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Cytokine gene expression profiles in kidney medulla and cortex of obese hypertensive dogs

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Cytokine gene expression profiles in kidney medulla and cortex of obese hypertensive dogs.

Background. The molecular mechanisms linking abnormal kidney function and obesity hypertension are poorly understood. This study compared gene expression profiles in the kidney medulla and cortex of obese and lean dogs.

Methods. Lean dogs ($N = 4$) were fed a standard kennel ration and obese dogs ($N = 4$) were fed the standard diet plus 0.5 to 0.9 kg of cooked beef fat per day for 10 weeks. The dogs were instrumented for continuous monitoring of mean arterial pressure (MAP), heart rate, glomerular filtration rate (GFR), and effective renal plasma flow (RPF). The relative mRNA levels of 375 genes in renal cortex and medulla were determined simultaneously using cDNA membrane arrays (R&D Systems).

Results. The high fat diet increased body weight by 57% and MAP increased by 24 mm Hg (112 ± 1 mm Hg vs. 88 ± 3 mm Hg) in obese compared to lean dogs. In obese dogs, expression of 11 and 13 genes changed significantly ($N = 4$; $P < 0.05$) in the renal medulla and the cortex, respectively, relative to the lean dogs. Differences in renal gene expression profiles between lean and obese dogs were closely related to functional pathways, including those associated with sympathetic activation, inflammatory response, matrix formation, angiogenesis, endothelial dysfunction, attenuated actions of leptin, and attenuated cell survival.

Conclusion. A high fat diet in dogs is associated with marked changes in renal gene expression profiles that provide potential molecular links to pathways associated with altered renal function and structure in obesity hypertension.

Obesity affects more than one third of American adults [1] and causes multiple target organ injury [2]. Some pathologic consequences of obesity include hypertension, type 2 diabetes, hyperlipidemia, hypercoagulation, stroke, and renal disease [2]. Epidemiologic studies suggest that more than 50% of the risk for human essential hypertension cases is related to obesity [3]. Studies in animals and humans show that weight gain raises blood pressure and that weight loss reduces blood pressure in both normotensive and hypertensive subjects [4–6]. Obesity may also be an important risk factor for renal disease, not only through its effects to cause hypertension and type II diabetes, but also through additional mechanisms that are poorly understood [7].

Although the mechanisms of obesity-associated hypertension are complex and multifactorial, increased renal tubular reabsorption and a hypertensive shift of pressure natriuresis play a key role in raising arterial pressure [7]. Obesity enhances renal tubular reabsorption by increasing renal sympathetic nerve activity, activating the renin-angiotensin system (RAS), and by physical compression of the kidneys [7]. Our previous studies suggest that obesity increases renal extracellular matrix (ECM) formation in the renal medulla and in glomeruli, and that these changes may contribute to a hypertensive shift of pressure natriuresis [8]. We found that dogs fed a high fat diet for 7 to 9 weeks or 24 weeks exhibited early changes in renal structure and function [9]. These changes include increased glomerular filtration rate (GFR) and renal plasma flow (RPF), elevated arterial blood pressure, expansion of Bowman's capsule, increased cell proliferation in the glomerulus, and thickening of the mesangial matrix and Bowman's capsule basement membranes. However, there are no studies, to our knowledge, that have examined the changes in renal gene expression that may be involved in the pathophysiology of obesity-associated hypertension and renal disease.

The present study examined potential molecular links between obesity hypertension and abnormal kidney function by comparing gene expression profiles in kidney cortex and medulla of lean dogs (fed the standard diet) and obese dogs (fed a high fat diet). We also examined changes in mean arterial pressure (MAP) and renal function associated with obesity induced by feeding a high fat diet in dogs, a model that that closely mimics cardiovascular, renal, endocrine, and metabolic changes that occur in obese humans [5]. Moreover, obesity has been shown to be a proinflammatroy state in animal models and humans. Thus, we focused on cytokines in the present study. Cytokine cDNA membrane arrays containing 375

Key words: gene expression, cDNA membrane array, renal medulla, renal cortex, obese dog, blood pressure.

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cytokine-related genes and 9 housekeeping genes were used to compare renal gene expression profiles in obese and lean dogs. In addition, a reliable data analysis method was developed based on the normalization of gene expression data by stable internal housekeeping genes.

METHODS

Animal protocol

The protocols were carried out according to the guidelines for the care and use of laboratory animals implemented by the National Institutes of Health and the Guidelines of the Animal Welfare Act and were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee. We used eight conditioned male mongrel dogs in this experiment. Four dogs became obese after 10 weeks of being fed the high fat diet, which included an additional 0.5 to 0.9 kg of cooked beef per day added to the regular diet (standard kennel ration). Four control lean dogs were fed the same standard kennel ration but were not given the beef fat supplement. Total sodium intake, including sodium in the food and intravenous saline infusions, was maintained constantly at approximately 76 mmol/day for both groups of dogs.

Physiologic measurements

Surgical procedures were performed on dogs during isoflurane anesthesia, using aseptic techniques. Tygon (Norton Plastics, Akron, OH, USA) catheters were implanted into the femoral arteries and veins for measurement of arterial blood pressure and blood sampling, respectively. After a recovery period of 1 to 2 weeks, the dogs were housed in individual metabolic cages and instrumented for continuous monitoring of MAP, heart rate, GFR, and effective RPF, as previously described [5]. Plasma renin activity, plasma sodium concentration, and plasma potassium concentration were also determined as previously described [5].

RNA isolation

Kidney tissues of the cortex and medulla were immediately dissected and frozen for total RNA preparation using a modified guanidinium-based method (Totally RNA Isolation Kit) (Ambion, Austin, TX, USA), as previously described [10]. The genomic DNA was removed by treating with Rnase-free Dnase I for 45 minutes at 37°C followed by phenol/chloroform extraction and ethanol precipitation. Absence of genomic DNA contamination was determined by polymerase chain reaction (PCR) amplification of the actin gene. RNA integrity was verified on a 3-[N-morpholine] propane sulfonic acid (MOPS)-formaldehyde agarose gel prior to cDNA synthesis. The RNA sample had an A_{260}/A_{280} ratio of 1.8 or above.

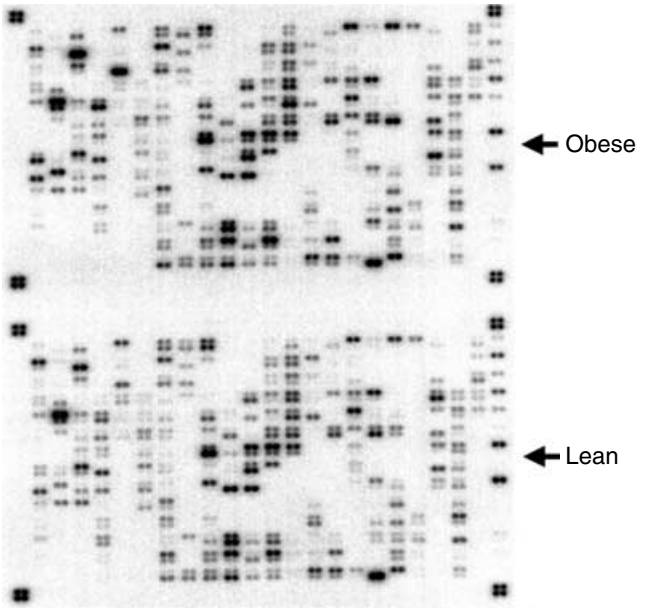

Fig. 1. The membrane images of expression profile arrays of 375 cytokine-related genes plus nine housekeeping genes in the kidney medulla of obese and lean dogs after 10 weeks of a diet program. Known genes (cDNA fragments) are immobilized in duplicate on a nylon membrane that is hybridized to a ^{32}P-labeled cDNA probe representing expressed genes in a particular tissue. The tetrad positive control spots at the four corners in both membranes are similar with minimal background noise around the gene spots, which indicate that the membranes have undergone thorough hybridization and washes. Quantitation of gene expression signals was performed on membrane images of duplicate spots representing each gene using a Phosphor-Imager (Molecular Dynamics, Eugene, OR, USA).

cDNA labeling and membrane-array hybridization

The cDNA array hybridization technique relies on the principle of reverse Northern blotting. Known genes (cDNA fragments) are immobilized in duplicate on a nylon membrane that is hybridized to a ^{32}P-labeled cDNA probe [generated by reverse transcription (RT)] representing expressed genes in a particular tissue. Human cDNA expression arrays were obtained from R&D Systems (Minneapolis, MN, USA) and all procedures were followed as recommended by the manufacturer's protocols. We utilized a step-by-step intensive wash method to minimize the background noise. After stringent washing, membranes were exposed to a phosphor screen (Molecular Dynamics, Eugene, OR, USA) (software ImageQuant 3.3) for 4 or 24 hours. Analysis was conducted after 4 or 24 hours of exposure not only to ensure that hybridization intensity was in the linear range, but also to collect data (signal intensity) on genes with low expression levels.

Analysis of array data

We analyzed the cDNA array membrane with minimal background noise. For example, Figure 1 shows the images of expression profile arrays of 375 cytokine-related

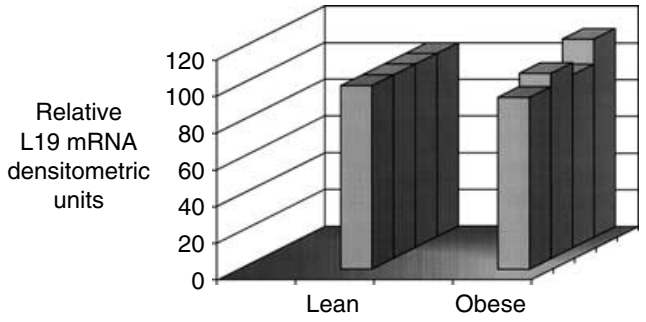

Fig. 2. The relative densitometric units of ribosome protein L19 mRNA in the renal medulla of lean dogs were very similar to those observed in obese dogs (100 vs. 99.16; $P = 0.8116$; $N = 4$). Analyzing the four sets of hybridization (one lean and one obese membrane for each set) for the renal medulla or cortex tissue, the data indicated that ribosome protein L19 mRNA was the most stable gene signal among the nine housekeeping genes and did not change between lean and obese groups. Therefore, L19 mRNA signal was chosen as the internal housekeeping gene to normalize the signals of all genes in each set of arrays.

genes in the kidney medulla of obese and lean dogs. Quantitation of gene expression signals was performed on membrane images of duplicate spots representing each gene using a Phosphor-Imager (Molecular Dynamics). Raw values of pixel intensity were extracted from membrane-array images using the software ImageQuant 3.3 (Molecular Dynamics). This yielded values for the mean signal intensity, which were adjusted by the local background area (same size as the spot area). The mean of their signal intensities and their standard deviation were calculated. A spot was defined as "nondetectable" if its mean signal intensity was less than the mean of the negative control signal intensities plus two times the negative control standard deviation for that same membrane. There were nine listed housekeeping genes in each array. By analyzing the four sets of hybridization (one lean and one obese membrane for each set) for the renal medulla or cortex tissue, the data indicated that ribosome protein L19 mRNA was the most stable gene signal among the nine housekeeping genes and did not change between lean and obese groups (Fig. 2). We also found that the relative densitometric units of the ribosome protein L19 signal in both renal medulla of lean vs. obese dogs or renal cortex of lean vs. obese dogs were very similar (99.2 ± 3.4 and 99.0 ± 2.9, respectively, compared to normalized ratios of 100 for medulla and cortex of lean dogs). The relative signal of the mRNA expression was defined as the densitometric ratio of gene-specific mRNA signal intensity over a housekeeping gene known as ribosome protein L19. The next step in data analysis was to compare the normalized signals (relative mRNA levels) on different arrays to identify expressed genes that were statistically different between two groups, lean and obese ($N = 4$). Statistical significance was defined as a two-tail Student t test value of $P < 0.05$. Unpaired Student t test was used to make comparisons between the two groups.

Northern blotting

Expression of vascular endothelial growth factor (VEGF) or transforming growth factor-β1 (TGF-β1) mRNA in the renal medulla tissues of obese and lean dogs was further examined by Northern blotting. The same total RNA samples were used for Northern blot analyses that were used in previously described methods [10]. The VEGF cDNA probe was a 580 bp *Eco*RI-*Bam*HI fragment of the murine VEGF cDNA that could have a cross-reaction with dog VEGF sequence [11]. The TGF-β1 cDNA probe was a 1.05 kb *Eco*RI-*Eco*RI fragment of human TGF-β1 cDNA that was cloned into pSP64 palsmid [12]. The VEGF mRNA or TGF-β1 expression was normalized against 28S rRNA in each sample.

Statistical analyses

All determinations were performed in duplicate. Data was presented as mean ± SE, as indicated. Differences were considered statistically significant when $P < 0.05$ by paired or unpaired t test. All statistical calculations were performed with StatView software (BrainPower, Calabasa, CA, USA). Control hemodynamic and renal function data obtained for dogs before initiation of the high fat diet period were compared with data obtained for the same dogs after the high fat diet period by using ANOVA and Dunnett's t test for multiple comparison [9].

RESULTS

Weight, hemodynamic, renal function, and hormonal data

Table 1 summarizes weight, hemodynamic, renal function, and hormonal data for dogs fed a high fat diet (obese group) or standard diet (control group) for 10 weeks. Body weight in obese dogs was 57% greater than in lean dogs. Kidney weight was increased by an average of 46% in dogs fed a high fat diet for 10 weeks, compared to dogs fed a standard diet. MAP was increased by 24 mm Hg (112 ± 1 mm Hg vs. 88 ± 3 mm Hg) in obese dogs compared to lean dogs ($P < 0.05$). Heart rate averaged 119 ± 2 beats/min and 69 ± 2 beats/min in obese and lean dogs, respectively. GFR and effective RPF were 71% and 58% greater, respectively, in obese dogs compared with lean dogs. There were no significant differences in plasma concentrations of sodium and potassium in obese dogs compared with lean dogs. Plasma renin activity was 1.8-fold higher in the obese group, compared to the control group.

Table 1. Weight, hemodynamics, renal function, and hormonal data for obese and lean dogsa

Parameter	Lean ($N = 4$)	Obese ($N = 4$)
Body weight *kg*	19.4 ± 1.6	30.4 ± 1.9[b]
Kidney weight *g*	41.9 ± 2.5	61.2 ± 3.1[b]
Mean arterial pressure *mm Hg*	88 ± 3	112 ± 1[b]
Heart rate *beats/min*	69 ± 2	119 ± 2[b]
Glomerular filtration rate *mL/min*	68 ± 3	117 ± 5[b]
Effective renal plasma flow *mL/mL*	166 ± 15	264 ± 17[b]
Plasma sodium concentration *mEq/mL*	149 ± 0.4	148 ± 0.5
Plasma potassium concentration *mEq/mL*	4.27 ± 0.1	4.04 ± 0.03
Plasma renin activity *ng angiotensin I/mL per min*	0.36 ± 0.10	0.63 ± 0.05[b]

[a]Data are means ± SEM; [b]$P < 0.05$ versus lean.

Table 2. Significantly altered expression of renal medullary genes between obese and lean dogs

Genes[a]	Relative mRNA levels (mean ± SE) Lean	Obese	% Change	P value[b]
FGR-14	0.24 ± 0.030	0.41 ± 0.060	+ 68	0.033
Endothelin-3	0.86 ± 0.130	1.40 ± 0.025	+ 61	0.012
GFR-α3	0.91 ± 0.075	1.35 ± 0.055	+ 47	0.002
STRL33	0.03 ± 0.005	0.07 ± 0.010	+ 95	0.040
IL-13Rα2	0.37 ± 0.065	0.57 ± 0.075	+ 52	0.049
P-selectin	0.07 ± 0.005	0.10 ± 0.010	+ 45	0.029
ENA-78	1.56 ± 0.135	1.04 ± 0.120	+ 49	0.016
MMP-3	.029 ± 0.007	.007 ± 0.005	−72	0.045
TGF-β1	0.03 ± 0.005	0.09 ± 0.011	+ 60	0.028
TGF-α	0.75 ± 0.265	1.48 ± 0.195	+ 98	0.037
VEGF-B	1.08 ± 0.140	1.48 ± 0.050	+ 37	0.032

Abbreviations are: FGF-14, fibroblast growth factor-14; GFR-α3, glial cell line–derived neurotrophic factor receptor-α3; IL-13Rα2, interleukin-13 receptor-α2; ENA-78, epithelial cell–derived neutrophil attractant-78; MMP-3, matix metalloproteinase-3; TGF-β1 and -α, transforming growth factor-β1 and -α; VEGF-B, vascular endothelial growth factor-B. [a]See **Results** section for more detailed gene descriptions; [b]The statistical significance was defined as a two-tail Student t-test value of $P < 0.05$ ($N = 4$).

Cytokine gene expression profiles in the renal medulla of obese and lean dogs

Figure 1 shows the images of expression profile arrays of 375 cytokine-related genes plus nine housekeeping genes in kidney medulla of obese and lean dogs after 10 weeks of a diet program. A complete list of genes contained in the array can be accessed at the web site (*www.rndsystems.com*). We divided the genes into groups:(*1*) adhesion molecules (22 genes); (*2*) angiogenic factors and their receptors (32 genes); (*3*) cell surface proteins (19 genes); (*4*) chemokines and their receptors (48 genes); (*5*) cytokines, cytokine receptors, and nitric oxide–related factors (57 genes); (*6*) binding proteins and integrins (27 genes); (*7*) epidermal growth factor (EGF) and TGF family (30 genes); (*8*) ephrins and their receptors (16 genes); (*9*) interleukins (IL) and their receptors (40 genes); (*10*) neurotrophic factors and orphan receptors (32 genes); (*11*) protease or related factors (21 genes); (*12*) tumor necrosis factor (TNF) family (31 genes); and (*13*) housekeeping genes (9 genes).

As previously mentioned, expression of a gene to abundance of mRNA transcripts was defined as "nondetectable" if its mean signal intensity was less than the mean of the negative control signal intensities plus two times the negative control standard deviation. Using these criteria, all genes indicated above were detectable in the renal medulla of obese and lean dogs.

Table 2 shows that expressed relative mRNA levels of 11 renal medullary genes were significantly changed among the 375 genes expressed in lean and obese dogs ($N = 4$, $P < 0.05$). In obese dogs, up-regulation occurred in 10 genes: endothelin-3 (61%, $P = 0.012$), fibroblast growth factor-14 (FGF-14) (68%, $P = 0.033$), glial cell line–derived neurotrophic factor receptor-α3 (GFR-α3) (47%, $P = 0.002$), STRL-33 (a member of the chemokine receptor family) (95%, $P = 0.040$), TGF-α (98%, $P = 0.037$), TGF-β1 (60%, $P = 0.028$), VEGF-B (37%, $P = 0.032$), P-selectin (45%, $P = 0.029$), epithelial cell–derived neutrophil attractant-78 (ENA-78) (49%, $P = 0.016$), and IL-13 receptor-α2 (IL-13Rα2) (52%, $P = 0.049$). Matrix metalloproteinase-3 (MMP-3) mRNA expression was down-regulated by 72% ($P = 0.049$) in the obese dogs compared with the lean control dogs.

Northern blot analysis TGF-β1 and VEGF mRNA expression in renal medulla of obese and lean dogs

The expressions of TGF-β1 and VEGF mRNA in the kidney medulla of obese and lean dogs were also determined by Northern blot analysis. We found that the expressions of TGF-β1 increased by 74% in the kidney medulla of obese dogs, compared to lean dogs ($P < 0.01$; $N = 4$) (Fig. 3). This result was consistent with the data resulted from cDNA membrane array analysis in which TGF-β1 mRNA expression increased by 60% in obese dogs, compared to lean dogs. We used a 580 bp *Eco*R1-*Bam*H1 fragment of a murine VEGF cDNA probe for Northern blot analysis. In our previous study [11], this VEGF probe was successfully used to determine VEGF mRNA expression in cultured dog coronary artery smooth muscle cells. Figure 3 indicates that VEGF mRNA expression increased by 1.9-fold in the kidney medulla obese compared to lean dogs ($N = 4$, $P < 0.01$). In the cDNA membrane array analysis, VEGF-B mRNA increased 1.4-fold in the renal medulla of obese compared to lean dogs.

Cytokine gene expression profiles in the renal cortex of obese and lean dogs

Using the threshold as described in the **Methods** section, we found that only 4 of 375 genes had nondetectable spots on the array membrane while analyzing the renal

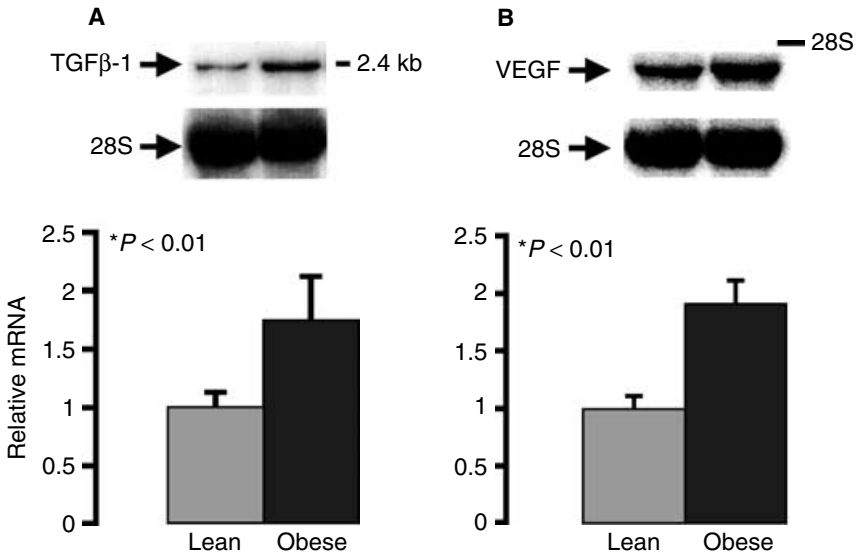

Fig. 3. Northern blot analysis of transforming growth factor-β1 (TGF-β1) (*A*) or vascular endothelial growth factor (VEGF) (*B*) mRNA expression in the kidney medulla of obese dogs compared to the lean dogs. To verify the relative amounts of total RNA, filters were hybridized with a ^{32}P-labeled 28S rTNA antisense oligonucleotide probe (Ambion). The relative mRNA expression of TGF-β1 increased by 74% in the kidney medulla of obese dogs, compared to lean dogs ($P < 0.01$; $N = 4$). The relative mRNA of VEGF increased by 1.9-fold in the kidney medulla of obese dogs compared to the lean dogs ($P < 0.01$, $N = 4$).

Table 3. Significantly altered expression of renal cortex genes between obese and lean dogs

	Relative mRNA levels (mean ± SE)			
Genes[a]	Lean	Obese	% Changes	*P* value[b]
Leptin R	.007 ± 0.001	.002 ± 0.001	−76	0.022
LIFR	.012 ± 0.004	.002 ± 0.001	−87	0.026
TIMP-3	0.25 ± 0.015	0.15 ± 0.020	−38	0.006
Ephrin-A1	0.06 ± 0.004	0.10 ± 0.015	+40	0.042
FGF-1	0.02 ± 0.003	0.01 ± 0.004	−46	0.044
FGF-3	.074 ± 0.004	.047 ± 0.007	−36	0.006
CTGF	0.09 ± 0.010	0.05 ± 0.010	−43	0.020
Integrin-α_3	1.40 ± 0.121	0.92 ± 0.111	−34	0.013
MIP-1δ	0.05 ± 0.010	0.02 ± 0.005	−56	0.018
BCA-1	0.01 ± 0.001	.007 ± 0.001	−32	0.033
OSM-Rβ	0.06 ± 0.008	0.04 ± 0.004	−34	0.041
VCAM-1	0.01 ± 0.004	.004 ± 0.001	−67	0.046
K-cadherin	0.03 ± 0.004	.012 ± 0.002	−55	0.008

Abbreviations are: LIFR, leukemia inhibitory factor receptor; TIMP-3, tissue inhibitor of metalloproteinase-3; FGF-1 and -3, fibroblast growth factor-1 and -3; CTGF, connective tissue growth factor; MIP-1δ, macrophage inflammatory protein-1δ; BCA-1, B-cell–attracting chemokine-1; OSM-Rβ, oncostatin M receptor β; VCAM-1, vascular cell adhesion molecule-1.

[a]See **Results** section for more detailed gene description; [b]the statistical significance was defined as a two-tail Student *t* test value of $P < 0.05$ ($N = 4$).

cortex gene profiles of obese and lean dogs. These genes included leptin, C-kit ligand, monocyte chemoattractant protein-2 (MCP-2), and IL-8. In analyzing four sets of hybridizations (one lean and one obese membrane for each set) for the cortex, the data also indicated that ribosome protein L19 mRNA was the most stable gene among the nine housekeeping genes listed. The relative densitometric units of ribosome protein L19 mRNA in the cortex were shown not to change between lean and obese groups (100 ± 0.0 vs. 99.0 ± 2.9; $P = 0.8023$; $N = 4$). Thus, we also used the ribosome L19 protein mRNA signal as the housekeeping gene to normalize signals of all the genes in each set of renal cortex arrays.

Table 3 indicates that expressed relative mRNA levels of 13 renal cortex genes were significantly different between lean and obese dogs among the 375 genes examined ($N = 4$, $P < 0.05$). The expression of ephrin-A1 mRNA increased by 40% ($P = 0.042$) in the kidney cortex of obese compared to lean dogs. In obese dogs, the 12 genes that were down-regulated included leptin receptor (76%, $P = 0.022$), leukemia inhibitory factor receptor (LIFR) (87%, $P = 0.026$), tissue inhibitor of metalloproteinase-3 (TIMP-3) (38%, $P = 0.006$), oncostatin M receptor β (OSM-Rβ) (34%, $P = 0.041$), FGF-1 (46%, $P = 0.044$), FGF-3 (36%, $P = 0.006$), connective tissue growth factor (CTGF) (43%, $P = 0.020$), integrin-α_3 (34%, $P = 0.013$), K cadherin (55%, $P = 0.008$), vascular cell adhesion modecule-1 (VCAM-1) (67%, $P = 0.046$), B-cell–attracting chemokine (BCA-1) (32%, $P = 0.033$), and macrophage inflammatory protein-1δ (MIP-1δ) (56%, $P = 0.018$).

Comparison of different gene expression patterns between renal medulla and cortex

We compared the expression profiles between the renal medulla and cortex in lean dogs (control group), as indicated in Table 4. Of the 375 genes in the array, there were 89 (24%) genes with significantly different ($P < 0.05$) expression in the renal medulla compared to the cortex. Seventy-one of the 89 genes were up-regulated and the other 18 genes were down-regulated in the medulla compared to the cortex. Some genes were highly expressed in the medulla compared to the cortex and some genes were highly expressed in the cortex compared to the medulla. For example, the relative mRNA level of interferon (IFN)-α/βRα was more than 10-fold higher ($P = 0.0199$) in the medulla compared to the cortex. The relative mRNA level of integrin-α_7 (1.133) was 6.8-fold higher ($P = 0.0233$) in the cortex compared to the medulla. Most genes in the FGF family, the integrin

Table 4. The relative mRNA levels (mean ± SE) of the genes differentially expressed between the kidney medulla and cortex of lean dogs

Gene Name	GeneBank Number	Medulla	Cortex	*P* value
CAD -11	L34056	1.057 ± 0.207	0.269 ± 0.153	0.0112
CAD -12	L34057	0.281 ± 0.035	0.092 ± 0.016	0.0203
N-cadherin	X54315	0.088 ± 0.026	0.019 ± 0.008	0.0225
ALCAM	L38608	0.213 ± 0.022	0.051 ± 0.027	0.0018
E-selectin	M30640	0.031 ± 0.022	0.149 ± 0.014	0.0003
FGF-6	X63454	0.165 ± 0.026	0.056 ± 0.021	0.0083
FGF-7	M60828	0.019 ± 0.004	0.071 ± 0.023	0.0387
FGF-12	U66197	0.055 ± 0.015	0.017 ± 0.009	0.0403
FGF-14	U66200	0.243 ± 0.029	0.084 ± 0.061	0.0292
FGF-16	AB009391	0.095 ± 0.031	0.012 ± 0.007	0.0193
FGF-17	AB009249	0.177 ± 0.051	0.054 ± 0.013	0.0309
Cysteine-rich FGF-R	U28811	0.412 ± 0.092	0.113 ± 0.039	0.0124
FGF-R2	U11814	1.041 ± 0.308	0.300 ± 0.173	0.0403
FGF-R3	M58051	0.551 ± 0.064	0.135 ± 0.029	0.0005
FGF-R4	L03840	0.505 ± 0.123	0.158 ± 0.044	0.0191
CD6	U34625	1.485 ± 0.339	0.259 ± 0.125	0.0182
CD8α	M12828	0.432 ± 0.056	0.122 ± 0.086	0.0118
ST2	D12763	0.237 ± 0.035	0.083 ± 0.032	0.0093
BCA-1	AF044197	0.032 ± 0.010	0.011 ± 0.001	0.0395
IP-10	X02530	0.404 ± 0.049	0.110 ± 0.045	0.0022
Fractalkine	U91835	0.213 ± 0.036	0.096 ± 0.019	0.0149
GRO-γ	M36821	0.562 ± 0.104	0.119 ± 0.067	0.0059
CXCR-1	L19591	0.105 ± 0.037	0.024 ± 0.002	0.0376
IGF-I	X56773	0.137 ± 0.026	0.047 ± 0.013	0.0119
LIF	X13967	0.079 ± 0.017	0.018 ± 0.009	0.0128
Oncostatin M	AH001516	0.358 ± 0.102	0.063 ± 0.024	0.0158
PDGF-A chain	X03795	0.078 ± 0.023	0.023 ± 0.009	0.0348
SARP-1	AF017986	0.179 ± 0.031	0.092 ± 0.011	0.0201
G-CSF R	M59818	0.206 ± 0.075	0.042 ± 0.012	0.0409
IFN-α/β Rα	J03171	1.569 ± 0.544	0.143 ± 0.028	0.0199
MSP-R	X70040	0.374 ± 0.073	0.147 ± 0.083	0.0435
PDGF-Rβ	M21616	0.383 ± 0.051	0.061 ± 0.017	0.0005
IGF binding P-7	L19182	0.506 ± 0.139	0.182 ± 0.051	0.0361
Integrin-α_1	X68742	0.370 ± 0.067	0.057 ± 0.029	0.0026
Integrin-α_2	M28249	0.490 ± 0.059	0.082 ± 0.034	0.0005
Integrin-α_4	X16983	0.351 ± 0.070	0.171 ± 0.037	0.0105
Integrin-α_5	X06256	0.125 ± 0.034	0.044 ± 0.009	0.0321
Integrin-α_6	X53586	0.317 ± 0.070	0.037 ± 0.021	0.0043
Integrin-α_7	AF072132	0.166 ± 0.046	1.133 ± 0.384	0.0233
Integrin-α_9	L24158	0.370 ± 0.073	0.077 ± 0.037	0.0061
Integrin-αE	L25851	0.349 ± 0.069	0.102 ± 0.026	0.0081
Integrin-αV	M14648	0.381 ± 0.041	0.103 ± 0.022	0.0005
Integrin-β_1	X07979	0.567 ± 0.076	0.097 ± 0.048	0.0009
Integrin-β_3	M20311	0.735 ± 0.090	0.130 ± 0.020	0.0191
Integrin-β_5	X53002	0.376 ± 0.092	0.054 ± 0.016	0.0071
Integrin-β_6	M35198	0.502 ± 0.125	0.090 ± 0.055	0.0117
Integrin-β_7	S80335	0.793 ± 0.168	0.350 ± 0.138	0.0441
erbB3	M29368	0.119 ± 0.034	0.037 ± 0.004	0.0273
Activin RIB	NM_004302	0.260 ± 0.059	0.695 ± 0.126	0.0105
BMP-8	M97016	1.154 ± 0.127	0.775 ± 0.104	0.0325
TGF-β2	M19154	0.039 ± 0.012	0.143 ± 0.045	0.0365
TGF-β RI	AF054598	0.434 ± 0.256	1.637 ± 0.548	0.0471
Ephrin-A1	M57730	0.572 ± 0.193	0.069 ± 0.003	0.0205
Ephrin-A2	AJ007202	1.090 ± 0.397	0.170 ± 0.009	0.0299
Ephrin-A5	U26403	0.834 ± 0.207	0.211 ± 0.051	0.0135
Ephrin-B1	U09303	5.976 ± 2.467	1.635 ± 0.637	0.0404
EphA4	L36645	0.168 ± 0.055	0.049 ± 0.008	0.0396
EphB1	L40636	1.121 ± 0.345	0.229 ± 0.059	0.0236
EphB3	X75208	0.417 ± 0.123	0.068 ± 0.027	0.0151
EphB4	U07695	0.997 ± 0.308	0.184 ± 0.027	0.0196
IL-1β	M15330	0.053 ± 0.003	0.021 ± 0.011	0.0141
IL-10	M57627	0.037 ± 0.006	0.121 ± 0.015	0.0011
IL-1ra	M55646	0.061 ± 0.021	0.015 ± 0.003	0.0366
IL-2Rα	X01057	0.025 ± 0.004	0.089 ± 0.025	0.0261
IL-2Rβ	M26062	0.098 ± 0.009	0.574 ± 0.180	0.0129
IL-3Rα	M74782	0.620 ± 0.041	0.155 ± 0.092	0.0018
IL-15Rα	M96651	0.440 ± 0.125	0.147 ± 0.043	0.0352
IL-18R	U43672	0.024 ± 0.009	0.075 ± 0.022	0.0413
GFRα1	U97144	0.834 ± 0.209	0.040 ± 0.010	0.0045
GFRα3	NM_001496	0.690 ± 0.148	0.295 ± 0.093	0.0322
Neuropilin-2	AF022860	0.431 ± 0.053	0.138 ± 0.051	0.0037
beta-NFG	X52599	0.197 ± 0.029	0.097 ± 0.032	0.0324
Semaphorin F	U52840	0.097 ± 0.035	0.024 ± 0.009	0.0473
TrkA	M23102	0.094 ± 0.021	0.037 ± 0.011	0.0271
TrkB	U12140	0.095 ± 0.016	0.036 ± 0.007	0.0081
GFR-5	L36149	0.172 ± 0.018	0.078 ± 0.016	0.0038
STRL33	U73529	0.034 ± 0.004	0.052 ± 0.008	0.0456
SPC4	M80482	0.048 ± 0.006	0.075 ± 0.009	0.0241
MMP-1	X05231	0.062 ± 0.015	0.152 ± 0.041	0.0435
April	AF046888	0.445 ± 0.056	0.880 ± 0.019	0.0377
CD30L	L09753	0.265 ± 0.080	0.075 ± 0.016	0.0302
DR-6	AF068868	0.082 ± 0.022	0.307 ± 0.072	0.0121
Osteoprotegerin	U94332	0.019 ± 0.007	0.065 ± 0.018	0.0306
OX40L	D90224	0.026 ± 0.011	0.217 ± 0.077	0.0244
TRAIL	U57059	0.070 ± 0.010	0.031 ± 0.010	0.0176
Osteopontin	J04765	0.112 ± 0.016	0.036 ± 0.005	0.0023
GM-CSF Rα	X17648	0.066 ± 0.019	0.020 ± 0.003	0.0319

family, and the ephrin family were expressed to a greater extent in the medulla compared to the cortex.

We also found that endothelin-2 was highly expressed of the 375 genes in the kidney medulla as well as the cortex, where the relative mRNA levels of endothelin-2 were 6.20 ± 1.48 (SE) and 5.75 ± 030, respectively. The relative mRNA levels of endothelin-3 in the medulla and the cortex were 0.77 ± 0.22 and 0.57 ± 0.30, respectively. In contrast, the relative mRNA levels of endotheial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and neuronal nitric oxide synthase (nNOS) in the medulla were 0.15 ± 0.05, 0.04 ± 0.01, and 0.09 ± 0.01, respectively. Their expressions in the cortex were similar to those observed in the medulla.

DISCUSSION

In the present study, cDNA array analysis was used to identity altered molecular pathways associated with changes in renal function and structure in the early stages of dietary-induced obesity in dogs. We utilized cDNA membrane arrays containing 375 cytokine-related genes to compare the gene expression profiles in the renal medulla and cortex in lean and obese dogs. We focused specifically on cytokine-related genes since our previous studies suggested that accumulation of ECM and possible activation of inflammatory pathways may be involved in the early renal pathophysiology found in obesity [9].

Only a few candidate genes have been investigated as potential mediators of obesity-induced renal structural and functional changes. The primary goal of the present study was to examine changes in expression of a large

number of cytokine genes that may be involved in mediating obesity renal changes using a targeted cytokine gene array and examining cardiovascular and renal functional changes in a dietary model of obesity that closely mimics the changes observed in human obesity.

We have previously shown that dogs fed a high fat diet have cardiovascular, renal, endocrine, and metabolic changes that closely mimic those observed in obese humans. However, dog-specific gene arrays are not readily available and very few candidate genes from dogs have been sequenced. We therefore tested the hybridization of dog RNA-derived cDNA versus human DNA sequences immobilized on nylon membranes. Fortunately, this human array system worked well with dog kidney RNA as evidenced by the following. First, we were able to observe significant expression of most of the cytokine genes in both the kidney medulla and cortex of dogs. This suggests that the human cDNA probes on the array contained sufficient sequence identity to canine homologues. Second, there was a linear and quantitative response with a wide dynamic range more than five to six orders of magnitude for detecting the expression of 375 cytokine-related genes in the dog tissues. Third, all statistically significant changes in renal gene expression profiles between lean and obese dogs were interpretable and were closely related to certain functional pathways in relation to obesity hypertension. Finally, using Northern blot analysis, we found that human TGF-β1 cDNA probe could detect TGF-β1 mRNA expression in dog tissues. Therefore, it is likely that most of the human cytokine cDNA probes on the array have substantial cross-reaction with dog cytokine genes.

Assessing the cDNA membrane array results

The cDNA array technologies provide an effective means to simultaneously monitor the expression of a large number of genes in biologic systems. However, problems associated with background noise, normalization, and false positive results have caused substantial concerns in the application of the cDNA microarray technologies [13, 14]. To ensure the interpretability of the experimental results generated using cDNA array technologies, certain minimum information should be reported. In this study, we followed the Minimum Information About a Microarry Experiment (MIAME) standards [15].

The high-density membrane array system used in the present study not only gives a linear and quantitative response with a wide dynamic range of five or six orders of magnitude, but it also has the advantage of low background noise as well as direct and more exact measurements [13]. In order to further reduce background noise, the background subtraction was performed locally (taking the background around each gene spot) when doing image analysis.

There were nine listed housekeeping genes in the array membrane used. We found that some of the listed housekeeping genes were not stable between the two groups. For example, the signal intensity of β-actin in the renal cortex of obese dogs was 40% higher than that observed in lean dogs. Using a cDNA membrane array system, Lyn et al [16] also found that the β-actin signal intensity increased more than twofold in the myocardium after ischemia compared to the control, while the signal intensity of ribosomal protein S29 was very similar between the two groups. The L19 mRNA signal did not differ in obese and lean dogs and served as a stable internal housekeeping gene to normalize signals of all the genes in each set of arrays in the present study.

The next critical step was to determine whether changes of gene expression were statistically significant. The cDNA microarray data analysis system usually sets up the thresholds of >twofold or >fourfold changes of gene expression over the control in order to determine differential gene expression [13, 14]. However, these high thresholds may not allow detection of many expressed genes that are physiologically relevant. In this study, the data analysis was based upon the comparison of multiple lean ($N = 4$) and obese dogs ($N = 4$). All statistically significant changes in renal gene expression profiles between lean and obese dogs were closely related to certain functional pathways in relation to obesity hypertension (Table 5). Future studies are needed to elucidate the role of each these genes in mediating renal changes associated with obesity and to extending these analyses to earlier time points to identify candidates for regulatory genes that show altered expression even before histologic changes take place during the development of obesity hypertension.

Identification of differentially expressed renal genes related to possible functional pathways in obesity hypertension

The magnitude of tubulointerstitial injury is an important prognostic marker of renal outcomes in many forms of renal diseases. Accumulation of ECM in the renal interstitium and glomerulus is a characteristic pathologic feature of kidney disease [17]. However, there are a few studies examining the effects of obesity on renal ECM formation, especially in the early stage before development of diabetes mellitus. Table 2 indicates that after only 10 weeks of a high fat diet there was a significant up-regulation of TGF-β1 mRNA expression and down-regulation of MMP-3 mRNA expression in renal medulla of obese dogs, compared to lean dogs. TGF-β has been identified as a key mediator of ECM synthesis and accumulation during the progression of renal disease [18].

Table 5. Functional clustering of renal cytokine genes related to obesity hypertension

Possible functional pathways	Alteration of the renal genes	References
Sympathetic activation	Up-regulation of FGF-14, GFR-α3, endothelin-3, and STRL33	21–24
Inflammatory response	Up-regulation of ENA-78, P-selectin, and IL-13Rα2; down-regulation of OSM-Rβ and LIFR	25–29
Matrix formation	Up-regulation of TGF-β1 and down-regulation of MMP-3	16–19
Angiogenesis	Up-regulation of VEGF, TGF-α, TGF-β1, P-selection, and ephrin A1; Down-regulation of MMP-3 and TIMP-3	30–34
Endothelial dysfunction	Up-regulation of endothelin-3 and P-selectin	35–37
Attenuated actions of leptin	Down-regulation of leptin receptor and LIFR	38–40
Attenuated cell survival	Down-regulation of FGF-1, FGF-3, Integrin-α_3, K cadherin, VCAM-1, BCA-1, and MIP-1δ	41–49

Abbreviations are: FGF, fibroblast growth factor; GFR-α3, glial cell line–derived neurotrophic factor receptor-α3; ENA-78, epithelial cell–derived neutrophil attractant-78; IL-13Rα2, interleukin-13 receptor-α2; OSM-Rβ, oncostatin M receptor β; LIFR, leukemia inhibitory factor receptor; TGF-β1, transforming growth factor-β1; MMP-3, matix metalloproteinase-3; VEGF-B, vascular endothelial growth factor-B; TIMP-3, tissue inhibitor of metalloproteinase-3; VCAM-1, vascular cell adhesion molecule-1; BCA-1, B-cell–attracting chemokine-1; MIP-1δ, macrophage inflammatory protein-1δ.

Among different TGF-β isoforms, TGF-β1 is the most highly expressed within the kidney [18]. MMPs are enzymes that degrade ECM components. Uchio et al [19] reported that decreased MMP activities caused abnormal accumulation of ECM in the kidneys of hereditary nephrotic mice. Studies have also shown important roles of increased TGF-β1 and decreased MMP in renal disease [18, 19]. However, their roles in obesity-associated renal disease are unknown. Further studies are needed to determine if up-regulation of TGF-β1 mRNA expression and down-regulation of MMP-3 mRNA expression in renal medulla of obese dogs may contribute to increased ECM formation obesity-induced renal injury.

Several observations suggest that increased renal sympathetic activity is a major factor contributing to obesity hypertension [5–8]. For example, bilateral renal denervation greatly attenuated sodium retention and hypertension in obese dogs fed a high fat diet for 5 weeks [20]. In the present study, we found increased mRNA expressions of FGF-14, GFR-α3, endothelin-3, and STRL33 in renal medulla of obese dogs compared to lean dogs. FGF-14 is a neuro-isoform of fibroblast growth factor that is up-regulated in the early stages of limb development when innervation is taking place [21]. GFR-α3 is a receptor component primarily related to artemin, a glial cell line–derived neurotrophic factor that can function for sympathetic neurone generation, survival, and growth [22]. Endothelin-3 is a neuro-isoform of endothelin that influences salt and water homeostasis through its multiple effects on hormone system and sympathetic activation [23]. Previous studies also indicate that sympathetic activation enhances endothelin-3 release [23]. STRL33 is an orphan receptor which also functions as chemokine receptors in the nervous system [24]. Although the role of each of these pathways in obesity hypertension is unknown, the data from our study provide important clues for further investigation of FGF-14, GFR-α3, endothelin-3, and STRL33 in the renal structural and functional changes associated with sympathetic activation in obesity hypertension.

CONCLUSION

We found that expression of 11 and 13 genes changed significantly in the renal medulla and the cortex of obese dogs, respectively, relative to the lean dogs. Potential functional pathways that may link these differentially expressed genes to obesity hypertension are shown in Table 5. These functional pathways include sympathetic activation [21–24], inflammatory responses [25–29], ECM formation [16–19], angiogenesis [30–34], endothelial dysfunction [35–37], attenuated actions of leptin [38–40], and attenuated cell survival [41–49]. Functional clustering of genes identified as differentially expressed by using cDNA membrane-arrays analysis further suggests that the mechanisms of obesity hypertension and renal injury are complex and multifactorial. These functional genomic data will enhance the understanding of potential molecular links among obesity, hypertension, and abnormal kidney structure and function. An interesting aspect of the present studies is that many of the changes in gene expression were observed after only 10 weeks of a high fat diet, before severe metabolic changes were observed, and with only modest increases in blood pressure. Therefore, further studies are needed to investigate the time course of altered gene expression profile during the development of obesity hypertension.

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