Akt activation and augmented fibronectin production in hyperhexosemia

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In diabetes, several pathogenetic mechanisms secondary to hyperglycemia may cause activation of signaling molecules, leading to aberrant synthesis of macromolecules and subsequent structural changes. The established mechanisms include protein kinase C (PKC) activation, augmented polyol pathway, and advanced glycatated end product formation, among others (25, 36). Increased mitochondrial superoxide production may influence such pathways (4). These changes ultimately cause increased extracellular matrix (ECM) protein production and their impaired degradation, as well as apoptosis of vascular cells, damaging the microvasculatures in the retina, heart, and kidney.

Our group and others (6, 7, 14, 33) have demonstrated upregulation of fibronectin (FN) in several organs in diabetes and in endothelial cells exposed to glucose. FN is a highly specialized glycoprotein of 250 kDa. FN interacts with other matrix proteins and provides outside-in signaling via cell surface integrins (3, 18, 43). Our group (7, 20) has further demonstrated that glucose-induced FN production and that of its splice variant, EDB + FN, is regulated by protein kinase B (PKB, also known as Akt). In this study, we investigated the role of Akt1 in ECM protein production in the organs affected by chronic diabetic complications. We studied Akt1/PKBα knockout mice and wild-type control littermates. To avoid confounding effects of systemic insulin, we used 30% galactose feeding to induce hyperhexosemia for 8 wk starting at 6 wk of age. We investigated FN mRNA, EDB + FN mRNA, and transforming growth factor (TGF)-β mRNA expression, Akt phosphorylation, Akt kinase activity, and NF-κB and AP-1 activation in the retina, heart, and kidney. Renal and cardiac tissues were histologically examined. Galactose feeding caused significant upregulation of FN, EDB + FN, and TGF-β in all tissues. FN protein levels paralleled mRNA. Such upregulation were prevented in Akt1-deficient galactose-fed mice. Galactose feeding caused ECM protein deposition in the glomeruli and in the myocardium, which was prevented in the Akt knockout mice. NF-κB and AP-1 activation was pronounced in galactose-fed wild-type mice and prevented in the galactose-fed Akt1/PKBα-deficient group. In the retina and kidney, Ser473 was the predominant site for Akt phosphorylation, whereas in the heart it was Thr308. Parallel experiment in streptozotocin-induced diabetic animals showed similar results. The data from this study indicate that hyperhexosemia-induced Akt/PKBα activation may be an important mechanism leading to NF-κB and AP-1 activation and increased ECM protein synthesis in the organs affected by chronic diabetic complications.

Table 1. Oligonucleotide sequences for real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5′ → 3′) Forward/Reverse</th>
<th>PCR Temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>CTCCTAGGCCAACACAGTGCC-&lt;br&gt; CATGCTACCTCCTGTCTGCTG</td>
<td>Denaturation, 95°C-0 s; annealing, 58°C-5 s; extension, 72°C-9 s; signal acquisition, 83°C-1 s</td>
</tr>
<tr>
<td>FN</td>
<td>CCGTAGGACTCTTGAATCTCCT-&lt;br&gt; GATACATGACCCCTTCATTG</td>
<td>Denaturation, 95°C-0 s; annealing, 55°C-5 s; extension, 72°C-10 s; signal acquisition, 80°C-1 s</td>
</tr>
<tr>
<td>EDB FN</td>
<td>AGTTAGTGGCGAGGAGAA-&lt;br&gt; CCGCCATTAATGAGAGTGAT</td>
<td>Denaturation, 95°C-0 s; annealing, 55°C-5 s; extension, 72°C-8 s; signal acquisition, 81°C-1 s</td>
</tr>
<tr>
<td>TGF-β</td>
<td>GAGAGCCCTGGGATACCAACTACTCTG-&lt;br&gt; GTGTTGTTCCAGGCCCTGAGTTGAGTAG</td>
<td>Denaturation, 95°C-0 s; annealing, 55°C-5 s; extension, 72°C-25 s; signal acquisition, 84°C-1 s</td>
</tr>
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Initial denaturation was carried out at 95°C for 1 min. Ramp rate for all PCR phases was 20°C/s.

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tions in normal and disease states. All Akt isoforms contain a pleckstrin homology domain, a catalytic domain, and a putative regulatory domain. Akt is activated by phosphorylation, predominantly through a PI3K-dependent mechanism. Stimulus for such activation may include growth factors, insulin, and hormones. Via activation of transcription factors, Akt may modulate expression of several genes and influence a large number of signaling pathways, which may affect vital cellular functions such as survival, proliferation, differentiation, migration, and apoptosis (47). Our group (7) has previously demonstrated that in the endothelial cells, short-term glucose exposure may cause NF-κB- and AP-1-dependent upregulation of FN expression. Our group (22) also has demonstrated that such upregulation, at least in part, is dependent on Akt1 and TGF-β. However, it is not known whether a similar activation of Akt is present in the tissues affected in chronic diabetes and whether such activation leads to increased FN production via transcription factors. Akt1 is the predominant isoform in most tissues (47). Furthermore, Akt1 is the specific isoform that modulates adaptive angiogenesis (1). Hence, we focused on Akt1 in this study. Because it is difficult to sustain long-term diabetic animals without exogenous insulin, given that Akt activation is a major pathway in insulin signaling, we used galactose-fed mice as a model for majority of these studies. Use of this model allows the avoidance of possible conflicting results of insulin administration. Since there are no specific inhibitors of Akt, we

Table 2. Clinical monitoring of animals

<table>
<thead>
<tr>
<th></th>
<th>Akt+/+</th>
<th>Akt+/+ G</th>
<th>Akt−/−</th>
<th>Akt−/− G</th>
<th>Akt−/− D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>20.3±1.8</td>
<td>20.9±2.5</td>
<td>17.94±2.3</td>
<td>16.8±1.3</td>
<td>15.1±1.2*</td>
</tr>
<tr>
<td>Blood hexose, mM</td>
<td>8.1±2.0</td>
<td>11.5±2.5*</td>
<td>8.8±1.1</td>
<td>10.2±2.5</td>
<td>25.5±2.6*</td>
</tr>
<tr>
<td>GHb, %</td>
<td>7±0.5</td>
<td>13±1.4*</td>
<td>8±0.8</td>
<td>11.6±1.2*</td>
<td>12±2.4*</td>
</tr>
</tbody>
</table>

Values are means ± SD of 8 animals in each group (G, group on 30% galactose diet; D, group with STZ-induced diabetes). GHb, glycated hemoglobin. *P < 0.05 compared with Akt+/+.

Fig. 1. Quantitative RT-PCR analysis of fibronectin (FN), its splice variant (EDB FN), and transforming growth factor (TGF)-β mRNA expression. Hyperhexosemia-induced upregulation of FN, EDB FN, and TGF-β mRNA in cardiac, renal, and retinal tissues (relative to Akt wild type) was prevented in the Akt knockout animals. WT, wild type; Neg, Akt1 knockout; G, 30% galactose diet. *P < 0.05 compared with Akt WT; n = 8/treatment.
used Akt1 knockout mice for such experiments. Galactose-fed animals are a well-studied model for chronic diabetic complications, and biochemical and structural changes of chronic diabetic complications have been demonstrated in this model. Early and late lesions of diabetic retinopathy such as basement membrane thickening, pericyte loss, microaneurysms, acellular capillary formation, and angiogenesis have been characterized in these animals (2, 26). These animals demonstrate contractile abnormalities in the heart (9). In the kidneys, galactose feeding results in albuminuria, increased extracellular matrix protein synthesis, basement membrane thickening, and mesangial matrix protein expansion (5, 10). We have further confirmed the results in well-established streptozotocin-induced diabetic mice.

MATERIALS AND METHODS

Reagents. Unless otherwise stated, all chemicals were of reagent-grade quality and were purchased from Sigma Chemical (Oakville, ON, Canada).

Animals. Akt1/PKB knockout (Akt1−/−) mice (kindly provided by Dr. M. J. Birnbaum, University of Pennsylvania, Philadelphia, PA) and wild-type (WT) controls of a similar background (C57BL6; Jackson Laboratory, Bar Harbor, ME) were obtained. The experiments were performed in accordance with regulations specified by the Canadian Council on Animal Care. The University of Western Ontario Animal Care and Veterinary services approved all experiments. Beginning at 6 wk of age, Akt1−/− and WT animals were divided into two groups and fed with either a standard rodent diet containing 19% protein or a diet enriched with 30% galactose (Test Diet, Richmond, IN). Feeding of the groups was continued for 8 wk. During this period, the animals were monitored for changes in blood glucose, body weight, and glycated hemoglobin (GHB) levels. Parallel C57BL6 mice (WT) with or without chemically induced diabetes were investigated to assess Akt activation. Diabetes was induced by intraperitoneal injections (×2 on alternate days) of 50 mg/kg streptozotocin (STZ) in 50 mM citrate buffer (pH 5.6) and monitored for 4 wk. The same volume of vehicle was injected in the control animals. All diabetic animals were treated with small doses of insulin (0.5 units) as necessary to prevent ketoacidosis based on urine analysis (Uriscan Gluketo; Yeong Dong, Seoul, Korea). At the end of the follow-up, the animals were killed, and their organs were harvested and snap frozen. Small amounts of kidney and heart tissues were fixed in 10% neutral buffered formalin for histological analysis.

Protein analysis. Total mouse tissues (retina, heart, and kidney) were homogenized and isolated using complete RIPA buffer as previously described (41): 50 mM Tris·HCl, adjusted to pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS. Proteins were quantified using a Bio-Rad protein assay procedure (Bio-Rad Laboratories, Mississauga, ON, Canada). Akt/PKB protein activation was assessed using biological Akt and phospho-Akt (Ser473 and Thr308) antibodies (1:1,000; New England BioLabs, Pickering, ON, Canada). FN protein was assessed using anti-fibronectin polyclonal antibody (1:2,000; DakoCytomation California, Carpinteria, CA). Anti-rabbit immunoglobulin G conjugated with horseradish peroxidase was purchased from New England BioLabs (Ipswich, MA). Mouse monoclonal β-actin antibody and anti-mouse immunoglobulin G conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The detections were performed using the ECL Plus kit (Amersham Pharmacia Biotech, Piscataway, NJ). The blots were quantified by densitometry (20, 40, 42). The data are expressed as FN/β-actin or p-Akt/Akt ratios.

Akt kinase assay. The tissues were subjected to Akt kinase assay with the use of an Akt kinase assay kit (Cell Signaling, Beverly, MA) in accordance with the manufacturer’s recommendations. Briefly, kinase buffer supplemented with 200 μM ATP was added to the protein samples. The reaction was terminated with 20 μl of 3× SDS sample buffer. The samples were then centrifuged for 30 s, heated to 95–100°C for 2–5 min, and loaded on SDS-PAGE gel (12–15%) for sample analysis by Western blotting.

Real-time RT-PCR. RNA was extracted and subjected to cDNA synthesis as described (6, 7). Real-time quantitative RT-PCR for FN, EDB, FN, and TGF-β mRNA expression was carried out in LightCycler (Roche Diagnostics Canada, QC, Canada) using SYBR green I. PCR reactions were performed as previously described (6, 7). The reaction mixture consisted of 10 μl of ReadyMix SYBR (Sigma-
such ECM protein deposition was not seen in Akt1 knockout mice fed galactose (F). Magnification is the same for chow (D). Galactose feeding also caused interstitial ECM protein deposition (arrows) in the myocardium in WT mice (C) compared with mice on regular chow (B). Such mesangial expansion (arrows) in WT mice (A). Such mesangial expansion was prevented in Akt1 knockout mice fed galactose (C). Galactose feeding also caused interstitial ECM protein deposition (arrows) in the myocardium in WT mice (E) compared with mice on regular chow (D). Such ECM protein deposition was not seen in Akt1 knockout mice fed galactose (F). Magnification is the same for A–F.

RESULTS

Clinical monitoring. Akt−/− animals with or without galactose feeding had slightly lower body weight than the WT controls; however, this change was not statistically significant. Galactose feeding and diabetes caused increased reducing sugar levels as well as GHb levels (Table 2).

Hyperhexosemia induces FN, EDB+FN, and TGF-β mRNA upregulation via Akt/PKB activation. Analysis of retinal, renal, and cardiac tissues in the galactose-fed animals revealed significant upregulation of FN and EDB+FN mRNA in all three organs. The levels of upregulation were, however, higher in the retina and kidney compared with the heart. TGF-β mRNA was also similarly upregulated. Akt1 knockout had no effect on basal FN mRNA expression. However, Akt1 deficiency prevented galactose feeding-induced FN, EDB+FN, and TGF-β upregulation (Fig. 1).

Hyperhexosemia induces FN protein expression via Akt/PKB activation. We then investigated protein levels. Because the amount of available material was limited, we performed such analysis only on FN. Galactose feeding resulted in increased protein expression in kidney, heart, and retina. Akt1 gene knockout prevented the upregulation of FN protein in the galactose-fed mice (Fig. 2).

Hyperhexosemia causes ECM protein deposition via Akt/PKB activation. To further explore ECM protein deposition, we performed histological analyses on heart and kidneys, since more tissues were available from these organs. Galactose feeding caused increased PAS positivity in the mesangium of WT mice, indicative of an increase in ECM proteins, compared with similar mice on regular chow. Such changes were not seen in the Akt1−/− mice (Fig. 3). Furthermore, myocardium of the

Electrophoretic mobility shift assay. Nuclear extracts were prepared as described previously by our group (6, 7). Briefly, rapid detection of octamer binding proteins with “mini-extracts” was prepared from cells. The cells were washed, resuspended in PBS, an centrifuged, and the pellet was resuspended in 0.4 ml of cold buffer. The cells were allowed to swell on ice for 15 min. Twenty-five microliters of a 10% IGEPAL CA-630 were added. The homogenate was centrifuged, and the nuclear pellet was resuspended in ice-cold buffer. The samples were centrifuged again, and the supernatant was frozen at −70°C. The protein concentrations were measured. NF-κB and AP-1 consensus oligonucleotide DNA probes (Promega, Madison, WI) were prepared by end labeling with [γ-32P]ATP (Amersham, Quebec, Canada) using T4 polynucleotide kinase. The probes were purified by ethanol precipitation and resuspended in 10 mM Tris and 1 mM EDTA (pH 7.6). Five micrograms of nuclear proteins were incubated with 100,000 cpm of 32P-labeled consensus oligonucleotides. Protein-DNA complexes were resolved on a standard 6% (NF-κB) and 4% (AP-1) nondenaturing polyacrylamide gel and subjected to autoradiography. Specificity was confirmed by incubation with 100-fold unlabeled oligo. The blots were quantified by densitometry.

Histological analysis. Formalin fixed tissues were embedded in paraffin and sectioned at 5-μm thickness on positively charged slides. They were stained with hematoxylin-eosin, periodic acid-Schiff (PAS), and trichrome stains. The slides were examined by one of us blinded to the source of the specimen.

Statistical analysis. Results are means ± SE and were analyzed using ANOVA followed by the Bonferroni/Dunn test for multiple comparisons. Significance was defined at P < 0.05.

Fig. 3. Histological analysis of renal (top images, periodic acid-Schiff stain) and cardiac tissues (bottom images, trichrome stain). Galactose feeding caused renal mesangial expansion (arrows) in WT mice (B) compared with mice on regular chow (A). Such mesangial expansion was prevented in Akt1 knockout mice fed galactose (C). Galactose feeding also caused interstitial ECM protein deposition (arrows) in the myocardium in WT mice (E) compared with mice on regular chow (D). Such ECM protein deposition was not seen in Akt1 knockout mice fed galactose (F). Magnification is the same for A–F.

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WT mice fed galactose showed a mild increase in interstitial ECM protein deposition by trichome stain, which was also prevented in the Akt1−/− mice (Fig. 3).

Hyperhexosemia-induced Akt mediates transcription factor activation. Our group has previously demonstrated that glucose-induced FN expression in the endothelial cells and in the organs of diabetic rats is mediated via NF-κB and AP-1 (6, 7). Hence, we performed electrophoretic mobility shift assays (EMSA) in the mouse tissues. EMSA results revealed activation of NF-κB and AP-1 in the WT galactose-fed mice. Ablation of Akt1 prevented galactose-mediated NF-κB and AP-1 activation (Fig. 4). These findings suggest that both these transcription factors may be involved in Akt signaling pathway.

Activation of Akt in tissues. We also examined Akt activation in various organs of WT mice with or without galactose diet. Interestingly, the pattern of Akt activation varied in different organs. Ser473 Akt phosphorylation was increased in the kidney and retina of galactose-fed mice, but in the heart it showed a trend toward reduction. On the other hand, cardiac tissues of galactose-fed animals showed augmented Thr308 Akt phosphorylation, which was not pronounced in the kidney and retina (Fig. 5).

We further examined Ser473 and Thr308 phosphorylation in the tissues from STZ-induced diabetic mice after 4 wk of follow-up. The results of these experiments are similar to those for the galactose-fed animals. Increased Ser473 phosphorylation was seen in the retina and kidney, whereas increased Thr308 phosphorylation was present in the heart in the STZ-induced diabetic mice (Fig. 6).

Finally, experiments were carried out in all these tissue with respect to Akt activation by performing an Akt kinase assay.
The results confirmed Akt activation in the retina, kidney, and heart in both diabetic and galactose-fed mice (Fig. 7).

**DISCUSSION**

This study has demonstrated that hyperhexosemia-induced increased production of FN and its splice variant, EDB FN, is mediated, at least in part, by Akt activation. Akt may activate relevant transcription factors to produce increased FN and EDB FN. TGF-β may also play a significant role in this pathway.

We used a galactose-fed animal model for the majority of the investigation. However, in parallel, we demonstrated that similar Akt activation occurs in the tissue of diabetic animals. Galactose-fed rodents are well-recognized animal models for the study of diabetic complications. The majority of structural and functional changes of chronic diabetic complications have been reproduced in this model (2, 9, 26). Increased reducing sugar in the serum of these animals has been demonstrated to be galactose (12, 40). Galactose feeding has been demonstrated to cause tissue accumulation of galactose and its metabolites (2, 12, 26, 37, 40). Similarly to diabetes, galactose feeding has been shown to cause activation of PKC, MAPK, and PI3K-AKT, produce lipid peroxidation and oxidative stress, and lead to increased ECM protein production (5, 26, 30, 46). It has further been shown that even after termination of galactose feeding, chronic diabetic complications such as diabetic retinopathy may continue to progress (26). Increased ECM protein production is a characteristic early event in several chronic diabetic complications. Structurally, such increases manifest as thickening of microvascular capillary basement membrane, mesangial matrix expansion, and focal fibrosis (24). FN is a key ECM protein that is known to be upregulated in diabetes. Our group has previously demonstrated that diabetes causes increased production of a splice variant of FN, namely, EDB FN (22). This splice variant is capable of sending outside-in signals causing VEGF upregulation, endothelial proliferation, and differentiation and is regulated by TGF-β (23).

A plethora of molecular mechanisms may, however, be involved in the production of increased ECM protein in diabetes. Hyperglycemia-induced biochemical alterations are key mechanistic factors in the production of such abnormalities (25). PKC activation is known to cause increased ECM protein synthesis (39). Our group (42) and Tomlinson (39) have demonstrated that activation of MAPK is a possible downstream mediator of PKC activation causing increased ECM protein synthesis. There is, however, extensive interaction among several signaling molecules (25, 31). Several growth factors, unregulated in diabetes, may also activate signaling pathways (21).
In this study, we demonstrated activation of Akt in several tissues from hyperhexosemic animals. Various mechanisms may be responsible for such activation. Glucose-induced Akt activation may be caused, at least in part, by PKC as well as MAPK (41). In other systems, it has been demonstrated that TGF-β may also have a regulatory role in Akt activation via Smad proteins (8, 32). However, in this study, the demonstration of TGF-β downregulation in the Akt knockout mice indicates that Akt may possibly affect ECM protein via TGF-β. In some of the previous studies, Akt activation has been demonstrated in the retina and kidney, whereas hyperhexosemia in the heart caused Thr308 Akt phosphorylation. Interestingly, in a study of ischemia-reperfusion injury in the cardiomyocytes, Ser473 Akt phosphorylation was found to be an early event, whereas Thr308 Akt phosphorylation was found to be a late event (13). It is possible that, at least in the heart, in chronic hyperhexosemia we are seeing such a late phenomenon. In keeping with this study, other investigators have recently demonstrated differential phosphorylation of Ser473 and Thr308 in the diabetic rat heart Akt (28). In this study, a similar pattern of Akt activation in both diabetic and galactose-fed mice indicate that this pathway is of importance in chronic diabetic complications. It has further been postulated that a high-carbohydrate diet may cause cardiovascular damage via Akt activation. In addition, other possible coexisting mechanisms may include peroxisome proliferator-activated receptor-α activation due to high lipid levels (35). However, establishment of such concepts may need more experiments.

Our group has previously demonstrated the role of NF-κB and AP-1 in augmented FN synthesis in diabetes (6, 7). Similar to our studies in endothelial cells (41), the current investigation also demonstrated that Akt activation influences FN synthesis in tissues. Activated Akt in the target organs of diabetic complications via transcription factors may have several potential implication (44). Such activation via modulation of ECM production may cause tissue damage. In addition, Akt activation may have angiogenic effects, through VEGF (1, 29).

In summary, we have demonstrated that hyperhexosemia causes upregulation of FN and its splice variant, EDB⁺FN, at the transcriptional level in several tissues affected by chronic diabetic complication. The pattern of Akt activation, however, varied depending on the tissue microenvironment. Understand-
ing these mechanisms is important in identifying novel treatment targets.

GRANTS

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REFERENCES


