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Hepatic Expression Profiling Shows Involvement of PKC Epsilon, DGK eta, Tnfaip, and Rho kinase in type 2 Diabetic Nephropathy Rats

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ABSTRACT

Type 2 diabetes is associated with an increased risk for developing fatty liver disease, which results in an increased incidence of diabetic nephropathy. Hence, the present study was conceived to identify transcriptional changes in the liver that can provide molecular mediators for increased risk of developing nephropathy associated with type 2 diabetes. Type 2 diabetes was rendered in male SD rats using both high-fat diet and low dose of streptozotocin (35 mg/kg, intraperitonially, i.p.). Hepatic gene expression profiling was performed in animals after development of diabetic nephropathy. The gene expression data were validated by RT-PCR, protein expression, and immunohistochemistry. Gene expression profiling data revealed dramatic increase in expression of PKC epsilon, TNF-alpha-induced protein (four- to seven-folds), and decrease in the expression of DGK eta in the liver of diabetic nephropathic rats. Furthermore, there was an increase in expression of genes regulating Rho signaling pathway, which was further confirmed by increase in Rho kinase activity. To the best of our knowledge, this is the first report which shows the involvement of PKC epsilon, DGK eta, Tnfaip, and Rho kinase in the liver of type 2 diabetic rats and its association with diabetic nephropathy. J. Cell. Biochem. 111: 944–954, 2010.

KEY WORDS: LIVER; TYPE 2 DIABETES; PKC EPSILON; DIABETIC NEPHROPATHY; RHO KINASE

Liver is the main organ of glucose disposal and also the foremost organ to be affected in diabetes. The excess of free fatty acids and hyperinsulinemia found in the insulin-resistant state is known to be directly toxic to hepatocytes [Lewis et al., 2002]. Fatty liver is known to be associated with chronic kidney disease and is also an early marker for the progression of diabetic nephropathy [Targher et al., 2008a,b]. Patients with type 2 diabetes seem to be at increased risk for developing fatty liver disease and certainly have a higher risk for developing fibrosis and cirrhosis [Day, 2006; Tolman et al., 2007]. However, the underlying mechanisms by which prevalence of fatty liver in type 2 diabetes increase the risk for developing diabetic nephropathy are poorly understood. In liver, in addition to pathways that are known to be involved in maintaining glucose homeostasis, several other pathways are also affected, finally leading to type 2 diabetes [Saltiel and Kahn, 2001]. These effects are achieved not only by altering activities of key enzymes involved in various signaling cascades via phosphorylation but also by altering expression of certain genes via phosphorylation of certain transcription factors. Therefore, a global analysis of hepatic gene expression appears to be essential for understanding the molecular mechanisms underlying the development of type 2 diabetes and its complications.

DNA microarray technology has been widely used to gain insight into the overall effect on gene expression under various pathological conditions and provides a global view of the molecular events, which led to the development of these pathologies. This high

Abbreviations used: STZ, streptozotocin; NPD, normal pellet diet; HFD, high-fat diet; HFD/STZ, high-fat diet + streptozotocin; PGL, plasma glucose; PI, plasma insulin; PTCHL, plasma total cholesterol; PTG, plasma triglycerides; BUN, blood urea nitrogen and PCR, plasma creatinine levels; PKC, protein kinase C; TNF, tumor necrosis factor; DGK, diacylglycerol kinase.

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throughput technique has also proven useful for generating novel hypothesis regarding regulatory pathways or disease mechanisms [Liang et al., 2004]. The DNA microarray approach has been applied to study transcriptome responses in streptozotocin (STZ)-induced diabetic animals [Knoll et al., 2005; Kume et al., 2005], high-fat diet (HFD)-fed animals [Sparks et al., 2005], and many other genetic diabetic models [Heishi et al., 2006; Almon et al., 2009]. Also recently, studies have demonstrated the effect of various treatment regimens like lipoic acid [Yang et al., 2008], rosiglitazone [Rong et al., 2007], etc. in preventing diabetes-related symptoms by microarray technology.

However, feeding of HFD to normal rats leads to the development of in vivo insulin resistance, glucose intolerance and obesity without causing diabetes [Srinivasan et al., 2004]. One of the mechanisms proposed is the presence of high level of triglycerides due to excess fat intake, which could constitute a source of increased availability of fatty acids for oxidation. The preferential use of these fatty acids for oxidation blunts the insulin-mediated reduction of hepatic glucose output and reduces glucose uptake and utilization in skeletal muscle leading to compensatory hyperinsulinemia, which is a common feature of insulin resistance [Rosholt et al., 1994; Belfiore and Iannello, 1998]. However, injecting low dose of STZ along with feeding HFD (HFD/STZ, HS), produced hyperglycemia in the presence of less circulating insulin concentration that replicates the metabolic characteristics of human type 2 diabetes [Srinivasan et al., 2005].

Hence, there is a need of a study that profiles the transcriptional changes in liver of type 2 diabetic rats having persistent hyperglycemia with prevalence of diabetic nephropathy. Therefore, in the present study, we performed the cDNA microarray analysis in liver of type 2 diabetic rats that developed nephropathy at 24 weeks after dietary manipulation. Our objectives is to identify differential gene expression profiles in HS-fed rat liver which can help in better understanding the development of fatty liver disease associated with diabetic nephropathy.

MATERIALS AND METHODS

ANIMALS

The male adult Sprague–Dawley rats (160–180 g) were procured from the central animal facility of the institute, NIPER. They were maintained under standard environmental conditions and provided with feed and water ad libitum. All the animals were fed on normal pellet diet (NPD) 1 week prior to the experimentation. Our protocol is in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Environment, Government of India. Rules of CPCSEA are laid down as per ILAR (Institute of Laboratory Animal Resources, USA) guidelines and prior permission was sought from the institutional animal ethics committee for conducting the study. All experimental procedures had been approved by the local government authorities.

DEVELOPMENT OF EXPERIMENTAL TYPE II DIABETIC NEPHROPATHY

Type 2 diabetes in Sprague–Dawley rats were developed as described previously [Gaikwad et al., 2007]. Briefly, the rats were allocated into two dietary regimens either NPD (normal pellet diet) or HFD (58% fat, 25% protein, and 17% carbohydrate, as a percentage of total kcal), respectively, for an initial period of 2 weeks. After 2 weeks of dietary manipulations, the rats from HFD-fed group were injected with low dose of STZ (35 mg/kg) i.p., while the respective control rats (NPD fed) were given vehicle citrate buffer (pH 4.4) in a dose volume of 1 ml/kg, i.p. The rats were allowed to continue feeding on their respective diets until the end of the study (24 weeks). The body weight was measured and biochemical estimations were carried out after 24 weeks of dietary manipulations in rats. Diabetic nephropathy was assessed by measurements of blood urea nitrogen and creatinine levels.

ESTIMATION OF BIOCHEMICAL PARAMETERS

The blood samples were collected, plasma was separated and analyzed for glucose (PGL), triglycerides (PTG), total cholesterol (PTC), insulin (PI), blood urea nitrogen (BUN), and plasma creatinine (PCR) as described previously [Gaikwad et al., 2007; Tikoo et al., 2008a]. Insulin determination was made by ELISA kit using rat insulin as standard (Rat ELISA kit; Linco Research, USA).

WESTERN BLOTTING

Western blot analysis was performed as described previously [Tikoo et al., 2007a,b]. Briefly, proteins extracted from liver tissue were separated using SDS–PAGE and transferred to nitrocellulose membrane and probed with anti-PKC epsilon (rabbit 1:1,000; Santa Cruz Biotechnology), anti-p-Akt (Ser-473; rabbit 1:2,500; Santa Cruz Biotechnology), anti-TNF-α (goat 1:500, Santa Cruz Biotechnology), anti-p-Mypt-1 (Thr-853; rabbit 1:500, Santa Cruz Biotechnology), or anti-actin antibody (rabbit 1:2,500; Sigma). Proteins were detected with the enhanced chemiluminescence (ECL) system and ECL Hyperfilm (Amersham Pharmacia Biotech, UK, Ltd.). The immunoblots were quantified by densitometric scanning with NIH Image J software.

ISOLATION OF TOTAL RNA

Livers from control and type 2 diabetic rats were isolated after 24 weeks and used for the preparation of total RNA for both DNA microarray and RT-PCR analysis. RNA was isolated following the protocol of Chomczynski and Sacchi [1987]. Briefly, total RNA was extracted from each rat liver with TRIZOL reagent (Invitrogen) and purified according to the manufacturer's protocol using RNeasy kit (Auprep RNeasy mini kit; Life Technologies). RNA quality and integrity from each sample was assured using Nanodrop (ND-1000) by A260/280 absorbance ratio and using agarose gel electrophoresis, respectively. Total RNA prepared from individual animals was used for DNA microarray analysis.

PROBE LABELING AND HYBRIDIZATION

The mouse 7.4 K (Microarray centre, University Health Care, Toronto) used in the present study is a double-spotted array containing 7,407 mouse expression sequence tags (ESTs). We have used the mouse cDNA array to analyze the rat's transcriptome profile because these mammalian genomes have been predicted to encode similar numbers of genes [Gibbs et al., 2004]. Fluorescence-labeled cDNA probes from control and HS-fed group were prepared by using

20 μg of total RNA from each, using SuperScript III (Invitrogen Life Technologies), oligo dT primer (Invitrogen Life Technologies), and Cy3 or Cy5-labeled dCTP's (Amersham Biosciences). The Cy3 and Cy5 probes were mixed in equal amounts and hybridization was carried out at 43°C for 16 h on Hyb Array 12 hybridization station (Perkin Elmer). Hybridization was repeated three times with samples from different animals. Dye-swapped experiment was performed to improve the accuracy of the measurement and to rule out the non-specific signals.

ANALYSIS OF MICROARRAY DATA

Slides were scanned with a Scanarray Gx microarray scanner (Perkin Elmer). Raw data were subject to preprocessing by Scanarray software. Background subtracted mean spot intensities were logged (base 2) and good intensity spots were filtered out before normalization and flagged spots were excluded from analysis. In addition, buffer controls and positive controls were removed. The further analysis was performed using Avadis software (Strand Lifesciences). The signal intensities were normalized between Cy3 and Cy5 by performing lowess normalization. The log 2 value ratio for the signal intensity of each spot was calculated for each slide and the significant analysis was performed using student's *t*-test. Fold change was calculated as the ratio of normalized signal intensities of control and HS-treated rats. Genes showing more than 1.2-fold change were taken for further analysis. Genecard (www.genecards.org) and Pubmed (www.pubmed.gov) were used for assigning the genes to specific biological processes.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

To confirm the cDNA microarray analysis, RT-PCR was performed for certain genes. The first strand cDNA was generated from 20 μg of total RNA by using SuperScript III (Invitrogen, Life Technologies) and oligo dT primer (Invitrogen, Life Technologies) in a 30 μl reaction mixture. The quantitative real-time PCR reaction was performed using LightCycler 2.0 (Roche Diagnostics) in a final volume of 20 μl containing 50 ng of cDNA, 15 μl of reaction buffer from LightCycler FastStart DNA master plus kit (Roche Diagnostics) and specific forward and reverse primers (Midland Certified Reagent Company, Inc.; Table I). After amplification, a melting curve analysis was performed to verify the specificity of the reaction. The analysis was performed using LightCycler software (Roche Diagnostics). Relative gene expression was assessed using the comparative C_t (ΔC_t) method and normalized to GAPDH. The list of the primers used is available in Table I below.

TABLE I. Sequence of Primers Used for mRNA Quantitation by RT-PCR

Transcript name	Sense primer	Antisense primer
TNFAIP1	CACTTGGCTGAGAGGAAAGG	CAAATGAGTGTCCCGCAGA
Alkbh3	GGCTAACGAAAGTCGGAAACT	GATTTCGTTCCCCATTCAGA
Creb	CTAGTGCCCAGCAACCAAGT	GGAGGACGCCATAACAACTC
Pkc epsilon	TCTACCCTGTCTGGCTTAGCA	CGGGTTCTTGGTCATGAAAG
GAPDH	CTTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGAG

HISTOPATHOLOGICAL EVALUATION AND IMMUNOHISTOCHEMISTRY

Parts of the liver and kidney were fixed in 10% formalin in PBS and embedded in paraffin. Two micrometer sections were cut and mounted on slides coated with 3-aminopropyl triethoxy-silane and were stained with hematoxylin and eosin. Glomerular damage was assessed using a semi-quantitative score: 0 = no lesion, 1 = <25% damage, 2 = 25–49% damage, 3 = 50–74% damage, and 4 = 75–100% damage, respectively, as described [Ninichuk et al., 2008]. Ten glomeruli were analyzed per section. For immunohistological studies paraffin-embedded sections of liver and kidney were incubated with primary antibody: anti-PKC epsilon and anti-fibronectin, respectively (rabbit 1:1,000; Santa Cruz Biotechnology) as described earlier [Tikoo et al., 2008b]. Immunohistochemical changes were assessed in at least 10 randomly selected tissue sections from each group studied. The intensity of spot was graded from 1 to 4 (1, slight or no color; 2, very-low color; 3, moderate blue color; and 4, very intense blue color) [Ilnytska et al., 2006]. The immunohistochemistry score was expressed as mean ± SEM for each experimental group.

ESTIMATION OF RHO KINASE ACTIVITY

Studies have shown that increased MYPT1 phosphorylation at Thr853 directly correlates with Rho kinase activity. To determine Rho kinase activity, MYPT1 phosphorylation was detected by using a site-specific antibody recognizing p-Mypt-1 (Thr-853; rabbit 1:500; Santa Cruz Biotechnology) and Western blot analysis.

STATISTICAL ANALYSIS

Experimental values are expressed as mean ± SEM. Comparison of mean values between two groups was performed by using student *t*-test. *P*-value <0.05 was considered to be significant.

RESULTS

CHANGE IN BODY WEIGHT, PLASMA GLUCOSE (PGL), TOTAL CHOLESTEROL (PTC), TRIGLYCERIDE (PTG), INSULIN (PI), BLOOD UREA NITROGEN (BUN), AND PLASMA CREATININE (PCR) LEVELS IN CONTROL AND TYPE 2 DIABETIC RATS

Treatment of STZ (35 mg/kg, i.p.) produced significant reduction in the body weights of the HFD-fed rats along with increase in kidney weight. Moreover, injection of STZ (35 mg kg, i.p.) significantly ($P < 0.001$) increased PGL in HFD-fed rats, thus producing frank

TABLE II. Development of Renal Failure in Type 2 Diabetic Animals

Parameters	NPD	HFD/STZ
Body weight (g)	512 ± 9.53	355 ± 14.05***
Kidney weight (g)	2.76 ± 0.19	3.42 ± 0.17*
PGL (mg/dl)	106 ± 1.35	495 ± 14.97***
PTCHL (mg/dl)	46 ± 2.87	243 ± 14.78***
PTG (mg/dl)	46 ± 2.2	196 ± 25.91***
PI (pmol/ml)	184 ± 32	88 ± 7***
BUN (mg/dl)	20 ± 0.57	43 ± 1.38***
PCR (mg/dl)	1.50 ± 0.08	2.64 ± 0.06***

Body weight, kidney weight, and different biochemical parameters like plasma glucose (PGL), blood urea nitrogen (BUN), plasma creatinine (PCR), plasma total cholesterol (PTCHL), plasma triglycerides (PTG), and plasma insulin (PI) were estimated after 24 weeks. All the values were represented as mean ± SEM (n = 10), ***$P < 0.001$, *$P < 0.05$ versus NPD-fed group.

hyperglycemia. Insulin levels were also measured and they show decrease along with a significant ($P < 0.001$) increase in basal PTG and PTC levels of HS-treated rats as compared to control rats Table II). Thus, increase in plasma glucose (PGL), triglycerides (PTG), total cholesterol (PTC) levels shows the development of type 2 diabetes.

DEVELOPMENT OF NEPHROPATHY IN TYPE 2 DIABETIC RATS

After 24 weeks of dietary manipulation, there is significant increase in plasma BUN and PCR levels (Table II) of type 2 diabetic rats. Increase in kidney weight and biochemical changes suggest development of nephropathy in these type 2 diabetic rats. We also observed glomerular damage in kidney of HS-fed group as compared to normal control rats by histopathological analysis (Fig. 1A,B). Additionally, nephropathy was further confirmed by expression of fibronectin in the kidney of these animals by immunohistochemical staining, suggesting the development of renal fibrosis in diabetic animals (Fig. 1C,D).

DIFFERENTIALLY EXPRESSED GENES IN THE LIVER OF TYPE 2 DIABETIC RATS

Only those genes whose mRNA levels were changed more than 1.2-fold with $P < 0.05$ were designated as differentially expressed genes.

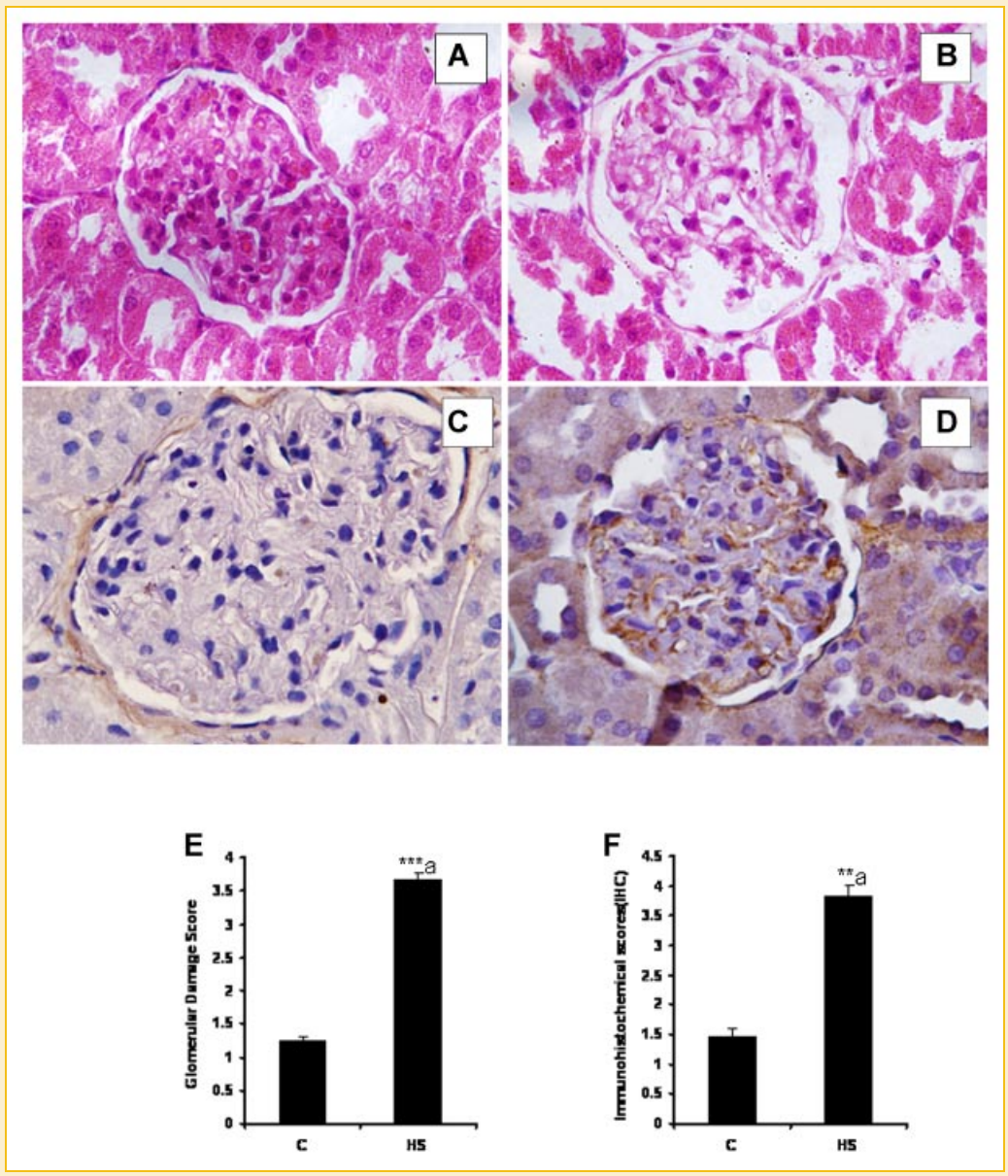

Fig. 1. Histopathological changes and immunostaining of fibronectin in kidney sections of control and HFD/STZ (HS)-fed rats. Renal sections from rats of control (A) and HS-fed rats (B) were stained with hematoxylin and eosin and scored for the extent of glomerulosclerosis as described in Materials and Methods Section. The graph (E) illustrates the mean percentage of each score ± SEM from all rats in each group (n = 10). For identification of the extent of glomerular fibrosis renal sections from control (C) and HS-fed rats (D) were processed for immunostaining with anti-fibronectin antibody as described in "Materials and Methods" Section. The graph (F) illustrates the mean percentage of immunohistochemical score ± SEM from all rats in each group (n = 10). Each value is represented as mean ± SEM and a versus control $^{***}P < 0.001$ and $^{**}P < 0.01$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

According to these criteria, 111 genes were found to be differentially expressed in the liver of HS-fed group as compared with the control group, 32 of which showed a more than twofold difference in expression. Out of these 111 genes, which were found to be changed significantly ($P < 0.05$), 54 genes were up-regulated and 57 were found to be down-regulated. Although the overall numbers of up- and down-regulated genes were almost same but when categorized into different functional categories, they show a definite trend.

Genecard (www.genecards.org) and Pubmed (www.pubmed.gov) were used to assign the regulated genes into functional categories (Supplementary data). The regulated genes showed a broad range, but most of the up-regulated genes were mainly categorized into cell cycle, apoptosis, protein/amino acid metabolism, carbohydrate/fat metabolism and cell adhesion-related genes (Fig. 2). On the other hand, down-regulated genes mainly belonged to transcription, signal transduction, RNA processing, cytoskeletal organization, and DNA repair-related genes. In order of calculating the percentage of genes belonging to particular pathways (Fig. 2) to the total no. of significantly changing genes ($P < 0.05$), it was found that the maximum number of differentially expressed genes (31%) belong to signal transduction pathway. Suggesting that, signal transduction pathways are significantly altered in the liver of type 2 diabetic nephropathic rats.

Four genes were randomly picked up among the up- and down-regulated genes judged from the microarray data, and were analyzed by RT-PCR. The changes in the gene expressions of all the genes as observed by both microarray and RT-PCR were showing same trend further validate our microarray data (Fig. 3). However, the difference in RT-PCR and microarray estimated fold change values can also be attributed to the use of mouse cDNA array for hybridizing rat's cDNA.

CHANGE IN INSULIN SIGNAL TRANSDUCTION-RELATED GENES IN LIVER OF TYPE 2 DIABETIC RATS

Insulin signal transduction-related genes, such as diacylglycerol kinase eta (Dgkh), protein tyrosine phosphatase, receptor type T

A

Gene name	Microarray	RT-PCR
Alkbh3	-4.03185	-2.584 ± 0.07
Tnfaip1	6.733733	6.24 ± 0.26
Creb1	-1.85139	-3.694 ± 0.19
Pkc-epsilon	4.024347	2.337 ± 0.24

B

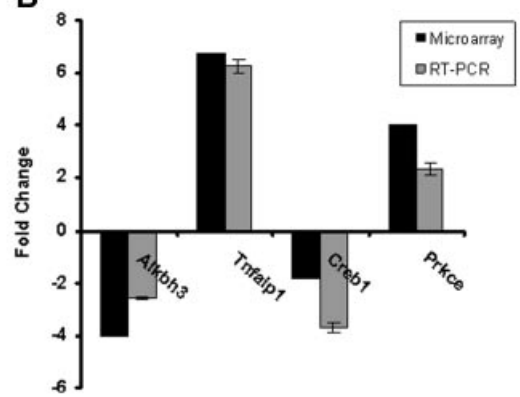

Fig. 3. Confirmation of microarray results by RT-PCR analysis. The fold change in mRNA levels of randomly selected genes were determined by RT-PCR using a light cycler 2.0 on total RNA preparation from livers of control and HFD/STZ-fed rats. Rat GAPDH was used as a control for equivalent RNA template among two groups (A). Table showing comparison of fold change by respective microarray and RT-PCR analysis (B) Graph showing validation of microarray data by RT-PCR. All the values were represented as mean ± SEM ($n = 3$).

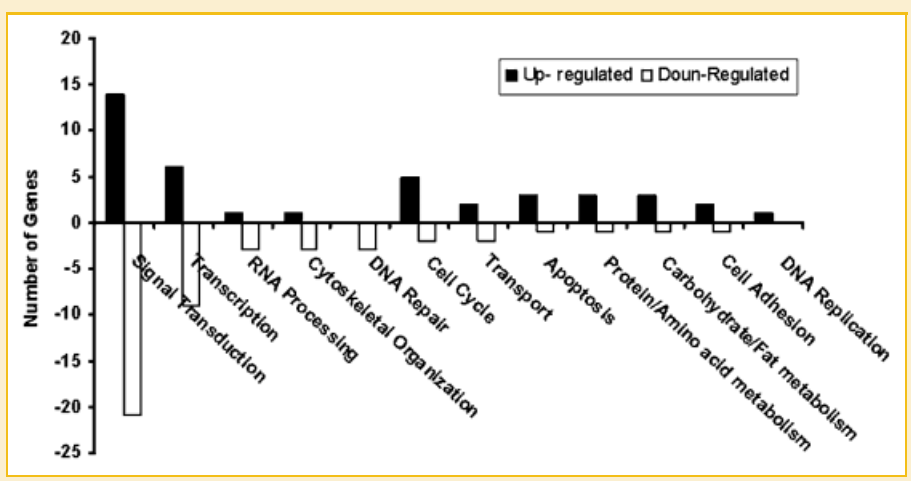

Fig. 2. cDNA microarray analysis for differentially regulated genes in liver of HFD/STZ-fed rats as compared to control. Bar diagram showing differentially expressed genes toward various functional pathways as analyzed by microarray analysis. The figure is derived from list of genes (Supplementary data) that are statistically significantly changing with $P < 0.05$, and assigned to a corresponding biological process as described in "Materials and Methods" Section.

(ptprt), protein tyrosine phosphatase, receptor type M (ptprm), protein tyrosine phosphatase, receptor type D (ptprd) and phosphatidyl ionositol transfer protein, and cytoplasmic (pitpnc1), which are the major elements of PI3 kinase pathway, show decreased levels of expression in type 2 diabetic rats than in control group. A number of polymorphisms associated with them are known to confer an increased risk of developing type 2 diabetes [Langberg et al., 2007]. Decreased expression of these genes indicates their role in the development of type 2 diabetes. In contrast, PKC epsilon, TNF-alpha-induced protein and several genes encoding mediators of Rho signaling pathway, displayed spectacular increased expression (four- to seven-folds) in type 2 diabetic rat liver. In addition many genes encoding for proteins that regulates oxidative stress, DNA repair, protein degradation, and fat metabolism were also found to show altered expression.

Additionally, microarray also demonstrates that glutathione-s-transferase (gstt3) mRNA levels are increased in livers of type 2 diabetic rats, implying that the tissue is affected by oxidative stress [Raza et al., 2004]. Also there is an increased expression of CD36 gene in liver of HS-fed rats. CD36 or fatty acid translocase (FAT) is a multiligand receptor; the over-expression of which in muscle tissues is associated with hyperglycemia and hyperinsulinemia [Ge and Elghetany, 2005]. In addition, genes encoding for proteins that regulates DNA repair such as Alkbh3, that repairs alkylated DNA by oxidative deamination, Rad15, that is involved in the post-replication repair of UV damaged DNA and Nhej 1, involved in end

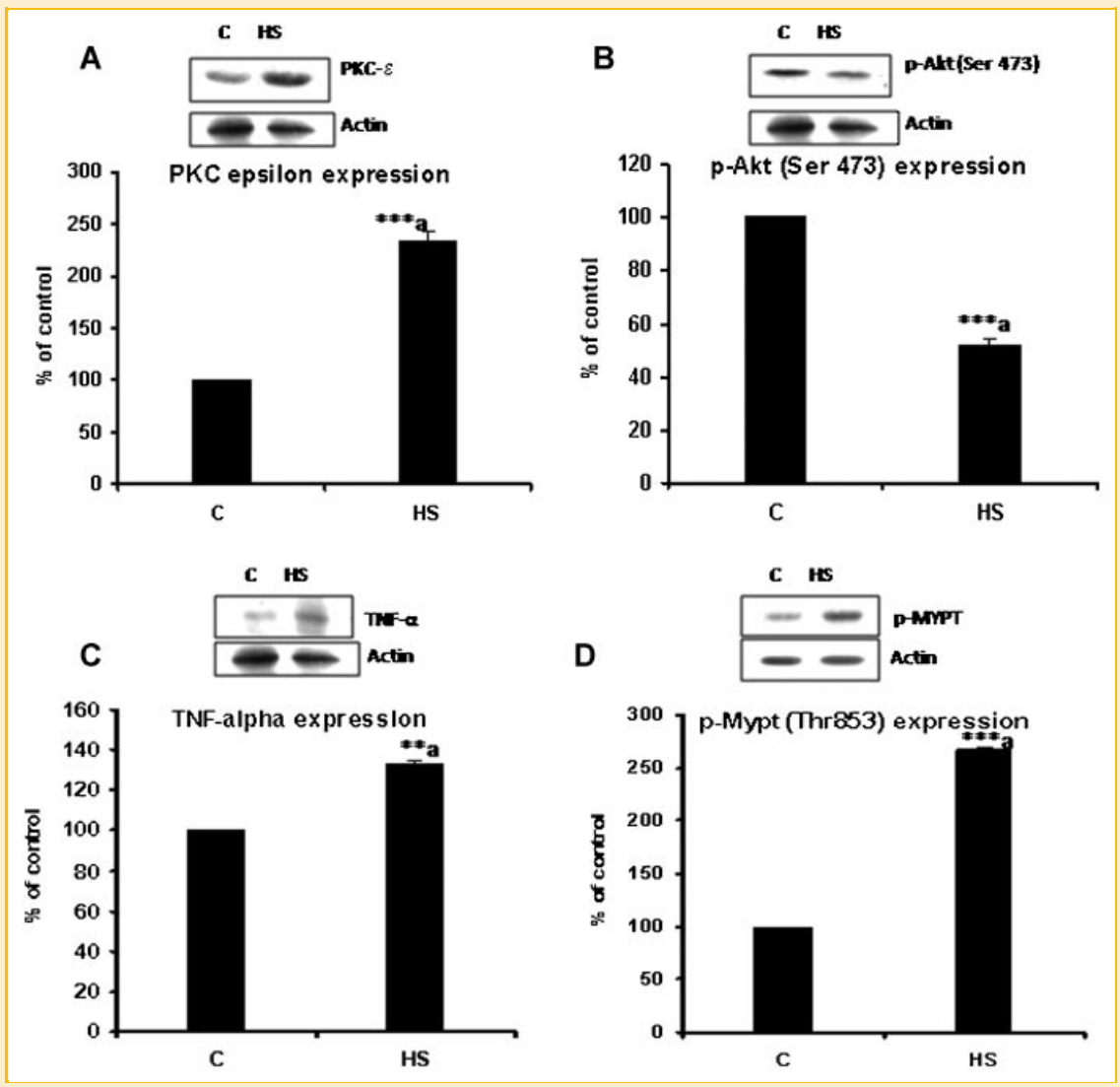

Fig. 4. Western blot analysis for change in protein expression of PKC epsilon, TNF-α, p-Akt (Ser 473), and p-MYPT-1 (Thr 853) in liver of HFD/STZ-fed rats as compared to control. Liver tissues were lysed with lysis buffer and processed for immunoblotting using specific antibodies as described in "Materials and Methods" Section. Immunoblots of (A) PKC epsilon (B) Akt (Ser 473) phosphorylation (C) TNF-α, and (D) MYPT-1 (Thr 853) phoshorylation in the liver of control and HFD/STZ-fed rats. Where (C) control (NPD-fed rats) and HS: HFD/STZ-fed rats. Results were normalized with respect to actin as respective control. Similar results were obtained in three independent set of experiments. All the values were represented as mean ± SEM (n = 3), $^{***}P < 0.001$ and $^{**}P < 0.01$, a versus control.

joining, required for double strand break repair, have also shown decreased expression in type 2 diabetic rats liver.

CHANGE IN PROTEIN EXPRESSION OF PKC EPSILON, TNF-α AND P-AKT (473) IN LIVER OF TYPE 2 DIABETIC RATS

Decreased insulin stimulated Akt kinase activity has been previously reported in type 2 diabetic subjects [Krook et al., 1998]. Western blot analysis (Fig. 4) reveals decreased levels of phosphorylation of Akt at Ser 473 in liver of HS-treated rats, suggesting the development of type 2 diabetes. Further due to drastic increase in expression of genes encoding PKC epsilon, TNF-alpha-induced protein and mediators of Rho signaling pathway (four- to 7-fold) in type 2 diabetic rat liver as observed by our microarray data, we intend to observe their protein expression by Western blot analysis to further confirm our results. We observed increased protein expression of PKC epsilon and TNF-α in liver of HS-treated rats by Western blot analysis, thus further validating the microarray results. Immunohistochemical staining also shows increase in protein expression of PKC epsilon as observed by intense staining of cytoplasm in liver of diabetic nephropathic rats as compared to control rats (Fig. 5).

INCREASED RHO KINASE ACTIVITY IN LIVER OF TYPE 2 DIABETIC RATS

Active RhoA exerts its effects by binding to its effector molecules such as Rho kinase and inducing their activation. Rho kinase in turn phosphorylates its substrates such as MYPT-1 at threonine 696 and

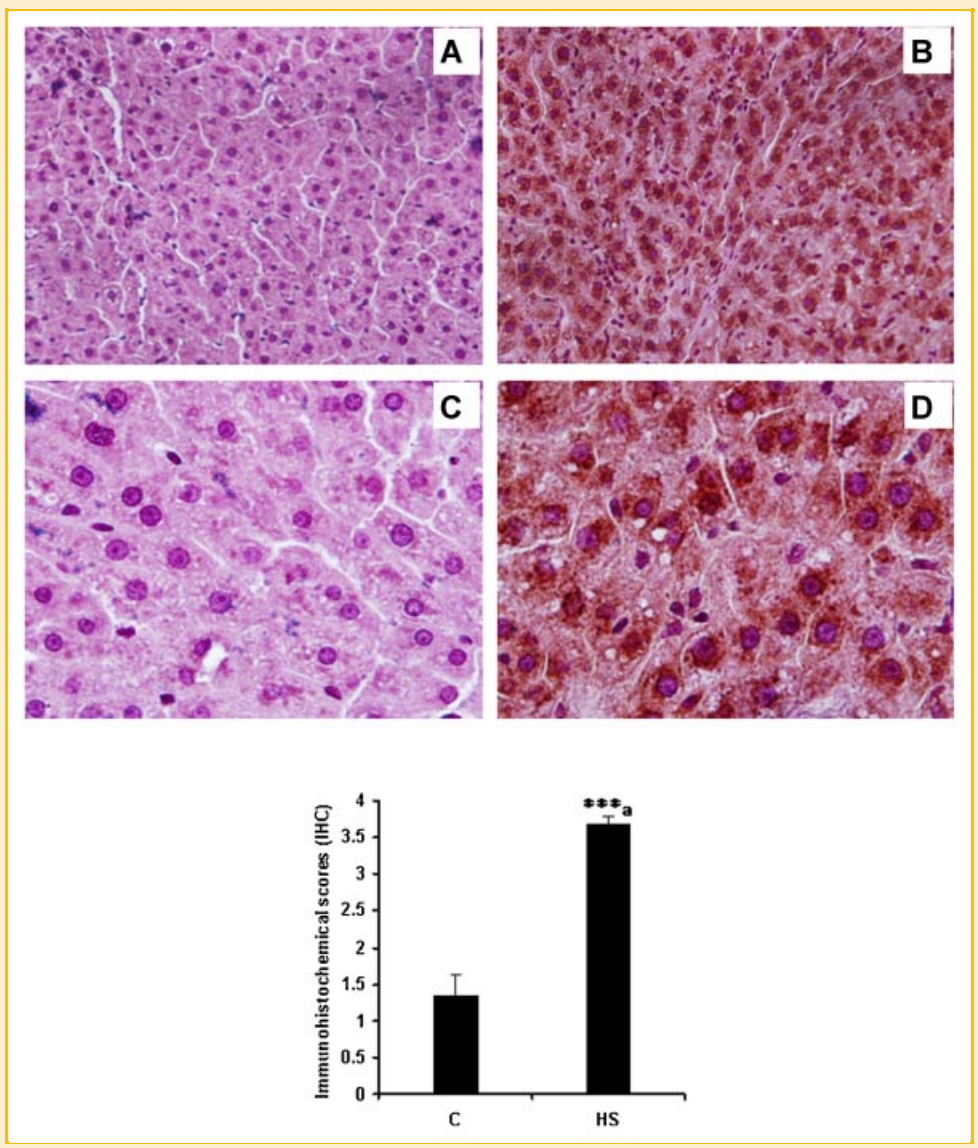

Fig. 5. Immunostaining of PKC epsilon in liver sections of control and HFD/STZ (HS)-fed rats. Liver sections from control (A,C) and HS fed rats (B,D) were processed for immunostaining as described in "Materials and Methods" Section. Magnification is at 10× for (A,B) and 100× for (C,D). Similar results were obtained in three independent set of experiments. Each value is represented as mean ± SEM and a versus control $^{***}P < 0.001$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

853. Thus, phosphorylation at threonine 853 of MYPT1 (phospho-MYPT1) by Rho kinase serve as a marker for Rho kinase activity [Gien et al., 2008]. We found an increase in threonine 853 phosphorylation of MYPT1 thus showing increased Rho kinase activity in the liver of type 2 diabetic nephropathic rats as compared to the control rats (Fig. 4D).

DISCUSSION

This is the first prospective study aimed at assessing the transcriptional changes in the liver of type 2 diabetic rats that developed nephropathy after 24 weeks of dietary manipulations. Previous studies reflect that fatty liver disease associated with diabetes worsens whole-body insulin resistance and hyperglycemia, which may in turn contribute to the progression of chronic kidney disease [Rossing, 2006; Tan et al., 2007; Targher et al., 2007]. An increase in kidney weight, blood urea nitrogen and plasma creatinine levels show the development of nephropathy in these type 2 diabetic rats. We have used global microarray analysis to identify potential novel candidate genes involved in the development of fatty liver associated with diabetic nephropathy. We try to find out the possible molecular mediators in liver that confer an excess risk of developing nephropathy associated with type 2 diabetes.

With coherent changes in the expression of 111 genes, we have observed an increase in the expression of PKC epsilon gene by around fourfold by microarray analysis and this was further confirmed by increase in protein expression of PKC epsilon in liver of type 2 diabetic rats as compared to control rats by Western blot analysis as well by immunohistochemical staining. The serine/threonine-specific protein kinase C, PKC, has been particularly implicated in the pathogenesis of insulin resistance [Shmueli et al., 1993; Qu et al., 1999]. There are evidence that hyperglycemia-induced insulin receptor inhibition is mediated by PKC [Berti et al., 1994]. PKC has many isoenzymes with different biochemical characteristics. Considine et al. [1995] have shown increased PKC epsilon levels in liver of Zucker diabetic fatty rats and the work by Schmitz-Peiffer et al. [1997] also show chronic activation of PKC (epsilon) in skeletal muscles of high-fat-fed Wistar rats. In addition, PKC epsilon is also shown to be activated in non-alcoholic fatty liver disease [Samuel et al., 2004]. However, all these studies are conducted at short duration of dietary manipulation. This is the first report that shows an increase in PKC epsilon mRNA and protein expression in liver of diabetic animals that are under chronic hyperglycemia, i.e., 24 weeks. Therefore, these changes in PKC epsilon expression in liver can easily be linked to the development of type 2 diabetes that may result in an increased risk for the development of diabetic nephropathy.

There are reports suggesting that hyperglycemia stimulates Rho kinase activity in endothelial cells via PKC-dependent pathway [Rikitake and Liao, 2005]. Rho signaling pathway in muscle cells has

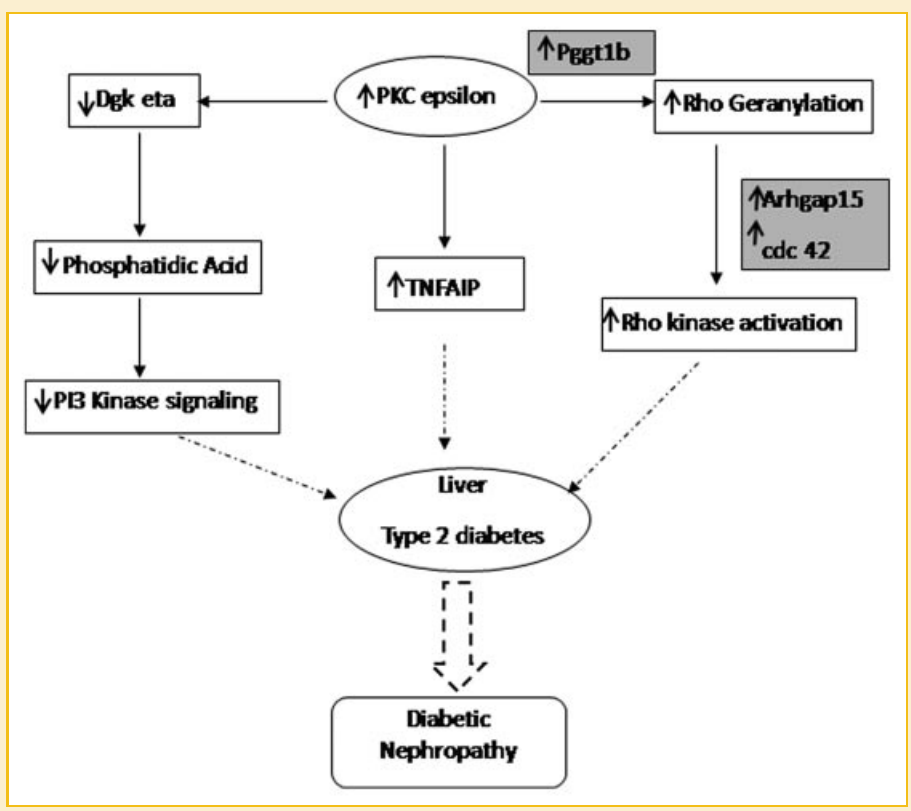

Fig. 6. Proposed pathway-depicting mechanisms of development of type 2 diabetes in HFD/STZ-fed rats.

been implicated in the pathogenesis of hypertension as well as diabetes [Hsueh and Law, 1999; Sandu et al., 2000]. In these cells, Rho activation requires its translocation from cytosol to plasma membrane where it is converted from the inactive GDP bound form to the active GTP bound form [Gong et al., 1997]. This localization to the membrane is regulated by post-translational modifications by geranylgeranylation and phosphorylation of Rho [Moomaw and Casey, 1992; Sauzeau et al., 2000]. Insulin inhibits Rho signaling by inhibition of isoprenylation of Rho A via reduction in geranyl geranyl transferase 1 activity in smooth muscle cells [Begum et al., 2002]. In this study, we have found an increase in the expression of protein geranyl geranyl transferase 1, beta subunit (Pggt1b) by around fourfolds in liver of type 2 diabetic rats. Also the expression of Arhgap15 that is a Rho GTPase-activating protein and cdc 42, a Rho GTPase is found to be increased by around twofold. In addition, we have also demonstrated an increase in the Rho kinase activity in liver of type 2 diabetic rats by increased phosphorylation of MYPT1 at Thr853. Suggesting that, up-regulation of PKC epsilon in liver stimulates Rho kinase pathway that may be implicated in the development of type 2 diabetes.

Additionally, there are evidence that PKC (epsilon) enhances the inhibitory effect of TNF-α on insulin signaling in rat fibroblasts [Kellerer et al., 1997]. We have found an increase in the expression of TNF-α-induced protein (tnfaip1) by almost sevenfolds. In addition, our result demonstrates an increase in TNF-α protein expression in liver of type 2 diabetic rats. Thus, enhanced PKC (epsilon) in liver of type 2 diabetic rats may down-regulate insulin signaling via enhancing the inhibitory effect of TNF-α.

Diacylglycerol (DG) kinase (DGK), that phosphorylates DG to yield phosphatidic acid (PA), has many isotypes that are phosphorylated in vivo by PKC [Diaz-Flores et al., 2003]. DGK modulates the balance between two signaling lipids, diacyl glycerol, and PA [Kanoh et al., 2002; Luo et al., 2003]. DGK is also known to initiate downstream signal transduction pathways through the generation of PA, a potential second messenger that can activate phosphatidylinositol-4-phosphate kinase [Jones et al., 2000; Luo et al., 2004]. As phosphoinositide pathway plays a key role in liver insulin signal transduction pathway, the decreased PA formation thus may be involved in the development of type 2 diabetes. PKC epsilon activation have been suggested to negatively regulate DGK activity [van Baal et al., 2005]. Our data show decreased expression of DGK eta, in liver of type 2 diabetic rats. Highlighting the critical role of PKC epsilon in the development of type 2 diabetes by increasing Rho signaling pathway, by decreasing DGK eta-dependent generation of PA and by enhancing the inhibitory effect of TNF-α on insulin signaling in liver of diabetic nephropathic rats (Fig. 6).

In conclusion, the present study demonstrates that in non-genetic model of type 2 diabetic nephropathy there is a profound change in the mRNA expression levels of key genes. We provide evidence for the involvement of novel genes that are associated with diabetic nephropathy and fatty liver. However, further studies are required to elucidate whether these changes in liver are cause or consequence of diabetic nephropathy. Understanding of which can have profound clinical implications in elucidating pathogenesis of type 2 diabetic nephropathy.

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REFERENCES

Almon RR, DuBois DC, Lai W, Xue B, Nie J, Jusko WJ. 2009. Gene expression analysis of hepatic roles in cause and development of diabetes in Goto-Kakizaki rats. J Endocrinol 200:331–346.

Begum N, Sandu OA, Duddy N. 2002. Negative regulation of rho signaling by insulin and its impact on actin cytoskeleton organization in vascular smooth muscle cells: Role of nitric oxide and cyclic guanosine monophosphate signaling pathways. Diabetes 51:2256–2263.

Belfiore F, Iannello S. 1998. Insulin resistance in obesity: Metabolic mechanisms and measurement methods. Mol Genet Metab 65:121–128.

Berti L, Mosthaf L, Kroder G, Kellerer M, Tippmer S, Mushack J, Seffer E, Seedorf K, Haring H. 1994. Glucose-induced translocation of protein kinase C isoforms in rat-1 fibroblasts is paralleled by inhibition of the insulin receptor tyrosine kinase. J Biol Chem 269:3381–3386.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Considine RV, Nyce MR, Allen LE, Morales LM, Triester S, Serrano J, Colberg J, Lanza-Jacoby S, Caro JF. 1995. Protein kinase C is increased in the liver of humans and rats with non-insulin-dependent diabetes mellitus: An alteration not due to hyperglycemia. J Clin Invest 95:2938–2944.

Day CP. 2006. Non-alcoholic fatty liver disease: Current concepts and management strategies. Clin Med 6:19–25.

Diaz-Flores E, Siliceo M, Martinez AC, Merida I. 2003. Membrane translocation of protein kinase Ctheta during T lymphocyte activation requires phospholipase C-gamma-generated diacylglycerol. J Biol Chem 278:29208–29215.

Gaikwad AB, Viswanad B, Ramarao P. 2007. PPAR gamma agonists partially restores hyperglycemia-induced aggravation of vascular dysfunction to angiotensin II in thoracic aorta isolated from rats with insulin resistance. Pharmacol Res 55:400–407.

Ge Y, Elghetany MT. 2005. CD36: A multiligand molecule. Lab Hematol 11:31–37.

Gibbs RA, Weinstock GM, Metzker ML, Muzny DM, Sodergren EJ, Scherer S, Scott G, Steffen D, Worley KC, Burch PE, Okwuonu G, Hines S, Lewis L, DeRamo C, Delgado O, Dugan-Rocha S, Miner G, Morgan M, Hawes A, Gill R, Celera, Holt RA, Adams MD, Amanatides PG, Baden-Tillson H, Barnstead M, Chin S, Evans CA, Ferriera S, Fosler C, Glodek A, Gu Z, Jennings D, Kraft CL, Nguyen T, Pfannkoch CM, Sitter C, Sutton GG, Venter JC, Woodage T, Smith D, Lee HM, Gustafson E, Cahill P, Kana A, Doucette-Stamm L, Weinstock K, Fechtel K, Weiss RB, Dunn DM, Green ED, Blakesley RW, Bouffard GG, De Jong PJ, Osoegawa K, Zhu B, Marra M, Schein J, Bosdet I, Fjell C, Jones S, Krzywinski M, Mathewson C, Siddiqui A, Wye N, McPherson J, Zhao S, Fraser CM, Shetty J, Shatsman S, Geer K, Chen Y, Abramzon S, Nierman WC, Havlak PH, Chen R, Durbin KJ, Egan A, Ren Y, Song XZ, Li B, Liu Y, Qin X, Cawley S, Cooney AJ, D'Souza LM, Martin K, Wu JQ, Gonzalez-Garay ML, Jackson AR, Kalafus KJ, McLeod MP, Milosavljevic A, Virk D, Volkov A, Wheeler DA, Zhang Z, Bailey JA, Eichler EE, Tuzun E, et al. 2004. Genome sequence of the Brown Norway rat yields insights into mammalian evolution. Nature 428:493–521.

Gien J, Seedorf GJ, Balasubramaniam V, Tseng N, Markham N, Abman SH. 2008. Chronic intrauterine pulmonary hypertension increases endothelial cell Rho kinase activity and impairs angiogenesis in vitro. Am J Physiol Lung Cell Mol Physiol 295:L680–L687.

Gong MC, Fujihara H, Somlyo AV, Somlyo AP. 1997. Translocation of rhoA associated with Ca^{2+} sensitization of smooth muscle. J Biol Chem 272: 10704–10709.

Heishi M, Ichihara J, Teramoto R, Itakura Y, Hayashi K, Ishikawa H, Gomi H, Sakai J, Kanaoka M, Taiji M, Kimura T. 2006. Global gene expression analysis in liver of obese diabetic db/db mice treated with metformin. Diabetologia 49:1647–1655.

Hsueh WA, Law RE. 1999. Insulin signaling in the arterial wall. Am J Cardiol 84:21J–24J.

Ilnytska O, Lyzogubov VV, Stevens MJ, Drel VR, Mashtalir N, Pacher P, Yorek MA, Obrosova IG. 2006. Poly(ADP-ribose) polymerase inhibition alleviates experimental diabetic sensory neuropathy. Diabetes 55:1686–1694.

Jones DR, Sanjuan MA, Merida I. 2000. Type Ialpha phosphatidylinositol 4-phosphate 5-kinase is a putative target for increased intracellular phosphatidic acid. FEBS Lett 476:160–165.

Kanoh H, Yamada K, Sakane F. 2002. Diacylglycerol kinases: Emerging downstream regulators in cell signaling systems. J Biochem 131:629–633.

Kellerer M, Mushack J, Mischak H, Haring HU. 1997. Protein kinase C (PKC) epsilon enhances the inhibitory effect of TNF alpha on insulin signaling in HEK293 cells. FEBS Lett 418:119–122.

Knoll KE, Pietrusz JL, Liang M. 2005. Tissue-specific transcriptome responses in rats with early streptozotocin-induced diabetes. Physiol Genomics 21:222–229.

Krook A, Roth RA, Jiang XJ, Zierath JR, Wallberg-Henriksson H. 1998. Insulin-stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects. Diabetes 47:1281–1286.

Kume E, Aruga C, Ishizuka Y, Takahashi K, Miwa S, Itoh M, Fujimura H, Toriumi W, Kitamura K, Doi K. 2005. Gene expression profiling in streptozotocin treated mouse liver using DNA microarray. Exp Toxicol Pathol 56:235–244.

Langberg EC, Gu HF, Nordman S, Efendic S, Ostenson CG. 2007. Genetic variation in receptor protein tyrosine phosphatase sigma is associated with type 2 diabetes in Swedish Caucasians. Eur J Endocrinol 157:459–464.

Lewis GF, Carpentier A, Adeli K, Giacca A. 2002. Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. Endocr Rev 23:201–229.

Liang M, Cowley AW, Greene AS. 2004. High throughput gene expression profiling: A molecular approach to integrative physiology. J Physiol 554:22–30.

Luo B, Prescott SM, Topham MK. 2003. Association of diacylglycerol kinase zeta with protein kinase C alpha: Spatial regulation of diacylglycerol signaling. J Cell Biol 160:929–937.

Luo B, Prescott SM, Topham MK. 2004. Diacylglycerol kinase zeta regulates phosphatidylinositol 4-phosphate 5-kinase Ialpha by a novel mechanism. Cell Signal 16:891–897.

Moomaw JF, Casey PJ. 1992. Mammalian protein geranylgeranyltransferase. Subunit composition and metal requirements. J Biol Chem 267:17438–17443.

Ninichuk V, Clauss S, Kulkarni O, Schmid H, Segerer S, Radomska E, Eulberg D, Buchner K, Selve N, Klussmann S. 2008. Late onset of Ccl2 blockade with the Spiegelmer mNOX-E36-3'PEG prevents glomerulosclerosis and improves glomerular filtration rate in db/db mice. Am J Pathol 172:628.

Qu X, Seale JP, Donnelly R. 1999. Tissue and isoform-selective activation of protein kinase C in insulin-resistant obese Zucker rats—effects of feeding. J Endocrinol 162:207–214.

Raza H, Prabu SK, Robin MA, Avadhani NG. 2004. Elevated mitochondrial cytochrome P450 2E1 and glutathione S-transferase A4-4 in streptozotocin-induced diabetic rats: Tissue-specific variations and roles in oxidative stress. Diabetes 53:185–194.

Rikitake Y, Liao JK. 2005. Rho-kinase mediates hyperglycemia-induced plasminogen activator inhibitor-1 expression in vascular endothelial cells. Circulation 111:3261–3268.

Rong JX, Qiu Y, Hansen MK, Zhu L, Zhang V, Xie M, Okamoto Y, Mattie MD, Higashiyama H, Asano S, Strum JC, Ryan TE. 2007. Adipose mitochondrial biogenesis is suppressed in db/db and high-fat diet-fed mice and improved by rosiglitazone. Diabetes 56:1751–1760.

Rosholt MN, King PA, Horton ES. 1994. High-fat diet reduces glucose transporter responses to both insulin and exercise. Am J Physiol 266: R95–R101.

Rossing P. 2006. Prediction, progression and prevention of diabetic nephropathy. The Minkowski Lecture 2005. Diabetologia 49:11–19.

Saltiel AR, Kahn CR. 2001. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414:799–806.

Samuel VT, Liu ZX, Qu X, Elder BD, Bilz S, Befroy D, Romanelli AJ, Shulman GI. 2004. Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. J Biol Chem 279:32345–32353.

Sandu OA, Ragolia L, Begum N. 2000. Diabetes in the Goto-Kakizaki rat is accompanied by impaired insulin-mediated myosin-bound phosphatase activation and vascular smooth muscle cell relaxation. Diabetes 49:2178–2189.

Sauzeau V, Le Jeune H, Cario-Toumaniantz C, Smolenski A, Lohmann SM, Bertoglio J, Chardin P, Pacaud P, Loirand G. 2000. Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced Ca^{2+} sensitization of contraction in vascular smooth muscle. J Biol Chem 275:21722–21729.

Schmitz-Peiffer C, Browne CL, Oakes ND, Watkinson A, Chisholm DJ, Kraegen EW, Biden TJ. 1997. Alterations in the expression and cellular localization of protein kinase C isozymes epsilon and theta are associated with insulin resistance in skeletal muscle of the high-fat-fed rat. Diabetes 46:169–178.

Shmueli E, Alberti KG, Record CO. 1993. Diacylglycerol/protein kinase C signalling: A mechanism for insulin resistance? J Intern Med 234:397–400.

Sparks LM, Xie H, Koza RA, Mynatt R, Hulver MW, Bray GA, Smith SR. 2005. A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. Diabetes 54:1926–1933.

Srinivasan K, Patole PS, Kaul CL, Ramarao P. 2004. Reversal of glucose intolerance by pioglitazone in high fat diet-fed rats. Methods Find Exp Clin Pharmacol 26:327–333.

Srinivasan K, Viswanad B, Asrat L, Kaul CL, Ramarao P. 2005. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: A model for type 2 diabetes and pharmacological screening. Pharmacol Res 52:313–320.

Tan AL, Forbes JM, Cooper ME. 2007. AGE, RAGE, and ROS in diabetic nephropathy. Semin Nephrol 27:130–143.

Targher G, Bertolini L, Rodella S, Tessari R, Zenari L, Lippi G, Arcaro G. 2007. Nonalcoholic fatty liver disease is independently associated with an increased incidence of cardiovascular events in type 2 diabetic patients. Diabetes Care 30:2119–2121.

Targher G, Bertolini L, Rodella S, Zoppini G, Lippi G, Day C, Muggeo M. 2008a. Non-alcoholic fatty liver disease is independently associated with an increased prevalence of chronic kidney disease and proliferative/laser-treated retinopathy in type 2 diabetic patients. Diabetologia 51:444–450.

Targher G, Chonchol M, Bertolini L, Rodella S, Zenari L, Lippi G, Franchini M, Zoppini G, Muggeo M. 2008b. Increased risk of CKD among type 2 diabetics with nonalcoholic fatty liver disease. J Am Soc Nephrol 19:1564–1570.

Tikoo K, Bhatt DK, Gaikwad AB, Sharma V, Kabra DG. 2007a. Differential effects of tannic acid on cisplatin induced nephrotoxicity in rats. FEBS Lett 581:2027–2035.

Tikoo K, Tripathi DN, Kabra DG, Sharma V, Gaikwad AB. 2007b. Intermittent fasting prevents the progression of type I diabetic nephropathy in rats and changes the expression of Sir2 and p53. FEBS Lett 581:1071–1078.

Tikoo K, Meena RL, Kabra DG, Gaikwad AB. 2008a. Change in post-translational modifications of histone H3, heat-shock protein-27 and MAP kinase p38 expression by curcumin in streptozotocin-induced type I diabetic nephropathy. Br J Pharmacol 153:1225–1231.

Tikoo K, Tamta A, Ali IY, Gupta J, Gaikwad AB. 2008b. Tannic acid prevents azidothymidine (AZT) induced hepatotoxicity and genotoxicity along with change in expression of PARG and histone H3 acetylation. Toxicol Lett 177:90–96.

Tolman KG, Fonseca V, Dalpiaz A, Tan MH. 2007. Spectrum of liver disease in type 2 diabetes and management of patients with diabetes and liver disease. Diabetes Care 30:734–743.

van Baal J, de Widt J, Divecha N, van Blitterswijk WJ. 2005. Translocation of diacylglycerol kinase theta from cytosol to plasma membrane in response to activation of G protein-coupled receptors and protein kinase C. J Biol Chem 280:9870–9878.

Yang RL, Li W, Shi YH, Le GW. 2008. Lipoic acid prevents high-fat diet-induced dyslipidemia and oxidative stress: A microarray analysis. Nutrition 24:582–588.