Thiol-Related Genes in Diabetic Complications

A Novel Protective Role for Endogenous Thioredoxin 2

Mingyu Liang, Jennifer L. Pietrusz

Objective—Our laboratory and others have found that deficiencies in cellular thiols may be importantly involved in the development of diabetic complications. However, the role for specific thiol-related genes in diabetic complications is unclear.

Methods and Results—We began the present study by systematically determining the expression level of 11 thiol-related genes in three tissues from rats with streptozotocin-induced diabetes. Several thiol-related genes were found to exhibit diabetes-associated, time-dependent differential expression. Thioredoxin 2, a mitochondrion-specific thioredoxin whose role in diabetes was unknown, was suppressed in the aorta from rats with two weeks of diabetes. When thioredoxin 2 expression in human umbilical vein endothelial cells was knocked-down by small interfering RNA, high-ambient glucose-elicited substantial injurious effects (n=5 to 9, P<0.05), including increases in cytosolic cytochrome c (by 2.2±0.6-fold), lipid peroxidation (by 40±8%), fibronectin expression (by 35±7%), and oxidized glutathione, and decreases in endothelial nitric oxide synthase expression (by 79±15%), basal accumulation of nitrite/nitrate (by 68±16%), total free thiols (by 42±8%), and glutathione (by 6±1%). In the absence of thioredoxin 2 knockdown, high-ambient glucose did not have significant effects on any of these measurements. The effect of thioredoxin 2 knockdown appeared to be associated with increases in glucose consumption and glucose transporter 1 expression.

Conclusion—These results provided the first expression profile of thiol-related genes in a model of diabetes and demonstrated a novel role for endogenous thioredoxin 2 in protecting cells against high ambient glucose. (Arterioscler Thromb Vasc Biol. 2007;27:77-83.)

Key Words: type 1 diabetes • functional genomics • vascular biology • nitric oxide • oxidative stress

Hyperglycemia is the main factor leading to the development of many chronic complications in diabetes mellitus. However, the mechanism by which hyperglycemia leads to cell and tissue injury remains to be fully understood. Among several pathways that have been associated with the development of diabetic complications, cellular thiols have emerged as playing an important role.

The thiol group is present in the amino acid cysteine, which can be found in many cellular peptides or proteins. Thiol groups can be oxidized to form disulfide. The oxidation of the thiol groups in several peptides or proteins is specifically coupled with the reduction of other molecules. These thiol antioxidative peptides or proteins, such as glutathione, thioredoxin, and so on, play an important role in the cellular antioxidative defense as well as the regulation of cellular functions involving the thiol–disulfide exchange. In addition to the genes directly encoding thiol antioxidative proteins, several other genes also contribute to the regulation of thio-related mechanisms by encoding proteins that are involved in the action and metabolism, such as synthesis and recycling, of the thiol antioxidative peptides or proteins.

Disturbances of thiol-related mechanisms have been observed in diabetes. For instance, plasma levels of protein-bound thiols were found to be lower in type 2 diabetic patients compared with their controls. Consistent with an important role of cellular thiols in the development of diabetic complications, treatment with α-lipoic acid, a disulfide-containing compound that can be reduced to form dihydrolipoic acid, has been shown to ameliorate diabetic complications. Moreover, several thiol related genes have been found to be differentially expressed between diabetic subjects and their controls, or between cells exposed to high and normal levels of ambient glucose.

A major gap in the knowledge regarding the role of cellular thiols in diabetic complications is the lack of a systematic view of thiol-related mechanisms and genes during the progression of diabetic complications. Furthermore, the specific role of many thiol related genes, particularly the newly discovered members of this gene family, in diabetic complications remains poorly understood. The goal of the present study was to begin to address these questions using the combination of a targeted profiling approach and an in-depth exploration of new functional roles of selected genes suggested by the targeted profiling.
Methods and Materials

Streptozocin Model of Diabetes
Male Sprague-Dawley rats (Harlan, Indianapolis, Ind) weighing 250 to 300 grams were treated with streptozocin (55 mg/kg body weight) to produce type 1 diabetes as we described previously. Insulin treatment was started 24 hours after the streptozocin injection in one half of the streptozocin-treated rats that were confirmed to have become diabetic. Insulin treatment was given in the form of Linplant, a sustained release insulin implant (LinShin Canada, Scarborough, Ontario). The rats were anesthetized with isoflurane and one and one-half pieces of Linplant were implanted subcutaneously between the shoulder blades following the manufacturer’s instructions. The implantation was repeated after six weeks for the long-term treatment group. Blood glucose and body weight were monitored throughout the experiment and summarized in supplemental Figure I (available online at http://atvb.ahajournals.org). Rats were euthanized 2 or 12 weeks after the streptozocin treatment, and the abdominal aorta, the renal cortex, and the retina were harvested for further analysis. The time points selected represented initial and established stages of diabetes, and the tissues analyzed were known to be the main targets of diabetic complications. In total, 35 rats divided into 6 groups (control, diabetes, and insulin-treatment, for 2 or 12 weeks) were analyzed.

Real-Time Polymerase Chain Reaction
Total RNA was extracted using TRizol (Invitrogen, Carlsbad, Calif). Quantitative real-time polymerase chain reaction (PCR) analysis using the Taqman method and the ABI Prism 7900HT system (Applied Biosystems, Foster City, Calif) was performed as we described previously.9 The sequences of the primers and probes are shown in supplemental Table I; 18S rRNA was measured in parallel with each performed in duplicate or triplicate cell preparations. Data shown are mean±SEM. Student t test or analysis of variance was performed when appropriate. P<0.05 was considered significant.

Results
Expression Profile of Thiol-Related Genes in Streptozocin Diabetes
As the first step for investigating the role of thiol related genes in diabetic complications, we performed a targeted profiling of the mRNA expression level of thiol related genes in rats with streptozocin-induced diabetes. We used the Taqman real-time PCR technique to analyze 11 genes in the abdominal aorta, the renal cortex, and the retina from 35 rats (control, diabetes, or insulin treatment, at 2 or 12 weeks after the streptozocin injection, n=4 to 7). The study involved nearly 3000 Taqman real-time PCR reactions. A gene was considered exhibiting diabetes-associated differential expression if the differential expression between control and diabetes groups was significantly attenuated by the insulin treatment.

As shown in Figure 1, thiol-related genes exhibited robust differential expression associated with streptozocin diabetes. In the aorta, thioredoxin 2, glutathione reductase, and thioredoxin reductase were suppressed by 2 weeks of diabetes, whereas thioredoxin interacting protein, known to inhibit thioredoxin,14 and thioredoxin 2 reductase were upregulated by 12 weeks of diabetes. In the renal cortex, glutathione peroxidase 1, glutathione reductase, and glucose-6-phosphate dehydrogenase were upregulated by 2 weeks of diabetes. By 12 weeks, most of the adaptational response in the renal cortex was lost, and thioredoxin interacting protein was upregulated. In the retina, thioredoxin interacting protein was upregulated in diabetic rats at both 2 and 12 weeks. The complete set of the real-time PCR data are shown in supplemental Table II.
Endogenous Thioredoxin 2 Protects Cells Against High-Ambient Glucose

We decided to further investigate the functional role of thioredoxin 2. As shown in Figure 1 and supplemental Table II, thioredoxin 2 (Trx-2) mRNA was suppressed by 2 weeks of streptozotocin diabetes in the aorta, which was normalized by the insulin treatment. Similar changes occurred at the protein level (Figure 2C and 2D). Thioredoxin 2 is a thioredoxin-like protein specifically localized to mitochondria and has been shown to participate in the regulation of apoptosis, oxidative stress, and mitochondrial bioenergetics. We hypothesized that endogenous thioredoxin 2 was...
Thioredoxin 2 siRNA (si-Trx-2) Exacerbated the Injurious Effect of High-Ambient Glucose on HUVECs

<table>
<thead>
<tr>
<th>SiRNA, Glucose Level</th>
<th>si-Luc, Normal</th>
<th>si-Luc, High</th>
<th>si-Trx-2, Normal</th>
<th>si-Trx-2, High</th>
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<td>Total free thiol, nmol/mg cell protein</td>
<td>230±25</td>
<td>190±17</td>
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<td>Glutathione, nmol/mg cell protein</td>
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<td>Oxidized glutathione, nmol/mg cell protein</td>
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<td>TBARS, pmol/mg cell protein</td>
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<td>8-isoprostane (cell), pg/mg cell protein</td>
<td>222.8±40.8</td>
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<td>8-isoprostane (medium), pg/mg cell protein</td>
<td>103.7±5.9</td>
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<td>Fibrinectin, % of siLuc/normal glucose</td>
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<td>Cytosolic cytochrome c, % of siLuc/normal glucose</td>
<td>100±11</td>
<td>110±19</td>
<td>182±12</td>
<td>401±109</td>
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</table>

*Exhibiting significant difference between the effect of high glucose after si-Trx-2 treatment and that after si-Luc (n=5 to 9). Other differences are described in the text.

High-ambient glucose elicited significant injurious effects on HUVEC after thioredoxin 2 was knocked down. As shown in the Table, after thioredoxin 2 knockdown (si-Trx-2), high-ambient glucose significantly decreased the level of total cellular free thiols by 42±8% and glutathione by 6±1% and increased the level of thiobarbituric acid reactive substance by 40±8%, fibronectin expression by 35±7%, and cytosolic cytochrome c, an index of apoptosis, by 2.2±0.6-fold. After thioredoxin 2 knockdown, the level of oxidized glutathione was undetectable when the cells were cultured with normal glucose, but became readily detectable when exposed to high glucose. After the control siRNA (si-Luc) treatment, high-ambient glucose did not have significant effects on any of these measurements except there appeared to be decreases in total free thiols and oxidized glutathione, neither of which reached statistical significance. Levels of 8-isoprostane in the cells or the culture medium were not altered by high glucose after either control siRNA (si-Luc) or thioredoxin 2 siRNA (si-Trx-2). Treatment with si-Trx-2, independent of high glucose, significantly increased cytosolic levels of cytochrome c, which was consistent with the anti-apoptotic effect of Trx-2 previously reported.16–19 Paradoxically, si-Trx-2 decreased cellular levels of 8-isoprostane.

Nitric oxide generated by endothelial nitric oxide synthase (eNOS) is important for normal endothelial function. The knockdown of thioredoxin 2 permitted high-ambient glucose to substantially suppress eNOS expression by 79±15% (Figure 3A), which was accompanied by a 68±16% decrease in the basal accumulation of nitric oxide metabolites, nitrite and nitrate, in the culture medium (Figure 3B). It should be noted that thioredoxin 2 knockdown per se, independent of high glucose, decreased the eNOS expression level by ~30%. The ability of thioredoxin 2 to protect eNOS expression from high glucose was not limited to HUVECs. In human dermal microvascular endothelial cells, thioredoxin 2 siRNA reduced...
In human aortic endothelial cells, thioredoxin 2 contributes to the injurious effect of high ambient glucose. Interestingly, byproducts is believed to be the main mechanism mediating ways in the cell and the subsequent generation of toxic byproducts. Increased glucose flux through its various metabolic pathways in the cell and the subsequent generation of toxic byproducts is believed to be the main mechanism mediating ways in the cell and the subsequent generation of toxic byproducts. High-ambient glucose increased glucose consumption and stimulated the downregulation of glucose transporter 1 induced by high ambient glucose, compared with L-glucose control, was minimal (Figure 4A). Such increase became significant (79±15%) only after thioredoxin 2 was knocked down by siRNA (Figure 4A). Similar results were obtained when glucose consumption was normalized by cell number. Concomitantly, high-ambient glucose significantly downregulated the expression level of glucose transporter 1, which is the main glucose transporter in many endothelial cell types, by 62±14%. Thioredoxin 2 knockdown significantly attenuated the downregulation of glucose transporter 1 induced by high ambient glucose (Figure 4B). Some cell types can deplete glucose in the culture medium containing a normal level of glucose over a few days, confounding the interpretation of the effect of high glucose. That did not appear to be the case for HUVECs because ≈60% of the glucose remained in the medium after 48 hours if the cells were 80% to 95% confluent and 1 mL of the regular medium (containing 5 mmol/L D-glucose) was used for every 5 cm² of growth area.

**Discussion**

The present study has made 2 major contributions. It provided the first systematic expression profile of the thiol-related genes in a model of diabetes, and demonstrated a novel protective role for thioredoxin 2 against high ambient glucose.

We and others have examined the global gene expression profile in various diabetic models mainly using the DNA microarray technology. The present study took a different approach of targeted profiling focused on a specific group of genes, namely, the thiol-related genes. The targeted profiling approach allowed us to take advantage of the specificity, sensitivity, and high-throughput capability of the Taqman real-time PCR technique, the putative gold standard in mRNA quantification. The genes we examined were a broad and substantive, although not exhaustive, representation of thiol-related genes. The result was a clear view of the robust response of thiol-related genes to the streptozotocin model of diabetes as shown in Figure 1. One should be cautious in extrapolating mRNA levels, examined in the present study, to protein or enzymatic activity levels. Nevertheless, the robust response of thiol-related genes observed in the present study supports the presence of an important role for cellular thiols in the development of diabetic complications that has been suggested by previous pharmacological and biochemical analyses.

The response of the thiol-related genes to the streptozotocin diabetes was largely tissue specific. Suppression of thiol anti-oxidative genes was observed only in the aorta after 2 weeks of diabetes. The renal cortex appeared to attempt to adapt by upregulating antioxidative thiol genes at 2 weeks, consistent with our previous findings. The adaptation appeared to diminish by 12 weeks. The only change common to all tissues examined was the up-regulation of thioredoxin-containing protein at 12 weeks of diabetes. Thioredoxin-containing protein has been shown to be upregulated in models of hyperglycemia in vivo and in vitro, and may contribute to hyperglycemia-induced oxidative stress and other damages. The tissue specificity in the response of thiol genes seen in the present study was consistent with the finding in our previous study of gene expression profiles on the near-genome scale. While the precise mechanism underlying the tissue specificity is not clear, the data indicate the presence of distinct adaptational or maladaptational responses to streptozotocin diabetes in different organs, possibly in part because of the difference in their cell type compositions.

Mammalian thioredoxin 2 is a recently discovered member of the family of thiol-related genes and has been characterized primarily to a limited extent. Mammalian thioredoxin 2 contains the conserved thioredoxin-active site, Trp-Cys-Gly-Pro-Cys, and a mitochondrial signal sequence at the N-terminus.
termination that explains its specific localization to mitochondria. Thioredoxin 1 is usually localized to the cytosol and the nucleus. Overexpression of human thioredoxin 2 has been shown to increase mitochondrial membrane potential in the human cell line HEK-293, and protect against oxidant-induced apoptosis in 143B human osteosarcoma cells. Similarly, overexpression of chicken thioredoxin 2 was shown to confer resistance to mitochondria-dependent apoptosis in a chicken B-cell line. In human umbilical vein endothelial cells, thioredoxin 2 was shown to interact with apoptosis signal-regulating kinase 1 in mitochondria and suppress apoptosis induced by the kinase. Homozygous loss of thioredoxin 2 in mice was lethal, and the mutant embryos showed abnormal development and massive apoptosis.

The present study examined for the first time the role of thioredoxin 2 in the effect of high-ambient glucose. Although overexpression or knockdown studies were useful for identifying the potential functional role of a gene, the alteration of thioredoxin 2 in physiological or pathophysiological conditions in vivo may be more subtle as in the case of the aorta from rats with streptozotocin diabetes shown in the present study. It is therefore particularly relevant that a partial knockdown of thioredoxin 2 achieved by RNA interference, mimicking the downregulation in the aorta of the streptozotocin rats, was sufficient to permit or exacerbate the injurious effects of high-ambient glucose on human endothelial cells as shown in the present study. Overexpression of mouse mitochondrial thioredoxin 2, thioredoxin reductase 2, or both in mouse Neuro2A cells, monkey COS-7 cells, or human Hela cells did not confer added resistance to several pro-oxidant or nonoxidant apoptotic stimuli. These data together suggest that thioredoxin 2 might have to be present at levels above a critical threshold in order to protect cells, while excessive thioredoxin 2 may or may not have additional benefits depending on the specific cellular environment.

A few previous studies have reported increases of apoptosis or fibronectin expression in HUVECs treated with high-ambient glucose, although the evidence is not nearly as abundant as in glomerular mesangial cells. It was reported that high-ambient glucose, compared with normal glucose, increased DNA fragmentation, an index of apoptosis, in HUVECs. The difference, however, was not significant at either 48 or 72 hours when the cells treated with high glucose were compared with the mannose control. As shown in the present study, the increase of glucose consumption by HUVECs treated with high-ambient glucose for 48 hours was small, if any, which is consistent with the absence of substantial injurious effects. Variability seems to exist between different isolations of HUVECs, which may explain some of the injurious effects seen in previous studies.

High-ambient glucose has been shown to either decrease or increase the expression of eNOS and nitric oxide production depending on the experimental setting. Nitric oxide generated by eNOS is importantly involved in a wide variety of normal cellular function. The suppression of endothelial nitric oxide synthase by high ambient glucose, possibly involving protein kinase C, might contribute to endothelial injuries that are often observed in diabetic complications. The present study suggested that the endogenous thioredoxin 2 could buffer the suppressive effect of high-ambient glucose on eNOS.

A full investigation of the mechanisms underlying the protective role of thioredoxin 2 against high ambient glucose is beyond the scope of the present study. Nevertheless, initial efforts to identify such mechanisms have indicated an interesting role for thioredoxin 2 in the regulation of glucose uptake and/or metabolism. Glucose transporter-1 (Glut1) is the main glucose transporter found in several endothelial cell types. The data from the present study was consistent with previous findings that high-ambient glucose decreased the expression level of Glut1 protein in bovine aortic endothelial cells and bovine retinal endothelial cells. The reduction of Glut1 expression, perhaps with additional contributions from changes in intracellular glucose metabolism, might explain why glucose consumption did not increase substantially when HUVECs were treated with high-ambient glucose. Thioredoxin 2 appeared to contribute to this apparent negative feedback response because knockdown of thioredoxin 2 attenuated the reduction of Glut1 expression and increased glucose consumption in response to high-ambient glucose.

It remains to be investigated how thioredoxin 2 contributes to the regulation of glucose metabolism and Glut1 expression. A tangible link between thioredoxin 2 and glucose metabolism is the effect of thioredoxin 2 on mitochondrial membrane potential. It is possible that thioredoxin 2 affects glucose metabolism through effects on the mitochondrial bioenergetics. It is also possible that thioredoxin 2 influences the oxidative stress caused by the initial exposure to high-ambient glucose, which in turn elicits the apparent negative feedback response described.

In addition to further investigating the mechanism underlying the protective role of thioredoxin 2 against high-ambient glucose, future studies should further explore the significance of thioredoxin 2 in the development of diabetic complications in vivo. For example, it would be interesting to determine if maintaining or increasing the level of thioredoxin 2 expression could ameliorate diabetic complications. Detailed studies of the functional role in diabetic complications of other thiol-related genes identified by the present targeted profiling study will also be valuable.

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Disclosures
None.

References
Liang and Pietrusz

Thiol-Related Genes in Diabetic Complications

83


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**Thiol related genes in diabetic complications: A novel protective role for endogenous thioredoxin 2**

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Supplemental Figure 1. Characteristics of the rats with the streptozotocin model of diabetes. Rats were treated with a control injection, streptozotocin (STZ), or streptozotocin followed by insulin implants as described in Methods (n = 4 to 7). Blood glucose (A) and body weight (B) were monitored throughout the experiment, and right kidney weight (C) were obtained at the time of tissue harvest. *, P<0.05 vs. control or insulin groups.
### Supplemental Table 1. Primer and probe sequences for real-time PCR

<table>
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<th>Gene</th>
<th>GenBank Accession</th>
<th>Primer/probe sequences</th>
<th>Amplicon position</th>
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</table>
| GPX1  | X07365            | Forward: TGAGAAGTGCAGGTGAATGG  
Reverse: GTGCTGGCAAGGCATTC  
Probe: FAM-AAAGGCTCACCCGCTCTTACCTTCCTC-TAMRA | 681-751 |
| PRDX6 | NM053576          | Forward: GCAAGAAATACCTCCGTATTACG  
Reverse: GCTGGTCGAGGACTTGCA  
Probe: FAM-CCCAGCTTTAAGTCTGCGGGAACG-TAMRA | 674-745 |
| GSR   | NM053906          | Forward: CCGGAAACTCGCCATAGA  
Reverse: TGAAGACCAGCTGGGTATGTT  
Probe: FAM-AGTCCAACCTCCGTTCCCTAGCAGAG-TAMRA | 1029-1108 |
| TrxR  | NM031614          | Forward: AACGTGGGCTGCATACCTAAA  
Reverse: TAGTGGCGTGAAGTCTTCAAGCCTT | 314-390 |
| TrxR2 | NM022584          | Forward: AGCATATAGAGGTTTACCATACCTATTACA  
Reverse: GAAGTGGCAGCCTGCTTTCAGAGCTT  
Probe: FAM-CCCGAGCTCGCAGCTGGAACCTCTTGG-TAMRA | 1298-1428 |
| Trx   | X14878            | Forward: TCCAATGTGGTTCTTCTTTTGAAGTAA  
Reverse: GGCAATGTGCGTCTCTTACG  
Probe: FAM-CGTTGGACTCCAGGATGTTCC-TAMRA | 208-284 |
| Trx-2 | U73525            | Forward: AGCAGGGGAAGGTGCTTGGG  
Reverse: CGAGACACCCTCAGCTTACG  
Probe: FAM-CAAGGAGCAGATCCACACAGACCT-TAMRA | 375-450 |
| Txnip | NM001008767       | Forward: TGCAAACAGACCTTTGCAGTCTATT  
Reverse: TCTCATGATCACCAGACAGACAGCCT | 377-466 |
| G6PD  | NM017006          | Forward: CCATCTGCACCTTCAGCTTCC  
Reverse: CTATGCTCAGCTTGTTCCACCA  
Probe: FAM-CCCTTTACCTACCTTACCCCTACCTATTGGACTTGG-TAMRA | 1606-1714 |
| GSS   | NM012962          | Forward: CGCCGGAAGATTGAAGATGTC  
Reverse: GTAGCCTACCTGAGCTAAGACA  
Probe: FAM-GCAACTCTCCGTTCCCTCATACAACAGGCCT-TAMRA | 750-854 |
| GCL   | NM012815          | Forward: ACTCAGACCGTGTGAAAGACCA  
Reverse: CAGATTCATTTGCTGAAAGGACGCTT  
Probe: FAM-CCTACAGCTTCCAGGTTCCACACAGCTG-TAMRA | 2023-2107 |
<p>| Trx-2 | NM 012473         | Forward: CAGCCCAACAGGTTTCTGTAAGGTC | 868-943 |</p>
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Abbreviations: GPX1 = glutathione peroxidase 1, PRDX6 = peroxiredoxin 6, GSR = glutathione reductase, TrxR = thioredoxin reductase, TrxR2 = thioredoxin reductase 2, Trx = thioredoxin, Trx-2 = thioredoxin 2, Txnip = thioredoxin interacting protein, G6PD = glucose-6-phosphate dehydrogenase, GSS = glutathione synthetase, GCL = glutamate cysteine ligase, FN = fibronectin 1.
Supplemental Table 2. Effect of streptozotocin diabetes on the mRNA expression level of thiol related genes.

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<thead>
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Note: The mRNA expression levels were analyzed using Taqman real-time PCR as described in Methods and Results. Expression levels are shown here as folds relative to the control group for each gene in each tissue at each time point. Comparisons between tissues or time points were not made because only samples from the same tissue and the same time point were analyzed in parallel on a 384-well PCR plate. Values shown in bold and larger font indicated significant differences between the streptozotocin (STZ) diabetic group and both the control and the insulin-treated groups. See Supplemental Table 1 for gene name abbreviations.