Kinetic analysis of cardiac transcriptome regulation during chronic high-fat diet in dogs

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ABSTRACT

OBESITY IS HIGHLY PREVALENT in developed countries and will be a major health concern in the future mainly because of its impact on cardiovascular morbidity and mortality (22, 27, 35, 49). Duration of obesity has been shown to be a determinant of the incidence and severity of obesity-associated cardiac morbidity (4, 36), raising the probability that the recent increase in prevalence of childhood obesity will in the future result in a marked rise in the appearance of cardiovascular diseases (14, 34, 44). Cardiovascular morbidity in obesity is, in part, a consequence of arterial hypertension (33) as well as endocrine and metabolic disorders such as insulin resistance, diabetes mellitus, or dyslipidemia. However, increased adiposity enables secretion of multiple adipokines (53) that may act on target organs such as kidney, brain, and heart (19).

Our group and others have investigated the cardiovascular adaptations to obesity in dogs fed a hyperlipidic, hypercaloric diet (HFD) (17, 43, 54). This model closely mimics human obesity and is associated with hypertension, cardiac dysfunction, and metabolic abnormalities. This is in contrast to many mouse and rat genetic models of obesity with defects in leptin synthesis or leptin signaling. In most of these models obesity is not associated with sympathetic nervous system activation or hypertension. Moreover, dietary models of obesity, especially those produced by feeding an HFD, mimic the diet of Western countries where excess fat is a major environmental factor responsible for increased prevalence of obesity (41).

We previously observed that an HFD induces abdominal obesity, hyperinsulinemia, and arterial hypertension within a few weeks, whereas marked left ventricular hypertrophy (LVH) develops only after about 20 wk of HFD (32, 54). In a recent study, we also demonstrated that short-term HFD in dogs caused substantial cardiac molecular adaptations (38). LVH in other models of hypertension has also been studied at the transcriptome (21) and proteome level (6). However, most studies provide only a static view of cardiovascular adaptation to arterial hypertension, and no study, to our knowledge, has examined the molecular mechanisms involved in long-term cardiac remodeling during dietary-induced obesity. Moreover, the time course of cardiac transcriptome changes during HFD-induced obesity is unknown. Therefore, the present study was designed to investigate the molecular processes induced by 9–24 wk of hyperlipidic and hypercaloric diet in obese, hypertensive dogs.

METHODS

Experiments were conducted in 20 chronically instrumented mongrel dogs that were conditioned before study. All experimental protocols were carried out according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and according to the guidelines of the Animal Welfare Act.

Surgical procedures were performed with the dogs under pentobarbital sodium anesthesia and using aseptic methods. Arterial and venous catheters were implanted for continuous monitoring of arterial pressure, blood sampling, and intravenous infusions as previously described (17). An electromagnetic flow probe was implanted on the ascending aorta for continuous monitoring of cardiac output (17). After recovery from surgery, antibiotics were administered daily and rectal temperatures were monitored to ensure that the dogs were afebrile throughout the studies.
After a 10- to 14-day recovery period, the dogs were placed in individual metabolic cages in a temperature-controlled room with a 12:12-h light/dark cycle and fitted with harnesses containing pressure transducers for measurements of arterial pressure. Analog signals were sampled at a rate of 400 per second in bursts of 12 s each minute, 24 h per day, digitized, and processed by the computer to determine systolic, diastolic, and mean arterial pressures and heart rate. Cardiac output signals were monitored with an electromagnetic flow meter (Zepeda Instruments) 24 h per day, at 400 samples per second in bursts of 12 s each minute. Aortic flow signals were processed to determine stroke volume, cardiac output, and peak aortic pressure, and total peripheral resistance was computed on a beat-by-beat basis by use of the cardiac output and arterial pressure signals. The averages of arterial pressure, heart rate, cardiac output, and total peripheral resistance for each day were calculated from values recorded during an 18-h period between 1:00 PM and 7:00 AM. The daily values were averaged for each week of recording.

All dogs received two cans of a sodium-deficient diet each day of the prescription diet (H/D; Hills Pet Products, Topeka, Kansas; 542 kcal/418 g can) that provided a balanced and constant amount of carbohydrates, fat, and protein and ∼7 mmol of sodium and 65 mmol of potassium per day. A more detailed description of the diet can be found at the Hill’s Pet Nutrition web site (http://www.hillspet.com/). The dogs were also given 5 ml of vitamin syrup (VAL syrup, Fort Dodge Laboratories) each day. Total sodium intake, including sodium in the food, was held constant throughout the study at ∼76 mmol/day by continuous intravenous infusion of 450 ml/day of sterile isotonic saline through one of the femoral vein catheters. Lean control dogs (n = 5) were maintained throughout the study on the regular diet of two cans of H/D food. Obesity was induced in some of the dogs (n = 15) by supplementing their regular diet with cooked beef fat (0.5–0.9 kg), and this diet was maintained for 9–24 wk. After 9, 10, 15, or 24 wk of normal diet or HFD, the dogs were anesthetized with pentobarbital sodium, and surgery was quickly performed to remove the hearts. Left ventricular tissue samples were frozen in liquid nitrogen and maintained at −80°C until preparation of the RNA.

**RNA extraction, DNA array hybridization, and expression analysis.** RNA was extracted by disrupting cells in TRIzol reagent (Invitrogen) and prepared according to recommended procedures. The concentrations of RNA were monitored using RiboGreen ( Molecular Probes) and a plate fluorometer (Fluoroskan, Labsystems). RNA integrity and concentration were further checked with an Agilent Bioanalyzer 2100 apparatus. We used 1 µg of total RNA to produce cDNA 32 P-labeled probes. Labeling with [γ-32P]dATP (NEN) of mRNA from obese hypertensive or control dogs was performed using the Strip-EZ kit (Ambion; http://www.ambion.com/techlib/prot/hp/1360.pdf), and hybridization to the membranes (NYH+, Millipore) and washes were performed as described in the PCR select differential screening kit manual (Clontech; http://www.bdbiosciences.com/clontech/techinfo/manuals/PDF/PT3138-1.pdf). Labeled probes were hybridized to our arrays containing 2,400 heart dog cDNA clones spotted in duplicates and that were described previously (38). Membranes were exposed for ∼1–3 h in a cassette and analyzed with a Storm 860 PhosphorImager (Molecular Dynamics). Signal intensity was analyzed with X-dot reader software (COSE), duplicates were averaged, and data were filtered for signal above 1.5-fold the background and normalized to the mean intensity from all the measured values. Results are shown as ratio of obese/control of normalized data. Complete data are available in the Gene Expression Omnibus (GEO) database under the accession number GPL974.

**Real-time PCR analysis of gene expression.** A set of 20 genes was randomly chosen from 63 differentially expressed genes for real-time PCR validation of the differential expression (40). In addition, a set of genes involved in the TGF-β pathway was chosen for real-time PCR mRNA quantification, according to GenMAPP software and known target genes to complement TGF-β data acquired by DNA array analysis. Oligonucleotides were synthesized by Proligos and designed with Primer Express software (Applied Biosystems) (Table 1). Real-time PCR was performed with the SYBR Green master mix reagent (Applied Biosystems) in a GeneAmp 5700 apparatus. The standard curve method was used for relative quantification of the PCR products, and gene expressions were normalized to 18S RNA quantification, which has been found to be a reliable internal control gene (1, 47). Real-time PCR data were statistically analyzed with SigmaStat software (SPSS Science).

**Western blot analysis of TRIP-1.** Cardiac left ventricle tissue was disrupted with mammalian MCL-1 cell lysis kit solution (Sigma) in the presence of a mix of protease inhibitors, and procedures were performed according to the manufacturer’s protocol. Sixty micrograms of protein was loaded on a 10% polyacrylamide-SDS gel that was blotted on a 0.45-µm nitrocellulose membrane BA85 (Schleicher and Schuell). MultiMark Multi-Colored standard (Invitrogen) was used to determine size of the proteins. Nitrocellulose membranes were blocked for 2 h in TBS (7 mM Tris, pH 7.5; 150 mM NaCl) with 0.1% Tween 20 and 3% nonfat dry milk. Hybridization of the anti-TRIP-1 serum was performed in TBS-Tween 0.1% during 2 h. After three washes in TBS-Tween 0.1%, horseradish peroxidase conjugate (10−4 dilution) was incubated for 2 h with the membrane in TBS-Tween 0.1%, 3% nonfat dry milk. Blots were washed three times in TWEEN-20 and one time in TBS; then hybridizations were revealed with SuperSignal West Pico chemiluminescent substrate according to the manufacturer’s protocol (Pierce). Each blot comprised a set of dilutions of sera to ensure semiquantitative quantification. Quantification of the detected TRIP-1 protein was performed by scanning densitometry and using ImageQuant software (Molecular Dynamics).

**Statistical analysis and identification of differentially expressed genes.** All results are depicted as means ± SE. Multiple comparisons were analyzed using ANOVA followed, when appropriate, by the Dunnett post hoc test using Statview 4.5 software (Abacus Concepts). Single comparisons were performed using unpaired Student’s t-test with a value of P ≤ 0.05 considered as significant.

Analysis of differentially expressed genes was done for cDNA arrays by ANOVA test using NIA Array Analysis Tool (http://lgsun.grc.nia.nih.gov/ANOVA/).

**Hierarchical clustering and principal component analysis (PCA).** Hierarchical clustering was performed on normalized X-dot reader data set with Cluster software and drawn with TreeView software (13). The clustering was performed on the average linkage clustering method for the genes and the arrays. PCA analysis was done using a software available online at http://lgsun.grc.nia.nih.gov/ANOVA/.

**RESULTS**

**Hemodynamic and hormonal effects of HFD.** Table 2 shows the physiological variables measured in lean control dogs and in dogs fed HFD for 9, 10, 15, and 24 wk. Body weight increased by ∼50% after 9–24 wk of the HFD. Mean arterial pressure also increased significantly, averaging 104–112 mmHg during the HFD, compared with 91 ± 2 mmHg in lean control dogs. Heart rate increased to 103–123 beats/min during the HFD, compared with 76 ± 6 beats/min in lean control dogs. The HFD also caused marked increases in cardiac output, which averaged 4.7 ± 0.7 l/min after 24 wk of HFD compared with 2.5 ± 0.2 l/min in lean control dogs.

Plasma renin activity increased to 1.05 ± 0.41 ng ANG I·ml−1·h−1 after 24 wk of HFD, compared with 0.42 ± 0.16 ng ANG I·ml−1·h−1 in lean control dogs. There were no major changes in plasma sodium or potassium concentrations in dogs fed an HFD compared with lean control dogs.
**Table 1. PCR primer sequence used for real-time RT-qPCR quantification**

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>rRNA 18S</td>
<td>5'- TCTGATTTGGCCGCGTGAAG-3'</td>
<td>5'- TGAACATCTGTCGAGAACAA-3'</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis 2 (ALS2CR3) Homo sapiens</td>
<td>5'- TTCCGAAAAGAAAAGGTTGA-3'</td>
<td>5'- TTCGACTAGCTTGCATCGAACAA-3'</td>
</tr>
<tr>
<td>Collagen type I a1 (COL1A1)* Canis familiaris</td>
<td>5'- CGCTCTTGGCTGAAATACAT-3'</td>
<td>5'- TGGCCAGTTCGCTTAAAG-3'</td>
</tr>
<tr>
<td>Cytochrome c oxidase subunit I (COI) Canis lupus</td>
<td>5'- AGGAATAGTGAACAGCAAGCGC-3'</td>
<td>5'- CTCGGTGGGATGAGGAT-3'</td>
</tr>
<tr>
<td>EGF receptor Homo sapiens</td>
<td>5'- TAGTATGTGCTGGTACACATGTAG-3'</td>
<td>5'- AGTGGTGGCTTGCAGCTT-3'</td>
</tr>
<tr>
<td>Heat shock 70 protein (HSP70) Homo sapiens</td>
<td>5'- TGCGAAGCTGGGAAATACAT-3'</td>
<td>5'- CCACAGAAATGACATCGGA-3'</td>
</tr>
<tr>
<td>Integral membrane protein 1 (ITIM) Homo sapiens</td>
<td>5'- ATGGCCAGATTTCAGCAGT-3'</td>
<td>5'- TTGGTCTGCGACATTGA-3'</td>
</tr>
<tr>
<td>Karyopherin a4 (importin o) Homo sapiens</td>
<td>5'- GGCCTGAGACCAAGAATA-3'</td>
<td>5'- CTTCTCTCCTCCGTTGACA-3'</td>
</tr>
<tr>
<td>Lectin-3 (LEC3) Homo sapiens</td>
<td>5'- GGAAAGCGGCTCTCTGAAGA-3'</td>
<td>5'- TCTGGTAAAGGATGATG-3'</td>
</tr>
<tr>
<td>Mitochondrial phosphate carrier protein precursor Homo sapiens</td>
<td>5'- CCAAAGTAAAGGCTTTATTAACG-3'</td>
<td>5'- GAGCCATCTCTTACACCAAA-3'</td>
</tr>
<tr>
<td>Matrix metalloproteinase-9 (MMP-9) Canis familiaris</td>
<td>5'- CCGCTGAGACCAAGAATA-3'</td>
<td>5'- AAGCCCTGAGGATGAAAGAT-3'</td>
</tr>
<tr>
<td>Myostatin (GDF 8)</td>
<td>5'- TCTGAGACTACGATGGTAA-3'</td>
<td>5'- TGCCGCTTGGGAAAATG-3'</td>
</tr>
<tr>
<td>ANP receptor A (NprA)* Homo sapiens</td>
<td>5'- TGAGCCAGCCGCAATGATG-3'</td>
<td>5'- CCATACCCGAGGGAAGATT-3'</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor (PAI-1)* Canis familiaris</td>
<td>5'- TGGAGGACCAACACCTTCA-3'</td>
<td>5'- CAATAGGAAGCCAGACT-3'</td>
</tr>
<tr>
<td>Prostaglandin D2 synthase (PGDS) Homo sapiens</td>
<td>5'- CGTCTCTTGGCTCAGAAATCCT-3'</td>
<td>5'- GCTGTCTGATGAAATCTGT-3'</td>
</tr>
<tr>
<td>Phospholipase C beta4</td>
<td>5'- CTGGCTCTGATGAAATCTGT-3'</td>
<td>5'- ACATTTCCAATGCGCCAGAA-3'</td>
</tr>
<tr>
<td>Phospholamban Canis familiaris</td>
<td>5'- AAAATCTCAAGGCTCTCAATG-3'</td>
<td>5'- CCAATACAAGGCTCTCAATG-3'</td>
</tr>
<tr>
<td>Rydnoedine receptor 2 (RyR2)* Muc musculus</td>
<td>5'- TGGAGGACCAACACCTTCA-3'</td>
<td>5'- CAATAGGAAGCCAGACT-3'</td>
</tr>
<tr>
<td>SERCA2a Canis familiaris</td>
<td>5'- TCGAAGCTTCCGAGCAATGCA-3'</td>
<td>5'- TCAACAGATCCGAGCATGTC-3'</td>
</tr>
<tr>
<td>Smad 2* Homo sapiens</td>
<td>5'- CAGGGCTCGGCTGAA-3'</td>
<td>5'- CGAAGATTTACGCTGTCGA-3'</td>
</tr>
<tr>
<td>Smad 3* Homo sapiens</td>
<td>5'- TCGGCTGAGATGGAATGTA-3'</td>
<td>5'- CTTGGTCTGATGAAATCTGT-3'</td>
</tr>
<tr>
<td>Smad 4* Homo sapiens</td>
<td>5'- AGCACTGACGCCGCAATGTA-3'</td>
<td>5'- GCTGTCTGATGAAATCTGT-3'</td>
</tr>
<tr>
<td>Smad 6* Homo sapiens</td>
<td>5'- ACCGTGCTACTGCCGACATG-3'</td>
<td>5'- TGAAGTAGATGATCAGGACCA-3'</td>
</tr>
<tr>
<td>Smad 7* Homo sapiens</td>
<td>5'- TGTCACTACAAGGCTTTTGAG-3'</td>
<td>5'- TCTAGGTTGCTGAGCAGAC-3'</td>
</tr>
<tr>
<td>β-Actin Canis familiaris</td>
<td>5'- GACCAGATGATGATGATGATG-3'</td>
<td>5'- AGCCCTGATGAGCCAGTA-3'</td>
</tr>
<tr>
<td>Adrenergic, β1, receptor (ADRB1)* Homo sapiens</td>
<td>5'- GCACTGATGATGATGATGATG-3'</td>
<td>5'- GCAAGTGGGGCGTTGAA-3'</td>
</tr>
<tr>
<td>Adrenergic, β2, receptor (ADRB2) Homo sapiens</td>
<td>5'- GCACTGATGATGATGATGATG-3'</td>
<td>5'- GCAAGTGGGGCGTTGAA-3'</td>
</tr>
<tr>
<td>Adrenergic, β3, receptor (ADRB3)* Homo sapiens</td>
<td>5'- GCACTGATGATGATGATGATG-3'</td>
<td>5'- GCAAGTGGGGCGTTGAA-3'</td>
</tr>
<tr>
<td>Tensin Homo sapiens</td>
<td>5'- GGTATCTTCCGAGGATGATG-3'</td>
<td>5'- CATGACGCTGCTGACATG-3'</td>
</tr>
<tr>
<td>TGF-β1* Homo sapiens</td>
<td>5'- GCCGCGTCTGGATGATGATG-3'</td>
<td>5'- AGCCGCGTCTGGATGATGATG-3'</td>
</tr>
<tr>
<td>TGF-β receptor type 2 (TGFBR2) Homo sapiens</td>
<td>5'- CTGGGCTGAGGAGGGGGT-3'</td>
<td>5'- TTCGGGAGGAGGGGGT-3'</td>
</tr>
<tr>
<td>Thioredoxin interacting protein Homo sapiens</td>
<td>5'- TTCGGGAGGAGGGGGT-3'</td>
<td>5'- TTCGGGAGGAGGGGGT-3'</td>
</tr>
<tr>
<td>TGF-receptor-interacting protein 1 (TRIP-1) Homo sapiens</td>
<td>5'- ATGGGCTGAGGAGGGGGT-3'</td>
<td>5'- GCTGGAGGAGGGGGT-3'</td>
</tr>
<tr>
<td>Voltage-dependent anion channel 2 (VDAC 2) Sus scrofa</td>
<td>5'- ATGGGCTGAGGAGGGGGT-3'</td>
<td>5'- TTAAGTCCAGGCGCTTACCA-3'</td>
</tr>
</tbody>
</table>

*Additional chosen genes involved in the TGF-β pathway to complement DNA arrays data.

Differential gene expression in left ventricles of obese and lean dogs. We used our tissue-specific cDNA array to analyze transcriptome modulations during 9–24 wk of the HFD. ANOVA analysis revealed 63 significantly differentially expressed genes in the ventricles of dogs fed HFD, compared with lean control dogs (Supplemental Table S1, available at the Physiological Genomics web site).1 Differentially expressed genes were grouped by hierarchical clustering organization (Fig. 1) that showed groups of coregulated genes specifically regulated at each study period. In addition, to provide a clear picture of the adaptive process in heart, genes were grouped by functional classes, encompassing TGF-β and TNF-α signaling, metabolism, structure, remodeling, cell proliferation, transcription and translation, ionic currents, stress, signal transduction, and a set of 14 genes of unknown function (Supplemental Table S1).

From these differentially expressed genes, 20 were randomly chosen for differential expression verification by real-time PCR quantification of the mRNA. Differential expression of 17 genes (85%) in obese compared with lean dogs was confirmed by real-time PCR, although induction or repression was often even more apparent by real-time RT-quantitative PCR (real-time RT-qPCR) (Supplemental Table S1).

Hierarchical clustering provided a global view of gene expression and sets of coregulated genes within clusters. Clusters were numbered from 1 to 6 (Fig. 1). Cluster 1 grouped nucleoprophism (NPM), a gene involved in cell proliferation (55), with genes involved in structure such as β-actin or in energy metabolism, such as NADH dehydrogenase. Cluster 2 grouped genes involved in the TGF-β pathway, such as TRIP-1 and the TGF-β receptor II, and a target gene of this pathway, phospholamban. Cluster 3 encompassed genes involved in the protection to remodeling such as myostatin (28) and ANP (23). Cluster 4, which contains SERCA2, elongation factor-1, Homo sapiens Ser Thr kinase, HSP 70, ATP synthase Fo subunit, EGF receptor, mitochondrial phosphate carrier protein precursor, and SOD AL-2, constitutes a group of genes involved in stress and cellular hypertrophy. Also, genes involved in energetic metabolism (ATP synthase, mitochondrial phosphate carrier protein precursor) were present in this cluster.

Cluster 5 did not show a particular signature for cellular or biochemical regulation but clusters among unrelated function genes, such as the matrix metalloproteinase-9 (MMP9) gene, which is well known to be involved in remodeling process. Cluster 6 contains 4 genes out of 10 that encode for mitochon-
Table 2. Changes in body weight, hemodynamics, and hormones induced by 9–24 wk of HFD compared with lean control dogs

<table>
<thead>
<tr>
<th>Weeks of HFD</th>
<th>Control (n = 5)</th>
<th>9 (n = 3)</th>
<th>10 (n = 5)</th>
<th>15 (n = 3)</th>
<th>24 (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>23±1</td>
<td>39±1*</td>
<td>34±1*</td>
<td>36±3*</td>
<td>36±0.4*</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>91±2</td>
<td>112±0.4*</td>
<td>104±2*</td>
<td>NA</td>
<td>104±4*</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>76±6</td>
<td>103±3*</td>
<td>114±1*</td>
<td>NA</td>
<td>123±1*</td>
</tr>
<tr>
<td>Cardiac output, l/min</td>
<td>3.5±0.2</td>
<td>6.0±0.1*</td>
<td>4.3</td>
<td>NA</td>
<td>4.7±0.7*</td>
</tr>
<tr>
<td>Total peripheral resistance, dyn/cm²</td>
<td>27.57±1.46</td>
<td>19±0.33</td>
<td>22.8</td>
<td>NA</td>
<td>24.22±3.94</td>
</tr>
<tr>
<td>Plasma glucose, mg/ml</td>
<td>111.3±4.1</td>
<td>NA</td>
<td>96.6±3.2*</td>
<td>NA</td>
<td>104±2.4</td>
</tr>
<tr>
<td>Plasma renin activity, ng ANG I·ml⁻¹·h⁻¹</td>
<td>0.42±0.16</td>
<td>0.8±0.37</td>
<td>NA</td>
<td>1.05±0.41</td>
<td></td>
</tr>
<tr>
<td>Plasma Na⁺, meq/l</td>
<td>147±0.31</td>
<td>147.3±0.98</td>
<td>150.2±0.8*</td>
<td>NA</td>
<td>149.2±1.1*</td>
</tr>
<tr>
<td>Plasma K⁺, meq/l</td>
<td>4.26±0.12</td>
<td>4.5±0.25</td>
<td>4.1±0.10</td>
<td>NA</td>
<td>4.15±0.09</td>
</tr>
</tbody>
</table>

Values are means ± SE. Data for cardiac output and total peripheral resistances at 10 wk of the high-fat diet (HFD) are from one dog. NA, not available. *P<0.05, ANOVA and Dunnett.

Fig. 1. Hierarchical cluster analysis of differential expression. Measurements values are shown in duplicates. Genes close to each other harboring correlated expressions are illustrated by the tree on the left. Numbers in the tree indicate cluster names in the results.
drial proteins or expressed by the mitochondrial genome: cytochrome c oxidase subunit III, VDCA 2, 16S RNA, D18b mitochondrial control region, and thioredoxin. This cluster showed upregulation during the HFD.

This clustering representation of gene expression also provides a color-coded view of gene expression levels a different time points during the HFD. In the 9 wk HFD group, downregulation of TRIP-1, a recently discovered regulator of the TGF-β pathway (9) remained downregulated in obese dogs after 15 and 24 wk of HFD.

Myostatin, a TGF-β family member, known as a negative modulator of muscle mass (28), was also strongly downregulated after 15 wk (arrays) and 24 wk of the HFD (real-time PCR). The TGF-β receptor 2 was also downregulated and found in the same cluster as TRIP-1. Moreover, SERCA2 and phospholamban, two TGF-β-regulated target genes, were also downregulated. Semiquantitative Western blot analysis (Fig. 2, A and B) showed significant downregulation of TRIP-1 after 10, 15, and 24 wk of HFD that was concomitant to a downregulation of the mRNA level for TRIP-1 (Fig. 2 C).

PCA analysis also displayed regulation of SERCA2, phospholamban, TRIP-1, and myostatin in the same group (not shown). This result was confirmed for SERCA2 and TRIP-1 by correlation analysis (see Fig. 4).

Since the TGF-β pathway seemed to be involved in the transcriptome adaptations in obese heart, we used GenMAPP, a new bioinformatics tool, for viewing and analyzing array data from biological pathways (11). As subtractive libraries used for the cDNA arrays cannot be considered as exhaustive, we also considered the possibility that we could have missed some genes involved in the TGF-β pathway that were proposed by GenMAPP and not tested. Therefore, real-time RT-qPCR data were added in GenMAPP for a defined set of genes, and this software allowed us to organize gene expression in the TGF-β pathway (Fig. 3). GenMAPP showed globally significant upregulation of the TGF-β1 gene, downregulation of TGF-β2 receptor, Smad 2, Smad 4, and TRIP-1, and upregulation of Smad 6. Smad 7 expression was not significantly altered by the HFD. GenMAPP also displayed altered regulation of a set of 10 target genes for the TGF-β pathway. Upregulated genes included the atrial natriuretic peptide receptor, which was strongly induced by the HFD, the β1-, β2-, β3-adrenergic receptors, the ryanodine receptor, and collagen 1. Collagen 1 gene, however, was upregulated only at 9 wk of the HFD.

TGF-β pathway target genes that were downregulated by the HFD included SERCA2 and phospholamban. Surprisingly, plasminogen inhibitor activator 1 gene expression was not significantly altered by the HFD. M2 muscarinic receptor was upregulated after 10 and 15 wk and downregulated at 24 wk of the HFD. Moreover, a positive correlation was observed between expression of SERCA2 and TRIP-1 (R = 0.70, P = 0.0006 with the Spearman test) and also between TGF-β type 2 receptor (TGF-β R2) and SERCA2 (R = 0.63; P = 0.008) (Fig. 4).

**DISCUSSION**

Results from previous studies indicate that dogs fed HFD closely mimic the systemic hemodynamic, renal, hormonal, and metabolic changes observed in obese humans (16, 17, 43, 54). As shown in the present study and in previous studies, dogs fed an HFD have marked increases in heart rate, cardiac output, and blood pressure as well as mild activation of the renin-angiotensin system, hyperinsulinemia, insulin resistance, and other metabolic changes that are characteristic of changes observed in human obesity. Thus this model appears to be an excellent one with which to investigate the molecular mechanisms associated with obesity-induced cardiovascular disease.

To our knowledge there has been only one report using a functional genomic approach in this animal model (38). One of the difficulties in studying dogs has been the lack of tools for functional genomic studies. Therefore, we prepared our own

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**Fig. 2.** A: representative Western blot analysis of TRIP-1 protein in dog hearts. Sixty micrograms of protein was loaded on each lane of a 10% polyacrylamide-SDS gel. The right area of the blot contained several dilutions of heart proteins to be used to draw a standard curve for quantification. B: quantification of the Western blots by densitometry; protein level was normalized to control. HFD, high-fat diet. *P < 0.05.
cDNA arrays for dogs with the goal of investigating the cardiac molecular adaptations that occur during chronic obesity induced by feeding an HFD for 9–24 wk. This also enabled us to investigate the time course of the molecular adaptations in the left ventricle associated with a chronic HFD.

Hierarchical clustering organization provided us both a global view of the changes in cardiac gene expression induced by the HFD and also permitted us to group genes that were regulated in a similar way. Interpretation of the data is challenging for some clusters, because of a limited number of genes differentially expressed, which limited cluster size. Other pattern discovery methods such as PCA analysis was not more informative (not shown). Thus cluster 1 showed clearly a coregulation of genes involved in cell proliferation and structure. Cluster 2 grouped several genes involved in the TGF-β pathway, and cluster 6 associated mostly genes involved in mitochondria function. One outcome of this clustering of gene expression is a color-coded graphical view of gene expression that clearly showed that the duration of obesity had a profound impact on cardiac transcriptome regulations. Moreover, this global representation illustrated that genes are up- or downregulated in the heart depending on the duration of the HFD.

This analysis indicates that molecular mechanisms are involved in the heart to coordinate controlled expression of groups of genes during HFD, probably by means of a defined set of cardiac transcription factors. Thus our observations point out a dynamic model for cardiac molecular adaptations in dietary-induced obesity.

Our array analysis revealed 63 genes that were up- or downregulated in left ventricles of dogs made obese and hypertensive by HFD. Confirmation of the array data was obtained in 85% of the genes by real-time RT-qPCR (17 confirmed out of 20 tested genes), thus indicating, as previously reported, that arrays can be reliable indicators of altered gene expression. Despite this measure of reliability, arrays can generate some artifacts, as we have shown in our comparison of expression profiles between arrays and real-time PCR for MMP9 at 15 wk. Even taking into account limitations, our data globally show that in dogs fed a chronic HFD, a continuous gene-regulated reprogramming occurs in the left ventricle. Major changes in gene expression were noted after 9 wk of HFD, even though major ventricular macroscopic alterations in the left ventricle are generally not observed at this time (38).

This finding suggests that changes in cardiac gene expression may occur very early and participate in tissue remodeling that is seen later after many weeks of an HFD (32).
At 9 wk of HFD, we observed some differences when comparing gene expression data in left ventricle from our previous study (38). At this stage of HFD we did not find in the present study a significant induction of MMP9, although we observed significant downregulation of SERCA2 and phospholamban not previously observed in the left ventricle of dogs fed an HFD. This might be explained by the different pedigrees of the animals in the present study compared with our previous study (38). We also found upregulation of mRNA levels encoding proteins of the mitochondrial respiratory chain such as cytochrome c oxidase subunit I and ATP synthase subunit 6. These changes may reflect increased energy and ATP consumption secondary to increased cardiac work load and arterial hypertension. Similar upregulation of ATP synthesis has also been described in left ventricular mitochondria from spontaneous hypertensive rats (7, 48).

After 24 wk of HFD, we observed downregulation of the gene for myostatin, a factor that inhibits cardiac muscle growth (28). Deficiency of myostatin has been shown to cause muscle hypertrophy (56), an observation that is in accordance with our finding of downregulation of myostatin and with previous studies showing cardiac hypertrophy in dogs after 22 wk of HFD (32).

We also observed a significant reduction of N-acetylgalactosaminyl transferase mRNA only after 15 wk HFD. This enzyme mediates acquisition of carbohydrate side chains in O-glycosidic linkage to either Thr or Ser and modifies the structure of polypeptide backbone and heavily O-glycosylated proteins involved in cell interactions such as mucin glycoproteins (52). In addition, O-glycans function as ligands for receptors mediating tumor cell adhesion (45). mRNA levels encoding lectomedin-3 were also found to be downregulated by RT-qPCR. This protein harbors a galactose binding domain and is a G protein-coupled receptor that also regulates cell adhesion via an inside-out effector signaling pathway. Decreased expression of this protein can lower adhesion to ligands present in the extracellular matrix or on opposing cells resulting in chemotaxis and extravasation (20).

Reorganization of tissue structure is often concomitant with cell proliferation (5). We observed upregulation of the expression of a set of genes involved in cell proliferation that have, to our knowledge, not been previously studied in heart. First, NPM, which is known to accumulate in nuclei of exponentially growing HeLa cells (55) and is strongly upregulated during estrogen-induced cell proliferation in MCF-7 breast cancer cells (50), was continuously upregulated after 10 wk of HFD. NPM has several potentially important roles in regulating cell function and signaling. Specifically, NPM is a chaperone for nuclear import of proteins (12, 51). This feature lends itself to chromatin dynamics (26) and likely plays an important role in the regulation of cellular mitogenesis. In this same group of genes, we also found downregulation of mRNA levels for ERK3, a mitogen-activated protein kinase present in the nucleus (9). ERK3, also called p97 or MAPK6, is responsive to different growth factors during activation of the p38 pathway (57). Downregulation of ERK3 may be a feedback control mechanism of cellular proliferation which attempts to slow down cellular proliferation during the process of ventricular hypertrophy.

One striking observation in the present study is that multiple components of the TGF-β pathway were altered during the HFD. Using cDNA arrays, we observed that several members of the TGF-β pathway were differentially regulated in lean and obese dogs. Since our cDNA arrays contained 2,400 clones that were derived from subtractive libraries and since we cannot consider these as exhaustive, we decided to further focus on

![Fig. 4. Correlation of expression of TRIP-1 and SERCA2 genes and TGF-β R2 and SERCA2 assessed by real-time RT-qPCR. A: expression of TRIP-1 (x-axis) and SERCA2 (y-axis) are plotted. B: expression of SERCA2 (x-axis) and TGF-β R2 (y-axis) are plotted. Vertical bars delineate the duration of HFD for the data pairs [from 24 weeks (24w) to regular diet (Lean)].](http://physiolgenomics.physiology.org/)

Physiol Genomics • Vol. 19 • www.physiolgenomics.org
this signaling pathway by real-time PCR mRNA level evaluation of the Smad genes, well-known effectors of this pathway, and to assess the mRNA levels of a set of target genes for the TGF-β pathway. We observed upregulation of the transcript for TGF-β in the left ventricle of obese compared with lean dogs. TGF-β has been proposed as an autocrine/paracrine factor responsible for myocardial fibrosis. Several experimental models of pathological myocardial growth associated with increased arterial pressure on volume overload also demonstrated upregulation of the TGF-β gene. Blockade of TGF-β function by injection of neutralizing antibodies prevented myocardial fibrosis and diastolic dysfunction in pressure-overloaded rats (24). Moreover, increased TGF-β has been associated with increased body mass index and hypertension in humans, and it has been proposed that adipose tissue may be an important determinant of plasma TGF-β levels, possibly by a leptin-dependent pathway (3, 39).

TGF-β has been shown to be a potent inducer of plasminogen activator inhibitor 1 (PAI-1) (43), although TGF-β did not modify expression of PAI-1 in cardiac fibroblasts (2). TGF-β1 administered in mice increased PAI-1 activity in plasma and PAI-1 mRNA expression in adipose and heart tissue (46) or human cardiac myocytes in vitro at the mRNA level (30). In our study, PAI-1 expression of the left ventricle was not altered by obesity despite increased levels of TGF-β1 and collagen 1, a well-known target gene of TGF-β (29), which was upregulated transiently at 9 wk HFD. This is in accordance with the level of the transcript for TGF-β1 that reached its maximum induction (29-fold increase) at 9 wk and the observation that high levels of TGF-β are required for induction of collagen in cardiac tissues (37). These controls for mRNA levels may occur through a specific regulatory mechanism for PAI-1 and collagen in hearts of obese hypertensive dogs. This regulation may be a consequence of the level of TGF-β or of the pattern of expression of the genes involved in the TGF-β1 pathway. As Smad 6, B1, B2, B3, and RYR receptor reached maximal induction at 9 wk and since they are known TGF-β target genes, we suggest that these genes may be upregulated by high levels of TGF-β, whereas NpRA receptor is strongly upregulated by lower levels of TGF-β (i.e., at 10 wk HFD). Since we found that the negative regulator of the TGF-β pathway, Smad 6, was upregulated, we propose that this protein may block the expression of a set of defined genes. In fact, Smad 6 has been recently shown to be predominantly expressed in heart and vessels, is involved in the control of gene expression during heart development (15), and is proposed to be a corepressor of transcription by recruiting histone deacetylase (8).

mRNA from genes involved in cardiac excitation-contraction coupling such as phospholamban and SERCA2 were downregulated in the left ventricle of obese compared with lean dogs. SERCA2 has been proposed to be regulated by the TGF-β pathway (31). We also observed downregulation of TRIP-1, a negative regulator of the TGF-β pathway (10) in heart ventricle. This downregulation of an inhibitory factor for the TGF-β pathway may be responsible for the activation of a set of specific genes such as NpRA, B1 and B2 AR, and RyR2. The positive correlations found between expression of SERCA2 and TRIP-1 (Fig. 4A), and also between TGF-β R2 and SERCA2 (Fig. 4B), emphasize potential links between the regulation of these genes. Since increased expression of TRIP-1 is associated with increased expression of SERCA2, it is likely that TRIP-1 acts as a positive factor on SERCA2 or that TRIP-1 and SERCA2 share common regulatory sequences. Comparison of 10 kb of 5′ regulatory sequence of TRIP-1 and SERCA2 displayed a segment of about 300 bp of 82% of homology (data not shown). Within this DNA segment we could identify DNA binding sites for SREBP, a transcription factor involved in lipids homeostasis (42). Binding of this transcription factor on its cognate DNA binding site will be investigated in future studies.

In summary, a long-term HFD in dogs led to increased body weight and multiple cardiovascular adaptations including increases in blood pressure, cardiac output, and heart rate, as well as profound modifications of the cardiac transcriptome. Because we analyzed cardiac transcription modifications at several time points during the HFD, the observed changes offer a dynamic picture of obesity-related pathological adaptations in the myocardium. The molecular adaptations to dietary-induced obesity observed in the present study also suggest novel regulatory pathways that may mediate cardiac remodeling in obesity.

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