite motif-containing protein 72; 5) α-enolase; 6) succinyl-CoA ligase (ADP-forming)-β; 7) isocitrate dehydrogenase [NAD-α]; 8) isocitrate dehydrogenase [NAD-α]; 9) ATP synthase delta chain; 10) serum albumin.
RESULTS

DIGE. DIGE was applied in control and diabetic heart, skeletal muscle, and liver protein homogenates as an initial screen. Heart and skeletal muscle demonstrated considerably less variation between the diabetic and control tissues compared with liver (Fig. 1). The protein content differences are presented in Supplemental Table S1 along with any changes detected by IEV (labeled as shift) with diabetes. The liver revealed numerous changes in protein expression in diabetes and numerous posttranslational modifications reflected in changes in the generation of IEV (Fig. 1). The DIGE approach provided a visual overview of the protein differences and detected some proteins not found in the MS studies with our conservative criterion. The major advantage of the DIGE approach was the screening of posttranslational modifications based on IEV generation. There were consistent alterations in the IEV patterns of aldehyde dehydrogenase 1 (liver), uricase (liver), fumarase (skeletal muscle, heart, liver), HSP70 (liver), enolase (skeletal muscle, heart), tripartite motif containing protein 72 (heart, skeletal muscle), and glycogen phosphorylase (heart).

LC-MS. On examination of the DIGE data, many spots were non-Gaussian, with highly variable background levels making absolute quantitation difficult, especially with the low-abundance proteins, where “bleed over” from more abundant proteins was problematic. In addition, any protein greater than 100 kDa or with an isoelectric point outside pH 3–10 was not detected by these methods. Because of these limitations and the...
large number of changes that were identified, we chose to rely on a semiquantitative LC-MS approach mostly focused on the liver: 8,735 liver peptides were identified and quantified, resulting in 4,686 protein identifications. Of these, 555 proteins were identified with high confidence: 365 proteins had significant changes with \( P < 0.05 \), and 194 proteins were \( >20\% \) different, including 65 changes not previously reported in the diabetes literature to our knowledge. In the heart, there were several protein changes that were of interest. Pyruvate dehydrogenase kinase isoform 4 (PDHK4) was strongly upregulated, as previously demonstrated (67), in addition to several proteins that have not previously been associated with diabetes. We confirmed the increase in PDHK4 and other PDHK isoforms by Western blotting on the crude tissue homogenates (Supplemental Fig. S2).

To better understand the functional implications of the protein changes with diabetes, the liver data were used to generate graphic canonical models of represented metabolic pathways. Reasonable coverage of the proteins associated with glucose, fatty acid, tricarboxylic acid, urea, reactive oxygen species (ROS), and oxidative phosphorylation was seen in this screen (Figs. 2–4). We utilized a canonical approach that essentially assumes that the capacity of a given reaction step and metabolic pathway is proportional to the enzyme concentration. Naturally, this is a simplification because of the effects of posttranslational modifications, substrate/product/inhibitor interactions, and numerous allosteric effectors; however, it does provide a method of building hypotheses from this data set. What we sought to determine is whether the protein expression values would recapitulate the metabolic data that have been collected in diabetes and generate hypotheses about the metabolic pathway that have not previously been appreciated in studies that were more focused in their data collection methods.

**DISCUSSION**

Proteomics was used in this study to semiquantitate changes in protein expression patterns that might reveal metabolic changes in type 1 diabetes that are currently not appreciated. The intent was to obtain leads from this broad data collection method that could form the basis of hypotheses for future
In the diabetes model used, the most dramatic proteomic alterations were observed in the liver, with more subtle effects observed in skeletal muscle and heart. These data imply that the major adaptations occurring in this model of type 1 diabetes were occurring in the liver, with more modest changes occurring in the heart and skeletal muscle. These results are consistent with the known large alterations in liver metabolism associated with a lack of insulin mimicking systemic starvation. The general modifications to the proteome and likely function within the heart and skeletal muscle are relatively smaller.

In general, alterations in a given metabolic pathway were reflected by net changes in enzymes of the whole pathway rather than just a few “rate-limiting” reactions, with the exception of the citric acid cycle and glycolysis. This is intuitive, as these pathways have multiple metabolic input and output nodes. This contributed to the large number of liver protein alterations observed (Fig. 1), since many proteins associated with each pathway were altered. This observation is consistent with the previous screen of the mitochondrial metabolic pathways between different tissues with different metabolic requirements that also reflected entire metabolic pathways alterations (32, 33).

For many of the proteins that were altered in the diabetic animal at levels >50%, we found excellent correlation with gene expression data from individual experiments looking at effects of insulin as well as screening studies in different diabetic animals (see Supplemental Table S2 and references). Since no gene expression screen has been conducted on the BB-DP rat, a direct comparison between the proteomic data and screens is difficult. It is interesting to note that many of the screen studies in diabetic models (13, 15, 64) did not detect gene expression changes in many genes that have been found to be altered in focused studies on similar diabetic models (for example, see Ref. 45). This observation suggests that the statistical power of these particular gene expression screens may not be approaching the sensitivity of the more selective studies or the present proteomic approach. It is also important
to note that gene expression measurements are not dependent on the expressed protein concentration, only the generation of mRNA. Thus gene expression techniques detected production changes in low-concentration signaling proteins and transcription factors that were not detected in our protein screens. This discrepancy is likely due to the poor sensitivity of the MS detection in these complex samples.

To aid in the discussion of the predicted biochemical consequences of the protein changes detected, we have consolidated the data into several canonical pathways. The remaining discussion is focused on what the proteomics data suggest about these metabolic pathways.

Oxidative phosphorylation. It is well established that the net oxygen consumption of the liver cell is increased in diabetes, consistent with an increase in ATP turnover secondary to increased metabolic work in the liver. For example, Exton and colleagues (18) demonstrated a 1.74-fold increase in oxygen consumption with diabetes, corresponding to a 2-fold increase in urea synthesis. Consistent with these observations, our proteomic data indicate that the capacity of the electron transport chain to produce ATP, and potentially therefore ROS, was upregulated in the liver at steps including complex 1, complex 3, complex 4, ANT1, ANT2, FoF1 complex, and the electron transport flavoprotein (ETF) (Fig. 2). These data are consistent with an increase in the ATP-generating capacity in the diabetic liver cell resulting from a general increase in the overall enzymatic content of this reaction sequence. The generalized increase in oxidative phosphorylation enzymes is consistent with an increase in mitochondria biosynthesis associated with the increase in PPARγ coactivator (PGC)-1α that occurs in the diabetic liver (25).

Citric acid cycle. It has been demonstrated in the literature that the citric acid cycle is downregulated in diabetes (38). However, the specific modifications to the citric cycle enzymes have not been described in detail. According to the proteomic data collected in these studies, only two citric acid enzymes were downregulated in diabetes (Fig. 2): succinyl-CoA ligase and citrate synthase (CS). A posttranslational modification to fumarase was observed with an acid shift in its IEV, consistent with protein phosphorylation. The nature of this posttranslational modification of fumarase is unknown. There was an increase in both cytosolic (c) and mitochondrial (m) aspartate transaminase (AT). The latter two enzymes could decrease the mitochondrial matrix oxaloacetate by facilitating the export of oxaloacetate from the matrix for gluconeogenesis (discussed below). Together, these modifications could impede the citric acid cycle by both decreasing the entry of acetyl-CoA via CS and decreasing matrix oxaloacetate. CS levels have also been suggested to influence metabolic changes associated with aging (28, 57) and in caloric restriction (26). Finally, CS concentration is frequently used as a global measure of mitochondrial content/activity (37). However, as demonstrated in this study, CS is specifically downregulated in diabetes compared with enzymes involved in oxidative phosphorylation or fatty acid oxidation (FAO). Thus the reliance on CS concentration to quantitate mitochondrial content or as a standard in relation to mitochondrial function is tenuous.

Fatty acid oxidation. Consistent with the previous documentation of an increase in FAO in the diabetic liver (20, 43), numerous enzymes associated with FAO were increased in the diabetic liver, including most of the acyl-CoA dehydrogenases, the three enzymes responsible for unsaturated fatty acid oxidation, carnitine palmitoyltransferase 2, the trifunctional enzyme complex (TFEC), and the previously mentioned ETF (Fig. 2). Thus the higher liver ATP generation capacity indicated by enzymes of oxidative phosphorylation is likely supported by FAO in diabetes. Interestingly, Krebs predicted that the acetyl-CoA generated via FAO is likely not entering the citric acid cycle but shunted to ketone bodies (20, 36), which has been supported by numerous subsequent studies that show that low matrix oxaloacetate inhibits citric acid flux under these conditions (17). Our data suggest that the enhanced diabetic ketone body formation from acetyl-CoA may be due in part to the downregulation of CS and the increased export of oxaloacetate from the matrix by the two isoforms of AT. Furthermore, a substantial change was apparent in the competition for acetyl-CoA between CS for citric acid cycle activity and hydroxymethylglutaryl-CoA (HMG-CoA) synthase for ketone body synthesis. As shown by our LC-MS standard curves, CS decreased by 60% while HMG-CoA synthase increased by >300% in diabetes, suggesting that ketone body formation would favorably compete for acetyl-CoA in these livers.

Short-chain acyl-CoA dehydrogenase (SCAD) was not upregulated in diabetes, despite the requirement that all FAOs must be processed through this enzyme for completion. The FAO pathway was upregulated with the exception of SCAD. This could potentially cause the buildup of short-chain fatty acyl-CoA, which in conjunction with an increase of carnitine acyltranslocase (lower-quality ID but significantly increased P value of 0.000025) suggests that short-chain acyl-carnitine may be released from the diabetic liver, allowing the recycling of CoA in the presence of decreased flux through SCAD. There is evidence that short-chain acyl-carnitines are increased in the serum of diabetic and fasted patients and that patients with diabetes with ketones in their breath (12, 30). It is also of interest to note that the short-/branched-chain CoA dehydrogenase was upregulated in the heart, which would allow the handling of the increased short-chain acyl-carnitine released by the liver. To our knowledge, this is the first study that has demonstrated a differential regulation of acyl-CoA dehydrogenases, and it suggests a novel mechanism for the liver to deliver reducing equivalents and carnitine to the periphery via short-chain fatty acids.

Glycolysis. A decrease in glycolysis and an increase in gluconeogenesis in diabetes are well documented (48). We observed a decrease in pyruvate kinase, one of the key enzymes in the control of glycolysis flux, enolase, and a single peptide identification of glucokinase (GK) (Fig. 3). A surprising decrease in glucokinase regulatory protein (GKRP) was measured in the diabetic animals in the DIGE studies. Since GKRP is generally believed to inhibit GK by sequestration in the nucleus, its downregulation is counterintuitive in a state in which glycolysis is suppressed. However, GKRP and GK contents have been directly correlated in GKRP knockout studies (24). Supporting the notion that GKRP is suppressed in diabetes, overexpression of GKRP in diabetic mice increases GK protein levels, increases GK activity, and improves glucose tolerance (59). The specific mechanisms related to the coregulation of GKRP and GK have yet to be resolved but may involve the protection of both proteins from degradation in their combined state.
In the heart, the IEVs for glycogen phosphorylase shifted to more acid positions consistent with phosphorylation and activation of the enzyme in response to low insulin signaling and potentially modifications of cellular Ca\(^{2+}\) handling in diabetes (42, 51). These results support the contention that the Ca\(^{2+}\) signaling apparatus may be dysfunctional in the diabetic heart.

In both heart and skeletal muscle, enolase was downregulated in the diabetic rat and the enolase IEV moved to a more acidic range consistent with phosphorylation. This phenomenon has not been previously reported in the literature and suggests that enolase is undergoing a posttranslational modification that could suppress this highly active enzyme. There is evidence that enolase expression is downregulated in a genetic diabetic mouse model (58) and in a dilated cardiomyopathy model (21). Thus altered expression and posttranslational modification of enolase may play a significant role in muscle energetics and diabetic adaptation.

**Gluconeogenesis.** In the pseudostarved state of diabetes the liver is producing large amounts of glucose and urea from lactate, glutamine, and alanine generated by catabolism in other tissues. In diabetes, gluconeogenesis can be increased as much as 12-fold (71). Gluconeogenesis from lactate requires 6 ATP per glucose generated, while glucose generation from alanine also requires 6 ATP per glucose generated plus 4 more ATP to generate urea from the residual nitrogen (11, 34). With regard to urea synthesis, a 70% increase in urea synthetic rate has been reported in a rat model of diabetes (3, 50) as well as in human insulin-dependent diabetes mellitus (2) that correlates with the overall cachectic state. These observations suggest that urea and glucose synthesis are major driving forces for the increased capacity to generate ATP via oxidative phosphorylation and FAO in diabetes. This is graphically represented in Fig. 4.

With respect to gluconeogenesis there was a large increase in two key rate-limiting steps, phosphoenolpyruvate carboxykinase and pyruvate carboxylase, consistent with prior studies (4, 9), while the export of oxaloacetate from the mitochondria for glucose synthesis may be enhanced by the previously mentioned increase in cAT and mAT (Fig. 3). The remaining detected enzymes in gluconeogenesis catalyze near equilibrium, bidirectional reactions participating in both glycolysis and gluconeogenesis. However, diabetes increased glyceraldehyde-3-phosphate dehydrogenase, which is somewhat surprising for an enzyme that participates in both gluconeogenesis and glycolysis.

We also observed upregulation of the enzymes associated with scavenging of GABA for gluconeogenesis. The liver takes up plasma GABA (50) under control conditions with a liver-specific GABA transporter (8). The scavenging pathway for GABA to succinate via aminobutyrate transaminase (ABT) was enhanced in diabetes, where plasma GABA concentration is decreased (1, 22, 41). These data suggest that GABA may be a substrate for gluconeogenesis in diabetes.

**Urea cycle.** There was a general upregulation of the enzymes responsible for the elaboration of urea, including carbamoyl phosphate synthase (CPS), ASS, and ASL, with the exception of arginase and ornithine carbamoyltransferase (OCT) (Fig. 4), consistent with many previous studies in a variety of models (7, 40). OCT can support increased flux without an increase in enzyme level, possibly because of the activation by Mn\(^{2+}\) (68). Arginase also demonstrates linear first-order kinetics to increasing concentrations of plasma arginine (65). Thus the present studies confirm a large body of work demonstrating upregulation of urea cycle enzymes in diabetes.

A striking isoelectric focusing shift in urocanase, catalyzing the second step of histidine degradation, in the liver, is of interest for future study. This may be related to the general upregulation of amino acid oxidation in the diabetic state. No posttranslational modifications of urocanase have been reported. Other upregulated enzymes associated with amino acid oxidation included glutamate/oxaloacetate transaminase, argininosuccinate synthase, 4-aminobutyrate aminotransferase, glycine methyltransferase, serine/threonine dehydratase, glutaminase, phenylalanine-4-hydroxylase, glutaryl-CoA dehydratase, aminoadipate semialdehyde synthase, and alanine aminotransferase.

![Fig. 5. Overall metabolic summary of focus of the liver in the diabetic state.](http://ajpendo.physiology.org/)

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**Fig. 5.** Overall metabolic summary of focus of the liver in the diabetic state. Yellow areas concern the conversion of energy into ATP; red arrows represent upregulated ATP hydrolysis reactions generating work.
**Reactive oxygen species.** Protein oxidation and oxidative damage have been previously associated with diabetes (16, 55). The enzymes responsible for scavenging ROS in the cytosol [catalase, PRX-6-1-4, Cu-superoxide dismutase (CuOD), and glutathione S-transferases] were downregulated or unchanged without any significant change in enzymes responsible for scavenging ROS in the mitochondrial matrix [PRX-5, Mn-SOD, and glutathione peroxidase (GPX)-1] (Fig. 4). These data suggest that the cytosol would be more susceptible to ROS damage in diabetes due to a downregulation of ROS scavenging pathways. The liver ROS production would likely be higher (44), because of a high concentration of reduced substrates and enhancement of electron transport elements. The suppression of cytosolic ROS scavenging pathways may again reflect the disconnected signaling of the pseudostarved state of the liver. In the normal starved state ROS production is reduced (10, 69), while ROS scavenging mechanisms are unaffected or decreased (23). On the basis of these observations, we speculate that in the altered signaling milieu of diabetes the cytosolic ROS scavenging enzymes are downregulated in anticipation of a normal starved state reduction of ROS production. In diabetes the liver is adjusting its ROS scavenging capacity as if in a starved state, while the ROS production rate is actually increasing. This would result in the marked increase in free radical protein damage associated with diabetes (16, 55). As such, ROS metabolism dysregulation could contribute to the excessive protein oxidation pathology associated with diabetes.

**Methionine cycle.** The proteomic data revealed upregulation of many enzymes of the methionine cycle. This may relate to the body wasting characteristics of uncontrolled type 1 diabetes and a need for increased capacity for the catabolism of choline derived from the breakdown of phospholipids (Fig. 4). The net effect of the observed upregulation of enzymes of the methionine cycle would support this catabolism. These findings suggest that this could be an important adaptation in the cachectic diabetic state. The observed upregulation of choline oxidase and betaine hydroxymethyltransferase would increase the transfer of methyl groups of choline into the methionine cycle. Upregulation of choline oxidoreductase (CO) and betaine-homocysteine S-methyltransferase (BHMT) shunts methyl groups into the methionine cycle. Upregulation of glycine-N-methyltransferase (GNMT) advances the synthesis of sarcosine, which must be converted to glycine with formation of a one-carbon THF derivative. In the presence of adequate THF, the cycle would net one serine and either two formate or two formyl-CoA per choline metabolized. Since serine is a gluconeogenic amino acid and serine dehydratase was found to be upregulated, metabolism of choline produced by wasting of tissues of the body supports the synthesis of glucose by this pathway. If THF should happen to be limiting, then the result would be one serine plus two molecules of formaldehyde per choline. This is of interest since formaldehyde is elevated in serum and urine of diabetic patients (14) and is suggested to play a role in retinopathy and vasculopathy that accompany diabetes (70). The source of formaldehyde at present is believed to be increased activity of the polyol pathway in peripheral nerves generating high concentrations of fructose that nonenzymatically glycosylate proteins that are broken down to formaldehyde (6, 62). It is of note that neither aldose reductase inhibitors (29, 54) nor sorbitol dehydrogenase inhibitors (56) have been shown to ameliorate all of the complications attributed to this pathway, except in concentrations high enough to prevent all flux through the sorbitol pathway with aldose reductase inhibitors (46). In addition, in this data set there was decreased sorbitol dehydrogenase as well as aldose reductase (lower-confidence identification), and both were highly significant ($P < 0.0053$). These data demonstrate another potential source of the formaldehyde in diabetes that warrants evaluation, and suggest a possible metabolic adaptation that has been unrecognized in the literature. There are studies that have demonstrated upregulation of elements of the methionine cycle (52, 66), but these studies were unable to evaluate the entire pathway concurrently and that is a particular strength of the present data.

**Protein differences not associated with canonnals.** The advantage of this study was evaluation of multiple proteins and associated metabolic pathways simultaneously in one diabetic setting. There were many previously unrecognized protein expression differences identified in this data set; specifically, there were 38 proteins that were significantly differentially regulated in the liver and 12 in the heart that have not previously been reported. Several of these proteins were discussed in the canonical pathway discussion above; however, some novel observations might warrant further investigation. As an example, the tripartite motif-containing protein 72 was altered in both heart and skeletal muscle with diabetes. This protein has no known function but contains zinc finger domains that may imply a transcription factor activity. 3-Oxo-5β-steroid 4-dehydrogenase is necessary in bile acid synthesis (31) and as such may have a role in the hypercholesterolemia that accompanies diabetes. D-Dopachrome tautomerase was downregulated in diabetes and is an inflammatory mediator important in T cell and macrophage migration inhibition (47, 63) with no established role in diabetes beyond that ketones are effective inhibitors of this enzyme (19). Liver calcium/calmodulin-dependent 3′,5′-cyclic nucleotide phosphodiesterase 1C (CDCDP) was significantly downregulated in diabetes, suggesting a modification of the calcium signaling/handling pathways in diabetic liver as described for the heart.

**Conclusions.** These data give an overall impression of the enzyme perturbations and global function of the liver during the diabetic state. An overview of metabolism of the diabetic liver is graphically depicted in Fig. 5, in an attempt to generate a more systems-oriented examination of the consequences of diabetes in the liver. The overall increase in ATP production capacity is used for glucose and urea synthesis utilizing the incomplete oxidation of fatty acids to ketone bodies and short-chain fatty acids likely due to a downregulation of CS. The increase in ROS generation associated with oxidative capacity is not balanced by an increase in ROS scavenging pathways. This may lead to an increase in ROS and protein oxidative damage. The processing of choline from phospholipids is enhanced and may fuel the generation of formaldehyde through the methionine cycle. Like most discovery approaches, more hypotheses were generated by these data than solutions to problems; however, this screening information is critical for the validation of existing models and provides novel insights for the formulation of new hypotheses on the biochemical adaptations associated with diabetes.
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