Gene expression analysis of hepatic roles in cause and development of diabetes in Goto-Kakizaki rats

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Abstract

Progression of diabetes was studied in male Goto-Kakizaki (GK) spontaneously diabetic rats between 4 and 20 weeks of age, and compared with Wistar-Kyoto (WKY) controls. Five animals from each strain were killed at 4, 8, 12, 16, and 20 weeks of age. Body weight, plasma glucose, and plasma insulin were measured. WKY rats showed a significantly larger weight gain than GK animals from 8 weeks of age onward. Plasma glucose was relatively stable in WKY. By contrast, plasma glucose was higher in GK than WKY even at 4 weeks and continued to increase up to 12 weeks and then maintained a hyperglycemic plateau throughout the remainder of the experiment. Plasma insulin was relatively stable in WKY from 8 weeks onward but was sharply elevated in GK between 4 and 8 weeks. After 8 weeks,

insulin declined in GK with GK concentrations lower than WKY at 20 weeks, suggesting β -cell failure. Gene expression in liver was explored using Affymetrix 230-2 gene arrays. Data mining identified 395 probe sets out of more than 31 000 that were differentially regulated. Excluding unidentifiable probe sets and considering duplicate probe sets, there were 311 genes that were expressed differently in the liver of the two strains. A functional analysis of these genes indicated that disruption of lipid metabolism in the liver is a major consequence of the chronic hyperglycemia in the GK strain. In addition, the results suggest that chronic inflammation contributes significantly to the development of diabetes in the GK rats.

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Introduction

Although rare forms of human type 2 diabetes are associated with defects in individual genes, more common forms appear to involve a complex interaction between predisposing genetic backgrounds and environmental factors. While epigenetic factors such as fetal programming may contribute, major aspects of the development of type 2 diabetes appear to result from interactions between the expression and/or structure of multiple genes with environmental lifestyle factors. The hallmark of type 2 diabetes is peripheral insulin resistance; however, the associated phenotypes in humans are quite heterogeneous. Other characteristics that may be, but are not always, associated with this form of diabetes are central obesity, dyslipidemia, hypertension, abnormal insulin secretion in response to glucose, hyperphagia, and excessive hepatic gluconeogenesis. At present, it is not clear the degree to which the various sub-phenotypes of human type 2 diabetes are the result of genetics, epigenetics, or environment (Sedova *et al.* 2007).

Several animal models have been developed for the study of type 2 diabetes. Many of these models such as the leptin-deficient (ob/ob) mouse, the leptin receptor-deficient (db/db) mouse, and the leptin receptor-deficient (fa/fa) rat are, like rare forms of

the human type 2 disease, the result of single-gene defects. In fact, there are a large number of genes that can either be knocked out or overexpressed to cause an insulin-resistant phenotype (Almon *et al.* 2005a). In contrast to the single-gene defect models, Goto-Kakizaki (GK) rats are a polygenic diabetic rat model. GK rats have been widely used as a surrogate in type 2 diabetes studies. Originally developed in the early 1970s in Japan from multiple-generation breeding of Wistar-Kyoto (WKY) rats selected for high population values of glucose intolerance, these rats exhibit a spontaneous form of diabetes (O'Rourke *et al.* 1997, Portha 2005). Although many conflicting reports exist in the literature as to other characteristics of GK rats, elevated blood glucose, peripheral insulin resistance, and a non-obese phenotype are consistent features of this animal model. While no single animal model is a perfect surrogate for the human disease, the polygenic nature of inheritance and the previously demonstrated insulin resistance affecting muscle, adipose tissue, and liver in GK rats makes them a valid model for the human disease. However, since obesity is a factor in some but not all type 2 diabetic humans, the GK rat model is similar to only a subset of human type 2 diabetic patients in this regard (Prando *et al.* 1998).

The liver plays a central role in systemic energy metabolism and therefore is important in diseases such as diabetes where

systemic energy substrate flow is altered. Liver is the only tissue that stores glucose in the form of glycogen; this glucose can be released in response to glucagon or epinephrine to maintain systemic concentrations. In addition to glucose storage and release, liver can also synthesize glucose *de novo* through the process of gluconeogenesis. In addition to carbohydrate metabolism, the liver is central to whole body lipid metabolism. About one-half of the cholesterol in the body is produced in the liver, and the liver is also central to the control, storage, and distribution of apolipoproteins.

The contributing role of the liver to hyperglycemia appears to be a variable in human type 2 diabetes. There is clear evidence that in some, but not all, cases, excessive gluconeogenesis in the liver contributes to hyperglycemia (Gastaldelli *et al.* 2000, Cohen & Goedert 2004). Conversely, it is clear that chronically elevated blood glucose also has a significant impact on the liver itself. In contrast to skeletal muscle that primarily obtains glucose through the insulin-dependent GLUT4 transporter, the liver takes up glucose through the non-insulin-dependent GLUT2 (Bloomgarden 2003). As a result, uptake of glucose into the liver is highly controlled by the blood glucose concentration. Therefore, chronic hyperglycemia causes the liver to take up large amounts of glucose. Other than energy generation, the only two ways of using this glucose are glycogen synthesis and lipid synthesis. There are reports of excessive glycogen deposition in the liver associated with human diabetes (Vallance-Owen 1952, Clore *et al.* 1992). Much more common is the excessive buildup of fat in the liver (steatosis; Lonardo *et al.* 2005).

In the present study, we sought to carefully characterize disease progression (e.g., blood glucose and insulin) in the taconic colony of GK rats from shortly after birth through mid-adulthood. To that end, groups of GK and WKY rats were maintained in our facility from weaning through 20 weeks of age, with subgroups killed at 4, 8, 12, 16, and 20 weeks. Livers were removed from these animals and used for an extensive time-series analysis of gene expression using Affymetrix 230-2 gene arrays that contain more than 31 000 probe sets. The results provide not only indications as to the cause of diabetes in these animals but also changes in liver gene expression that are the consequence of chronic hyperglycemia.

Materials and Methods

Experimental design

This study involved 30 GK spontaneously diabetic and 30 WKY control male rats obtained from Taconic Farms (Germantown, NY, USA). Our research protocol adheres to the 'Principles of Laboratory Animal Care' (NIH publication 85-23, revised in 1985) and was approved by the University at Buffalo Institutional Animal Care and Use Committee. Animals were received at 21 ± 3 days of age. For experimental purposes, all animals were considered 22 days old at the time of arrival. Rats were maintained in a separate room in our animal facilities under stringent environmental

conditions that included strict adherence to 12 h light:12 h darkness cycles. All animal manipulations and animal care were carried out between 1.5 and 3.5 h after lights on. Animals were housed in individual cages and body weights measured twice weekly on all animals. Five animals from each strain were killed at five different ages: 4, 8, 12, 16, and 20 weeks. At the time of killing, animals were anesthetized with ketamine (80 mg/kg)/valium (5 mg/kg) i.p. Blood glucose was measured from whole blood at the time of killing. Animals were killed by aortic exsanguination using EDTA (4 mM final concentration) as anticoagulant. Plasma was prepared from blood by centrifugation (2000 g, 4 °C, 15 min), aliquoted, and stored at -80 °C. Liver was harvested, weighed, rapidly frozen in liquid nitrogen, and stored at -80 °C for future studies.

Assays

Blood glucose Blood glucose was measured from whole blood at the time of killing using a BD Logic blood glucose meter (BD Medical, Franklin Lakes, NJ, USA).

Plasma glucose Plasma glucose was measured by the glucose oxidase method (Sigma GAGO-20). The manufacturer's instructions were modified such that the assay was carried out in a 1 ml assay volume, and a standard curve consisting of seven concentrations over a 16-fold range was prepared from the glucose standard and run with each experimental set. Experimental samples were run in triplicate.

Plasma insulin

Insulin was measured in plasma samples using a commercial RIA (RI-13K Rat Insulin RIA Kit, Millipore Corporation, St Charles, MO, USA). All assays were carried out according to the manufacturer's directions with standards run in duplicate and experimental samples run in triplicate.

Liver triglycerides

Frozen liver samples were extracted in chloroform:methanol (2:1), dried, and resuspended in butanol as described previously (Hundal *et al.* 2000). Extracted triglycerides were assayed colorimetrically with commercial reagents (WAKO Chemicals, Richmond, VA, USA).

Statistical analysis

For statistical comparisons, two-way ANOVAs were carried out using SigmaStat 3.5 software (Systat Software, Point Richmond, CA, USA) with Tukey *post hoc* tests. Since data were not normally distributed, ANOVAs were performed on rank-transformed data.

Microarrays

Liver samples from each animal were ground to a fine powder in a mortar cooled by liquid nitrogen and 100 mg was added

to 1 ml pre-chilled TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA extractions were carried out according to the manufacturer's directions and were further purified by passage through RNeasy mini-columns (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols for RNA cleanup. Final RNA preparations were resuspended in RNase-free water and stored at -80°C . The RNAs were quantified spectrophotometrically, and purity and integrity assessed by agarose gel electrophoresis. All samples exhibited 260/280 absorbance ratios of approximately 2.0, and all showed intact ribosomal 28S and 18S RNA bands in an approximate ratio of 2:1 as visualized by ethidium bromide staining. Isolated RNA from each liver sample was used to prepare target according to the manufacturer's protocols. The biotinylated cRNAs were hybridized to 50 individual Affymetrix GeneChips Rat Genome 230-2 (Affymetrix, Inc., Santa Clara, CA, USA), which contained more than 31 000 probe sets.

Data mining

Affymetrix Microarray Suite 5.0 (Affymetrix) was used for initial data acquisition and analysis. The signal intensities were normalized for each chip using a distribution of all genes around the 50th percentile. The dataset was then loaded into a data mining program, GeneSpring 7 (Silicon Genetics, Redwood City, CA, USA), for further analysis. The generated dataset was submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/projects/geo/>) database (GSE13271). In order to screen for probe sets with different expression levels in the GK versus WKY, each probe set on the five GK chips at each time point was normalized to the average of the same probe set on the five WKY chips. Thus, if there is no difference between GK and WKY the result is a value of 1. If GK is greater than WKY the value is greater than 1, and if GK is less than WKY, then the value is less than 1.

In order to objectively identify probe sets of interest, the entire dataset was filtered with criteria similar to the ones applied to previous gene array datasets (Almon *et al.* 2005b, 2007a,b). This approach does not select for probe sets but rather eliminates those probe sets that do not meet certain criteria, leaving the remainder for further consideration. The first filter was designed to eliminate probe sets for genes that are not expressed in the liver. This filter employed a function in the Affymetrix Microarray Suite 5.0. During the initial data analysis, a 'call' of present (P), absent (A), or marginal (M) for each probe set on each chip was determined based on the intensity comparison of the matched and mismatched probe sequence pairs. The first filter eliminated all probe sets that did not have a call of P on at least five of the GK chips. Starting with 31 099 probe sets this filter eliminated 13 460 probe sets leaving 17 639 probe sets for further consideration. We repeated this filter, but in this case normalizing the WKY to the average of each probe set for the GK. This yielded an additional 708 probe sets not on the list when GK was

normalized to WKY. The reversal and refiltering considered the possibility that a probe set could be absent on the chips of one strain but expressed on the chips of the other strain. This filtering step left a remainder of 18 347 probe sets to be carried forward into the next step.

The second level of filtering eliminated probe sets that could not meet the basic criterion of having different expression levels in GK and WKY rats. We employed two filters that eliminated probe sets that were neither lower nor higher in GK relative to WKY. The first filter started with the 5P-filtered list to remove all probe sets that did not have average values below 0.5 for at least three time points. Those probe sets retained thus had expression levels in which WKY is at least twice that of GK. The next filter again started with the 5P-filtered list, and eliminated all probe sets that did not have average values above 2 for at least three time points. Those probe sets retained by this filter thus had expression levels at least twice as high in GK relative to WKY.

Quantitative real-time RT-PCR

Because gluconeogenesis in the liver contributes to hyperglycemia in many humans with type 2 diabetes, the array results for the enzyme phosphoenolpyruvate carboxykinase (PCK1) were confirmed by real-time quantitative RT-PCR. PCK1 is the rate-limiting enzyme in gluconeogenesis. The quantity of liver *Pck1* mRNA along with gene-specific *in vitro*-transcribed cRNA standards was determined by quantitative real-time RT-PCR using TaqMan probes. Briefly, primer and probe sequences were designed using PrimerExpress software (Applied Biosystems, Foster City, CA, USA) and custom synthesized by Biosearch Technologies, Inc. (Novato, CA, USA). The RT-PCR was performed using Brilliant QRT-PCR Core Reagent Kit, 1-Step (Stratagene, La Jolla, CA, USA) in a Stratagene MX3005P thermocycler according to the manufacturer's instructions. A standard curve was generated using the *in vitro*-transcribed sense cRNA standards. Primer and probe sequences are as follows: forward primer (5'-CCGGGCACCTCAGTGAAG-3'), reverse primer (5'-CACGTTGGTGAAGATGGTGT-3'), probe (5'-FAM-ATCCGAACGCCATTAAAGACCATC-BHQ-3'). Additional minus reverse transcriptase controls were run for each RNA sample analyzed; all controls exhibited lack of amplification. Intra- and interassay coefficient of variation values were less than 15%.

Results

Animals

Figure 1 presents mean body weights of all animals throughout the course of this study. As expected, both strains of animals exhibited continual increases in body weight throughout the study period. Significant differences in strain were present from 8 weeks of age onward. WKY animals were heavier than GK animals by 8 weeks ($P=0.004$), and the

Figure 1 Body weights of GK and WKY rats from 3 to 20 weeks of age. Symbols represent means and error bars 1 s.d. of the mean. Closed circles, GK; open circles, WKY.

disparity in weight became more pronounced at later ages ($P < 0.001$).

Figure 2 (top panel) shows the change in absolute liver weight as a function of time. There is a large increase in the weight of the livers in both GK and WKY between 4 and 8 weeks followed by slow asymptotic increases over 20 weeks. **Figure 2** (bottom panel) shows liver weight as a percentage of total body weight. By 8 weeks and continuing throughout the remainder of the experiment, the liver was a significantly larger percentage of total body weight in GK when compared with WKY. Measurement of liver triglycerides showed slight age-dependent increases, but no statistical differences between GK and WKY animals at any age (data not shown). Glucose was measured in whole blood at the time of killing using a glucometer. However, at later ages, many GK rats had blood glucose values that exceeded the range of the meter (600 mg/dl). Therefore, glucose concentrations were also measured in plasma by an enzymatic assay. The ratio of individual values of blood glucose:plasma glucose had a mean of 1.03 ± 0.16 , and the correlation between the two values was 0.89. **Figure 3** presents mean plasma glucose values at the time of killing.

Plasma glucose concentrations increased with age in both strains, reaching an apparent plateau between 8 and 12 weeks of age. Not unexpectedly, GK animals had higher plasma glucose compared with control WKY animals at all ages ($P < 0.001$ at all ages). Plasma insulin determined from RIA analyses is presented in **Fig. 4**. Variation between animals within a group exhibited high s.d. This high variability was likely due to true animal variation rather than experimental error, since inter-assay variability averaged $4.3 \pm 3.3\%$ for all samples, and variability of quality control samples (intra-assay variability) was under 6%. In addition, the samples were also analyzed by an ELISA method, which, despite higher inter-assay variability in our hands, resulted in almost identical insulin profiles (data not shown). The general trend in the data appears to indicate an increase in plasma insulin between 4 and 8 weeks of age in both strains, with GK generally higher

Figure 2 Mean liver weights of GK and WKY rats as a function of age. Top panel represents weights of liver in grams, and bottom panel liver weights as a percentage of animal body weight. Error bars represent 1 s.d. of means.

than WKY values between 8 and 16 weeks. However, at 20 weeks of age, GK values appear lower than WKY controls. These strains tested as significantly different (P at least < 0.05) at all ages except 16 weeks.

Figure 3 Plasma glucose concentrations of GK and WKY rats as a function of age. Error bars represent 1 s.d. of means.

Figure 4 Plasma insulin concentrations of GK and WKY rats as a function of age. Error bars represent 1 s.d. of means.

Data mining

The Affymetrix R230-2 chip contains more than 31 000 probe sets. The initial problem is to identify probe sets of interest for further attention. Because the chip can measure an expression range of over three to four orders of magnitude, the first problem is to bring all probe sets into a common range. To accomplish this, we divided the signal for each probe set on each chip for GK by the average of that probe set on the five WKY chips. The result is that no difference yields a value of 1. If GK is greater than WKY, the value is greater than 1, and if WKY is greater than GK, the value is less than 1. Using this normalized dataset, we eliminated probe sets that were not expressed in the liver by filtering using the Affymetrix call flag. Previously, we used a similar filtering as an approach to eliminate probe sets not expressed in a tissue (liver, muscle, and kidney) in previous Affymetrix gene array time-series datasets (Almon *et al.* 2005b, 2007a,b). Eliminating probe sets that did not get a call of P on at least five chips on either GK or WKY left a remainder of 18 347 probe sets. We then eliminated probe sets in which the expression was not different in GK and WKY. We sought to identify two types of probe sets. The first were probe sets for genes that are causative to the difference in physiology between GK and WKY. Our assumption was that the expression of these genes should be different at all five ages. The second were probe sets that reflect the consequence of chronic hyperglycemia in GK rats. These should correspond to the development of hyperglycemia in GK and should be different in at least three time points. We therefore chose to retain probe sets different at three, four, or five time points. Elimination of all probe sets in which GK were not twice as high as WKY at three to five time points left a remainder of 174 probe sets. Conversely, eliminating all probe sets in which WKY were not at least twice as high as GK at three to five time points left a remainder of 221 probe sets. Together, the two lists yielded 395 probe sets for further consideration. Figure 5 shows a gene tree of all probe sets on the 230-2 chip (left panel) compared with the gene tree of the 395

probe sets not eliminated by the filters (right panel). In this presentation, the probe sets are displayed vertically as a tree and their expression dynamics over time are displayed horizontally in colors with yellow in the graph representing an expression ratio around 1 or no difference. Colors progressing toward red indicate normalized values greater than 1 (GK greater than WKY by at least twofold), and colors toward blue indicate values less than 1 (WKY greater than GK by at least twofold). The brightness of the colors reflects the original signal intensities or expression levels before normalization. The more abundantly expressed mRNAs exhibit brighter colors. The *x*-axis provides the five ages studied. The *y*-axis represents the list of the probe sets in order of similarity. Supplementary Table 1, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol200/issue3/> provides raw data for each of the 395 probe sets in all 50 samples.

Data analysis

Affymetrix provides an accession number for the sequence from which each probe set was built. We submitted the accession number of each selected probe set to the NCBI Basic Local Alignment Search Tool (BLAST) to identify, as closely as possible, its gene. Of the 395 probe sets, there were 55 probe sets that could not be identified by the BLAST of their accession numbers. In addition, in several instances, there were multiple probe sets for the same gene. This left 306 individual genes. These genes were then submitted to NCBI 'across database search' primarily to identify all aliases and alternate symbols. The preferred symbol was submitted to NCBI AceView as well as extensive PubMed Boolean logic searches to ascertain the function of the gene in liver. Based on these searches, we separated the genes into groups based on their function in the liver. These groups were as follows: transcription/translation; signaling; protein processing; lipid metabolism; immune/inflammatory; small molecule metabolism; cell cycle control; mitochondrial; and transport. An additional 24 genes that did not readily fit into these categories were grouped as miscellaneous, and the 55 probe sets not identifiable by BLAST were listed as ESTs. Clearly, such functional categorizations are not perfect because overlaps exist. For example, a transcription factor such as SREPB that is central to the regulation of lipid metabolism would be placed in lipid metabolism not transcription/translation, or a cytokine placed in the immune/inflammatory group rather than in the signaling group. Supplementary Table 2, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol200/issue3/> provides a list of all differentially regulated genes organized by these functional categories, and includes their probe set ID, accession number, gene name, symbol, and which strain was higher in how many conditions (ages). The functional analysis indicated that the genes that are differentially expressed in the GK and WKY strains are heavily weighted towards four functions. These functional groups are immune/inflammatory (67 probe sets),

Figure 5 Gene tree representations of probe sets on individual Affymetrix R230-2 gene chips hybridized to total RNA prepared from the livers taken from GK and WKY rats as a function of age. The left panel represents all probe sets on the chip, and the right panel represents the 395 probe sets identified as differentially regulated in GK compared with WKY animals. The x-axis represents the age of animals at killing. The y-axis represents the list of probe sets grouped by similarity using Pearson's correlation. The yellow color indicates no change from controls, red indicates probe sets with enhanced expression relative to controls, and blue indicates suppressed expression relative to controls.

signal transduction (48 probe sets), transcription/translation (57 probe sets), and lipid metabolism (46 probe sets).

Normalization of GK to WKY and WKY to GK provided the ratios we used in the gene selection process. Once genes were selected, we restructured the dataset to directly examine the basis for the difference in the ratio. For example, the ratio data for probe set 1 377 672 showed that with increasing age there is steadily increasing difference in the ratio, with WKY > GK. The direct comparison (Fig. 9B) demonstrates that the expression is increasing in WKY while staying essentially constant in GK. The change in the ratio could just as plausibly be due to WKY remaining constant while GK declined. Using this direct view, we were able to gain insight into both the underlying cause of the diabetes in the GK strain and the gene expression consequences of chronic hyperglycemia.

Immune/inflammatory processes

This functional category of differentially regulated genes (67 probe sets) indicates a generally altered inflammatory state in GK animals. Out of the 67 probe sets, 45 were higher in WKY when compared with GK animals, while 22 were higher in GK when compared with WKY. A large number of these genes are involved in antigen processing and presentation, while many others are involved in cytokine/chemokine signaling pathways (Supplementary Table 2). Selected examples of genes in this category are presented in Fig. 6. Figure 6A shows the expression profile of CD14 antigen (*Cd14*). CD14 is an initiator of the natural

immunity signaling cascade responsible for the induction of the inflammatory response caused by bacterial cell wall lipopolysaccharide (LPS; Kubota *et al.* 2004). The expression of *Cd14* is much higher in the livers of diabetic GK rats when compared with WKY at all ages examined. Adding to the picture of chronic inflammation in the GK rats are the expression patterns of two interferon-induced genes: Fig. 6B, interferon-induced protein with tetratricopeptide repeats 1 (*Ifit1*) and Fig. 6C, interferon-inducible GTPase (*Iigp1*; Boehm *et al.* 1998, Ogawa *et al.* 2005). Both of these interferon-induced genes are much higher in the livers of diabetic GK animals than in control WKY rats at all ages. Interferons (IFNs) are involved in natural anti-virus immunity. In addition to suggesting a heightened inflammatory state in these animals, excessive circulating concentrations of interferon are also associated with insulin resistance (Mofredj *et al.* 2002, Li *et al.* 2005). Another gene suggesting chronic inflammation in the GK rats is chemokine (C-X-C motif) ligand 14 (*Cxcl14*). Nara demonstrated that disruption of *Cxcl14* greatly reduced obesity-induced insulin resistance in mice, and that restoring the gene restored the insulin resistance (Nara *et al.* 2007). Figure 6D shows that *Cxcl14* is chronically higher in GK when compared with WKY.

Signal transduction

Signal transduction is integral to both regulation of energy metabolism and immune modulation, and not unexpectedly many differentially regulated genes in GK liver are involved with

Figure 6 Examples of differentially regulated Immune/Inflammatory-related genes in GK and WKY rats as a function of age. The y-axis represents raw, non-normalized probe set intensities and the x-axis animal age. Symbols represent mean values of chip data from five animals and error bars 1 s.d. of the mean.

signal transduction pathways. Examples of genes in this functional grouping are presented in Fig. 7. Several of these are directly involved in or have been associated with insulin signaling. For example, the phosphatidylinositol 3-kinase (PI 3-kinase), regulatory subunit, polypeptide 1 (p85 alpha) (PIK3R1) pathway is one of two signaling cascades central to the actions of insulin on peripheral tissues including liver (Matsumoto *et al.* 2003). Alterations in the PIK3R1 central hub of insulin metabolic actions have been linked to insulin resistance both through allelic variation and expression (Almind *et al.* 2002, Ueki *et al.* 2003). PIK3R1 is a heterodimer containing a regulatory subunit and a catalytic subunit. There are several isoforms of both the catalytic and regulatory subunits. Figure 7A and B reflects significant differences between GK and WKY in the expression of one of the regulatory isoforms and one of the catalytic isoforms. Figure 7A shows the profiles of PI 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha; *Pik3r1*). The expression of *Pik3r1* is higher in WKY than GK at all ages. Figure 7B shows the expression profiles of a catalytic subunit, phosphoinositide-3-kinase, class 3 (*Pik3c3*). Although at 4 weeks the message for this gene is more highly expressed in GK than WKY, starting at 8 weeks and for the remainder of the experiment, *Pik3c3* message is more highly expressed in WKY than GK.

PFTAIR protein kinase 1 (*Pfk1*), which is a cell cycle-related kinase (Shu *et al.* 2007), is another gene whose expression is remarkably different in GK and WKY. As shown in Fig. 7C, the expression of *Pfk1* is substantially higher in WKY when compared with GK, suggesting that cell proliferation may be reduced in the livers of GK rats. The last gene in these examples is myotubularin-related protein 7

Figure 7 Examples of differentially regulated genes related to signal transduction in GK and WKY rats as a function of age. The y-axis represents raw, non-normalized probe set intensities and the x-axis animal age. Symbols represent mean values of chip data from five animals and error bars 1 s.d. of the mean.

(*Mtmr7*). MTMR7 has been shown to dephosphorylate PtdIns, a central component to insulin signaling (Mochizuki & Majerus 2003). The much higher expression of this gene in GK relative to WKY may provide another mechanism of interference with insulin action (Fig. 7D).

Transcription/translation

This functional group contains a large number of differentially regulated genes. Many of these gene products are themselves controllers of gene expression (i.e., transcription factors). Many of these transcriptional regulators are associated with control of energy metabolism or inflammation. For example, signal transducer and activator of transcription 3 (STAT3) is a transcription factor that is involved in a variety of cellular functions. Employing knockout experiments, it has been observed that STAT3 plays a critical role in controlling inflammation and that deletion of *Stat3* causes an exacerbated inflammatory response (Kano *et al.* 2003). Figure 8A shows that *Stat3* expression is much higher in the livers from WKY when compared with GK animals. Figure 8B presents expression patterns of crystallin, mu (*Crym*), a gene that plays pivotal roles in capturing and transporting thyroid hormone (T_3) into the nucleus (Suzuki *et al.* 2007). Thyroid hormone influences the expression of many genes that are also influenced by insulin. Figure 8B shows that the expression of *Crym* is much lower in GK compared with WKY.

The third example, cellular repressor of E1A-stimulated genes 1 (CREG1), is a glycoprotein produced and secreted by the liver that binds to the insulin-like growth factor 2 receptor

Figure 8 Examples of differentially regulated genes related to transcription/translation in GK and WKY rats as a function of age. The y-axis represents raw, non-normalized probe set intensities and the x-axis animal age. Bars represent mean values of chip data from five animals. Symbols represent mean values of chip data from five animals and error bars 1 s.d. of the mean.

and generally inhibits growth and thus energy expenditure by the animal (Bauer *et al.* 2004). CREG1 is strongly upregulated by starvation and calorie restriction (Bauer *et al.* 2004). Therefore, it is interesting that *Creg1* is consistently more highly expressed in GK than WKY animals (Fig. 8C). Figure 8D presents the expression pattern of the transcription factor RAB. RAB, a member of RAS oncogene family-like 3 (*Rabl3*), participates in the regulation of genes involved in vesicular trafficking and its upregulation in the liver has been associated with obesity (Wilton & Matthews 1996). The expression of *Rabl3* is much higher at all ages in GK relative to WKY rats (Fig. 8D). The examples presented here further support the conclusion that there are alterations in insulin signaling capacity, altered energy metabolism, and altered immune responses in the livers from diabetic animals.

Lipid metabolism

Figure 9 provides examples of genes involved in various aspects of lipid metabolism that are differentially expressed in the GK and WKY strains. Figure 9A presents expression of cytoplasmic, epoxide hydrolase 2 (*Ephx2*). EPHX2 is an important enzyme in the metabolic breakdown of arachidonic acid-derived eicosanoids (Lee *et al.* 2006). Eicosanoids are involved in a variety of processes ranging from blood pressure regulation and calcium homeostasis to the systemic response to inflammation (De Caterina & Basta 2001). *Ephx2* is much more highly expressed in the livers from WKY than GK animals. Polymorphisms of EPHX2 are associated with

Figure 9 Examples of differentially regulated genes related to lipid metabolism in GK and WKY rats as a function of age. The y-axis represents raw, non-normalized probe set intensities and the x-axis animal age. Bars represent mean values of chip data from five animals. Symbols represent mean values of chip data from five animals and error bars 1 s.d. of the mean.

predispositions for a variety of dyslipidemic states such as hypercholesterolemia as well as hypertension and insulin resistance (Sato *et al.* 2004).

Figure 9B provides the expression profile for sulfotransferase family, cytosolic, 1C, member 2 (*Sult1c2*). SULT1C2 is involved in the sulfate conjugation of many endogenous and exogenous compounds. Of particular relevance to the liver is the degradation of bile acids (Vemula *et al.* 2004). It is also strongly downregulated as part of the complex series of reactions known as an acute phase response (Vemula *et al.* 2004). The livers from WKY animals show a continuous increase in expression of *Sult1c2* with age. By contrast, this age-related enhancement fails to occur in GK rats. These data also support the hypothesis that chronic activation of the inflammation-related acute phase response is occurring in the GK animals.

Figure 9C provides the expression profiles of ATP-binding cassette, sub-family A, member 1 (*Absc1*), which is a cholesterol efflux pump in the cellular lipid removal pathway. *ABCA1* controls the rate-limiting step in high-density lipoproteins (HDL) particle assembly by mediating efflux of cholesterol and phospholipids from cells to lipid-free APOA1, which forms nascent HDL particles (Tanaka *et al.* 2008). The expression of this gene is much higher in the livers from GK compared with WKY rats. Figure 9D shows the expression of apolipoprotein A-II (*Apoa2*). *Apoa2* is also much higher in GK relative to WKY at all ages. APOA2 is the second most abundant apolipoprotein in HDL and has a role in the regulation of lecithin cholesterol acetyltransferase (LCAT). LCAT is the major enzyme involved in the esterification

of free cholesterol present in circulating plasma lipoproteins and is a major determinant of plasma HDL concentrations (Weng & Breslow 1996). These examples are not inclusive of all differentially expressed genes categorized as related to lipid metabolism (Supplementary Table 2). However, they are meant to illustrate that there are differences in the expression of genes involved in many aspects of lipid metabolism (bile acid, fatty acid, triglyceride, and cholesterol metabolism) in liver between GK and WKY rats.

Other regulated genes of potential interest

There are many additional genes that also warrant attention because of their differential expression in GK and WKY rats. The first two examples presented here are biliverdin reductase B (*Blvre*) and glutathione peroxidase 2 (*Gpx2*). The expression profiles for these two genes are shown in Fig. 10, and we have classified both under the functional grouping of Small Molecule Metabolism. Both BLVRB and GPX2 have antioxidant capabilities and are up regulated in response to oxidative stress (Wang & Ortiz de Montellano 2003, Yan & Chen 2006). Figure 10A and B demonstrates that both genes are more highly expressed at all ages in GK when compared with WKY. Another example is *Resp18*. Although initially thought to be endocrine tissue specific, regulated endocrine-specific protein 18 (RESP18) has been found in many tissues including liver. This gene is involved in secretory processes and seems to have tissue-specific regulation (Bloomquist *et al.* 1994, Schiller *et al.* 1995).

Figure 10 Examples of differentially regulated genes in other functional categories in GK and WKY rats as a function of age. The y-axis represents raw, non-normalized probe set intensities, and the x-axis animal age. Bars represent mean values of chip data from five animals. Symbols represent mean values of chip data from five animals and error bars 1 S.D. of the mean.

As shown in Fig. 10C, *Resp18* is more highly expressed in GK relative to WKY and that the difference seems to increase with age after 8 weeks. The last gene in these examples is one whose sequence blasts to sequences for α -2u globulin and major urinary protein 5 (*Mup5*; Rowland *et al.* 2005). Although it is not clear exactly what this protein does and its relationship to diabetes, the profile of this lipid-binding protein is quite interesting. Figure 10D shows the expression profiles of this gene in both GK and WKY rats. At 4 weeks of age, the expression is hardly measurable in both strains. However, at 8 weeks, it is strongly expressed in both strains with GK higher than WKY. The difference between GK and WKY increases with age. Out of the 341 currently identifiable probe sets that are differentially expressed in the livers of diabetic animals, 219 are functionally grouped in one out of the four categories (immune/inflammatory, signaling, transcription/translation, or lipid metabolism).

However, an additional 122 probe sets were found to be regulated in our experiment, and the four profiles presented in Fig. 10 are simply examples of these. Raw data for all probe sets identified as differentially regulated can be found in Supplementary Table 1. In addition, the entire dataset is available through the NCBI GEO repository (GSE13271).

Phosphoenolpyruvate carboxykinase 1 (*PCK1*)

There are reports that in a subset of human type 2 diabetic patients, excessive gluconeogenesis by the liver significantly contributes to the hyperglycemia (Hundal *et al.* 2000). *PCK1*, which converts oxaloacetate into phosphoenolpyruvate and carbon dioxide, is the rate-limiting enzyme for gluconeogenesis. The array contained a probe set for *PCK1* (1372264_at). Although this probe set did not meet our selection criteria of having a twofold difference in three out of five time points, we did examine the data directly. The array results indicated that GK was significantly higher than WKY ($P < 0.001$) only at 4 weeks. We reexamined this result using QRT-PCR. The highly significant difference between GK and WKY at 4 weeks was confirmed by QRT-PCR. However, QRT-PCR did indicate that GK was significantly higher with a lesser P value of < 0.05 at three other times as well (8, 12, and 20 weeks). The results, presented in Fig. 11, do not indicate a great difference between GK and WKY in the *Pck1* expression except at 4 weeks when measured with either methodology. While in general agreement, the difference in results obtained by the two methods reflects the greater precision of QRT-PCR.

Discussion

The liver is the central hub in the flow of systemic energy substrates. In sedentary circumstances, about 75% of the blood entering the liver comes from the hepatic portal vein. Following eating, this blood rich in glucose and GLUT2 will mediate the uptake of large quantities of glucose into

Figure 11 Expression of *Pck1* mRNA in liver from GK and WKY rats as a function of age measured by kinetic-based real-time quantitative RT-PCT (A) and by gene arrays (B). Symbols represent mean values from five animals and error bars 1 s.d. of the mean.

hepatocytes. Insulin effects on the liver are several, most of which are designed to promote storage and inhibit glucose release. In the liver, insulin activates hexokinase activity and inhibits glucose-6-phosphatase that 'captures' the glucose in the cells. Insulin stimulates both glycogen synthesis and lipogenesis while inhibiting gluconeogenesis and glycogen breakdown. Most of the remaining excess glucose that passes the liver into the general circulation is taken up by the musculature through the insulin-dependent transporter, GLUT4. To a great degree, insulin resistance in muscle involves the inability of the musculature to remove glucose from circulation. A consequence is that blood circulating to the liver remains rich in glucose and glucose continues to flow into the liver through GLUT2 (Bloomgarden 2003). Because water follows glucose into cells, the free glucose must be disposed of in some way. Besides energy generation, there are only two ways to dispose of the glucose: substrate-driven lipogenesis and glycogen synthesis. Lipogenesis leading to steatosis is common in human type 2 diabetes and excessive glycogen storage has also been reported (Vallance-Owen 1952, Clore *et al.* 1992, Lonardo *et al.* 2005).

In the present study, we compared the post-natal growth and development of GK rats with control WKY rats from 4 to 20 weeks of age. Although GK rats were derived from WKY, the two groups of animals are clearly different in body weights, with WKY being significantly heavier (Fig. 1). There is a substantial increase in the weight of the livers of both strains between 4 and 8 weeks of age, with slower increases throughout the remainder of the experimental period. However, the increases in the livers of the GK rats were proportionately larger than those in WKY (Fig. 2), despite the absence of excessive fat deposition in the GK livers as indicated by liver triglyceride concentrations. One distinct possibility to be examined in the future is that the proportionately larger weight of the GK livers is due to deposition of glycogen resulting from their chronic hyperglycemia, as illustrated in Fig. 3. It has previously been reported that with increasing age, GK rats develop β -cell failure (Portha 2005).

Plasma insulin concentrations (Fig. 4) tend to support this observation. By 8 weeks of age, the GK rats have higher circulating insulin, which is consistent with the hyperglycemia, but by 20 weeks insulin concentrations are higher in WKY than GK, suggesting that the β -cells of the GK are no longer capable of fully responding to the hyperglycemia.

We globally examined the gene expression in the livers of diabetic GK rats compared with control WKY rats as a function of disease progression from 4 to 20 weeks of age. Data mining yielded 395 probe sets out of more than 31 000 on the chip (less than 1.3%) that had at least a twofold difference in expression between the two strains for at least three ages. Based on an extensive review of the literature, we divided these probe sets into functional groups and examined these groups for genes whose expression can provide insights into the pathology. Gene arrays simply provide a snapshot of the concentration of mRNAs at one point in time. The results reflect the potential available at that time for the production of proteins. When used in a time series as we have done in this experiment, we are able to observe how that potential changes with disease development and progression. By design, the process by which we obtained RNA from the liver was as heterogeneous as possible, sampling all cell types. Thus, we cannot determine whether a particular message is from the various populations of hepatocytes, the vasculature of the liver, or the extensive population of immune cells that reside in the liver. Although we cannot directly ascertain whether steady-state concentrations of message are due to mRNA production or destruction, we can obtain insight from examining messages for proteins involved in functional processes that can either contribute to hyperglycemia or may reflect the impact of hyperglycemia on the liver.

The liver resides at the end of the hepatic portal system and normally receives about 70% of its blood supply as blood that has circulated first to the small intestine, which is both a source of nutrients and a potential source of infection. In the normal situation, 10–15% of the total blood volume is in the liver, with roughly 60% of that in the sinusoids. Because of its

location and large blood volume, the liver is very important for systemic immune surveillance and protection. Liver macrophages, Kupffer cells, are very actively phagocytic. The liver also produces approximately half of the lymph formed in the body. Chronic inflammation is linked to the development of insulin resistance, and chronic hyperglycemia is also linked to chronic inflammation (Wellen & Hotamisligil 2005). If systemic inflammation is present one would expect to observe it in the liver. The profiles presented in Fig. 6 illustrate the presence of chronic inflammation in GK rats. As part of the natural immune response to bacteria, there is a rapid systemic response to LPS, a carbohydrate–lipid structure expressed on their outer cell wall of gram-negative bacteria. CD14 is a protein expressed on the surface of many cells (especially macrophages) that serves as a coreceptor for LPS (Kubota *et al.* 2004). In addition, liver secretes into circulation a soluble form of CD14 that can confer LPS responsiveness to other tissues (Kubota *et al.* 2004). To some degree, LPS is always present in circulation if for no other reason than arising from gut bacteria. Hyperresponsiveness to LPS has been observed in both human and animal forms of type 2 diabetes (Yang *et al.* 1997, Cani *et al.* 2007, Amar *et al.* 2008). The profiles for CD14 indicate chronic high levels of message for CD14 in GK rats relative to WKY (Fig. 6A).

IFNs are protein cytokines produced by immune cells in response to a variety of ‘foreign’ agents, in particular double-stranded RNA. Relationships between high levels of IFNs and insulin resistance have been reported in both humans and animals (Mofredj *et al.* 2002, Li *et al.* 2005). High levels of IFNs can occur for a variety of reasons, ranging from infections such as hepatitis to interferon therapy. The relationship between elevated IFN and insulin resistance is not absolute. Rather, some investigators hypothesize that insulin resistance in response to IFN only occurs in type 2 diabetic-prone humans and animals (Mofredj *et al.* 2002, Li *et al.* 2005). *Ifit1* and *Iigp1* are two IFN-induced, proinflammatory genes that are chronically far more highly expressed in the livers of GK rats than in the livers of control WKY animals (Fig. 6B and C). CXCL14 is a secreted protein involved in immunoregulatory and inflammatory processes (Nara *et al.* 2007), which is also chronically upregulated in the livers of GK rats (Fig. 6D). CXCL14 is involved in attracting dendritic cells and it has been reported that this chemokine may be specific for *Cd14*-positive cells (Meuter *et al.* 2007), which is relevant, given the high expression of *Cd14* in the livers of GK rats discussed earlier. Just as there are processes that promote inflammation, there are those that inhibit inflammatory signaling. SOCS2, which is a member of a family of suppressor of cytokine signaling proteins, is more highly expressed in the livers of WKY animals (Supplementary Table 2). SOCS2 interacts with another negative regulator of cytokine signaling, CIS (Uytendaele *et al.* 2007), which also has a lower expression profile in GK (Supplementary Table 2). The final example is *Traf6*, which is more highly expressed in the livers of WKY rats (Supplementary Table 2). TRAF6 has been shown to interfere with the

IL1B-induced signaling pathway involved in inflammation (Matsumura *et al.* 2004).

Clearly, defects in insulin signaling are involved in some types of human type 2 diabetes. With respect to the GK rat model of type 2 diabetes, the results are rather mixed. In this experiment, we observed a number of differences in the expression of genes in the livers of GK rats versus WKY, which could significantly contribute to the diabetic phenotype. Four examples of differences in expression profiles between GK and WKY are shown in Fig. 7. PI 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha) (*Pik3r1*) is a regulatory subunit that interacts with a type 1a catalytic subunit. This catalytic subunit is directly downstream from IRS proteins in the insulin signaling pathway. Decreased expression of *Pik3r1* suggests a possible decrease in insulin signaling at the level of PI3-kinase (Fig. 7A). Sandu *et al.* (2000) reported that in vascular smooth muscle, the signal transfer between IRS1 and PIK3R1 is disrupted in GK rats (Almind *et al.* 2002, Ueki *et al.* 2003, Matsumura *et al.* 2004). However, they attributed this to a close downstream step involving myosin-bound phosphatase (MYPT).

On the chip, there are probe sets for *Ppp1r12a*, *Ppp1r12b*, *Ppp1r16a*, *Mypt4*, and *Mypt5*. However, a review of the results for these probe sets showed that there was no difference between GK and WKY for any MYPT isoforms. PI 3-kinases are a family of enzymes that are involved in regulating a large number of diverse processes in virtually all tissues by generating lipid second messengers. There are three general types of PIK3R1s. The aforementioned PIK3R1 is in a type 1a pathway, which is activated by tyrosine kinase-associated receptors (Almind *et al.* 2002, Ueki *et al.* 2003, Matsumura *et al.* 2004). The second gene in Fig. 7 is *Pik3c3*, also known as *Vps34*, which is a catalytic subunit in a type 3 pathway (Almind *et al.* 2002, Ueki *et al.* 2003, Matsumura *et al.* 2004). PIK3C3 does not interact with PIK3R1, but rather interacts with the p150 regulatory subunit. The profile for *Pik3c3* showed that the expression for this gene was considerably higher in WKY relative to GK (Fig. 7B). This result would suggest that in the livers of GK rats, there is reduction in the intracellular movement of membrane proteins because type 3 pathways are involved in vesicle trafficking. *Pf1k1* codes for a cyclin-dependent kinase (Shu *et al.* 2007), which is chronically much more highly expressed in the livers of WKY than GK (Fig. 7C). This result suggests that cell proliferation is reduced in the livers of GK rats relative to WKY. As discussed above, one of the functional themes that distinguish GK from WKY is inflammation. In exploring possible relationships, we found that IFNs, whether pathologically or therapeutically elevated, inhibit cell proliferation in the liver (Degenhardt *et al.* 2006) and may relate to the lower expression of *Pf1k1* in the livers from GK rats. The last example presented in the signal transduction group is *Mtmr7*, which is a lipid phosphatase that removes phosphate groups on the phosphorylated inositol head group (Mochizuki & Majerus 2003). This phosphatase is chronically upregulated in the livers of GK rats when compared with WKY (Fig. 7D).

This is particularly interesting because *Mtmr7* is counter-regulatory to *Pik3c3*, which is much lower in GK relative to WKY (Fig. 7B). Together, the two results indicate that there is much lower activity in GK relative to WKY rats in this pathway involved in regulating vesicle trafficking. Other genes of interest in this functional category include *Igfbp1* and *Dyrk1a* (Supplementary Table 2). IGFBP1 is a hepatocyte-derived secreted protein. The primary role of IGFBP1 appears to be binding and sequestering insulin-like growth factor 1 (IGF1), thus reducing its availability (Deegenhardt *et al.* 2006). IGF1 is structurally and functionally related to insulin and contributes to glucose disposal from circulation. The expression of *Igfbp1* is substantially higher in the livers of GK relative to WKY rats. Data are available demonstrating that elevating circulating IGFBP1 elevates the circulating glucose concentration (Deegenhardt *et al.* 2006).

Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2 (DYRK2) is a cytoplasmic member of a family of priming kinases. These enzymes phosphorylate residues important to the phosphorylation of adjacent serine residues. Although at present the role of DYRK2 in the liver is not well understood, the members of the DYRK family have been shown to play an important role in the activation/inactivation of both the translation initiation factor EIF2B and glycogen synthase kinase 3 beta (GSK3B; Cohen & Goedert 2004, Wilson *et al.* 2005). Insulin is a prominent regulator of the activity of both EIF2B and GSK3B, which are keys to the regulation of protein and glycogen synthesis respectively. Therefore, it may be important that in WKY there is a steady increase in the expression of *Dyrk2* with age, but that this increase does not occur in the livers of GK rats.

Gene arrays only provide mRNA concentrations for a large number of genes at the time of killing. However, since the expressions of specific genes are involved in producing proteins, the results do provide indications concerning the availability of groups of proteins. For this reason, we have constructed a functional group that includes transcription and translation, and present examples of a number of differentially regulated genes in this category that may have relevance to the diabetic phenotype. STAT3 is a member of the family of transcription factors known as 'signal transducer and activator of transcription'. STATs are activated by tyrosine phosphorylation followed by dimerization and translocation into the nucleus where they bind to cognate sites in the promoter regions of responsive genes (Maritano *et al.* 2004). STAT3 mediates quite diverse functions in different tissues. In vascular endothelial cells, it mediates the expression of genes that protect against inflammation induced by LPS (Kano *et al.* 2003, Maritano *et al.* 2004). In hepatocytes, it mediates the aspects of the acute phase response promoting the expression of both pro- and anti-inflammatory proteins (Maritano *et al.* 2004). Similarly, its effect on the various immune cells is both pro- and anti-inflammatory being involved in both IL6 and IL10 signaling. Because of the heterogeneous population of cells contributing to our array results, it is difficult to interpret the results for this gene except that regulation of inflammation

is relevant in all cell types. Therefore, it is significant in that the expression of *Stat3* is chronically much lower in GK rats when compared with WKY (Fig. 8A).

A second example is provided by *Crym*, which is relevant to thyroid hormone function. Thyroid hormone receptor, like the glucocorticoid receptor, is a member of the nuclear hormone receptor superfamily and participates in the regulation of a subset of genes. The activating ligand is 3,3',5-triiodothyronine (T₃). CRYM is a NADPH-dependent cytosolic T₃-binding protein that participates in the regulation of the availability of T₃ to thyroid hormone receptor (Suzuki *et al.* 2007). Figure 8B demonstrates that the message for *Crym* is chronically higher in the liver of WKY when compared with GK rats. Given the role of thyroid hormone in regulating the expression of genes involved in energy metabolism, this difference in gene expression may be relevant to diabetes in GK rats.

Another transcription factor chronically elevated in GK samples is CREBL2. AMP-activated protein kinase (AMPK) is a major sensor of cellular energy charge. The general role of AMPK is to enhance ATP production and to reduce ATP utilization. In the liver, phosphorylation of targets by AMPK inhibits lipogenesis and gluconeogenesis while promoting the oxidation of fatty acids. Both enzymes such as HMG-CoA reductase (the rate-limiting enzyme in cholesterol biosynthesis) and transcription factors are targets of AMPK (Thomson *et al.* 2008). CREBL2 is a transcription factor whose activity is influenced by AMPK and several other kinases. CREBL2 is a member of the CREB family of transcription factors that bind to cAMP-response element (CRE) in the promoter regions of responsive genes (Thomson *et al.* 2008). Phosphorylation of CREBL2 activates it and promotes its translocation into the nucleus and binding to CREs. Thus, it may be relevant that this transcription factor has chronically higher expression in the livers from GK rats relative to WKY. In general, this transcription factor would be expected to participate in the inhibition of gluconeogenesis in the liver. Based on studies treating GK rats with troglitazone, O'Rourke *et al.* (1997) concluded that gluconeogenesis in the liver contributes to hyperglycemia in this model, much as it appears to do in some human type 2 diabetics. The chronically elevated levels of *Crebl2* seen here appear contrary to that conclusion. Cellular repressor of E1A-stimulated genes 1 (*Creg1*) was placed in this functional group instead of signal transduction because it influences the expression of a distinct set of genes (Tsuchiya *et al.* 2004). The expression profiles of this gene in the GK and WKY livers suggest that the observed difference may be a consequence of hyperglycemia. Specifically, there is not much difference at 4 weeks of age, but with increasing age GK becomes increasingly higher than WKY, with a pattern similar to the increased hyperglycemia (Fig. 8C). CREG1 is a secreted phosphoglycoprotein that represses E1A-stimulated genes. These genes stimulate cell division and inhibit differentiation. Specifically, during the cell cycle, they promote the transition from G1- to S-phase and the start of

DNA synthesis (Veal *et al.* 1998). The last gene in this group is *Rabl3* (Fig. 8D). Following translation, a membrane protein must be both folded properly and inserted into the membrane. *Rabl3* is a member of the RAB family of GTPases, which is involved in membrane trafficking to tight junctions in hepatocytes (Wilton & Matthews 1996). It has been reported that LPS disrupt tight junction in the liver (Sheth *et al.* 2007). The chronic higher expression of *Rabl3* in the livers of GK compared with WKY rats may reflect chronic repair of the damage caused by chronic inflammation. Another example in this functional category is nocturnin (Supplementary Table 2). Nocturnin, also known as CCR4 carbon catabolite repression 4-like (*S. cerevisiae*) (*Ccrn4l*), is a deadenylase and as such promotes the degradation of mRNAs. Nocturnin is important to regulating the concentration of messages for proteins involved in energy metabolism (Green *et al.* 2007). What is interesting about this gene is that its expression has a distinct circadian pattern with the highest expression early in the active feeding period of these nocturnal animals (Green *et al.* 2007). It is therefore important that these animals were killed early in the inactive rest period when the expression of nocturnin should be at its lowest point. The expression of this gene is consistently higher in the livers of GK when compared with WKY rats. Because we only measured expression at one time within the 24-h cycle, we cannot determine whether the difference between GK and WKY reflects a disturbance in circadian rhythms in GK rats, which itself has been shown to contribute to insulin resistance and metabolic syndrome, or whether the difference occurs throughout the circadian rhythm. However, it is relevant that knocking out nocturnin in mice confers resistance to diet-induced hepatic steatosis and obesity (Green *et al.* 2007).

Part of our criteria for identifying genes that are different between GK and WKY was a twofold difference in at least three out of five time points. PCK1, the rate-limiting enzyme for gluconeogenesis, clearly did not meet this criterion. However, because of the importance of this enzyme to glucose homeostasis, we directly looked at the probe set for PCK1. The array results showed that only at 4 weeks was the expression of *Pck1* mRNA substantially higher in GK relative to WKY. Additionally, we measured the expression of *Pck1* mRNA using quantitative fluorescence-based real-time RT-PCR. The results confirmed the substantially higher expression in GK at 4 weeks. However, smaller differences at three other times also tested significant at a much lower probability. Thus, our data suggest that excessive gluconeogenesis may only contribute significantly to the less than twofold elevation in glucose seen in GK rats at the earliest time point (4 weeks), but not to the several fold elevation in glucose seen at later time. Therefore, the results suggest that impaired glucose disposal is most likely the basis of the chronic hyperglycemia at the later ages.

Chronic hyperglycemia due to insulin resistance places the liver in a situation where its blood supply is chronically rich in glucose. In this situation, large amounts of glucose flow into

the liver through the insulin-independent GLUT2. The two primary ways of disposing of this excess glucose are substrate-driven lipid synthesis and glycogen synthesis. It is well documented that many type 2 diabetics develop steatosis (Lonardo *et al.* 2005). One of the most populated functional groups we found was lipid metabolism. The four examples from the lipid metabolism group shown in Fig. 9 suggest that alteration in lipid metabolism in the liver is a major consequence of the chronic hyperglycemia in GK rats. The first of these genes is *Ephx2*. As shown in Fig. 9A, the expression of this gene is very low in GK relative to WKY. This is important because a defect in this gene is associated with a type of hereditary hyperlipidemia, familial hypercholesterolemia, insulin resistance, and hypertension, suggesting that the activity of this gene is important for the normal control of cholesterol (Sato *et al.* 2004, Lee *et al.* 2006). Similarly, the expression in *Sult1c2*, which is involved in the degradation of bile acids through sulfation and therefore participates in cholesterol metabolism (Vemula *et al.* 2004), is much lower in GK relative to WKY (Fig. 9B). However, in this case, the expression of the gene increases with age in the WKY but remains low in GK.

By contrast, citrate lyase beta like (*Clybl*), which is involved in fatty acid biosynthesis (Morikawa *et al.* 2001), becomes progressively higher in GK relative to WKY with age due to the decline of this enzyme in WKY (Supplementary Table 2). Consistent with a picture of elevated lipid biosynthesis in GK is the higher amount of mRNA for ELOVL family member 6 (*Elo2*), which is involved in the elongation of long-chain fatty acids (Kobayashi *et al.* 2007), and acyl-CoA synthetase medium-chain family member 2 (*Acsn2*), which is involved in the production of medium-chain acyl-CoA (Lindner *et al.* 2006; Supplementary Table 2). The expression of both *Abca1* and *Apoa2* are much higher in GK at all ages (Fig. 9C and D). ABCA1 functions as a cholesterol efflux pump in the cellular lipid removal pathway, and is rate limiting in HDL particle assembly (Tanaka *et al.* 2008) while APOA2 is the second most abundant apolipoprotein in HDL (Weng & Breslow 1996). The elevations in message for these two genes suggest elevation in circulating HDL. An elevation in HDL may help explain the resistance of GK rats to atherosclerosis reported by Ragolia *et al.* (2004). Adding to the picture of resistance to atherosclerosis in GK rats is the lower expression of *Apobec1* (Supplementary Table 2), which is the catalytic component of the *Apob* mRNA editing enzyme complex. Lowering the expression of this gene should lower LDL (Dutta *et al.* 2003). *Fabp7* is consistently lower in GK relative to WKY, while the expression of *Fabp2* (Supplementary Table 2) is elevated in GK relative to WKY. These differences are interesting because dysregulation of fatty acid-binding proteins is thought to provide a link between lipid metabolism and chronic inflammation, which often accompanies type 2 diabetes in humans (Linton & Fazio 2003).

More than half of the 306 identified genes are encompassed by the functional groups discussed earlier. However, there are genes not in these groups that may provide insights into both

the cause and the consequences of diabetes in GK rats. Some examples are provided here. The first two are *Blurb* and *Gpx2*, both of which are more highly expressed in the livers of GK relative to WKY (Fig. 10A and B). These two antioxidant enzymes are markers of oxidative stress that is attendant on diabetes (Wang & Ortiz de Montellano 2003, Yan & Chen 2006). Another, *Cyp3a25*, whose expression is influenced by both GH and testosterone (Kawai *et al.* 2000) shows an age-related progressive increase in the expression in WKY that does occur in GK (Supplementary Table 2). One consequence of chronic hyperglycemia on the liver is to compromise liver function. The difference in the expression of *Cyp3a25* most likely reflects the impact of diabetes on liver function. Similarly, the higher expression of *Uqcrb*, a mitochondrial respiratory chain enzyme is probably consequential and may be related to oxidative stress (Supplementary Table 2). RESP18 is a protein, the glycosylated form of which is secreted. This gene seems to be involved in regulating systemic stress responses (Bloomquist *et al.* 1994). The higher expression in GK relative to WKY probably reflects a consequence of diabetes (Fig. 10C). *Mup5* is a member of the lipocalin family whose expression is highly androgen dependent and has been reported to only be expressed at about 40 days of age in male rats (Rowland *et al.* 2005). Consistent with that observation, our data show that the message for this protein is virtually absent in the livers of both strains at 4 weeks but appears in both at 8 weeks (Fig. 10D). However, the expression in the livers of WKY declines with age while remaining rather high in GK. Lipocalins bind and transport hydrophobic molecules and the difference between the two strains may reflect the altered lipid metabolism in GK rats discussed earlier.

The results of this study strongly support the hypothesis that chronic inflammation causally contributes to the development of diabetes in GK rats just as it does in a subset of humans with type 2 diabetes. Continued testing of this hypothesis will involve the analysis of other tissues from these animals as well as physiological measurements relevant to inflammation. Each animal in this study had a unique identifier and all samples are indexed to this identifier. Supplementary Table 1, which provides the raw data for each individual probe set identified as differentially regulated in these livers, also provides an animal code for each individual chip. It is our intent to provide this code information for all reported measurements in future publications from our ongoing studies, whether they be gene arrays, biochemical, or physiological. From a systems biology perspective, this will make these studies most useful to other investigators.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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