Cytokine-induced Patterns of Gene Expression in Skeletal Muscle Tissue*

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Tumor necrosis factor α (TNF-α) and other cytokines induce a state of negative energy balance leading to the breakdown of skeletal muscle. Leptin, another member of the cytokine superfamily, also induces a state of negative energy balance but does not alter lean body mass. The transcription profile of skeletal muscle was compared in animals treated with TNF-α or leptin or in animals pair-fed over a 7-day time course using 11,000-gene microarrays (Affymetrix, Santa Clara, CA). Ten clusters of skeletal muscle genes were identified, each of which showed significantly different expression between TNF-α treatment and pair feeding. Studies comparing leptin treatment and pair feeding revealed that both activate nearly identical programs of gene expression in skeletal muscle. These data indicate that the effects of leptin on skeletal muscle are markedly different from those of TNF-α and that the effects of leptin on skeletal muscle can be largely ascribed to its anorectic effects. Subtle differences between leptin and pair feeding were evident only after 7 days of treatment. In general, pair feeding altered gene expression after the 7-day treatment, whereas leptin did not. The effects of TNF-α on skeletal muscle are distinct from those of pair feeding, a result consistent with its known catabolic effects on this tissue. Analyses of the data from food-restricted animals also identified a set of transcriptional changes associated with this state. Further studies of many newly identified leptin-, TNF-α-, and starvation-regulated genes and the apparent coordinate regulation of these clusters may reveal important insights into the different effects of cytokines on skeletal muscle.

Members of the cytokine family of hormones including tumor necrosis factor α (TNF-α)1 and leptin (an adipocyte-derived hormone) are known to have significant effects on energy metabolism. The level of expression of both TNF-α and leptin in adipose tissue is increased with obesity, and both molecules decrease food intake and body weight as well as regulating other aspects of metabolism (1–12). However, TNF-α and leptin differ in other respects. Most notably, TNF-α leads to the loss of muscle mass and cachexia, whereas chronic treatment with high doses of leptin spares lean body mass. TNF-α also reduces insulin sensitivity in skeletal muscle, whereas leptin enhances insulin action (3, 4, 8, 11, 13, 14). Both leptin and TNF-α have been shown to act directly and indirectly on skeletal muscle to modulate muscle physiology (15–24). TNF-α stimulates muscle catabolism leading to the loss of muscle function in human diseases that range from cancer to heart failure, from arthritis to AIDS (13, 14, 25–27). Leptin acts directly on skeletal muscle to increase fatty-acid oxidation via an 5'-AMP-activated protein kinase-dependent inhibition of acetyl coenzyme A carboxylase (28). Indirect effects of leptin on skeletal muscle via the brain have also been reported. The molecular correlates of the response of skeletal muscle to leptin and TNF-α are largely unknown.

In this study, we have utilized oligonucleotide microarrays (Affymetrix, Santa Clara, CA) to characterize the transcription response to both TNF-α and leptin in treated wild-type mice and in pair-fed controls. To group genes that show similar gene expression patterns, an analytical method using K-means clustering was previously developed by our group (29). Clustering analysis of the TNF-α-treated wild-type mice using this K-means program showed that TNF-α activates a program of gene expression in skeletal muscle tissue that is distinct from food restriction. The large number of TNF-α-specific effects on skeletal muscle emphasizes the dynamic response of skeletal muscle tissue to this cytokine. The molecular basis for the coordinate regulation of genes in the TNF-α-regulated clusters is yet unknown.

Whereas TNF-α activates distinct programs of gene expression in skeletal muscle tissue, leptin and food restriction produce similar, and in the majority of cases, nearly identical patterns of gene expression. Whereas the expression level of some genes was different between leptin and pair feeding after 7 days of treatment, the data indicate that in contrast to TNF-α, leptin has minimal effects on skeletal muscle beyond those associated with food restriction. Thus despite the fact that leptin has structural features characteristic of the cytokine family of ligands, it does not exert a typical cytokine-like effect on skeletal muscle.

MATERIALS AND METHODS

Animals—Female C57Bl/6J (18–20 g) purchased from Jackson Laboratories were used for all experiments. Alzet 2002 mini-osmotic pumps (Alza Corp., Palo Alto, CA) were filled with phosphate-buffered saline (PBS) (control and pair-fed groups) or 400 ng/g leptin (Agen, Thousand Oaks, CA) soaked in sterile 0.9% NaCl at 37 °C for overnight incubation. The osmotic pumps were implanted subcutaneously under anesthesia. The mice were maintained in individual cages. Food intake and body mass were measured daily at 8:00 a.m. For the TNF-α studies, wild-type mice were injected intraperitoneally twice daily at 8:00 a.m. and 6:30 p.m. with 0.5 ml of PBS (control and pair-fed groups) or TNF-α (15 μg/100 g of body weight/injection). Body mass and food intake were monitored daily at 8:00 a.m. For both TNF-α and leptin treatment experiments, pair-fed animal groups were staggered 1 day behind the other groups. The pair-fed animals were fed an amount of food that was equal to the average amount of food.
consumed by the leptin group or by the TNF-α group the previous day. Statistical analyses were performed using an unequal variance Student’s t test (30). Animals were sacrificed by cervical dislocation, and skeletal muscle tissue was excised. The skeletal muscle tissue was frozen at −80°C for further processing.

Northern Blot Analysis—Total RNA from skeletal muscle tissue was extracted with TRIZol (Invitrogen) according to the manufacturer’s instructions. RNA samples (between 10 and 20 μg) were fractionated by agarose-formaldehyde gel electrophoresis and then transferred to nylon membranes by capillary transfer as described (31). The filters were incubated with 1 megacount per min [32P]dCTP-labeled DNA probe amplified by PCR at 65°C with Ultra-hyb (Ambion, Inc., Austin, TX). The filters were washed to high stringency, and autoradiography was performed as described (32).

Oligonucleotide Microarray Analysis—Total RNA from skeletal muscle tissue was pooled from four animals for each experiment including leptin, TNF-α, and pair-fed groups. Twenty μg of total RNA cleaned with RNeasy columns (Qiagen Inc., Valencia, CA) were used for biotin-labeled cRNA synthesis. Affymetrix Genechip Mu11000 probe arrays were hybridized, washed, and stained in an Affymetrix hybridization oven and fluidics station according to the Affymetrix technical manual. One Mu11000 probe array was run per pool. The arrays were scanned using a Hewlett Packard confocal laser scanner. Affymetrix Genechip 3.1 software was used for visualization.

K-means Cluster Analysis—The microarray data were analyzed using a modification of the K-means clustering program that we developed (29). The abundance of each gene was scored as an average difference value by comparing the intensity of hybridization to 20 sets of perfect match 25-mer oligonucleotides relative to 20 sets of mismatched oligonucleotides using Affymetrix Microarray Analysis Suite (version 4.0) software. Each experiment was compared with wild-type untreated base line to evaluate fold change and average difference change values. The complete data sets were filtered to include only those genes that met the following criteria in at least two time points: the fold change (>3.0) and the average difference change was >500 in one of the two samples (for increasing fold changes the numerator needs to be >500, and for decreasing fold changes, the denominator had to be >500). In addition, genes were not included in the analysis unless given a score of 1 (increased) or D (decreased) by the Affymetrix Microarray Analysis Suite (version 4.0) software in these same time points. Because version 4.0 of the Affymetrix software used an unusual transformation to symmetrize the fold change values, here we used the more usual log transformation, i.e. the fold change values were transformed as follows: logFC = log(FC) if FC > 0 or logFC = log(−1/FC) if FC < 0. Note values between −1 and 1 do not occur in the Affymetrix-fold change scale.

These logFC values were then used to create a d-dimensional vector (d = number of experiments included in the clustering) for each of N genes included from the filtered data set. These N, d-dimensional vectors were normalized to the unit sphere and used as input into the clustering algorithm. The standard Euclidean metric was used to measure the distance between genes in this space, but because we normalized the vectors prior to computing the distance, the effective metric is like the dot-product or angle metric. The overall scale of the individual vectors is removed, and it is only the difference in direction that determines the distance. Clustering was completed using a variation (29) of the K-means clustering algorithm (33, 34). To prevent the algorithm from getting trapped in a local minimum, we used a recursive technique (35) to initialize the process. This works by successively solving the clustering problem starting with just 1 mean and building up to K, by splitting the mean from the previous iteration that had the largest variance.

To further reduce the problem of poor initial conditions, the complete algorithm was run for 1000 iterations. To determine the optimal number of clusters, we used the Davies-Bouldin index (36), which is a measure of cluster separation divided by cluster width. This is calculated for each value of K (cluster number), and we chose values that represent its local minimum. The mean expression pattern was calculated from the normalized gene vectors in a cluster. For several of the clusters in which there was a large difference between the TNF-α-treated animals and the pair-fed controls, statistical significance was determined using an unequal variance Student’s t test (30).

RESULTS
Tumor Necrosis Reduces Food Intake and Body Weight in Wild-type Mice—Wild-type animals were treated with 300 μg/kg/day of TNF-α, a dose that is known to reduce food intake and body weight in normal mice without leading to significant mortality. Treatment of wild-type mice with this dose of TNF-α temporarily reduced food intake and body mass (Fig. 1), with the treated mice losing 13.6% of their pretreatment weight after 3 days of treatment. However, after this initial period of hypophagia, the treated mice resumed their pretreatment food consumption and regained their body weight (Fig. 1). In these studies, a separate group of pair-fed mice was also analyzed to differentiate specific effects of TNF-α from those attributable to a decrease in food intake. Pair feeding of wild-type mice resulted in a similar pattern of weight loss compared with TNF-α treatment (Fig. 1).

K-mean Cluster Analysis Indicates That TNF-α Activates Distinct Programs of Gene Expression in Skeletal Muscle Tissue—The analysis of gene expression was performed using RNAs isolated from wild-type skeletal muscle tissue from animals receiving 1, 3, and 5 days of TNF-α treatment or pair feeding. Pair-fed mice received chronic infusions of vehicle without TNF-α. A group receiving 1 day of PBS treatment was also included as an additional control for the experimental manipulation. The RNA was biotin-labeled and hybridized to 11,000-gene oligonucleotide microarrays (Affymetrix). The level of expression of these genes at each time point was determined using published protocols and compared with that in wild-type skeletal muscle from untreated mice. Genes with a fold change >3.0 relative to untreated skeletal muscle at one or more time points and an average difference of >500 were analyzed further. Note, when applying the average difference filter, we only filtered the numerator if the gene was scored as increasing and the denominator if it was scored as decreasing. A total of 1037 genes satisfied these selection criteria.
that are induced by TNF-C26/H9251/H9252 genes that are repressed by TNF-C22, and C27 (in Fig. 2) were composed of genes that are repressed by TNF-α but not by pair feeding. Still other clusters included genes that were repressed by pair feeding and either induced by TNF-α (Fig. 2, clusters C15, C24, and C28) or unchanged by TNF-α (Fig. 2, cluster C19). The genes in the remaining clusters behaved similarly in response to TNF-α treatment and pair feeding. The complete list of the genes in each of these clusters is available via the Internet at hal.rockefeller.edu/arrays/obesity/skeletalMuscle.

In aggregate, the clustering analysis indicates that TNF-α activates a novel program of gene expression in skeletal muscle tissue that is distinct from food restriction. The data also illustrate the complexity of the response to this cytokine.

Some of the salient features of the 10 kinetically distinguishable clusters that distinguish TNF-α and pair feeding in skeletal muscle are reviewed in Fig. 3. The genes in cluster 15 are induced by TNF-α but not by pair feeding at early times. The genes in this cluster were only transiently induced by TNF-α on days 1 and 3 with levels returning to base line by day 5 of treatment. Pair feeding decreased the level of expression of these same genes on day 5. This cluster includes genes that lead to growth arrest as well as cell cycle regulatory proteins, stress-induced proteins, and several transcription factors.

Members of this cluster include GASS (growth arrest-specific protein), heat shock 27-kDa protein, the cytokine-inducible transcription factor LRG-21, and LKLF (a Krüppel-like transcription factor).

Two kinetically distinguishable clusters are comprised of genes that are repressed by TNF-α treatment but not by pair feeding (Fig. 3C, clusters 5 and 27). These genes are selectively repressed by TNF-α treatment on day 3 and are further repressed on day 5 (Fig. 3C, cluster 27). An analysis of the genes in cluster 27 (in Fig. 3) demonstrates that TNF-α selectively represses the expression of a group of structural genes in skeletal muscle. Genes in this cluster include β1-globin, α-globin and β-globin complex DNA for three early embryonic β-globin genes, y, b60, bh1, and two adult globin genes b1 and b2.

An analysis of the genes in the TNF-α-regulated clusters also revealed that TNF-α selectively induced the expression of several genes that play a role in regulating protein breakdown. In this case, however, the TNF-α-regulated genes were dispersed among a number of different clusters. The following components of the ubiquitin/proteasome pathway were specifically induced: proteasome regulatory subunit p31, a non-ATPase subunit of the 19 S complex, and a gene that is similar to proteasome component C9 (a subunit of the 20 S proteasome) (see Fig. 3A, clusters 9 and 26). TNF-α was also shown to repress the expression of a probable ubiquitin carboxyl-terminal hydrolase (see Fig. 2, cluster C22).

TNF-α also specifically modulated the RNA level of a number of transcription factors. In cluster C15 (Fig. 2), TNF-α selectively induced the expression of two transcription factors including the cytokine-inducible transcription factor LRG-21 and LKLF, a zinc-finger domain transcription factor. In other TNF-α-regulated clusters, additional TNF-α transcription factors including the DNA-binding protein NFI-B (Fig. 2, cluster C22) and a helix-loop-helix transcription factor (Hed) (Fig. 2, cluster C24) were regulated.

Leptin and Food Restriction Activates Similar Programs of Gene Expression in Skeletal Muscle Tissue—The effect of leptin treatment and pair feeding on the gene expression profile of skeletal muscle tissue was tested in wild-type mice. As has been documented previously, leptin treatment of wild-type mice reduced food intake and body weight only until the adipose tissue mass was depleted at which point food intake returned to base-line levels (Fig. 4) (3, 37). In these studies, the effects of leptin on food intake were qualitatively and quantitatively different from those of TNF-α necessitating the use of a different pair-fed control group. Gene expression data from wild-type skeletal muscle tissue isolated after 3, 5, and 7 days of leptin treatment (a similar time course of pair feeding) and 7 days of PBS treatment was referenced to data from an untreated wild-type sample to compile a list of fold change values. A total of 686 genes satisfied the aforementioned selection criteria, and these genes were analyzed by K-means clustering (see above and under “Materials and Methods”).

K-means cluster analysis generated 22 distinguishable clusters (Fig. 5). The K-means cluster analysis results revealed that leptin treatment and pair feeding elicited remarkably similar programs of gene expression in skeletal muscle. In general, at all times before 7 days, the direction and magnitude of the differences in gene expression in response to leptin was similar to pair feeding, and leptin-specific patterns of gene expression could not be found. However, in three clusters, the effects of leptin were different from those of food restriction but only after 7 days of treatment (Fig. 5, clusters G, P, and I). These putative leptin-regulated genes were further analyzed in a pair-wise comparison of 7 days of leptin versus pair feeding.

Genes Differentially Expressed between 7 Days of Leptin Treatment and Pair Feeding in Skeletal Muscle—A pair-wise comparison of the patterns of gene expression in skeletal muscle after 7 days of pair feeding and 7 days of leptin treatment was performed using data from two independent experiments as follows. First, the fold change for each gene was compiled for leptin or pair feeding and free-fed animals given PBS for 7 days relative to skeletal muscle RNA from wild-type animals. These fold change values were then compared between the leptin and pair-fed groups. Of the 11,000 genes and expressed sequence tags that were analyzed, a total of 67 genes were differentially expressed 3-fold or more relative to PBS control samples in the 7-day pair-fed sample (data not shown). Of these 67 genes, 13 genes were altered by 7 days of pair feeding but were unaffected by leptin. The remaining 54 (of 67) genes were similarly affected by pair feeding and leptin treatment.

Of 13 genes that were altered by 7 days of pair feeding (but not by leptin) nine were specifically induced by pair feeding including a mouse cDNA similar to a rat proteasome subunit RC6-I, a GC-binding protein, a putative RNA helicase and an RNA-dependent ATPase, Hn1 mRNA, a fragment for fatty-acid synthase, a 3T3-L1 lipid-binding protein, and a cDNA clone similar to an RNA-binding motif protein (RNP1), as well as two unknown cDNA clones (see Fig. 6A). Four genes were down-
regulated by pair feeding including the \( \alpha_1 \) type I collagen, major urinary protein I (MUP1), COL3A1 gene for collagen \( \alpha_1 \), and adult cardiac muscle \( \alpha \)-actin (see Fig. 6B). A total of 38 genes were differentially expressed 3-fold or more after 7 days of leptin treatment with an average difference change of \( >500 \) (data not shown). Of these, two genes were specifically induced after 7 days of leptin treatment compared with pair feeding including a cDNA clone similar to “protective protein” Mo54 and one unknown cDNA clone (see Fig. 6C). The remaining genes were expressed at similar levels in both groups.
The Effects of Caloric Restriction on the Transcription Profile of Skeletal Muscle—In these studies, owing to the different effects of leptin and TNF-α on food intake, two different food restriction protocols were used. This provided an opportunity to compare the effects of severe food restriction (in the case of the animals pair-fed to the TNF-α group) to a more modest degree of food restriction (in the case of the animals pair-fed to the leptin-treated group). To compare the differential effects of different degrees of caloric restriction, the expression data from animals pair-fed to either leptin- or TNF-α-treated mice were analyzed. A total of seven kinetically distinguishable clusters comprised of genes that are differentially expressed between TNF-α and pair feeding in skeletal muscle were identified. These clusters included genes that are specifically induced or repressed by TNF-α or pair feeding. Northern blots confirmed the expression pattern observed in the microarray data, further validating the findings. The 18S ribosomal gene was used as a control for equal loading. *p < 0.005; †p < 0.05.
### Patterns of Gene Expression in Skeletal Muscle Tissue

#### Cluster 15

| Dist From Mean (SD) | Gene Bank Accession | Description                                                                 |
|---------------------|---------------------|-----------------------------------------------------------------------------|
| 0.983               | X61450              | clone E161                                                                  |
| 0.490               | AA039627            | Homologous to Heat Shock 27 KD Protein                                      |
| 0.518               | AA023309            | Homologous to Heat Shock 27 KD Protein                                      |
| 0.662               | X692728             | GASP Growth Arrest Spastic Protein                                          |
| 0.641               | M13706              | Fattin Large Subunit                                                        |
| 0.715               | W13156              | SV-40 Induced 2493 mRNA                                                     |
| 0.742               | AA096848            | Homologous to 78 KD Glucose Regulated Protein                              |
| 0.818               | U19121              | Transcription Factor 100-21                                                 |
| 0.856               | AA422566            | cDNA clone 846748 s, mRNA                                                   |
| 0.856               | W19431              | cDNA clone 385532 s                                                        |
| 0.856               | AA015426            | Homologous Heat Shock 27 KD Protein                                         |
| 0.923               | U85020              | Pax6                                                                         |
| 1.442               | JQ94179             | Chromatin Nonsense High Mobility Group Protein                             |
| 1.524               | U055986             | Knolal-Related Factor KLF mRNA, complete cds                              |
| 1.594               | W58767              | cDNA clone 387215 s                                                        |
| 1.713               | U29706              | 4F2 Antigen Heavy Chain                                                     |

#### Cluster 28

| Dist From Mean (SD) | Gene Bank Accession | Description                                                                 |
|---------------------|---------------------|-----------------------------------------------------------------------------|
| 0.5872              | M21590              | Lysozyme M gene, exons 4                                                   |
| 1.0138              | X58061              | Complement Component Subcomponent C1q Alpha-Chain                           |
| 1.0718              | X56295              | mRNA for C1q C- Chain                                                      |
| 1.2187              | W17141              | Homologous to Interleukin Factor 1A                                         |

#### Cluster 5

| Dist From Mean (SD) | Gene Bank Accession | Description                                                                 |
|---------------------|---------------------|-----------------------------------------------------------------------------|
| 0.4963              | AA032557            | cDNA clone 465199 s                                                        |
| 0.5317              | MUB1AT              | Marine b1                                                                  |
| 0.5848              | W37370              | Homologous to Signal Recognition Particle R                                |
| 0.6538              | AA203939            | cDNA clone 643893 s                                                        |
| 0.6920              | AA73018             | cDNA clone 1053993 s                                                       |
| 0.7967              | AA250419            | cDNA clone 636865 s                                                        |
| 0.8130              | AA160861            | Homologous to Adenylylkinase Isoenzyme 1                                   |
| 0.8614              | AA106126            | Homologous to Myosin Heavy Chain                                           |
| 0.9801              | R74626              | MDR0787: Stratifactor Mus Musculus cDNA                                     |
| 0.9999              | AA165986            | Homologous Pyruvate Kinase,M2 Isozyme                                       |
| 1.2314              | U81317              | Myosin-Associated/Oligodendrocyte Basic Protein                            |
| 1.2387              | P19527              | PEB RNA Helicase                                                           |
| 1.3349              | AA113936            | cDNA clone 574555 s                                                        |
| 1.3448              | AA221481            | Similar to DNA-Directed RNA Polymerase II                                  |
| 1.3613              | W08585              | Homologous to Extremain Precursor (cell wall hyd)                          |
| 1.4427              | AA165802            | Homologous to Tropomyosin Alpha Chain                                       |

#### Cluster 27

| Dist From Mean (SD) | Gene Bank Accession | Description                                                                 |
|---------------------|---------------------|-----------------------------------------------------------------------------|
| 0.5035              | V00772              | Beta-1-Globin                                                              |
| 0.6060              | V00774              | Alpha-Globin                                                               |
| 0.6823              | X14061              | Beta-Globin Complex DNA y, b(0), b(1), b(2) and b(2)                        |
| 0.8516              | C77469              | Similar to Mouse Alpha-1-Globin mRNA                                        |
| 1.0168              | C77755              | Similar to Mouse Musculus DNA for Alpha Globin                             |
| 1.1484              | AA1547486           | cDNA clone 634913 s                                                        |
| 1.6894              | U02239              | Fibroin                                                                    |

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**Fig. 3—continued**
Leptin and TNF-α are members of the cytokine superfamily of ligands, and both induce a state of negative energy balance. However, whereas TNF-α treatment is known to reduce lean body mass, leptin treatment does not. This suggests that leptin does not exert the same cellular effects on skeletal muscle that are characteristic of the typical cytokine response. To confirm this possibility and establish the molecular features of the skeletal muscle response to TNF-α and leptin, oligonucleotide microarrays were used to compare the transcription profiles of TNF-α and leptin. The data presented here provide the first global assessment of the in vivo response of skeletal muscle to cytokines and underscore the utility of large scale parallel gene expression analysis to study complex biological processes in higher organisms. The data further confirm that TNF-α activates specific programs of gene expression in skeletal muscle that are distinct from its known effects on food intake. This is in contrast to leptin, which with only two exceptions does not alter the transcriptional profile of muscle other than that attributable to its anorexigenic actions.

Analysis of the expression data further revealed some of the features of the biological response to cytokines and food deprivation. Comprehensive expression monitoring in TNF-α-treated and pair-fed wild-type skeletal muscle tissue identified a large number of genes in which expression was distinguished differently in TNF-α treatment than in that from food restriction (see Fig. 2, clusters C5, C9, C14, C15, C19, C22, C24, and C26–C28). This high level analysis of the transcription changes induced by TNF-α revealed that genes from many functional categories are differentially expressed in skeletal muscle after TNF-α treatment. These categories include genes that play a role in protein catabolism, the cellular stress response including acute phase proteins and inflammatory markers, structural genes, and genes regulating energy metabolism and transcription (see Fig. 3).

The identity of some of the genes that are specifically regulated by TNF-α also revealed unappreciated aspects of the response of skeletal muscle tissue to this molecule. Previous data have indicated that TNF-α stimulates catabolism by activating the ubiquitin/proteasome pathway (39, 40). Thus it has been shown previously that TNF-α can directly induce the expression of a ubiquitin-dependent proteolytic system in rat soleus muscle (40). Indirect evidence that TNF-α may activate the proteasome system is also provided by studies of tumor-bearing rats. Expression of MSSI (an ATPase subunit of the 19 S proteasome complex) was previously found to be increased in muscle from tumor-bearing rats, and this response was normalized when the animals were given pentoxifylline, which reduces the expression of TNF-α mRNA (41). In these studies, the animals were found to selectively up-regulate the expression of two genes in the ubiquitin/proteasome pathway, includ-
ing proteasome regulatory subunit P31 (a non-ATPase subunit of the 19 S complex) and a gene that is similar to proteasome component C9 (a subunit of the 20 S proteasome) (see Fig. 3A, clusters 9 and 26). The confirmation here that the expression of P31 and C9 subunits are induced by TNF-α, and not by some other aspect of the tumor-bearing state, provides a new avenue for studying the mechanism of TNF-α-induced muscle wasting. Further experiments can be used for a more in-depth understanding of the specific role of the individual gene products in regulation of protein catabolism.
FIG. 6. Genes differentially expressed between seven days of leptin treatment and pair feeding in wild-type skeletal muscle tissue. Each experimental group (PBS, leptin, and pair feeding) was compared with wild-type untreated baseline to evaluate fold change and average difference change values. Only genes whose fold change is >3 and average difference change is >500 are shown. Genes >3-fold different in abundance between 7 days of leptin treatment and pair feeding are shown. The expression of many of these genes was verified independently by Northern blotting as shown. A, 7-day pair-fed specific genes up-regulated 3-fold or more in wild type skeletal muscle. B, genes specifically repressed by 7 days of pair feeding in skeletal muscle. C, 7-day leptin specifically induced expression of genes in skeletal muscle. D, 20 μg of total RNA of wild-type day 7 of leptin, pair feeding, and PBS-treated skeletal muscle was analyzed by Northern blotting as described (see “Materials and Methods”). DNA fragments from several differentially expressed genes were used as probes.
Although we surmise that the genes in each cluster are coordinately regulated, the molecular mechanisms by which TNF-α regulates gene expression in the clusters is as yet unclear. In three such clusters (Fig. 2, C15, C22, and C24), TNF-α specifically modulated the RNA level of known transcription factors. In cluster C15 (Fig. 2), TNF-α selectively induces the expression of two transcription factors including cytokine-inducible transcription factors LRG-21 and LKLF (a zinc finger-containing transcription factor). In other TNF-α-regulated clusters, additional transcription factors were coordinately regulated such as DNA-binding protein NFI-B (Fig. 2, cluster C22) and helix-loop-helix transcription factor (Hed) (Fig. 2, cluster C24).

It is unclear why in some cases, such as in clusters 9, 22, and 26 (Fig. 2), genes known to play a role in protein breakdown are expressed in different clusters. It may be that such genes are in fact coordinately regulated by TNF-α but are placed in different clusters because they are differentially expressed under other conditions (such as the response to pair feeding or PBS). It is also possible that TNF-α activates distinct groups of functionally related genes by different transcriptional or post-transcriptional mechanisms. Further exploration of the molecular mechanisms underlying the regulation of the clustered genes should distinguish between these possibilities.

Cluster analysis of the genes that are regulated by leptin and food restriction indicates that leptin treatment and pair feeding activate nearly identical programs of gene expression in skeletal muscle (see Fig. 5). Both the pattern and the magnitude of differences in the response of wild-type mice to a change in plasma leptin concentration were remarkably similar to those of pair feeding, and leptin-specific patterns of gene expression could not be found. These data confirm that the effects of leptin have on skeletal muscle are largely secondary to its known anorectic actions.

In three clusters, however, gene expression after leptin treatment differed from food restriction after 7 days (see Fig. 5, clusters G, P, and I). Pair-wise comparison of the patterns of gene expression in skeletal muscle after 7 days identified 13 genes that were differentially expressed after 7 days of pair feeding versus leptin-treated genes of a total of 11,000 genes that were followed. The expression of nine mRNAs was increased after 7 days of pair feeding including a cDNA clone similar to rat proteasome subunit RC6-I, GC-binding protein, putative RNA helicase and RNA-dependent ATPase, Hn1 (a dimethyl nitrogen half-mustard mRNA), a fragment for fatty-acid synthase, 3T3-L1 lipid-binding protein, a cDNA clone similar to an RNA-binding motif protein (RNLP), and two unknown cDNA clones. The products of these nine genes belong to different functional protein types. Analysis of the up-regulated genes revealed that pair feeding selectively induced the expression of several genes that play a role in regulation of RNA metabolism including GC-binding protein (an “Sp1-like” sequence-specific DNA-binding protein that activates RNA polymerase II transcription from promoters that contain GC boxes) putative RNA helicase, RNA-dependent ATPase (a splicing factor that functions late in the pre-mRNA splicing pathway to facilitate spliceosome disassembly), and RNA-binding protein 3 (RNPL, a translation regulator). Pair feeding also specifically up-regulates the expression of lipid metabolism enzymes that are known to be responsive to nutritional regulation including 3T3-L1 lipid-binding protein, a facilitator of fatty-acid transport, and a fragment for fatty-acid synthase, which is an enzyme in the lipogenic pathway. In addition, the pair-fed animals were found to selectively up-regulate the expression of a cDNA clone similar to RC6-I, a subunit of the 20 S proteasome in the ubiquitin/proteasome proteolytic pathway. Previous studies have established the central role of the ubiquitin/proteasome system in the loss of skeletal muscle protein in many wasting conditions (14). Further studies can be used for the elucidation of the specific role of RC6-I in the ubiquitin/proteasome-dependent muscle proteolysis response. An analysis of the 7 days of pair feeding in down-regulated genes demonstrates that pair feeding selectively represses the expression of a group of structural genes in skeletal muscle including α1 type I collagen, COL5A1 gene for collagen α1, and adult cardiac muscle α-actin. Pair feeding also represses the expression of major urinary protein I (MUP1), which has a multiple role in the communication of information in urine-derived scent marks.

The expression of these genes was not altered by leptin treatment suggesting that, if anything, leptin has an even less dramatic effect on skeletal muscle than food restriction after an extended period of treatment. In addition, two genes were specifically up-regulated after 7 days of leptin treatment including cDNA clones, one of which is similar to the Mo54 “protective protein” and the other of which is unknown. The Mo54 “protective protein” is a glycoprotein that forms a complex with the β-galactosidase and neuraminidase of the lysosomal enzymes and inhibits them. This complex protects both β-galactosidase and neuraminidase from intralysosomal proteolysis. The potential role of Mo54 and this other leptin-regulated gene in protecting skeletal muscle can now be investigated. In aggregate, these results correlate well with the fact that caloric deprivation reduces skeletal muscle mass, whereas leptin treatment selectively reduces fat mass (3, 7). Recent data have indicated that leptin acts directly on skeletal muscle and indirectly via effects on the brain to modulate muscle physiology and metabolism (28). Whatever acute effects leptin has on skeletal muscle metabolism, these data show that chronic leptin treatment has minimal effects on skeletal muscle other than that attributable to its anorexigenic actions.

These studies clearly distinguish the effects of TNF-α treatment from leptin at the molecular level and indicate that TNF-α elicits a unique biological response that is independent of its effects on food intake. The larger number of TNF-α-specific effects on skeletal muscle compared with leptin is consistent with the fact that TNF-α is inextricably linked to muscle pathophysiology and is an important mediator of skeletal muscle degeneration associated with cachexia, a debilitating syndrome characterized by extreme weight loss and whole body wasting.

The mechanism by which TNF-α elicits its catabolic effects in vivo remains largely unknown and, as mentioned, may be advanced by these studies. It is also unclear to what extent the actions of TNF-α described here are direct or indirect. The available published evidence shows that TNF-α acts via both direct and indirect mechanisms (38, 42). These two possibilities can be resolved by studying the transcription profiles of skeletal muscle in tissue-specific knockouts of the TNF-α receptor. The dissection of the transcriptional mechanisms that control the synthesis of RNAs regulated by TNF-α as well as leptin and pair feeding may lead to the elucidation of the underlying signaling transduction pathways.

Conventional methods can be used for further understanding of the functional importance of the genes in each cluster. It is also likely that a number of the genes found in the clusters are regulated coordinately. Further studies of the apparent coordinate regulation of the genes in each cluster may lead to elucidation of the mechanisms by which cytokines act and the functions by which skeletal muscle tissue and energy metabolism are regulated.
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