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DIFFERENTIAL RNA EXPRESSION OF HEPATIC TISSUE IN LEAN AND OBESE MICE AFTER LPS-INDUCED SYSTEMIC INFLAMMATION

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TABLE OF CONTENTS

1. ABSTRACT

Mortality of obese patients with sepsis has been reported to be significantly higher than lean patients. The underlying basis for this difference is not currently known. However, it has been suggested that obesity is associated with an altered immune response to a septic or inflammatory insult. Since obesity is based on exclusion in many sepsis trials, little is known about how obesity affects mortality or whether obese individuals respond differently to therapeutic interventions. In this study, obese and non-obese mice were given intra-peritoneal injection of saline or LPS and the livers were harvested 2 hours later. RNA from these livers were subjected to DNA microarray. Analysis showed distinct differences in gene expression between lean and obese animals. The expression of one hundred and seventeen genes was found to be different among the groups. In the obese animals treated with LPS, the expression of 20 genes showed a significant change. Ontology analysis revealed increased expression of 15 genes and significant decrease in expression of 5 genes. This study shows different gene expression of liver in response to LPS in lean versus obese animals. These genes might play a role in the outcome of sepsis.

2. INTRODUCTION

Mortality of obese patients with sepsis is higher than in non-obese patients, and reported to be 23% in obese patients as compared to 6% in the non-obese (1). A study of mechanically ventilated obese patients found significant mortality increase in the obese patients and recommended that obesity be considered an independent risk factor of mortality (2). Although the mechanism of poor outcome in obese patients with sepsis or mechanical ventilation is unknown, it has been suggested that obesity is associated with an altered immune response to a septic or inflammatory insult (3-5). Since obesity has been an exclusion criterion in many sepsis trials, little is known about how obesity affects outcome or if the obese respond differently to therapy. In particular, little is known about how obesity affects liver function in sepsis. Because of the complex interactions associated with obesity and sepsis, standard investigational strategies do not allow examination of numerous tissues and biologic pathways simultaneously, and may not uncover the mechanisms by which obesity alters the immune response to a septic or inflammatory insult.

Because of the complex nature of the liver, the numerous cell types within the liver and co-existing health of the organ, studying the liver in a disease state is difficult. The level of complexity and difficulty grows exponentially when considering a variable such as obesity. Therefore, studying numerous pathways at one time can provide insights that more traditions methods may not. One technique of examining numerous biologic pathways and tissues simultaneously is to identify cellular RNA expression through microarray technology. This technology allows large-scale identification of the genes expressed

within a tissue, and may allow identification of pathways not previously associated with obesity, inflammation or the cellular response to an infectious or inflammatory insult.

In this study we compared RNA expression in hepatic tissue between obese and non-obese mice after LPS injection in an attempt to identify expression patterns that may provide clues to the hepatic dysfunction in sepsis, and those that may differentially expressed in obese subjects following the inflammatory insult.

3. METHODS

3.1. Animal Model

Obese transgenic mice (-actin promoter driving agouti) and non-transgenic lean littermates were used as the model for the presence and absence of obesity, respectively (6-8). This mouse strain appears to provide a model of obesity having less immunologic interference than leptin deficient mice (9). All mice were 6 weeks of age. The obese group mean weight was 51 grams and the lean was 29 grams.

3.2. Experimental Design

The study was designed as a two-way comparison between obese and non-obese mice (n=12), with 50% of animals in each group receiving saline and the other 50% receiving lipopolysaccharide (LPS).

Group 1: obese transgenic mice subjected to LPS challenge. Mice received 12 mg/kg of *E.coli* LPS (Sigma-Aldrich, St Louis, Mo) by intraperitoneal injection.

Group 2: obese transgenic mice subjected to saline challenge. Mice in this group were treated identically to group 1 above except they received an equal volume sterile saline rather than LPS intraperitoneally.

Group 3: non-transgenic littermates (non-obese) mice subjected to LPS challenge. Mice in this group were treated identically to group 1 above.

Group 4: non-transgenic littermates (non-obese) mice subjected to saline challenge. Mice in this group were treated identically to group 2 above.

3.3. Animal experimentation

All mice received either 12 mg/kg of E. coli LPS or equal volume of saline by intraperitoneal injection. The mice were observed and sacrificed two hours after receiving the injection. The liver was removed and cut in 0.5 cm sections. This tissue was subsequently placed in a RNA preservative (RNALater, Quiagen, Valencia, CA, http://www.qiagen.com) and frozen to -70°C for later RNA extraction and analysis. Animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

3.4. RNA isolation and microarray hybridization

The tissue was removed from the RNA preservative and homogyenized in a buffered solution. The isolation was performed utilizing the RNeasy® Mini Kit (Qiagen). A highly denaturing cell-lysis buffer containing guanidine isothiocyanate (GITC) immediately generates an RNase-free environment, stabilizes the RNA, and simultaneously releases the DNA. The cellular extract is prepared, the conditions adjusted to allow separation of RNA and DNA, and the extract loaded onto the QIAGEN-tip. Total RNA and a portion of the genomic DNA present in the sample bind to QIAGEN Resin while the remaining DNA passes through in the first flow-through fraction. Residual proteins, metabolites and low-molecular-weight impurities are removed by washing the QIAGEN-tip with a medium-salt buffer. Pure RNA is eluted in a high-salt buffer while DNA remains bound to the resin. The RNA is then concentrated and desalted by isopropanol precipitation.

For simultaneous isolation of genomic DNA and RNA from the same sample, the first flow-through is reapplied to the QIAGEN-tip after elution of the RNA in order to bind the rest of the genomic DNA. The column is washed again, and the genomic DNA is eluted. Concentration and desalting of the RNA and DNA by isopropanol precipitation can then be performed in parallel. RNA samples were test for quantity and quality utilizing photospectrometry. To reduce biologic and technical variability, the RNA was pooled by group and splint onto two oligo-chips as shown in Figure 1.

An initial test hybridization was performed, once these samples satisfied this quality control measure, they were hybridized to the MU 74 Av2® oligonucleotide chip (Affymetrix, Santa Clara, CA, http://www.affymetrix.com), hybridized and labeled. The chips were scanned with an Agilent Technologies (Palo Alto, CA, http://www.agilent.com) GeneArray® scanner.

4. DATA ANALYSIS

Initial data review and preparation was conducted with Microarray Suite 5.0 (Affymetrix, Santa Clara, CA, http://www.affymetrix.com). The acquired image files were visually inspected for aberrations. The mean intensity of the four quadrants of each image file was determined to ascertain that data acquisition on each chip was uniform (< 5% deviation). Grid alignment was manually verified on each sample before calculation of probe intensities. Cell expression values were obtained using the standard Affymetrix calculations. The values were transformed to a tab delineated text file and ported to the software package Genesis (TUGR). ANOVA was performed comparing he four groups after normalization and log transformation. The data subsequently underwent k-means analysis and self-organizing map (SOM) clustering.

5. RESULTS

Results of the filtering, normalization and ANOVA separated 117 genes with significant differences between groups. Figure 2 represents with hierarchical clustering of these genes separated and by groups. Figure 3 shows the SOM clustering of the genes with increased expression in the obese LPS group while Figure 4 shows

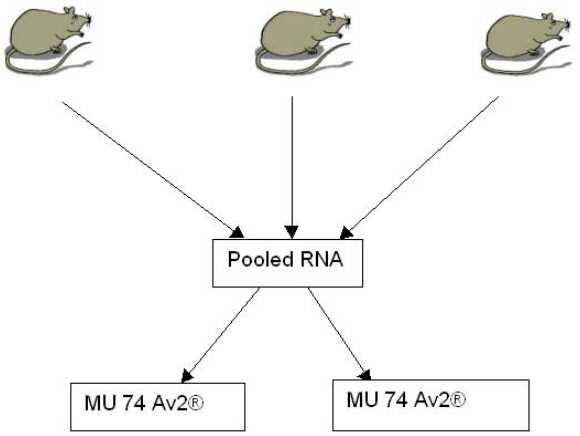

Figure 1. Experimental design that shows the RNA pooling of the liver tissue to reduce biologic variability. The pooled RNA is then separated onto separate oligonucleotide chips to reduce technical variability.

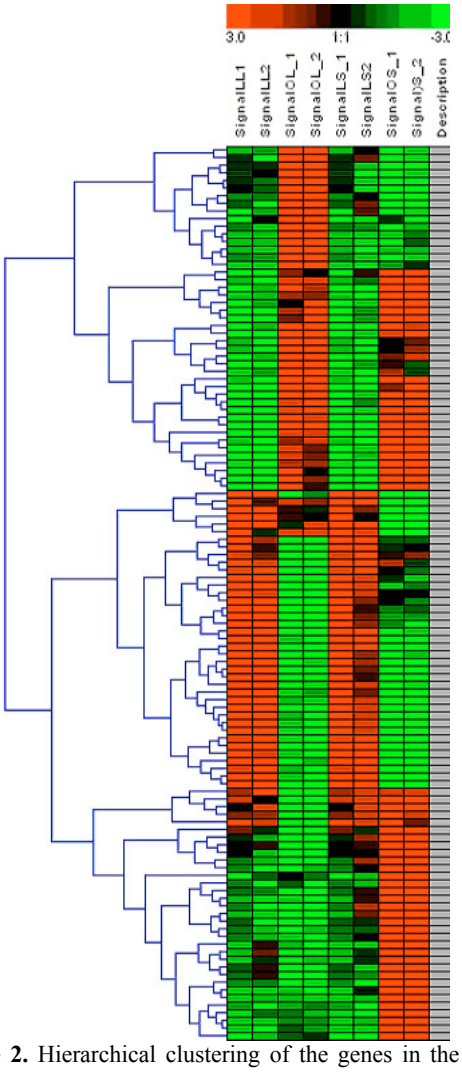

Figure 2. Hierarchical clustering of the genes in the lean and obese groups. This represents the genes of significance after ANOVA analysis which resulted in 117 genes showing significant differential expression ($p<0.01$).

the SOM clustering of the genes with significant decreased expression in the obese LPS group. Similar separation is performed using k-means clustering in Figure 5 genes with increased and decreased expression in obese LPS grouping. Table 1 lists the genes of increased expression in the obese LPS grouping while Table 2 lists the genes that are significantly decreased in expression in the obese LPS group.

Figure 6 shows the general ontologies of the genes of increased expression in the obese LPS grouping. This reveals that a majority of the genes are involved in the physiologic processes followed by cellular, biologic and then developmental. The physiologic processes are further broken down (bottom pie-chart). This shows catalytic activity and binding processes most active, followed by transporter genes, transcription regulation, signal transduction and finally structural genes. However, 25% of the genes have unknown activity.

6. DISCUSSION

Due to the liver's role in metabolism, its malfunction secondary to sepsis has significant impact on health. During sepsis, leukocytes enter the liver via liver sinusoids in response to numerous chemoattractants. As a result of this inflammatory mileu, platelets are recruited generating "clogging" of the hepatic sinusoids (10). This clogging leads to decreased blood flow through the sinusoids and inducing organ dysfunction.

Kupffer cells in the liver are bacterial scavengers leading to inactivation of bacterial products and clearance of inflammatory mediators. Hepatocytes, during inflammatory activation tend to modify their metabolic pathway toward gluconeogenesis and increased synthesis of coagulant and complement factors (11). This adds to an already procoagulant state, especially by inhibiting protein C synthesis. Activated Kupffer cells release chemokines that recruit blood neutrophils which lead to microcirculatory disturbances, fibrin deposition, and hepatocellular injury (12). Kupffer cells are activated by various bacterial stimuli, including bacterial lipopolysaccharide (LPS) and bacterial antigens, and produce interleukin (IL)-12. IL-12 and other cytokines lead to activation of natural killer (NK) cells. It has been suggested that inadequate activation of these cells can lead to bacteriemia and distal organ dysfunction and shock (13).

Hypoxia inducible factor 1, alpha subunit (HIF-1α) has gain increasing attention as an adaptive regulatory protein of hypoxia related genes, cell signaling and apoptosis. Furthermore, HIF-1α increases cellular adaptation to hypoxia by increasing glycolysis, erythropoietin and angiogenesis (14). In the endothelial cell, HIF-1α has been shown to induce cell cycle arrest which then decreases endothelial cell proliferation and apoptosis (15). In this model, HIF-1α is increased after LPS injection without evidence of hypoxia. Similar observations have shown that LPS can induce hypoxia related genes through the HIF-1α complex (16). HIF-1α is induced by

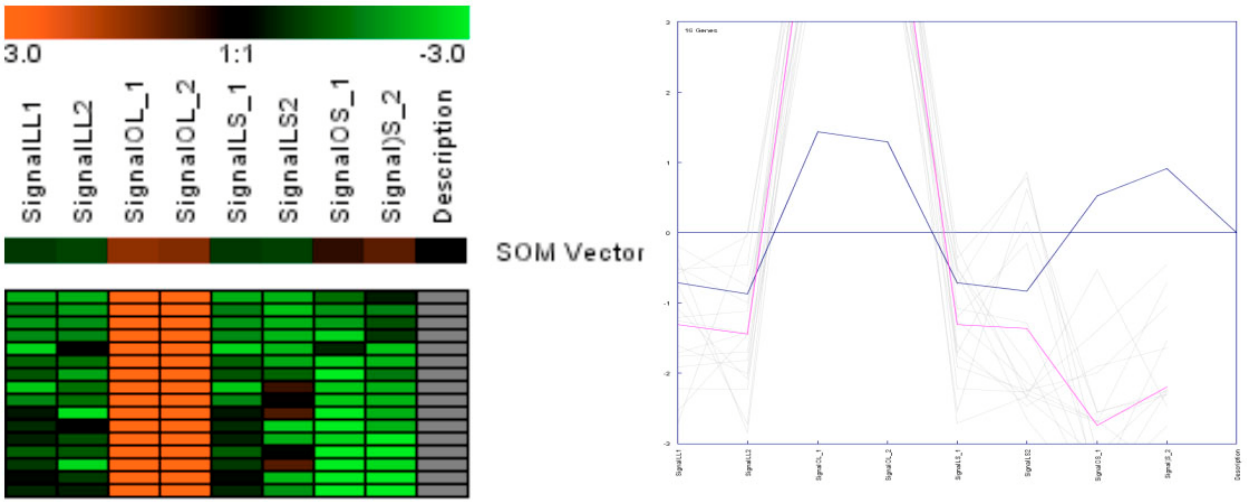

Figure 3. SOM map of the significant genes demonstrating increased expression in the obese LPS group

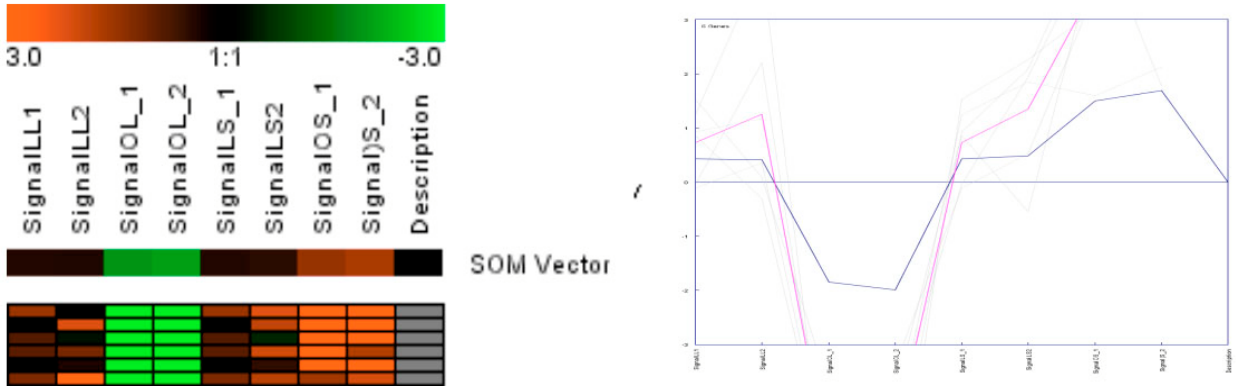

Figure 4. SOM map demonstrating the significant genes showing decreased expression in the obese LPS group compared the obese saline group and lean groups.

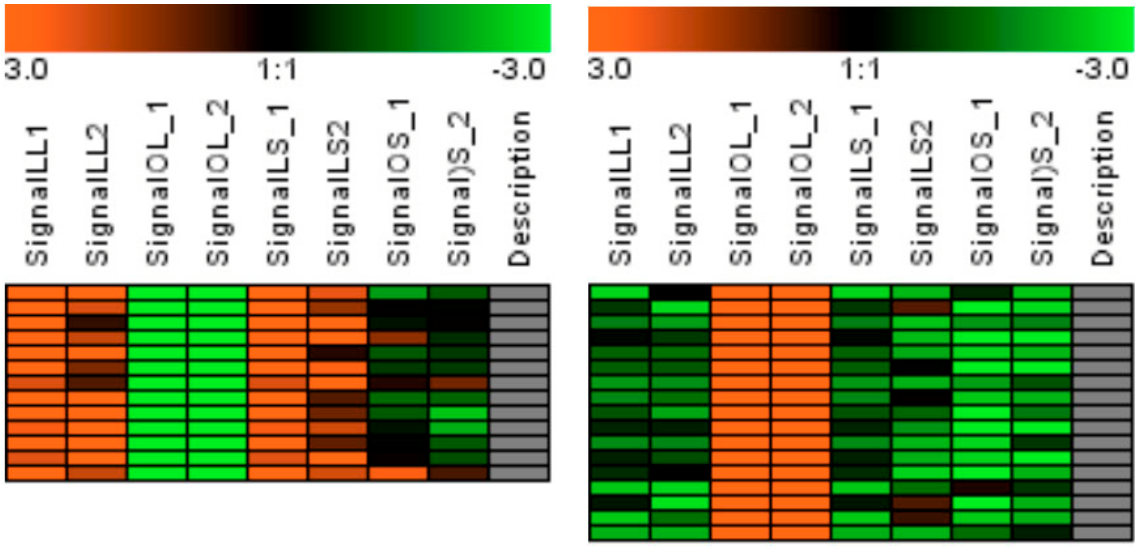

Figure 5. K-Means grouping demonstrating the genes that are increased and decreased expressed the obese LPS group. These genes match the SOM groups. The complete annotation are listed in Table 1 and 2.

Table 1. The list of genes that show unique increased expression in the obese-LPS group

AFFYID	LOCUSLINK	GENENAME	GENEONTOLOGY
95746_at	11964	ATPase, H+ transporting, V1 subunit A, isoform 1	ATP binding; ATP biosynthesis; ATP-binding and phosphorylation-dependent chloride channel activity; cytoplasm; energy coupled proton transport; hydrogen ion transporter activity; hydrogen-exporting ATPase activity, phosphorylative mechanism; hydrolase activity; proton transport.
161689_f_at	16178	interleukin 1 receptor, type II	ATP binding; ATP-binding cassette (ABC) transporter activity; cell surface receptor linked signal transduction; interleukin-1 binding; interleukin-1 receptor activity; interleukin-1, Type II, blocking receptor activity; membrane; receptor activity; transport; virus-host interaction
94758_s_at	12638	cystic fibrosis transmembrane conductance regulator homolog	ATP binding; ATP-binding cassette (ABC) transporter activity; chloride channel activity; integral to membrane; ion channel activity; ion transport; membrane; nucleotide binding; transport
94521_at	12581	cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	DNA binding; cell cycle; cyclin-dependent protein kinase inhibitor activity; kinase activity; negative regulation of cell cycle; perception of sound; regulation of transcription, DNA-dependent; transcription factor activity
98628_f_at	15251	hypoxia inducible factor 1, alpha subunit	DNA binding; flagellum (sensu Eukarya); nucleus; protein binding; regulation of transcription, DNA-dependent; response to hypoxia; signal transducer activity; signal transduction; transcription factor activity
162090_i_at	52231	DNA segment, Chr 1, ERATO Doi 161, expressed	DNA binding; regulation of transcription, DNA-dependent; transcription factor activity
104647_at	19225	prostaglandin-endoperoxide synthase 2	NOT nucleus; cytoplasm; extracellular space; membrane; oxidoreductase activity; acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen; peroxidase activity; prostaglandin biosynthesis;; regulation of blood pressure; response to oxidative stress
94357_at	20537	solute carrier family 5 (sodium/glucose cotransporter), member 1	Apical plasma membrane; glucose transport; glucose transporter activity; glucose:sodium symporter activity; integral to membrane; membrane; metanephros development; sodium ion transport; transporter activity
98474_r_at	21930	tumor necrosis factor alpha induced protein 6	Cell adhesion; extracellular space; hyaluronic acid binding
94752_s_at	20482	SKI-like	Cell differentiation; cell growth and/or maintenance; nucleus
161910_at	27398	mitochondrial ribosomal protein L2	Extracellular space; intracellular; mitochondrial large ribosomal subunit; protein biosynthesis; ribosome; structural constituent of ribosome
92449_at	14586	glial cell line derived neurotrophic factor family receptor alpha 2	Extracellular space; membrane; receptor activity
97681_f_at	14864	glutathione S-transferase, mu 3	Glutathione transferase activity; metabolism; transferase activity
94913_at	23881	RIKEN cDNA E430034L04 gene	Unknown function
92367_at	20460	Tal1 interrupting locus	Unknown function

Table 2. Gene list of the genes that demonstrate decreased expression in the obese-LPS group compared to the other groups

AFFYID	LOCUSLINK	GENENAME	GENEONTOLOGY
92190_at	22025	nuclear receptor subfamily 2, group C, member 1	DNA binding; ligand-dependent nuclear receptor activity; nucleus; proteolysis and peptidolysis; receptor activity; regulation of transcription, DNA-dependent; steroid hormone receptor activity; subtilase activity; transcription; transcription factor activity
92538_at	11556	adrenergic receptor, beta 3	G-protein coupled receptor activity; G-protein coupled receptor protein signaling pathway; adrenoceptor activity; beta-adrenergic receptor activity; integral to membrane; rhodopsin-like receptor activity
96549_at	11447	cholinergic receptor, nicotinic, delta polypeptide	Golgi apparatus; plasma membrane
97987_at	16005	insulin-like growth factor binding protein, acid labile subunit	Cell adhesion; extracellular space; insulin-like growth factor binding
100978_at	14300	FSHD region gene 1	Transport; transporter activity

adipocyte hypoxia and stimulation of HIF-1α may induce adipogenesis (17). How this relates to obesity and the biochemical milieu it produces, is unknown. More specifically, can this account for, at least in part, to the higher mortality levels seen in the obese that get sepsis or severe inflammation.

Prostaglandin-endoperoxide synthase 2, better known as cyclooxygenase-2 (COX-2), plays several key roles in inflammation through the metabolism of arachidonic acid into thromboxane and prostaglandins. COX-2 expression is primarily expressed in monocytes, macrophages, neutrophils, and endothelial cells after induction by a number of mediators including cytokines and endotoxin at sites of inflammation and/or injury (18). In the liver, COX-2 appears to up-regulate Kupffer cell IL-6 levels (19). Adiponectin, an adipocyte-derived hormone, can negatively influence lymphopoesis through the COX-2 pathway (20). How obesity affects these processes is again, largely unknown.

This study demonstrates the power of whole genome expression as a hypothesis generating tool. Although gene expression does not necessarily translate into protein function, it does provide a starting point. As complex as obesity and sepsis are independent of each other, combining the two raises the complexity to such a high level that traditional methods may not be appropriate. Developing a system knowledge of obesity and sepsis using large data sets, such as the ones presented here, may be the best approach to understand these two complex diseases. Obesity and sepsis occurring simultaneously seems well suited for a systems biology approach using genomic and proteomic information on a large scale to develop global theories to help direct more traditional methods of investigation.

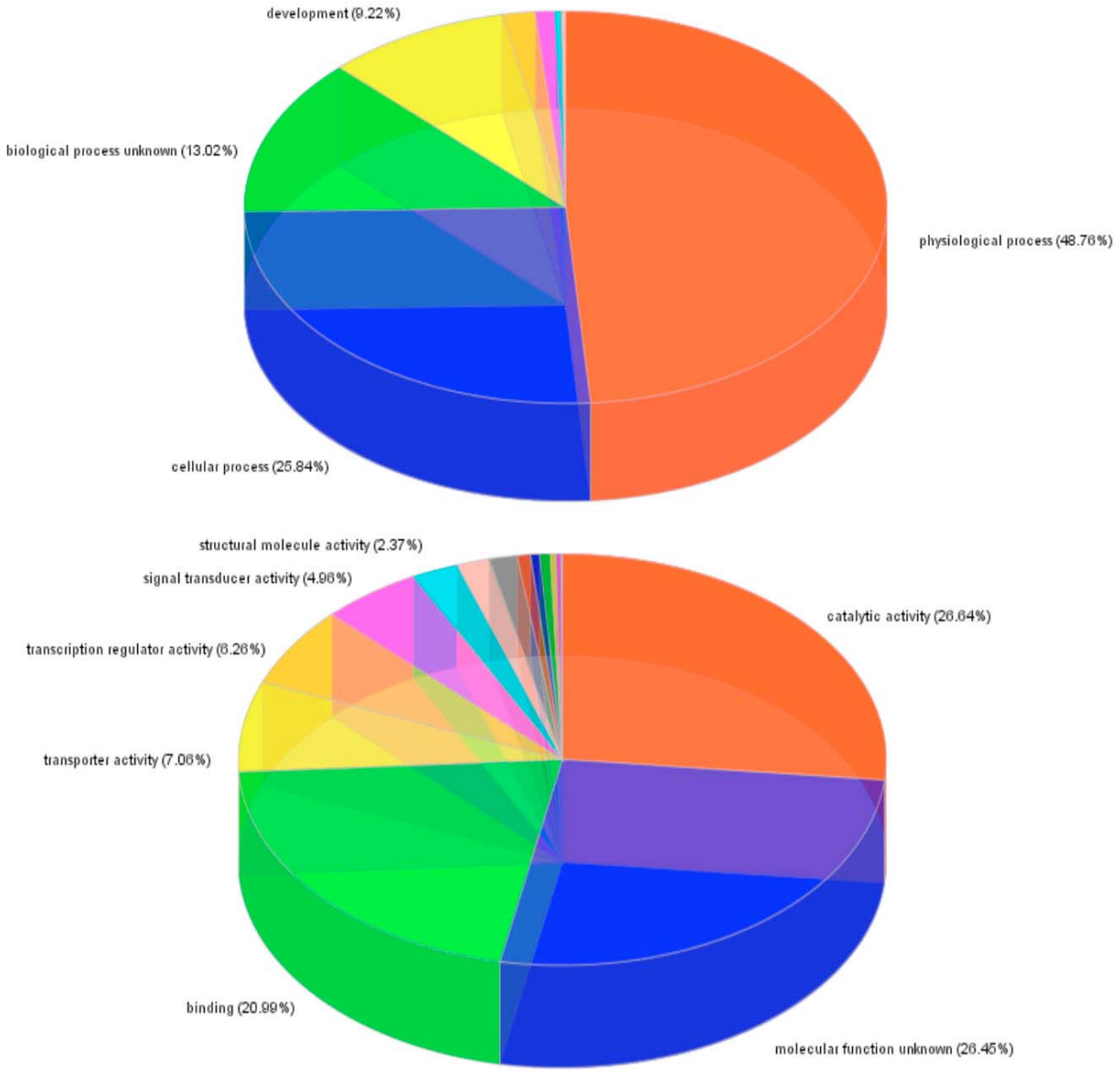

Figure 6. Chart representation of the annotation of the gene that shows increased expression in the obese LPS group. The top pie chart shows the basic biologic process while the bottom chart demonstrates the physiologic processes.

7. CONCLUSION

After LPS injection, hepatic tissue produces a unique genomic expression pattern in obese mice compared to lean mice. This study shows that microarray technology can demonstrate a unique genomic expression pattern in the obese after and inflammatory insult and may provide a useful tool for hypothesis generation that could help direct future investigations.

8. ACKNOWLEDGEMENT

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