Differential hepatic gene expression in a polygenic mouse model with insulin resistance and hyperglycemia: evidence for a combined transcriptional dysregulation of gluconeogenesis and fatty acid synthesis

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Abstract

New Zealand obese (NZO) mice exhibit severe insulin resistance of hepatic glucose metabolism. In order to define its biochemical basis, we studied the differential expression of genes involved in hepatic glucose and lipid metabolism by microarray analysis. NZO×F1 (SJL×NZO) backcross mice were generated in order to obtain populations with heterogeneous metabolism but comparable genetic background. In these backcross mice, groups of controls (normoglycemic/normoinsulinemic), insulin-resistant (normoglycemic/hyperinsulinemic) and diabetic (hyperglycemic/hypoinsulinemic) mice were identified. At 22 weeks, mRNA was isolated from liver, converted to cDNA, and used for screening of two types of cDNA arrays (high-density filter arrays and Affymetrix oligonucleotide microarrays). Differential gene expression was ascertained and assessed by Northern blotting. The data indicate that hyperinsulinemia in the NZO mouse is associated with: (i) increased mRNA levels of enzymes involved in lipid synthesis (fatty acid synthase, malic enzyme, stearoyl-CoA desaturase) or fatty acid oxidation (cytochrome P450 4A14, ketoacyl-CoA thiolase, acyl-CoA oxidase), (ii) induction of the key glycolytic enzyme pyruvate kinase, and (iii) increased mRNA levels of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase. These effects were enhanced by a high-fat diet. In conclusion, the pattern of gene expression in insulin-resistant NZO mice appears to reflect a dissociation of the effects of insulin on genes involved in glucose and lipid metabolism. The data are consistent with a hypothetical scenario in which an insulin-resistant hepatic glucose production produces hyperinsulinemia, and an enhanced insulin- and substrate-driven lipogenesis further aggravates the deleterious insulin resistance of glucose metabolism.

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Introduction

Insulin resistance and impaired glucose tolerance are early abnormalities in the development of type 2 diabetes. In a large part, glucose homeostasis is controlled by the liver, and hyperglycemia in type 2 diabetes reflects increased hepatic glucose production (Meyer et al. 1998) as well as reduced glucose uptake (Nielsen et al. 1998). At present, the cellular and molecular mechanisms of these abnormalities are not entirely clear. For several years, it has been assumed that free fatty acids modify hepatic glucose production and disposal, and that insulin resistance of adipose tissue produces an increased hepatic glucose output by this mechanism (Bergman & Ader 2000). However, the critical role of a direct action of insulin in the liver has been convincingly demonstrated with mice homozygous for a
liver-specific deletion of the insulin receptor (Michael et al. 2000). These mice exhibit severe abnormalities of glucose homeostasis, including postprandial hyperglycemia, glucose intolerance and insulin resistance. Similar abnormalities have been observed in mice heterozygous for combined null mutations of both insulin receptor and insulin receptor substrates IRS1 or IRS2 (Brüning et al. 1997, Kido et al. 2000, Previs et al. 2000).

Insulin resistance can also be a consequence of leptin deficiency, caused either by a mutation in the leptin gene (obese mice, Lepob/ob) or by lipodystrophy (transgenic aP2-SREBP1c mice) (Shimomura et al. 1998a). Interestingly, in both mouse models of leptin deficiency, the liver exhibits a mixed pattern of insulin resistance and sensitivity. In these mice, insulin fails to suppress hepatic glucose production and output, but continues to stimulate fatty acid synthesis (Shimomura et al. 2000). The combination of glucose overproduction and enhanced fatty acid synthesis leads to a further increase in insulin secretion and resistance, establishing a vicious cycle that may present a basic mechanism in the development of type 2 diabetes (McGarry 1992). It is presently unknown, however, whether the mechanisms derived from leptin-deficient mouse models are also responsible for insulin resistance associated with diet-induced obesity or leptin resistance.

New Zealand obese (NZO) mice present a polygenic metabolic syndrome of hyperphagia, obesity, dyslipidemia, hypertension and insulin resistance (Ortlepp et al. 2000). Furthermore, NZO mice have also been shown to exhibit marked leptin resistance (Halas et al. 1997, Igel et al. 1997). In the course of this syndrome, a large proportion of male mice develop hyperglycemia due to islet cell failure (Leiter et al. 1998). Hepatic glucose overproduction, which is resistant to insulin, has been shown to be a critical, early abnormality that precedes the overt fasting hyperglycemia of the NZO mouse (Rudorff et al. 1970, Veroni et al. 1991). Furthermore, NZO mice exhibit a reduced hepatic storage of glycogen, but an enhanced conversion of a glucose load to fat (Subrahmanyan 1960). Thus, the liver appears to play a central role in the pathogenesis of insulin resistance in the NZO mouse.

In order to genetically dissect its polygenic syndrome, we have recently established a backcross of the NZO mouse with the lean Swiss/Jackson Laboratory (SJL) strain (Ortlepp et al. 2000). With this backcross, we could identify separate susceptibility loci for obesity/insulin resistance and hyperglycemia/hypoinsulinemia (Kluge et al. 2000, Plum et al. 2000) and further supported the concept that diabetes in this mouse model is the result of a deleterious combination of obesity and diabetes genes (‘diabesity’ (Leiter & Herberg 1997, Leiter et al. 1998)). In addition, male NZO × F1 (SJL × NZO) backcross mice (subsequently referred to as NSZO) exhibited a heterogeneous metabolic phenotype, including normoglycemic/normoinsulinemic, hyperinsulinemic mice with (compensated) insulin resistance and diabetic (hyperglycemic/hypoinsulinemic) animals. This backcross population allows the comparison of metabolically different subgroups which have a comparable genetic background.

In the present study, we employed the NSZO backcross population in order to characterize the hepatic gene expression in a model of insulin resistance associated with leptin resistance and obesity. The data indicate that the metabolic syndrome of NZO mice is associated with a marked, inadequate upregulation of enzymes involved in lipogenesis, glycolysis and gluconeogenesis. Thus, an uncontrolled hepatic glucose production might lead to enhanced insulin secretion and insulin-driven lipogenesis, which, together with the additional nutritional lipid load, aggravates the deleterious insulin resistance.

Materials and methods

Animals

SJL (SJL/NBom) and NZO mice (NZO/HIBom) were obtained from Bomholtgard (Ry, Denmark). Female SJL and male NZO mice were used to found an F1 generation, and backcrosses (SJL × F1 and NZO × F1, at least 120 male or female mice in each group) were performed (Plum et al. 2000). A second independent backcross population (200 mice in each group) was generated 1 year thereafter (Plum et al. 2002). After weaning (3 weeks of age), mice received standard (Altromin, Lage, Germany; no. 1314, with 5, 48 and 22·5% fat, carbohydrates and protein respectively; 12·5 kJ/g metabolizable energy) or high fat (no. C1057, with 16·0, 46·8 and 17·1% fat, carbohydrates and protein respectively; 15·4 kJ/g) rodent chow. Throughout the study, mice had free access to food.
and water. Three to six mice were kept per cage (Macrolon, type III) in a temperature-controlled room (20 °C, 55 ± 5% relative humidity) with a 12 h light:12 h darkness cycle and lights on at 0600 h. Animals were killed at the age of 22 weeks, and livers were dissected and stored at −70 °C. Body weight, body mass index, and serum parameters were determined as described previously (Plum et al. 2000). Non-esterified fatty acids were assayed with a commercial kit from Roche Diagnostics (Mannheim, Germany). The principles of laboratory animal care were followed, and the study was approved by the committee for ethics of animal experimentation at the district administration in Cologne, Germany.

For the purpose of the present study, mice representative for the three metabolic phenotypes, normoinsulinemic/normoglycemic, hyperinsulinemic/normoglycemic (insulin-resistant) and hyperglycemic (diabetic), were selected from the backcross progeny. Liver RNA from these mice was isolated and pooled samples of RNA corresponding to normoinsulinemic, hyperinsulinemic or diabetic mice were set up by combining equal amounts of total RNA from each of the animals in the respective groups. The phenotypic characterization of these animals is presented in Table 1.

Isolation of RNA and Northern blot analysis

Total RNA from liver was prepared by the single-step method according to Chomczynski & Sacchi (1987). Total RNA (15 µg) was separated by denaturing formaldehyde electrophoresis on 1% agarose gel and transferred by capillary blot to positively charged nylon membranes (Hybond N+; Amersham, Freiburg, Germany). 32P-labeled cDNA probes were generated from the inserts of cDNA clones listed in Table 2. Blots were washed to a final stringency of 120 mM NaCl/12 mM sodium citrate/0.1% SDS at 55 °C. After probe removal by incubation with 1% SDS at 100 °C, blots were rehybridized with other cDNA probes.

Analysis of differential gene expression with high density cDNA filter arrays

Poly(A)-containing RNA was prepared with the help of oligo(dT)-coupled paramagnetic beads (Chemagen, Baesweiler, Germany) from the pooled RNA samples representing the normoinsulinemic, diabetic and hyperinsulinemic mice of backcross 1. Double-stranded cDNA was synthesized with a commercial kit (cDNA Synthesis System; Life Technologies, Karlsruhe, Germany). cDNAs were labeled with [35P]dCTP by random oligonucleotide priming to high specific activities (400–700 µCi/µg). Probes were hybridized to high-density filter arrays (colony filters; 22 × 22 cm) containing double-spotted cDNA clones of the Mouse Unigene 1 library (library no. 952; RZPD, Berlin, Germany). This library comprises a set of 24 960 non-redundant murine cDNA clones provided by the IMAGE consortium (Lennon et al. 1995). To control for variation between different filter arrays, each probe was hybridized with two different filters, and the same filter was subjected to hybridization with two probes. Differentially hybridizing cDNA clones were identified by visual inspection of autoradiograms. Signal intensity was evaluated with the help of a phosphoimager, and only transcripts showing a 3-fold or higher difference in signal intensity were considered differentially expressed.

Oligonucleotide microarray analysis

Aliquots of the same pools of total RNA that were analyzed by filter array hybridization (backcross 1 animals) were also subjected to microarray analysis. Synthesis of labeled cRNA, hybridization, washing and staining of Affymetrix Gene Chip arrays (Affymetrix, Santa Clara, CA, USA) were carried out according to the Affymetrix technical manual. Briefly, 10 µg total RNA were utilized to generate cDNA, using the Superscript Choice System for cDNA synthesis (Life Technologies). After purification by phenol:chloroform extraction and subsequent precipitation, the cDNA was used as a template for an in vitro transcription reaction (Enzo BioArray High Yield RNA Transcript Labeling Kit; distributed by Affymetrix) according to the manufacturer’s protocol. In each case 15 µg of the labeled cRNAs were than fragmented and hybridized overnight onto MG U74A GeneChip arrays (Affymetrix). The MG U74A GeneChip array contains probes for ~12 000 murine genes. After hybridization and removal of the hybridization solution, the GeneChip arrays were washed and stained according to the antibody amplification protocol.
Table 1 Body weight and serum parameters of the NSZO backcross and SJL mice selected for analysis of gene expression (means±S.E.M.)

<table>
<thead>
<tr>
<th>Backcross 1 (Fig. 1)</th>
<th>n</th>
<th>Body weight week 22 (g)</th>
<th>Weight gain 12–22 weeks (g)</th>
<th>BMI week 22 (g/cm²)</th>
<th>Blood glucose (mM)</th>
<th>Plasma insulin (ng/ml)</th>
<th>NEFA (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male NSZO, high fat diet</td>
<td></td>
<td>Normal</td>
<td>6</td>
<td>57.5±4.9</td>
<td>14.1</td>
<td>0.43</td>
<td>10.9±2.9</td>
</tr>
<tr>
<td>Diabetic</td>
<td>6</td>
<td>45.4±2.4</td>
<td>-4.6</td>
<td>0.36</td>
<td>32.2±1.6</td>
<td>0.3±0.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>Hyperinsulinemic</td>
<td>6</td>
<td>75.5±5.0</td>
<td>21.5</td>
<td>0.53</td>
<td>13.3±2.1</td>
<td>63.5±20.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>Male NSZO, high fat diet</td>
<td></td>
<td>Diabetic</td>
<td>7</td>
<td>53.0±8.1</td>
<td>11.6</td>
<td>0.42</td>
<td>6.6±2.4</td>
</tr>
<tr>
<td>Hyperinsulinemic</td>
<td>7</td>
<td>54.8±6.7</td>
<td>1.5</td>
<td>0.42</td>
<td>30.0±0.7</td>
<td>0.6±0.8</td>
<td>6.7±1.3</td>
</tr>
<tr>
<td>Female NSZO, high fat diet</td>
<td></td>
<td>Normal</td>
<td>7</td>
<td>45.3±5.4</td>
<td>7.6</td>
<td>0.37</td>
<td>7.3±1.2</td>
</tr>
<tr>
<td>Hyperinsulinemic</td>
<td>7</td>
<td>54.9±6.2</td>
<td>13.8</td>
<td>0.43</td>
<td>10.0±2.5</td>
<td>16.9±8.5</td>
<td>3.8±2.7</td>
</tr>
<tr>
<td>Male SJL, high fat diet</td>
<td></td>
<td>Normal</td>
<td>6</td>
<td>30.2±2.9</td>
<td>3.0</td>
<td>0.31</td>
<td>8.3±0.5</td>
</tr>
<tr>
<td>Female SJL, high fat diet</td>
<td></td>
<td>Normal</td>
<td>6</td>
<td>21.5±0.6</td>
<td>2.2</td>
<td>0.24</td>
<td>7.7±0.5</td>
</tr>
<tr>
<td>Male NSZO, standard diet</td>
<td></td>
<td>Normal</td>
<td>3</td>
<td>44.1±4.6</td>
<td>6.5</td>
<td>0.36</td>
<td>10.3±3.5</td>
</tr>
<tr>
<td>Diabetic</td>
<td>3</td>
<td>42.8±2.1</td>
<td>-5.9</td>
<td>0.34</td>
<td>33.2±0.1</td>
<td>1.3±0.7</td>
<td>10.1±3.1</td>
</tr>
<tr>
<td>Hyperinsulinemic</td>
<td>4</td>
<td>55.2±2.7</td>
<td>11.1</td>
<td>0.42</td>
<td>12.8±1.6</td>
<td>41.9±2.1</td>
<td>3.9±1.0</td>
</tr>
<tr>
<td>Female NSZO, standard diet</td>
<td></td>
<td>Normal</td>
<td>3</td>
<td>37.9±2.1</td>
<td>4.7</td>
<td>0.33</td>
<td>7.8±1.0</td>
</tr>
</tbody>
</table>

BMI, body mass index (body weight/body length²); NEFA, non-esterified fatty acid; n.d., not determined.

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protocol provided by Affymetrix. Finally, the GeneChip arrays were scanned with a confocal laser scanner (Hewlett Packard). GeneChip 4.0 software (Affymetrix) was used for quantitative analysis of the scanned images. Further data analysis including estimation of regulation and weighting of the statistical significance of those regulations was performed using proprietary software developed within Aventis.

Results

Characterization of insulin-resistant, diabetic and control mice

At 22 weeks of age, the male mice of the backcross population could be divided into three different metabolic ‘clusters’: normoinsulinemic/normoglycemic (controls), hyperinsulinemic/normoglycemic (insulin-resistant) and hypoinsulinemic/hyperglycemic (diabetic). The metabolic characteristics of these groups of mice are summarized in Table 1 (backcross 1); gene expression analysis of these animals is shown in Fig. 1. After completion of the first backcross population, we generated a second backcross for reproduction of the initial results, and for assessment of the effects of the dietary fat content. In backcross 2 (Table 1, gene expression shown in Fig. 2), mice were raised on either standard or high-fat diet, and male mice from the three metabolic clusters were selected at week 22. In order to assess gender differences, mice from two clusters of female mice (normoinsulinemic and hyperinsulinemic) were also selected (Fig. 3). As a particularly lean group, parental SJL mice were included in the comparison (Table 1).

Identification of differentially expressed genes by hybridization of cDNA arrays

For identification of genes differentially expressed in hyperinsulinemic or diabetic NSZO mice (male animals fed a high fat diet; backcross 1), we used two different cDNA arrays (high-density filter arrays containing ~25 000 non-redundant murine cDNA clones (mouse Unigene library; RZPD) and ~12 500 murine gene oligonucleotide microarrays (Affymetrix)) because it appeared possible that both arrays covered only a portion of the whole transcriptome. Both methods identified several genes that are known to be regulated by glucose or insulin (see below, e.g. glucokinase and stearoyl-
CoA desaturase 1 (SCD1)). The comparison of the data sets obtained with both types of arrays showed that some of the differentially expressed genes were detected by both methods (e.g. CYP4A14, SCD1, peroxisomal ketoacyl thiolase). In order to confirm the observed effects and to further analyze the transcriptional alterations that underlie the dysregulated hepatic metabolism in the diabetic and insulin-resistant mice, we selected 13 differentially expressed genes because of their known role in glucose or lipid metabolism for Northern blot analysis (Table 2). In addition, the insulin-regulated transcription factor SREBP1 (sterol response element binding protein 1) was analyzed as a known regulator of enzymes involved in hepatic lipogenesis. In these experiments, RNA samples from the individual animals were analyzed in parallel with the RNA pools (Fig. 1). In order to assess potential changes in the expression of gluconeogenesis-related enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6 Pase) were included in the

Figure 1 Northern blot analysis of differentially expressed transcripts identified by array hybridization – Northern blots contained samples (15 µg) of total liver RNA from individual male NSZO backcross mice (numbers) fed on a high fat diet, or pooled RNA samples (Pool) of the three experimental groups (N, normoglycemic; D, diabetic; H, hyperinsulinemic). Blots were hybridized with murine cDNA probes (expressed sequence tag clones) of genes identified by differential hybridization of cDNA arrays, and with cDNA probes for ferritin and GAPDH to control for equal loading (Table 2). Results obtained by pairwise comparisons of the filter array hybridization (FA, at least 3-fold difference) are indicated. Microchip data (MC) are specified as follows: >, at least 2-fold difference; ≥, 1.5–2 fold difference; =, difference smaller than 1.2-fold. Identification of the multiple transcripts hybridizing with SCP2/SCPx and HSD3b5 is indicated in Fig. 2 because the RNA species were better resolved in that set of experiments. The blot hybridized with the SREBP1 probe did not contain the pooled RNA samples.
analysis of backcross 2. Not all results obtained by the cDNA array hybridizations were confirmed by the Northern blot analysis (e.g. acyl-CoA oxidase, fatty acid binding protein (FABP1)), thus in the present study only data verified by Northern blotting were considered suitable for interpretation.
Induction of enzymes involved in hepatic lipid synthesis in hyperinsulinemic mice

Most strikingly, the analysis of the differential array hybridizations and the subsequent Northern blot analysis (Figs 1 and 2, Table 3) indicated that mRNA levels of several enzymes involved in lipid synthesis or lipid metabolism, e.g. fatty acid synthase (FAS), malic enzyme, SCD1, and phospholipid transfer protein (PLTP), were upregulated in the hyperinsulinemic, insulin-resistant mice. In agreement with the known regulation of these genes by insulin, their mRNA levels were markedly reduced in the diabetic cluster. Pyruvate kinase, a glycolytic enzyme, exhibited a similar pattern of expression. In contrast, mRNA levels of glucokinase were unaltered in hyperinsulinemia but reduced in diabetes.

Some of the transcripts that were upregulated in insulin resistance are known to be controlled by the insulin-regulated transcription factor SREBP1c (Shimomura et al. 1998a, Foretz et al. 1999a). A Northern blot indicated that SREBP1 mRNA was also upregulated in the hyperinsulinemic mice, and markedly reduced in four of the six diabetic mice (Fig. 1). However, two diabetic mice with markedly elevated levels of the SREBP1 transcript exhibited a dissociation of this transcript from the expression of the other insulin-controlled genes.

Since insulin resistance in NZO mice is markedly aggravated by a high-fat diet, we studied the effects of the dietary fat content in a second population of NSZO backcross mice. As is illustrated in Fig. 2 and Table 3, the induction of FAS, malic enzyme and SCD1 in insulin resistance was observed at both standard and high-fat dietary regimens. Surprisingly, a high-fat diet further increased mRNA levels of these transcripts. Again, PLTP and SREBP1 exhibited similar patterns of alterations.

![Figure 3](image_url)
Differential expression of genes involved in fatty acid oxidation

Four transcripts encoding enzymes involved in fatty acid oxidation were also increased in insulin-resistant, hyperinsulinemic mice. These genes are known to be induced by the transcription factor peroxisome proliferator-activated receptor α (PPARα) (Besnard et al. 1993, Lee et al. 1995), cytochrome P450 4A14 (CYP4A14), which catalyzes the microsomal oxidation of fatty acids, two enzymes involved in the peroxisomal β-oxidation (ketoacyl-CoA thiolase and acyl-CoA oxidase), and FABP1, a cytoplasmic lipid transport protein. Unlike the SREBP1-controlled genes, these genes were markedly induced in diabetic mice. Furthermore, the data identify peroxisomal phytanoyl-CoA hydroxylase as a novel diabetes-induced transcript (Fig. 1).

With reduced hepatic mRNA levels in insulin resistance and elevated levels in diabetes, SCPx exhibited a unique pattern of expression. SCPx is a splice variant of sterol carrier protein 2 (SCP2) and functions as a peroxisomal β-ketoacyl-CoA thiolase directed towards branched-chain fatty acids (Wanders et al. 1997, Seedorf et al. 1998). The elevated expression of SCPx in diabetes has been described previously (McLean et al. 1995) and is not unexpected for a peroxisomal enzyme. However, its downregulation in the hyperinsulinemic animals is in striking contrast to the other effects observed in this study.

As is illustrated in Fig. 2, similar patterns of alterations of CYP4A14 and SCPx were observed under standard and high-fat diet. In addition, high-fat diet produced a marked increase in CYP4A14, and a moderate increase in SCPx transcript levels.

Expression of gluconeogenic enzymes in hyperinsulinemic mice

NZO mice exhibit a markedly increased gluconeogenesis which is resistant to insulin (Rudorff et al. 1970). Accordingly, transcript levels of PEPCK and G6 Pase were found upregulated in the diabetic and, to a lesser extent, also in the insulin-resistant cluster of backcross mice (Fig. 2). As is also illustrated in Fig. 2, PEPCK mRNA levels were increased by high-fat diet. Surprisingly, the expression of G6 Pase exhibited a somewhat different pattern: mRNA levels were low in the normoglycemic, normoinsulinemic SJL mice, but were markedly elevated in all NZO-derived male and female mice, with little effects of the diet or of the metabolic state of the animals. Thus, enhanced expression of G6 Pase was seen in all mice with at least one set of NZO alleles.

Reduction of transcript levels of 3β-hydroxysteroid dehydrogenase 5 in both diabetic and hyperinsulinemic mice

Reduced transcript levels in both diabetic and hyperinsulinemic mice were observed for 3β-hydroxysteroid dehydrogenase (Hsd3b5). This enzyme belongs to a family of closely related proteins also containing enzymes with 3-ketosteroid reductase activity. Due to the high sequence similarity between the known isoforms (about 90% identical nucleotides over at least 250 bases), our probe might have cross-reacted with all members of the family. The tentative identification of the differentially expressed transcript (arrow, Fig. 3) as Hsd3b5 is based on the known expression of this gene in liver, and on the sexual dimorphism of its expression (see Fig. 3 (Abbaszade et al. 1995)). This isoform is thought to be involved in the inactivation of steroid hormones, e.g. dihydrotestosterone.

Effects of gender on the expression of hepatic genes in insulin-resistant NZO mice

The NSZO backcross population exhibits a marked sexual dimorphism of the metabolic aberrations (Table 1). Diabetes is observed in male mice only, and hyperinsulinemia in females is detected exclusively in animals fed a high-fat diet. Thus, we studied the effects of gender on the pattern of gene expression observed in the male NSZO backcross mice. Levels of most investigated transcripts were similar in both sexes (e.g. FAS, SCD1, malic enzyme, PLTP, pyruvate kinase, glucokinase, SREBP1), and small gender differences in mice on the standard diet reflected the lower degree of insulin resistance in females (data not shown). In contrast, a marked sexual dimorphism was observed for the expression of SCPx and HSD3b5: these transcripts were nearly undetectable in livers from normoinsulinemic females (Fig. 3). It should be noted that the high-fat diet produced a moderate upregulation of these transcripts in
females. In contrast to SCPx, the alternatively spliced transcripts (asterisks, Fig. 3) encoding SCP2 were essentially identical in both sexes. Although CYP4A14 exhibits a ‘female-specific’ pattern of expression in normoglycemic mice (also in SJL mice, data not shown), its mRNA levels in diabetic males were as high as in females under both high fat and standard diet conditions.

Discussion

The present study was designed to define alterations of the hepatic gene expression in morbidly obese, insulin-resistant NZO mice. A comparison of the inbred NZO strain with a normal strain, e.g. SJL, is hampered by the different genetic backgrounds of the strains. Thus, we used selected subpopulations of our NZO × F1 (SJL × NZO) backcross which provided an insulin-resistant, a diabetic and a normoinsulinemic/normoinsulinemic control group on a comparable genetic background. The results allow a comprehensive assessment of alterations of transcripts involved in the regulation of lipid metabolism and glucose output. Most importantly, the data reveal that marked changes in the hepatic gene expression take place in insulin-resistant mice, in particular an induction of genes involved in lipid synthesis in the insulin-resistant population. This profound dysregulation appears to reflect the biochemical basis of the previously described (Subrahmanyam 1960, Rudorff et al. 1970, Veroni et al. 1991) severe hepatic insulin resistance of the NZO mouse.

In the absence of an exact knowledge of the molecular defect(s) causing the polygenic syndrome, the interpretation of the present data is still ambiguous, but two scenarios may be discussed. First, it is conceivable that glucose homeostasis in NZO mice is disturbed primarily by an increased hepatic glucose output which is resistant to the inhibitory effect of insulin. The resulting hyperinsulinemia then stimulates lipogenesis and creates a vicious cycle in that it further enhances insulin resistance of hepatic glucose metabolism by production of fatty acids (Randle et al. 1963). This scenario is fully compatible with the present data, which suggest that lipid metabolism appears sensitive to increased serum insulin levels, since mRNA levels of SREBP1 and of other insulin-regulated genes of lipid metabolism were elevated. The scenario is strikingly similar to that observed in leptin-deficient ob/ob and in fatless mice, in which gluconeogenesis appears resistant, and lipogenesis is sensitive to insulin (Shimomura et al. 2000).

The dissociation of the regulation of hepatic glucose production from that of lipid synthesis has a molecular basis in diverse signaling pathways (Fig. 4). Gene expression of gluconeogenic enzymes (e.g. PEPCK, G6 Pase) is controlled by IRS2, phosphatidylinositol 3-kinase, Akt, and the transcription factor FKHR. This pathway is suppressed in hepatic insulin resistance because IRS2 is downregulated in rodents with sustained elevations in plasma insulin levels (Shimomura et al. 2000, Zhang et al. 2001). In contrast, the effect of insulin on the expression of lipogenic enzymes is mediated by the transcription factor SREBP1. SREBP1 appears to be induced by insulin independently of IRS2, since IRS2 knockout mice exhibit an increased expression of SREBP1 as well as of lipogenic genes (Tobe et al. 2001).

The above-described scenario, which postulates a primary defect in liver glucose production, is fully compatible with all previous data on the metabolic abnormalities of the NZO mouse. These data have convincingly demonstrated that the effect of insulin on glucose metabolism is dissociated from that on lipogenesis in the NZO mouse. Glucose is converted to fat, but not to glycogen (Subrahmanyam 1960, Rudorff et al. 1970). In contrast, alanine-dependent gluconeogenesis in the perfused liver is not inhibitable by insulin (Subrahmanyam 1960). Thus, the dysregulation of enzymes catalyzing hepatic glucose output, e.g. G6 Pase, may be one of the primary metabolic defects of the NZO mouse. Interestingly, overexpression of G6 Pase in the liver of normal rats has been described to produce a syndrome of glucose intolerance, hyperinsulinemia and increased peripheral triglyceride storage (Trinh et al. 1998), resembling the metabolic abnormalities of the NZO mouse. In addition, increased G6 Pase activity has also been found in liver samples from patients with type 2 diabetes (Clore et al. 2000). The increase in mRNA levels of pyruvate kinase in insulin-resistant backcross mice may be a consequence of the enhanced G6 Pase activity, which is regulated by hepatic intracellular glucose levels (Foretz et al. 1999b).

The alternative scenario assumes a primary defect in lipid metabolism, e.g. in the oxidation of
fatty acids. Increased levels of fatty acids might enhance expression of hepatic pyruvate dehydrogenase kinase 4 (PDK4), a key regulator of glucose oxidation (Holness et al. 2003). High levels of PDK4 inhibit pyruvate dehydrogenase, thereby suppressing glucose oxidation (Sugden et al. 2001). Indeed, transcript levels of PDK4 were 1.9-fold higher in the hyperinsulinemic vs normoglycemic NSZO mice (microarray analysis of backcross 1 animals), slightly below our threshold (2-fold) for considering the effect relevant. This scenario is analogous to the Randle hypothesis (Randle et al. 1963), which is based on the finding that oxidation of fatty acid inhibits glucose metabolism in muscle, and stimulates gluconeogenesis in liver. This mechanism cannot be readily dismissed by the argument that expression of lipogenic enzymes in the NZO mouse appears to be sensitive to insulin. It is still conceivable that a metabolite, e.g. acetyl-CoA, generated in excess because of the huge substrate load and a defect in β-oxidation, drives lipogenesis and simultaneously affects liver glucose metabolism. Ultimately, only the identification of the molecular defect will prove which of the possible scenarios is correct.

A striking finding of the present study is the stimulatory effect of high-fat diet on the expression of lipogenic enzymes, i.e. FAS, malic enzyme and SCD1 (Fig. 2). The induction appears to reflect the parallel induction of the key transcription factor SREBP1. This finding was unexpected, since SREBP1 is the key transcriptional activator of enzymes that catalyze the hepatic synthesis of fatty acids in rodents fed a carbohydrate-rich diet (Osborne 2000, Kawaguchi et al. 2001). Furthermore, in our backcross mice the high-fat diet resulted in the deposition of fat in almost all organs, in particular in liver. Thus, it might be speculated that the marked lipid load produces a key regulatory metabolite, possibly acetyl-CoA, through an increase in fatty acid oxidation, and that this metabolite accumulates and induces lipogenesis. Also, elevated serum insulin levels in mice fed a high-fat diet might have contributed to the induction. It should be noted that the induction of lipogenic enzymes by the high-fat diet further illustrates the metabolic dysregulation of liver metabolism in NZO mice, which exhibit reduced glucose storage and enhanced lipid synthesis. Among the lipogenesis-related enzymes, SCD1

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**Figure 4** Insulin effects on gene expression of enzymes involved in hepatic glucose and lipid metabolism. SREBP1c mediates the insulin-stimulated expression of enzymes that catalyze the conversion of glucose into fatty acids (glucokinase, pyruvate kinase, malic enzyme, FAS, SCD1). Expression of gluconeogenetic enzymes (G6Pase, PEPCK) is suppressed by insulin via an IRS2-dependent pathway. Hyperinsulinemic NSZO mice are resistant towards the insulin effect on gluconeogenesis but sensitive towards the insulin-dependent stimulation of lipogenesis-related enzymes.
appears to have a critical role in the generation of body fat, since the loss of SCD1 protects mice against obesity (Cohen et al. 2002, Ntambi et al. 2002).

In the present study, the pattern of expression of PLTP was strikingly similar to that of the known targets of SREBP1 (FAS, malic enzyme, SCD1, glucokinase, pyruvate kinase). PLTP is one of the main modulators of plasma high-density lipoprotein size and composition (Jiang et al. 1999, 2001) and its expression in the mouse is known to be regulated by PPARα (Bouly et al. 2001). However, the reduced mRNA levels of PLTP in the diabetic animals are in contrast to the elevated expression of other PPARα-regulated genes, indicating that other regulatory mechanisms must be responsible for this effect. It is interesting to note that the reduced expression of Hsd3b5 in hyperinsulinemic and diabetic mice appears to be caused by the activation of PPARα. Hsd3b5 mRNA levels in liver have recently been shown to be dramatically decreased upon exposure of mice to peroxisome proliferator (Wong et al. 2002).

The metabolic syndrome of the NZO mouse exhibits marked differences between males and females. Insulin resistance is more pronounced in males than in females, and a very low prevalence of diabetes is observed in females. Thus, all gender differences in gene expression are of particular interest, since they might help to elucidate the mechanism of the development of islet cell destruction in NZO mice. Since estrogens appear to exert a protective effect, a crucial role has been attributed to their metabolism (Leiter et al. 1991). In a recent study, we have shown that cytochrome P450 isozymes are differentially expressed in male and female backcross mice (CYP2B9 and CYP3A16 females > > males; CYP2B1 females < < males), and are also differentially expressed in diabetic vs non-diabetic mice (Pass et al. 2002). Here, we observed markedly higher mRNA levels of SCPx in male than in female mice, as was previously reported by Roff et al. (1992), possibly reflecting a sex difference in the oxidation of fatty acids. These findings appear consistent with the notion that islet cell destruction in obese mice is produced by a deleterious effect of lipid metabolites (‘lipotoxicity’, Unger 1995).

The analysis of the same cDNAs with two different types of arrays allows a comparison of these methods. The filter array contained clones for all but one (Hsd3b5) of the genes listed in Table 2, and one of these genes was not present on the Affymetrix array (Scp2). However, hybridization of the filter array with radioactively labeled cDNAs was less sensitive than the fluorescence-based detection of the Affymetrix array. Only 5–8% of the clones on the filter array, but 32–37% of the transcripts represented by the microarray yielded analyzable signals. In the present experiments, more informative data were obtained with the help of the Affymetrix array, although the filter array experiment allowed the identification of differentially expressed transcripts that were either absent on the microarray (SCPx) or showed no significant difference (peroxisomal acyl-CoA oxidase and phytanoyl-CoA hydroxylase).

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