Insulin immuno-neutralization in fed chickens: effects on liver and muscle transcriptome

Jean Simon,1 Dragan Milenkovic,2 Estelle Godet,1 Cedric Cabau,2 Anne Collin,1 Sonia Météayer-Coustard,1 Nicole Rideau,1 Sophie Tesseraud,1 Michel Derouet,1 Sabine Crochet,1 Estelle Cailleau-Audouin,1 Christelle Hennequet-Antier,1 Christian Gaspach,4 Tom E. Porter,5 Michel J. Duclos,1 Joëlle Dupont,6 and Larry A. Cogburn7

1Station de Recherches Avicoles, 2Système d’Information des Animaux d’Élevage, and 3Physiologie de la Reproduction et des Comportements, Institut National de la Recherche Agronomique (INRA), Nouzilly; 4Unité de Nutrition Humaine, INRA, Theix; 5Institut National de la Santé et de la Recherche Médicale, U673 and Université Pierre et Marie Curie Paris 6, Hôpital Saint Antoine, Paris, France; 6Department of Animal and Avian Sciences, University of Maryland, College Park, Maryland; and 7Department of Animal and Food Sciences, University of Delaware, Newark, Delaware

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In addition to providing a valuable source of human food, chickens exhibit several physiological peculiarities, particularly glucose metabolism and its endocrine control. For example, chickens exhibit constitutively high glycemia (1.8–2.0 g/l, in the fasted status) despite the presence of an endogenous hyperactive insulin circulating at rather “normal” levels (39). Additional hallmarks of avian metabolism are the requirement for a high dose of exogenous insulin to induce hypoglycemia and the survival of chickens following administration of a large dose of exogenous insulin, which would be lethal in mammals. Although normal for the chicken, these characteristics mimic Type 2 diabetes in mammals (11, 12). Furthermore, the mechanism of insulin signaling remains enigmatic or even provocative in chicken muscle. The early steps of insulin signaling [i.e., tyrosine phosphorylation of insulin receptor beta-subunit and substrate-1 and phosphatidylinositol-3 kinase (PI3K) activity] appear insensitive to insulin status, whereas steps downstream of PI3K are accordingly sensitive to insulin (11, 12).

Insulin immuno-neutralization in young fed chickens (i.e., a nutritional status with high demand in insulin) rapidly induces large increases in plasma levels of glucose (up to 4.3 or 7.5 g/l at 1 or 5 h, respectively), nonesterified fatty acids, and amino acids. Acute insulin deprivation alters several steps in the insulin signaling cascade and other components of the endocrine system: plasma glucagon, triiodothyronine (T3), and insulin-like growth factor binding protein-1 (IGFBP1) levels (11). Furthermore, several mRNAs coding for specific transcription factors or metabolic enzymes, selected a priori as potentially insulin sensitive, were altered at 5 h of insulin privation in liver and skeletal muscle (leg muscle). Some of these changes were insulin specific, since fasting for 5 h did not induce the same alterations in gene expression as did insulin deprivation. Therefore, insulin appears to also exert pleiotropic effects in fed chickens, which had been previously doubtful (12). Interestingly, the adenosine monophosphate kinase (AMPK) system was not activated in liver or muscle following insulin privation or fasting for 5 h. In other studies, the recruitment of the AMPK system in response to fasting requires a prolonged fasting time in the chicken (34). Together, these findings suggest that our experimental model of “diabetes” in the chicken is quite suitable for identification of the very early changes in liver and muscle transcriptome before development of long-term complications from insulin privation. Until now, microarray analysis of Type 2 diabetes or obesity have been studied mainly in rodent models or humans (42). In the present study, microarray analysis were performed in chicken liver and leg muscle samples from the insulin immuno-neutralization experiment, which we described earlier (11), to
gain a greater insight into the role of insulin in control of gene transcription in these two metabolic tissues and to evaluate whether the insulin-dependent pathways established in mammals have been conserved in the chicken. A dual benefit of this new knowledge on insulin control of metabolism in the chicken could be realized with genetic selection of leaner chickens as a healthier human food by favoring protein accretion, at the expense of lipogenesis and excessive body fatness.

MATERIALS AND METHODS

Chicken tissues and experimental conditions. Liver and leg muscle tissues were issued from our earlier study (11), where experimental conditions are described in detail. All procedures were approved by the French Agricultural Agency and the Scientific Research Agency and conducted in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching. Herein, changes in liver and leg muscle transcriptome were assessed in fed chickens at 5 h following insulin immuno-neutralization by comparing “diabetic,” insulin-deprived birds (n = 6), against the control group (n = 6 birds), which received injections of normal guinea pig serum. Total tissue RNA was extracted using RNeasy kits (Qiagen) according to manufacturer’s protocol. RNA samples were further purified using a final ethanol precipitation and washing step. RNA quality was determined using Agilent apparatus (2100 Bioanalyzer). RNA samples exhibited RNA integrity number >8.

Fluorescent cDNA labeling. Two samples of 5 μg RNA/chicken/ tissue were reverse-transcribed with 1 μl of random primers and 1 μl of oligo(dT) using the ChipShot Direct Labeling System kit (Promega), and fluorescent cDNA labeling was performed with either Cy3- or Cy5-dCTP (GE Healthcare). Labeled cDNAs were purified by application to an equilibrated filter cartridge using the ChipShot Membrane Clean-up System (Promega) as recommended by the manufacturer. Quantities and labeling efficiencies of labeled cDNAs were determined by measuring absorbencies at 260, 550, and 650 nm using a ND-1000 spectrophotometer (Nanodrop).

Hybridizations of microarrays. Hybridizations were carried out using the ChickenOligo 20.7K 70-mer microarray v1.0 oligo set, which was designed by the Roslin Institute/ARK Genomics and synthesized by Operon (http://www.Operon.com/Download/index.php) and spotted on Corning glass slides (lot 213) by the Centre de Ressources Biologiques, Génomique des Animaux Domestiques (http://crb-gadie.inra.fr/). Experimental conditions (“diabetic” vs. controls) were compared using six pairs of chickens (n = 6 chickens per experimental group) and a dye-swap hybridization design (i.e., cDNAs of each chicken pair were labeled and hybridized twice, inverting dye labeling). In all, 12 microarray slides were used for each tissue and simultaneously hybridized with a Ventana Hybridization System at 42°C for 8 h. Slides were subsequently washed twice in 2× saline sodium citrate (SSC) and 0.1× SSC at room temperature. The buffer remaining on the slide was removed by rapid centrifugation (4,000 g for 15 s). The fluorescence intensity was scanned using the Agilent Micro Array Scanner G2505B.

Image and data analysis. Images were analyzed as previously described (3). The signal and background intensity values for each spot in both channels were obtained using ImaGene 6.0 software. Data were filtered using the ImaGene “empty spot” option, which automatically flags low-expressed and missing spots to be removed from the analyses. The MIAME compliant raw microarray data have been deposited in the ArrayExpress database at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress/) under the accession number E-MEXP-3182. After base-2 logarithm transformation, data were corrected for systemic dye bias by Lowess normalization using GeneSight 4.1 software. Spot intensity ratios were then filtered in accordance with their variability among the six comparisons and genes (oligo spots) with high variability were removed from the analysis. Statistical analyses were performed using open source R 2.1 software (http://www.r-project.org). The log2 ratio between “diabetic” and control samples was analyzed with Student’s t-test and used to detect differentially expressed genes in the two experimental conditions. The probability values were adjusted using the Benjamini-Hochberg correction for multiple testing to control the rate of false positives [false discovery rate (FDR) <0.01]. Genes selected by these criteria are referred to as differentially expressed (DE) genes. At this level of FDR, no further arbitrary high cut-off for fold changes (FC) was applied. As shown in RESULTS, the abundance EGR1 mRNA decreased in muscle following insulin deprivation as indicated by both qRT-PCR (P < 0.03) and microarray (FC = 0.87) analyses, whereas liver IGFBP1 messenger increased (FC = 1.33 in microarray and P < 0.0001 in qRT-PCR) which accounts for the elevated levels of IGF-BP1 protein in plasma.

qRT-PCR analysis. Supplemental qRT-PCR measurements were performed for two additional genes (avUCP and SLC2A8) in the muscle tissue to complement of the 24 genes previously verified in our original study (11). Primer sequences were: CTATGGGATGAGAGGGAC-gRNA/chicken/ and CTGGAGAAATACCTGGAGGOC, forward (5’-3’) and CACCACATCAACTGGGACAA, reverse (5’-3’) for SLC2A8. Conditions for retro-transcription, PCR reactions, and internal references were identical to those previously used for the validation of our insulin immuno-neutralization model (11).

Assignment of DE genes to canonical pathways and visualization of gene interaction networks. To determine the major functional pathways altered by insulin privation, oligo probes having a corresponding human protein ID were classified using the Gene Ontology (GO) feature in Pathway Miner (http://www.biorag.org/index.php). Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, http://www.ingenuity.com/) was used to visualize the major gene interaction networks populated by DE genes in liver and muscle. The human protein identification (ID) corresponding to chicken orthologs and the chicken oligo (RIGG number) and log 2 FC (diabetics/controls) listed in Supplemental Table S1 (liver) and Supplemental Table S2 (muscle) were used as input data files for IPA.1 Although dozens of gene interaction networks were identified from microarray analysis of liver and muscle in insulin-deprived chickens, only a few representative gene interaction pathways are presented below.

RESULTS AND DISCUSSION

From the 20.7K oligonucleotide probes spotted on the array, intensity signals from 3,415 and 3,287 spots entered statistical analyses for liver and muscle, respectively. Insulin privation altered the abundance of 1,573 mRNAs in liver and 1,225 in leg muscle of fed chickens, using an FDR <0.01 as the threshold for significance. The lists of DE genes and the log ratio changes (FC, as diabetics/controls) are provided in Supplemental Materials (Supplemental Table S1 for liver and Supplemental Table S2 for muscle). The lists contain several ID numbers: the oligo IDs [Roslin Institute Gallus gallus (RIGG number)] and when available, the Ensembl G. gallus (ENSGALG) gene ID number and the corresponding human gene and protein IDs. These IDs were obtained from Ensembl (http://www.ensembl.org/index.html) with database versions available in September 2008: 50.2h for chicken and 50.36l for human genome. A total of 276 genes (oligo spots) in liver and 207 genes in muscle had no Ensembl gene ID or corresponding human protein ID. Elucidation of the information provided by these unknown or “orphan” RIGG oligos mRNAs awaits progress in resequencing and reassembly of the chicken genome.

1 The online version of this article contains supplemental material.
Table 1. *Number of differentially expressed (FDR < 0.01) genes found in liver and muscle after insulin immuno-neutralization*

<table>
<thead>
<tr>
<th>mRNAs</th>
<th>Liver</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>FC Range</td>
</tr>
<tr>
<td>Downregulated</td>
<td>850</td>
<td>0.91–0.48</td>
</tr>
<tr>
<td>Upregulated</td>
<td>723</td>
<td>2.42–1.10</td>
</tr>
</tbody>
</table>

Fold-change (FC) was determined as the ratio of “diabetics”/controls. FDR, false discovery rate.

Transcripts showing significant changes in their expression level were split about equally into down- or upregulated groups for both liver and muscle (Table 1). The FC ranges were also about of the same magnitude among expression groups and tissues.

Comparison between microarray data and qRT-PCR or protein level measurements. Liver FASN (FC = 0.46) and IGFBP1 (FC = 1.33) mRNAs were respectively down- and upregulated following insulin privation, confirming previous qRT-PCR analysis (11). And as we reported earlier (11), plasma IGFBP1 protein significantly increased. The decrease in abundance of DIO2 transcript, which accounts for the decrease in plasma T3 was significant in the qRT-PCR, although the microarray data did not reach the level of significance (FC = 0.94, FDR < 0.08). Other liver mRNAs, significantly different by qRT-PCR analysis, either did not enter statistical analysis (EGR1, PPARG, THRSPA) of microarray data or were not represented by an oligo probe on the microarray (DIO3 and SREBP1).

Previous qRT-PCR analysis of 24 genes showed fewer significant changes in muscle than in liver. Muscle microarray data confirmed lower EGR1 messenger (FC = 0.87). The increase in MURF1 messenger (E3 ubiquitin-protein ligase or TRIM63), which did not reach the level of significance in qRT-PCR analysis, was significant in microarray analysis. Atrogin-1 (FBXO32, F-box only protein32) messenger, also involved in protein degradation, significantly increased in qRT-PCR but did not enter microarray analysis. A discrepancy between qRT-PCR and microarray results was observed for avian UCP gene messenger (avUCP, related to human UCP3): an increase, although at the limit of significance, in qRT-PCR vs. a large decrease in microarray analysis (FC = 0.72). One hypothesis accounting for this discrepancy could be that several UCP3 transcripts exist in the chicken as in the case of humans. The oligo sequence printed on the microarray slide corresponds to the last exon of the avUCP gene. New qRT-PCR using primers designed within this exon confirmed previous qRT-PCR results (i.e., a slight increase, data not shown).

As a whole and apart from this unexplained discrepancy, our previous qRT-PCR analysis (or protein measurements) and the present microarray analysis are in good agreement for both tissues (liver and leg muscle). Furthermore, some mRNAs having an identified human counterpart were represented by two chicken oligo probes. For five of them, results of the two oligos agreed. For seven of them there was disagreement; some of these discrepancies may come from the existence of several transcripts.

**Ingenuity Pathway and Pathway Miner analyses.** To visualize the major canonical pathways altered by insulin privation, DE oligos having a corresponding human protein ID were first classified using Pathway Miner at Biorag (http://www.biorag.org).
The lists of these genes are given in Supplemental Table S3 for liver and Supplemental Table S4 for muscle. No assumption about cut-off limit was applied since relatively small changes could have important physiological consequences. For instance, as discussed earlier, an FC increase of 1.33 of liver IGFBP1 messenger was associated with a significant increase in plasma IGFBP1 protein levels in “diabetic” chickens. Furthermore, the decrease in muscle EGR1 messenger (FC 0.83) was confirmed by qRTP-CR analysis. Several genes may code for more than one protein; all protein IDs were maintained even though some forms are tissue specific in mammals and most likely in the chicken. In

![Gene interaction network in liver (A) showing genes involved in lipid metabolism and in leg muscle (B) showing genes involved in cellular growth and gene expression after insulin immuno-neutralization in chickens. Upregulated genes are indicated in red and downregulated genes are indicated in green; the number of genes in each category are shown in the legend. Solid lines indicate a direct relationship between genes and dashed lines represent an indirect association determined by Ingenuity Pathway Analysis (IPA).](image-url)

Fig. 1.

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Fig. 2. Gene interaction network in liver after insulin immunoneutralization in chicken showing genes involved in cell signaling (A) and the cell cycle (B). Upregulated genes are indicated in red, and downregulated genes are indicated in green; the number of genes in each category are shown in the legend. Solid lines indicate a direct relationship between genes and dashed lines represent an indirect association determined by IPA.
Fig. 3. Gene interaction network in leg muscle showing genes involved in the cell cycle (A) and cell apoptosis (B) after insulin immuno-neutralization in chickens revealed by IPA. Upregulated genes are indicated in red and downregulated genes are indicated in green; the number of genes in each category are shown in the legend. Solid lines indicate a direct relationship between genes and dashed lines represent an indirect association determined by IPA.
Fig. 4. G-coupled protein receptor/ligand gene interaction networks controlling cell signaling in liver (A) and leg muscle (B) after insulin immuno-neutralization in chickens. Upregulated genes are indicated in red, and downregulated genes are indicated in green; the number of genes in each category are shown in the legend. Solid lines indicate a direct relationship between genes and dashed lines represent an indirect association determined by IPA.
addition, both liver and skeletal muscles are heterogeneous organs, which allows for the possibility that some mRNAs altered by insulin privation may come from cells different than typical hepatocytes, myotubes, or myocytes. Future studies will have to address these issues and to identify the presence of potentially false positive results. Biogar classifies genes into two main categories: either metabolic pathways or cellular and regulatory pathways with subdivisions. The lists of the genes involved in the major pathways are presented in Supplemental Tables S5 and S6 for liver and Supplemental Tables S7 and S8 for muscle.

IPA software mapped a total of 1,846 pathway-eligible genes from both liver and muscle to the Ingenuity Knowledge Base (http://www.ingenuity.com/). And of these, 991 genes were unique to liver, 741 DE genes were unique to muscle, and 114 DE genes were common to both tissues. IPA emphasized the fact that major biological processes were altered by insulin deprivation in both liver and muscle (Table 2). Table 2 also shows the number of DE genes involved in the different biological processes. In liver, large numbers of genes were associated with metabolic disease (glucose metabolism disorder), endocrine disorders (diabetes mellitus), genetic disorder (obesity), metabolism of lipid (Fig. 1A), carbohydrate and protein, cell signaling (Fig. 2A), and cell cycle (Fig. 2B). In muscle, the largest number of genes was related to skeletal and muscular disorders, metabolic disease (glucose metabolism disorder), endocrine system disorders (diabetes mellitus), genetic disorder (amyotrophic lateral sclerosis), metabolism of lipid, and cell adhesion (Figs. 1B and 3, A and B). IPA identified numerous DE G protein-coupled receptors and growth factor interactions in liver (n = 28) and leg muscle (n = 21; Fig. 4, A and B, respectively).

An extensive review of DE genes classified in the various pathways revealed that at least 42 mRNAs have already been related to diabetes or obesity or involved in energy expenditure or sensing of metabolism in mice or humans (Table 3). It is worthy to note that three mRNAs from the oxidative pathway were decreased in muscle by the insulin deprivation: ATP synthase subunit beta (ATP5B), cytochrome c oxidase copper chaperone (COX17), and NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12 (NDUFA12). These genes belong to the “oxphos” gene complex (29, 32), which is less expressed in muscles from diabetic patients and has been identified following a gene set enrichment analysis of microarray data. Usual analyses of the same data concluded that no mRNAs were modified in muscles of diabetic humans. Changes appear more pronounced in the present chicken model, which could be accounted for by some physiological characteristics of birds, i.e., their high body temperature, 42°C (45), and high metabolic rate (20). In humans and mouse, the expression of “oxphos” genes is most likely coordinated by the transcription factors PGC-1A [peroxisome proliferator-activated receptor gamma, coactivator alpha 1 (PPARG-1A)] and nuclear respiratory factor 1 [NRF1 (29, 32, 40)]. Neither of these factors appeared in the DE gene lists issued from the present experiment and our earlier qRT-PCR analysis did not reveal any alteration for PGC-1A messenger at 5 h of insulin privation (11). Recent studies in humans have shown that alteration in oxphos gene transcription in diabetic humans is not a primary defect but results from dysregulation in metabolism (17).

Conclusions

Insulin privation rapidly and profoundly modifies global gene transcription in liver and muscle of fed chickens, despite several peculiarities observed in chicken for insulin sensitivity in vivo and insulin signaling in muscle (11, 12). The high number of genes altered by insulin deprivation may in part depend on two physiological features of chicken metabolism:

Table 3. Differentially expressed genes known to be associated with energy expenditure, sensing of metabolism, insulin resistance, obesity, or diabetes in mice or humans

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Gene Name</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>BMP-7 and PDE4B</td>
<td>43, 50</td>
</tr>
<tr>
<td>Muscle</td>
<td>NMUR1, HDAC3, AGTR2, ATF4 and PDE4B</td>
<td>6, 2, 28, 38, 50</td>
</tr>
<tr>
<td></td>
<td><strong>Insulin Resistance</strong></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>RSK2, ENTPD1, PTPN2, MAP4K4, PIK3R1 (or R2) and PTPN1 (or 9)</td>
<td>13, 14, 18, 35, 44, 9, 47</td>
</tr>
<tr>
<td>Muscle</td>
<td>MLK1, (2 or 3), PRKCE, LEPR, ADRB2, INSIG1 and OGT</td>
<td>21, 4, 19, 25, 48, 46</td>
</tr>
<tr>
<td></td>
<td><strong>Metabolism Sensors</strong></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>ITPR3</td>
<td>41</td>
</tr>
<tr>
<td>Muscle</td>
<td>CTBP2</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td><strong>Obesity</strong></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>BDKRB2, FGF19, PRKAR2B, HSPA5, F2R, PTK2B, IL15 and ADORA1</td>
<td>1, 5, 10, 22, 27, 49, 36, 33</td>
</tr>
<tr>
<td>Muscle</td>
<td>FGF19 and ADAM17</td>
<td>5, 16</td>
</tr>
<tr>
<td></td>
<td><strong>Type 1 Diabetes</strong></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>TNFRSF2</td>
<td>15</td>
</tr>
<tr>
<td>Muscle</td>
<td>TCF7L2, SLC2A9, WNT5B, VEGFA and OPRM1</td>
<td>7, 24, 23, 26, 37</td>
</tr>
<tr>
<td></td>
<td>ATP5B, COX17, NDUFA12, IGF1R, PK3R1, PPM1B and PPP1CB</td>
<td>29, 32, 31</td>
</tr>
</tbody>
</table>
high body temperature [42°C, (45)] and high metabolic rate (20). In both liver and muscle, changes concern components of multiple metabolic pathways, structural proteins, transporters, proteins of intracellular trafficking, major signaling pathways and elements of the transcription control, transcription machinery, and several other biological process. This further demonstrates pleiotropic effects of insulin in the chicken. If these genes are indeed activated, then we can hypothesize that insulin receptor and components of insulin receptor signaling cascade themselves play a major role within the nucleus. It has recently been shown that this complex is transiently activated during the turning off of the insulin signal. In the latter issue, one can hypothesize that insulin receptor and components of insulin receptor signaling cascade themselves play a major role within the nucleus. It has recently been shown that this complex is temporarily activated during the transcription process.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


