Temporal profiling of the transcriptional basis for the development of corticosteroid-induced insulin resistance in rat muscle

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Abstract

Elevated systemic levels of glucocorticoids are causally related to peripheral insulin resistance. The pharmacological use of synthetic glucocorticoids (corticosteroids) often results in insulin resistance/type II diabetes. Skeletal muscle is responsible for close to 80% of the insulin-induced systemic disposal of glucose and is a major target for glucocorticoid-induced insulin resistance. We used Affymetrix gene chips to profile the dynamic changes in mRNA expression in rat skeletal muscle in response to a single bolus dose of the synthetic glucocorticoid methylprednisolone. Temporal expression profiles (analyzed on individual chips) were obtained from tissues of 48 drug-treated animals encompassing 16 time points over 72 h following drug administration along with four vehicle-treated controls. Data mining identified 653 regulated probe sets out of 8799 present on the chip. Of these 653 probe sets we identified 29, which represented 22 gene transcripts, that were associated with the development of insulin resistance. These 29 probe sets were regulated in three fundamental temporal patterns. 16 probe sets coding for 12 different genes had a profile of enhanced expression. 10 probe sets coding for eight different genes showed decreased expression and three probe sets coding for two genes showed biphasic temporal signatures. These transcripts were grouped into four general functional categories: signal transduction, transcription regulation, carbohydrate/fat metabolism, and regulation of blood flow to the muscle. The results demonstrate the polygenic nature of transcriptional changes associated with insulin resistance that can provide a temporal scaffolding for translational and post-translational data as they become available.

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Introduction

A major function of the hypothalmic/pituitary/adrenal axis is control of blood glucose levels through the regulation of systemic gluconeogenesis. Aspects of this broad systemic function of glucocorticoids include increased gluconeogenesis in the liver and kidney, net muscle-protein breakdown to provide gluconeogenic substrates, insulin resistance of peripheral tissues including skeletal muscle, and lipid mobilization.

Glucocorticoids also suppress inflammatory/immune responses, and synthetic glucocorticoids (corticosteroids) are widely used therapeutically for this purpose (Schimmer & Parker 1996, Baxter 2000). The pathologies for which anti-inflammatory or immune-suppressing interventions by corticosteroids are used are numerous and include diverse types of organ transplantation. When corticosteroids are used pharmacologically aspects relating to their systemic glucose regulation are also amplified, resulting in numerous adverse effects (Frauman 1996, Bialis & Routledge 1998, Reynolds & Walker 2003, Tomono *et al.* 2002).

Corticosteroids cause insulin resistance, which occurs in conjunction with increased gluconeogenesis by the liver and kidney, and results in chronic hyperglycemia (Beck-Nielsen *et al.* 2002, Tomono *et al.* 2002, Reynolds & Walker 2003). Skeletal muscle is responsible for close to 80% of the insulin-directed glucose disposal by peripheral tissues. Therefore, muscle to a great extent is responsible for reduced glucose disposal and consequent hyperglycemia caused by peripheral insulin resistance (Koistinen & Zierath 2002). In skeletal muscle insulin directs glucose uptake through the GLUT4 transporter, and facilitates glucose disposal by both glycogen synthesis and glycolysis. Insulin also reduces fat utilization through β-oxidation in muscle. In addition, by way of its combined effects on both muscle and muscle vasculature, it increases blood flow through the musculature, facilitating glucose uptake (Rakugi *et al.* 2002). These effects of insulin in facilitating glucose disposal involve actions at the transcriptional,

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translational, and post-translational levels (Sugden & Holness 2002, Zierath & Wallberg-Henriksson 2002).

Unlike insulin, which has many of its effects through post-translational kinase cascades, glucocorticoids have most of their effects by altering transcription of specific genes. These transcriptional effects take two fundamental forms. Many regulated genes contain glucocorticoid-responsive elements in their regulatory sequences and their transcription is influenced directly. However, there are a large number of genes whose transcription is altered indirectly by glucocorticoids. In these cases glucocorticoids alter the expression or function of other transcription factors, which in turn alter the transcription of other genes (Sun *et al.* 1998*a*, 1998*b*, 1999, Almon *et al.* 2002, Rosmond 2003).

In the present study we used the rat model for corticosteroid-induced insulin resistance and employed Affymetrix gene arrays to profile the time course of changes in gene expression in skeletal muscle of adrenalectomized (ADX) male rats in response to a single bolus dose of the synthetic corticosteroid, methylprednisolone (MPL). ADX animals were used to eliminate circadian oscillation in corticosteroid-responsive genes, which facilitated data mining. The objective was to identify those changes in transcription induced by corticosteroids, which, if perpetuated by repeated dosing, would manifest as chronic insulin resistance. Using a filtering approach to eliminate probe sets that were not regulated we identified 653 probe sets out of 8799 with a high probability of being regulated (Almon *et al.* 2004). Of the 653 probe sets, 29 (representing 22 gene transcripts) directly related to insulin resistance. These probe sets can be divided into four functional categories. The first is signal transduction. The second is transcription regulation. The third is related to carbohydrate and fat metabolism. The fourth group is expressed either by the muscle, by the muscle vasculature, or both and influences the relationship between the muscle and blood flow. Together these results provide a broad temporal tableau of the polygenic nature of insulin resistance caused by corticosteroids.

Materials and Methods

Experimental design

Muscle samples (gastrocnemius) were obtained from a previously performed animal study in our laboratory (Sun *et al.* 1998a, 1999). All procedures involving experimental animals adhered to the *Principles of Laboratory Animal Care* (National Institutes of Health, 1985) and were reviewed by our institution's animal care and use committee. Male ADX Wistar rats (*Rattus rattus*) weighing 225–250 g were obtained from Harlan Sprague–Dawley (Indianapolis, IN). Animals were allowed free access to rat chow (RMH 1000; Agway, Harlan Teklad, Madison, WI, USA) and 0·9% NaCl drinking water. They were housed in a room with a 12 h light/12 h dark cycle and a constant temperature of 22 °C, and were allowed to acclimatize to this environment for at least 1 week. One day prior to the study, all rats were subjected to right external jugular-vein cannulation under light ether anesthesia. Cannula patency was maintained with sterile 0·9% NaCl solution. Four animals were designated as controls (i.e. 0 time samples) and were cannulated but received vehicle only. All remaining animals received a single 50 mg/kg dose of methylprednisolone sodium succinate (Pharmacia-Upjohn Company, Kalamazoo, MI, USA) via the cannula over 30 s. Rats (three per time point) were killed by exsanguination under anesthesia at 0·25, 0·5, 0·75, 1, 2, 4, 5, 5·5, 6, 7, 8, 12, 18, 30, 48, and 72 h after dosing. The sampling time points were selected based on previous studies describing glucocorticoid receptor (GR) dynamics and enzyme induction in skeletal muscle. In addition, four cannulated vehicle-treated rats were killed as controls (data represented as time 0). Gastrocnemius muscles were rapidly excised, flash-frozen in liquid nitrogen, and stored at −80 °C. Frozen muscle tissues were ground into powder using liquid-nitrogen-chilled mortars and pestles. Great care was taken to maintain tissues at temperatures of −80 °C or below at all times prior to RNA processing in order to prevent RNA degradation. In a companion study of six animals, blood samples were taken serially from the cannula into a heparinized syringe at 0·5, 1, 2, 4, 5, 6, 8, 10, 12, 24, 36, 48, and 72 h.

Assays

Plasma MPL concentrations were measured by a normal-phase high-performance liquid chromatography method with a quantitation limit of 10 ng/ml as described previously (Sun *et al.* 1998a, 1999). A modified enzymatic assay kit (Sigma Diagnostics, St. Louis, MO, USA) was used to determine plasma glucose according to the manufacturer's instructions. Plasma insulin concentrations were measured by a rat-specific enzyme-linked immunosorbent assay kit (1–2–3 Rat Insulin ELISA; ALPCO Diagnostics, Windham, NH, USA). The analysis was performed in duplicate according to the manufacturer's instructions and controlled by Mammalian/Rat Insulin Two-Level Control (ALPCO Diagnostics). The limit of quantitation for this assay was 0·07 ng/ml.

Microarrays

Muscle powder (100 mg) from each individual animal was added to 1 ml of pre-chilled Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA extractions were carried out according to the manufacturer's directions. Extracted RNAs were further purified by passage through RNeasy mini-columns (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols for RNA clean-up. Final RNA preparations were resuspended in nuclease-free water and stored at −80 °C. RNAs were quantified

spectrophotometrically and purity and integrity assessed by agarose gel electrophoresis. All RNA samples exhibited 260/280 ratios between 1·8 and 2·0, and all exhibited discrete ribosomal bands on agarose gels, indicating a lack of substantial sample degradation. We have found that tissues maintained carefully at ultra-low temperatures produce high-quality RNA, after even 10 years of storage.

Isolated RNA from each individual muscle was used to prepare target according to the manufacturer's protocols. The biotinylated cRNAs were hybridized to 51 individual Affymetrix GeneChips® Rat Genome U34A (Affymetrix, Santa Clara, CA, USA), which contained 8799 probe sets. Unlike the cDNA arrays used in a previous study (Almon *et al.* 2002), the high reproducibility of *in situ* synthesis of oligonucleotide chips allows accurate comparison of signals generated by samples hybridized to separate arrays. This entire data-set has been submitted to the NCBI Gene Expression Omnibus database (GSE490) and is also available online at http://pepr.cnmcresearch.org (Almon *et al.* 2004).

Array analysis

The approach to data mining was developed based on our use of gene arrays as a technique for high-throughput data collection within the context of a rigidly controlled time-series paradigm. The Affymetrix oligonucleotide microarrays use sequence information and photolithograpy-directed combinatorial chemical synthesis to develop probe sets for the genes of interest. Each probe set consists of a series of short oligonucleotide sequences and an identical partner sequence, except for a single base mismatch in the center. The mismatch sequence provides a unique background for each sequence in the series. The Affymetrix arrays used have the advantage of having multiple measurements per gene (11 probe pairs or more per transcript). However, this same redundancy leads to many different interpretations of probe sets to determine a signal for each gene (probe set algorithms), and controversy regarding the sensitivity and specificity of resulting signals exists, as do appropriate normalization methods (Tumor Analysis Best Practices Working Group 2004). We have used the Affymetrix MAS5·0 algorithm, and this places a relatively high penalty for mismatch hybridization, favoring specificity over sensitivity (Seo *et al.* 2004). In this first step, a 'call' of Present (P), Absent (A), or Marginal (M) was determined for each probe set on each chip based on the comparison of the matched and mismatched pairs for the gene sequence. The results were normalized for each chip using a distribution of all genes around the 50th percentile. The results from the first step were entered into the program GeneSpring 6·2 (Silicon Genetics, Redwood City, CA, USA). This robust software provides a number of tools for visualization and analysis of time-series data. One such tool for both hierarchical clustering and visualization is the gene-tree approach described by Eisen *et al.* (1998), as modified by the GeneSpring software. This algorithm can be used to construct a dendrogram of genes with similar patterns.

A negative aspect of this tool, and most time-series data-mining tools, is the assumption that the points in the time series are equally spaced. However, to design our 72-h time series in this manner would have ignored the richness of biological information during the early period following dosing of the drug. Notwithstanding this drawback, gene trees provide an excellent method of visualizing the data-set. In order for this and other tools to be used it was also necessary to transform the data so that the values for all probe sets were within the same range. To accomplish this, values for each individual probe set on each chip were expressed as a ratio of the mean of the four control values for that gene, which we refer to as normalized intensity. Thus the average of each probe set has a value of 1 at 0 time and either increases, decreases, or remains unchanged relative to controls over the time series. A series of filters was developed and applied to this data-set, which included filtering for expression of the probe set in the tissue, filtering for regulation of the probe set by the drug, and quality-control filtering. Genes selected as potentially regulated by drug using these filtering techniques were then subjected to a one-way ANOVA with a *post-hoc* Tukey's test ($P<0·05$). A detailed discussion of this approach to mining this data-set with lists of the identified probe sets and statistical analysis has been published (Almon *et al.* 2004). Extensive literature searches of the 653 probe sets identified by this mining method yielded 29 probe sets whose regulation is clearly related to insulin resistance. These 29 identified probe sets were then clustered using gene-tree analysis in GeneSpring 6·2.

Results

Figure 1A shows the plasma MPL concentrations in ADX rats as a function of time after the single iv bolus dose of 50 mg/kg. These data demonstrate that the drug is entirely eliminated by 7 h following dosing. Figure 1B shows a transient increase in the plasma glucose concentration that begins about 2 h following dosing and lasts for 9 h. Figure 1C demonstrates an increase in plasma insulin that begins between 2 and 3 h following dosing and does not return to baseline until between 30 and 48 h.

All glucocorticoid-regulated probe sets were identified using their accession numbers and an extensive literature search (Medline) was carried out on each one. In the course of this literature search 29 probe sets coding for 22 gene transcripts were found that relate to insulin resistance. Eight of these gene transcripts were down-regulated (Table 1A), 12 were up-regulated (Table 1B), and two showed biphasic regulation (Table 1C).

Figure 2 shows the gene tree derived from the GeneSpring program for these 29 probe sets (29 probe sets

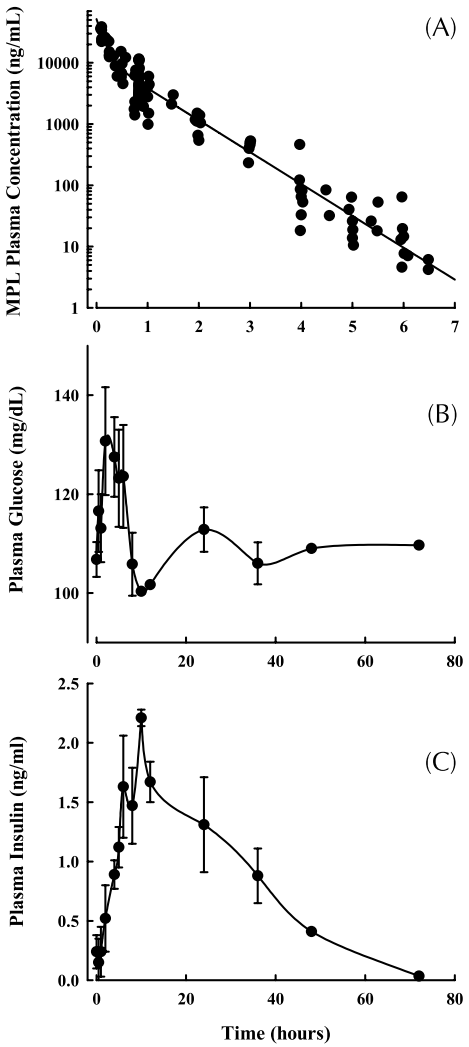

Figure 1 Plasma concentrations of (A) MPL, (B) glucose, and (C) insulin versus time after a 50 mg/kg i.v. bolus dose of MPL in rats.

at 17 time points). This tree was constructed using a Pearson correlation. The *x*-axis presents the 17 time points studied in rank order from left to right. Vehicle controls are nominally referred to as time 0. As pointed out above, each time point is equally spaced and therefore does not represent the true temporal relationship between points. The *y*-axis presents the mean of the normalized value at each time point for each of the individual probe sets, represented by color and clustered by similarity. In this view, the color yellow represents a value of 1, progression towards red represents values that exceed 1, and progression towards blue represents values that decline towards 0. The intensity of the color reflects the intensity of the original signal. To the immediate left of the gene tree is a schematic tree of the relationship of all probes to each other based on expression-pattern similarity (represented in green). Gene trees provide an excellent global view of all of the probe sets and their relationship to each other. At the bottom there is a group of 10 probe sets with sustained sequences of blue, which indicates down-regulation. An enlargement of this section of the tree (Fig. 3, left) shows that two probe sets for mitochondrial glycerol-3-phosphate dehydrogenase 2 (mGDPH) sit side by side and two probe sets for extracellular signal-related kinase 3 (ERK 3) also sit side by side. Above these 10 probe sets in Fig. 2 are 19 probe sets that in general appear to show enhanced regulation. However, three of these probe sets also show apparent down-regulation at the 30-, 48-, and 72-h points and thus appear to have a biphasic signature, a phenomenon we have described previously (Jin *et al.* 2003). An enlargement of the section of the tree containing these three probe sets (Fig. 3, middle) shows that two of the probe sets are for uncoupling protein 3 (UCP3) and one is for pyruvate dehydrogenase kinase isoenzyme 4 (PDK4). Figure 3 (right) shows an enlargement of the remainder of the tree containing the probe sets with enhanced regulation. This group contains two probe sets each for type-1A angiotensin II receptor (AT1A), p38 mitogen-activated protein kinase (p38 MAPK), peroxisome proliferator-activated receptor δ (PPARδ), and protein tyrosine phosphatase type 1B (PTP1B). In all cases except for p38 MAPK, the two probe sets for the same gene sit side by side, indicating an extremely high similarity of pattern. Interestingly, probe sets for CCAAT/enhancer-binding proteins (C/EBPs) β and δ also sit next to each other on the tree. Table 1 shows the names and probe-set identifiers for the down-regulated group, the up-regulated group, and the biphasic group.

The 29 probe sets presented in Figs 2 and 3 were grouped into four categories based on their function: those involved in signal transduction mechanisms, those that act as transcription factors, those that are related to carbohydrate or lipid metabolism, and those which influence blood flow. Figs 4–7 present mean normalized intensities (three animals per time point) for each gene at the 16 time points examined. Error bars represent standard deviations of those means. Figure 4 shows the temporal signatures of the six gene transcripts related to signal transduction. Two of these, insulin receptor substrate-1 (IRS-1) and ERK3, were down-regulated following MPL treatment. The remaining four transcripts in this group showed enhanced expression following corticosteroid treatment. Enhanced transcripts include p38 MAPK, PTP1B, leukocyte common antigen-related protein (LAR), and interleukin 6 receptor (IL6R1).

There were five transcription factors whose temporal patterns of regulation were related to insulin resistance (Fig. 5). Transcription factor transcripts which were down-regulated by corticosteroid treatment include steroid receptor-enhancing binding protein-1c (SREBP-1c) and retinoic acid X receptor γ-1 (RXRγ). Enhanced

Table 1 MPL-regulated transcripts relating to insulin resistance

(A)	**Down-regulated transcripts**		
	Gene transcript	Abbreviation	Functional category
Accession no.			
X58375	Insulin receptor substrate-1	IRS-1	Signal transduction
L16995	Steroid receptor enhancing binding protein-1c	SREBP-1c, ADD1	Transcription factor
AJ223083	Retinoic acid X receptor γ-1	RXRγ	Transcription factor
D89655	Scavenger receptor class B1	FAT, CD36	Metabolism
U36771	Glycerol-3-phosphate acyltransferase	GPAT	Metabolism
U67995	Stearyl-CoA desaturase 2	SCD2	Metabolism
X78593	Mitochondrial glycerol-3-phosphate dehydrogeanse 2	mGDPH	Metabolism
M64301	Extracellular signal related kinase 3	ERK3	Signal transduction
(B)	**Up-regulated transcripts**		
	Gene transcript	Abbreviation	Functional category
Accession no.			
L36664	Kininase II	ACE	Blood flow
M33962	Protein tyrosine phosphatase type 1B	PTP1B	Signal transduction
M60103	Leukocyte common antigen-related protein	LAR, LAR PTPase, Ptprf	Signal transduction
M64711	Endothelin-1	ET-1	Blood flow
M86912	Type-1A angiotensin II receptor	AT1A	Blood flow
U40064	Peroxisome proliferator-activated receptor δ	PPARδ	Transcription factor
M33648	3-Hydroxy-3-methylglutaryl-CoA synthase	HMG-CoA synthase	Metabolism
M24067	Plasminogen-activator inhibitor 1	PAI-1	Blood flow
S77528	CCAAT/enhancer-binding protein β	C/EBPβ, NFIL6	Transcription factor
AI045030	CCAAT/enhancer-binding protein δ	C/EBPδ, NFIL6β	Transcription factor
M58587	Interleukin 6 receptor	IL6R1	Signal transduction
U73142	p38 Mitogen-activated protein kinase	p38 MAPK	Signal transduction
(C)	**Biphasic-regulated transcripts**		
	Gene transcript	Abbreviation	Functional category
Accession no.			
AF030163	Uncoupling protein 3	UPC3	Metabolism
AF034577	Pyruvate dehydrogenase kinase isoenzyme 4	PDK4	Metabolism

expression following steroid treatment occurred with PPARδ, C/EBPβ, and C/EBPδ transcripts.

Seven regulated transcripts were found that relate to carbohydrate and lipid metabolism (Fig. 6). Four transcripts in this group exhibited corticosteroid-induced down-regulation: scavenger receptor class B1 (fatty acid transporter; FAT), mitochondrial glycerol-3-phosphate acyltransferase (GPAT), mGDPH, and stearyl-CoA desaturase 2 (SCD2). One transcript, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase), was enhanced following steroid treatment. In addition, both UCP3 and PDK4 showed biphasic regulation, with an initial enhanced expression followed by expression levels that fell below baseline.

The last functional group contained four transcripts, all of which showed enhanced expression and influence blood flow in the musculature (Fig. 7). These transcripts may be expressed in the muscle, the muscle vasculature, or both. These include kininase II, AT1A, endothelin-1 (ET-1), and plasminogen-activator inhibitor 1 (PAI-1).

Discussion

A population of ADX animals was injected with a single bolus dose of MPL, groups of three animals were killed at each of 16 time points over a 72-h period, and muscles from MPL-treated samples were compared with vehicle-treated controls. ADX animals were used to eliminate the circadian oscillation and provide a stable baseline. This allowed us to identify gene transcripts that deviated from the baseline following drug treatment and determine the duration of time it takes to return to that baseline. The drug was cleared 7 h following dosing. In another cohort of animals treated alike, elevated concentrations of glucose were found in the blood after about 9 h. The pattern of

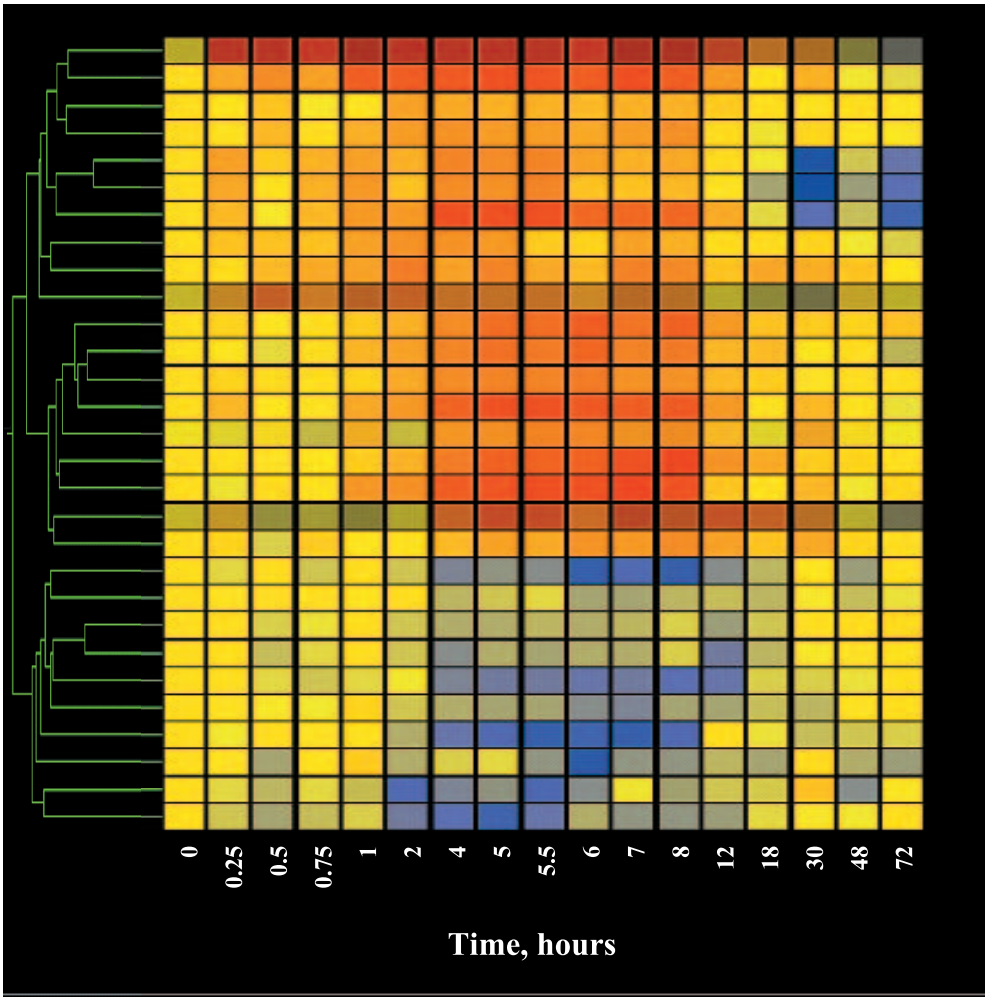

Figure 2 Gene-tree representation of the 29 probe sets identified to be related to insulin resistance in skeletal muscle. The gene tree represents the averaged normalized values of each of the 29 probe sets at 17 different time points following MPL treatment, grouped by pattern similarities. The *y*-axis represents individual probe sets, with color representing relative intensity (yellow represents no change from control, progression towards red indicates increased expression, and progression towards blue represents decreased expression versus control values). The *x*-axis ranks the samples in sequence of time following MPL treatment.

elevated glucose is consistent with the origin being corticosteroid-induced gluconeogenesis by the liver and kidney. Elevated concentrations of insulin were also found in the blood lasting about 48 h. The rise in insulin is consistent with the increased blood glucose. However, the long-lasting duration of the elevated insulin concentration was unexpected. At present it is not possible to determine if this persistence of plasma insulin is due to continued release from the pancreas or reduced elimination. In either case, because placebo controls did not demonstrate such a pattern, MPL must in some way be involved. Interestingly, such sustained elevation of insulin would contribute to insulin resistance, possibly through down-regulation of elements of the cascade leading to glucose transport. The fact that a single dose of MPL results in such a persistent hyperinsulinemia suggests that when dosing is repetitive there may be a cumulative effect.

The use of microarray technology within the context of a time-series approach allows one to evaluate the cascade of transcriptional events in the development and decay of different aspects of gene expression that relate to the complex physiological or pathological process being studied. Temporal profiles are particularly relevant in this case because 11 of the 22 gene transcripts were for proteins involved in signal transduction or transcription regulation. These temporal profiles should allow us to begin to evaluate downstream consequences of expression changes in these signaling cascades. We have organized these

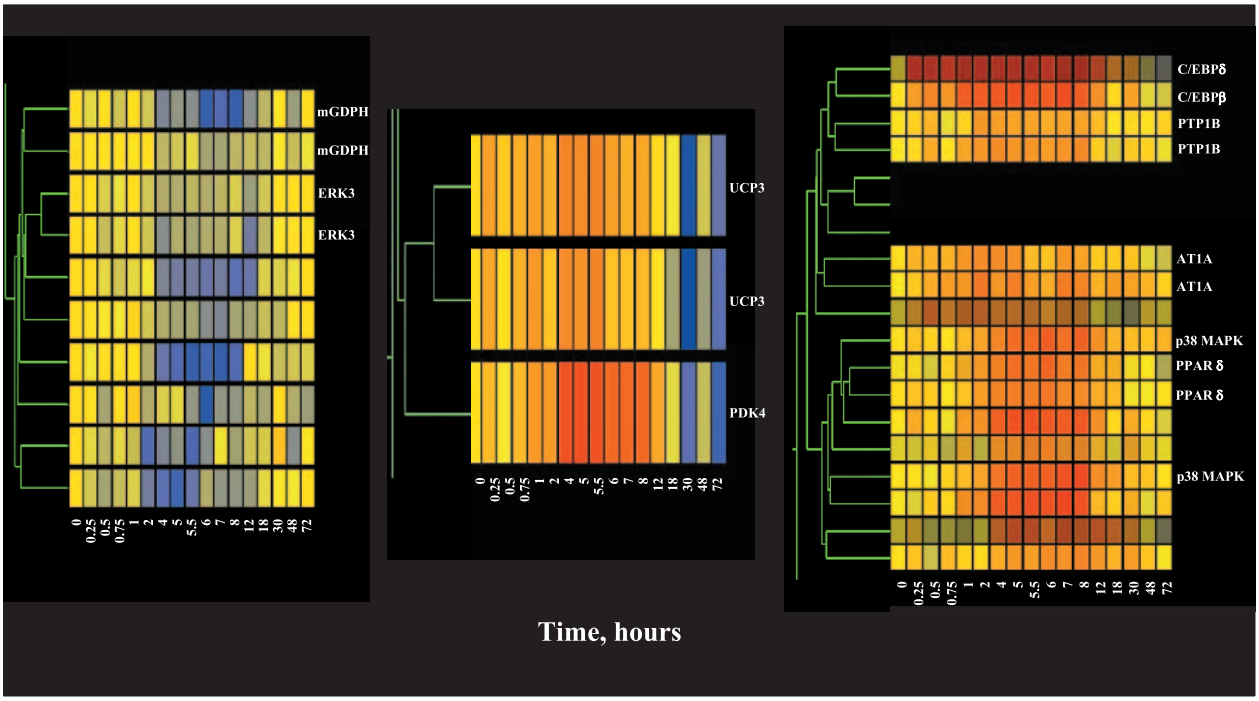

Figure 3 Magnified views of various regions of gene tree presented in Fig. 2. The left-hand panel shows a magnification of the down-regulated probe sets present on the gene tree in Fig. 2. The middle panel presents an enlargement of the three probe sets from Fig. 2 that exhibit biphasic regulation. The right-hand panel shows a magnified view of up-regulated probe sets. Abbreviations are given in Table 1.

22 transcripts relating to insulin resistance into four functional categories: signal transduction, transcription regulation, carbohydrate/fat metabolism, and regulation of blood flow.

Six gene transcripts related to signal transduction were regulated. These six transcripts either play a direct role in insulin signaling (IRS-1 and ERK3) or have been implicated in insulin sensitivity/insulin resistance (PTP1B, LAR, p38 MAPK, IL6R1). The decline in IRS-1, a central element in insulin signaling, will severely inhibit insulin-directed glucose uptake (Sesti 2000, Araki & Tsuruzoe 2002, Shirakami *et al.* 2002). It has been reported previously that chronic corticosteroid treatment reduces the amount of IRS-1 in skeletal muscle (Giorgino *et al.* 1993, Dupont *et al.* 1999), consistent with the down-regulation in the IRS-1 transcript seen here with a single bolus dose of MPL. Down-regulation of ERK3 transcripts following MPL administration should also contribute directly to reduced insulin signaling (Boulton *et al.* 1991, Turgeon *et al.* 2000). In contrast, expression of PTP1B, p38 MAPK, and LAR were increased. An increase in these transcripts would contribute to inactivation of insulin signaling at various levels and promote insulin resistance (Ahmad & Goldstein 1995, Ahmad *et al.* 1997, Chen *et al.* 1999, Egawa *et al.* 2001, Fujishiro *et al.* 2001, Goldstein 2001, Di Paola *et al.* 2002). The final transcript in this category is IL6R1, whose expression potentially enhances interleukin-6 sensitivity. Interleukin-6 has been shown to interfere with insulin-induced kinase cascades (Mooney *et al.* 2001, Senn *et al.* 2002), and elevated levels of plasma interleukin-6 are also associated with insulin-resistant states (Senn *et al.* 2002, 2003). Together, the changes in these six gene transcripts present a polygenic picture of greatly impaired insulin signaling in skeletal muscle.

The expressions of transcripts for five transcription factors with direct relevance to insulin resistance were also regulated. SREBP-1c, which was down-regulated following steroid treatment, is a helix-loop-helix transcription factor that mediates insulin effects on gene expression in liver, skeletal muscle, and other tissues. This gene at least in part mediates the insulin-induced increased expression of glycolytic and lipogenic enzyme genes (Guillet-Deniau *et al.* 2002, Shimano 2002). The early decreased expression of SREBP-1c following corticosteroid treatment would therefore presumably decrease some insulin-induced transcriptional effects. To our knowledge this is the first report demonstrating that corticosteroids regulate the expression of this gene. RXRγ, which was also down-regulated, is a transcription factor that functions as a homodimer or as a heterodimer with PPARα and PPARγ in skeletal muscle (Dowhan *et al.* 1994, Cha *et al.* 2001).

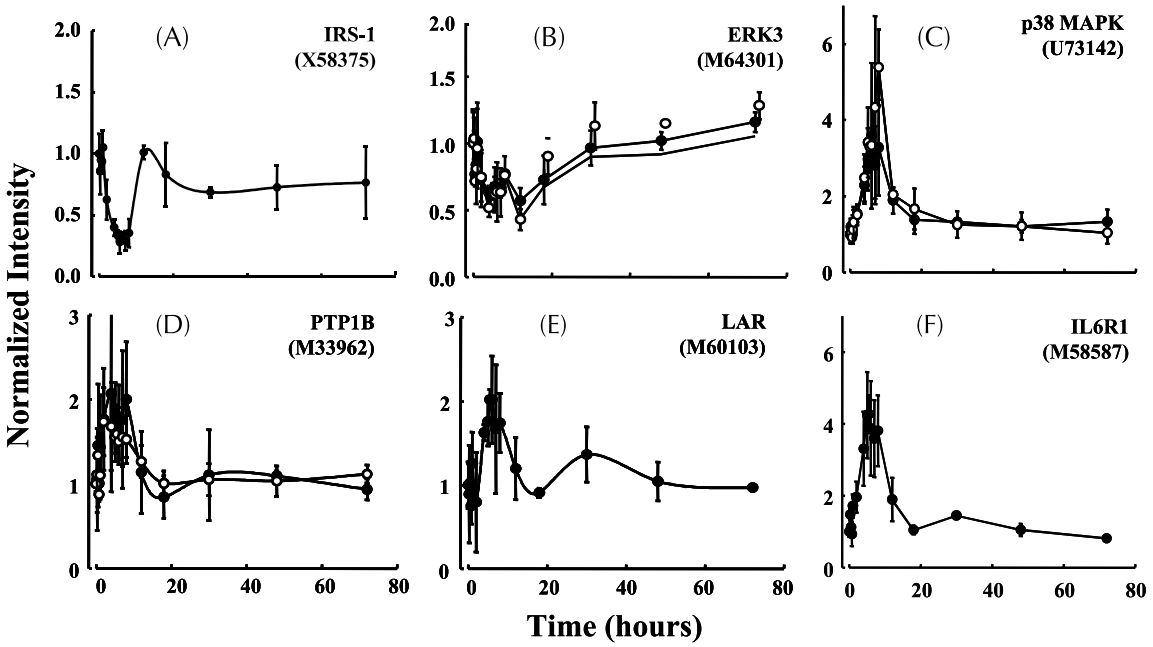

Figure 4 Linear plots of the six gene transcripts related to signal transduction as a function of time following MPL administration. The *x*-axis represents time after MPL administration, and the *y*-axis average normalized intensities from Affymetrix array analysis. Error bars represent standard deviations of the mean. Numbers in parentheses are accession numbers. Abbreviations are given in Table 1.

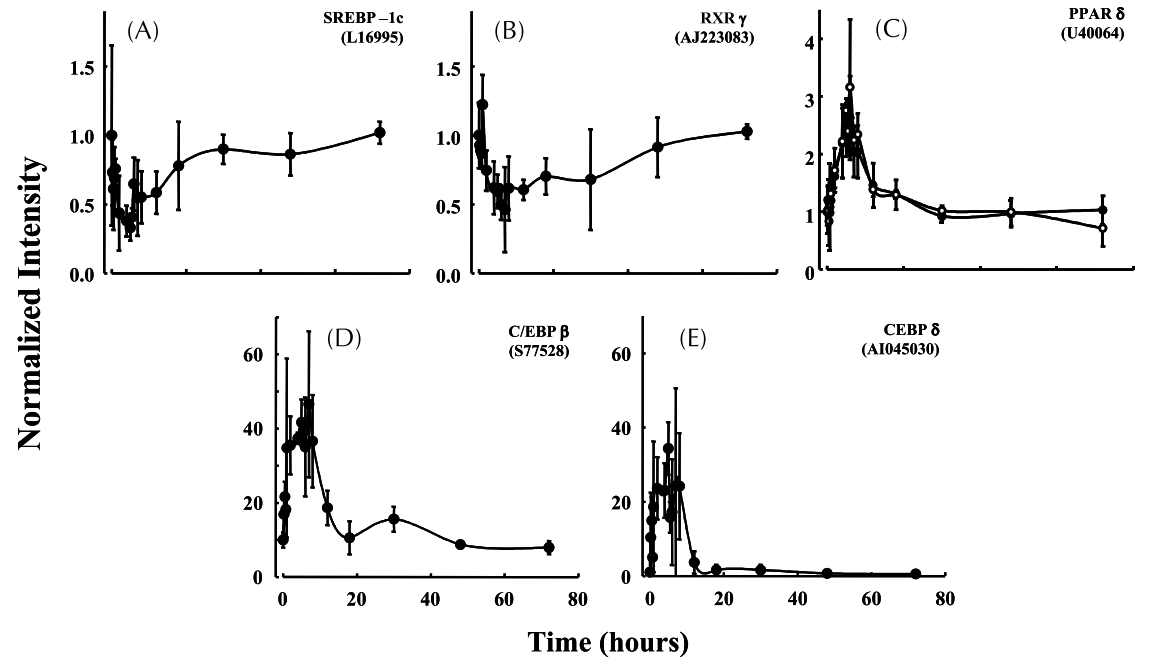

Figure 5 Linear plots of the five transcription factor gene transcripts as a function of time following MPL administration. Axes and symbols are as defined in Figure 4. Numbers in parentheses are accession numbers. Abbreviations are given in Table 1.

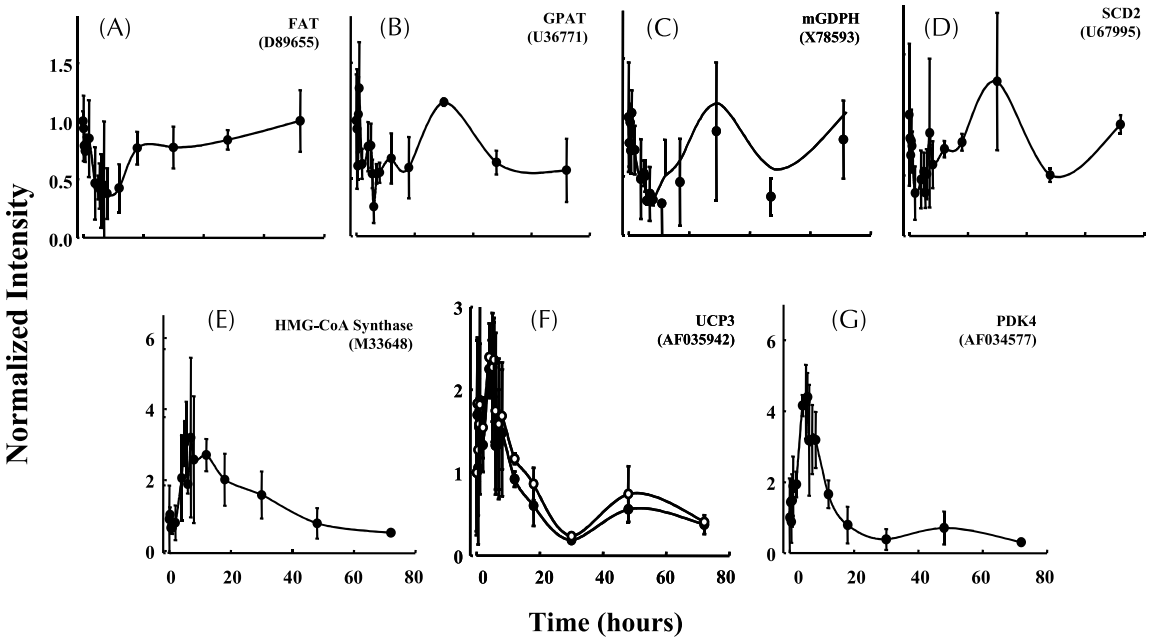

Figure 6 Linear plots of the seven gene transcripts related to carbohydrate and lipid metabolism as a function of time following MPL administration. Axes and symbols are as defined in Figure 4. Numbers in parentheses are accession numbers. Abbreviations are given in Table 1.

This transcription factor regulates several genes involved in energy metabolism, including FAT, and influences muscle insulin sensitivity (Cha *et al.* 2001, Singh Ahuja *et al.* 2001). Three transcription factors (PPARδ, C/EBPβ, and C/EBPδ) are up-regulated; up-regulation of all three are associated with increased lipid metabolism in skeletal muscle and have also been associated with insulin resistance (Zador *et al.* 1998, Muoio *et al.* 2002, Penner *et al.* 2002).

The next group includes genes related to carbohydrate and lipid metabolism. These probes, together with the transcription factors discussed above, present a picture of a shift in the muscle away from glycolysis towards β-oxidation, consistent with an insulin-resistant state. Four of the gene transcripts in this group are down-regulated (FAT/CD36, GPAT, SCD2, mGDPH), one is up-regulated (HMG CoA synthetase), and two show biphasic regulation (UPC3, PDK4). The expression of three of the four transcripts whose expression is decreased (FAT/CD36, SCD2, GPAT) relate to increased utilization of lipids for energy generation, and in some cases have been directly linked to decreased insulin sensitivity (Cha *et al.* 2001, Lewin *et al.* 2001, Mooney *et al.* 2001, Singh Ahuja *et al.* 2001, Kim *et al.* 2002). The regulation of all three of these genes is also mediated by SREBP-1c and RXR (Cha *et al* 2001, Ikeda *et al.* 2002). The last down-regulated transcript in this group is mGDPH. Down-regulation of this transcript is consistent with decreased glycolytic activity associated with insulin resistance (Brown *et al.* 1994). Two transcripts in this group (UPC3, PDK4) have a temporal signature suggesting biphasic regulation. Both transcripts show a rapid enhanced expression that returns to baseline by 12 h, then declines below baseline by 30 h, and remains low for the remainder of the 72-h period. The first is UCP3, which is a transporter protein that creates a leak across the mitochondrial inner membrane. This uncouples oxidative phosphorylation allowing the energy to be dissipated as heat (Dulloo *et al.* 2001). Enhanced expression of UCP3 in skeletal muscle has been associated with obesity and type 2 diabetes in humans (Bao *et al.* 1998), and elevated levels of UCP3 in skeletal muscle have been linked closely to insulin resistance in several insulin-resistant states (Bao *et al.* 1998, Samec *et al.* 1999, Oberkofler *et al.* 2000, Fabris *et al.* 2001, Strommer *et al.* 2001). In skeletal muscle increased expression of UCP3 is associated with the channeling of fuel utilization towards fat and away from carbohydrates (Weigle *et al.* 1998), and the initial up-regulation of this transcript is consistent with insulin resistance. The second biphasic transcript in this group is PDK4. This enzyme inactivates the mitochondrial pyruvate dehydrogenase complex by phosphorylation. Since the pyruvate dehydrogenase complex regulates the oxidative disposal of glucose, its inactivation by PDK4 can limit glucose use by skeletal muscle (Sugden *et al.* 1995). Insulin down-regulates the expression of PDK4 in skeletal muscle while high-fat diets tend to increase it (Majer *et al.* 1998). There is considerable interest in regulating PDK4 as an approach to the treatment of type 2 diabetes

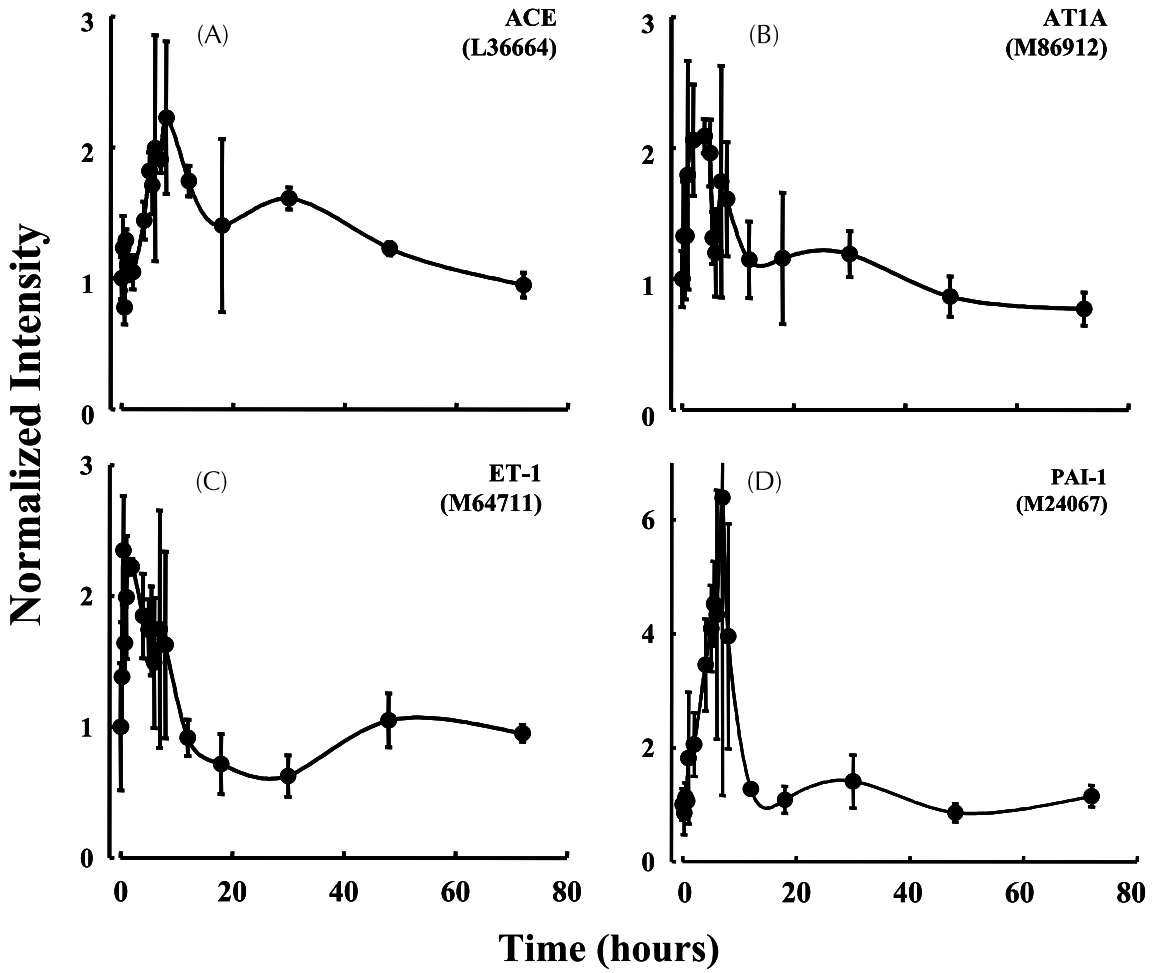

Figure 7 Linear plots of the four gene transcripts related to muscle blood flow as a function of time following MPL administration. Axes and symbols are as defined in Figure 4. Numbers in parentheses are accession numbers. Abbreviations are given in Table 1.

(Oshida *et al.* 1999, Sreenan *et al.* 1999, Sugden & Holness 2002). The last transcript in this group is HMG-CoA synthase, which shows enhanced expression. HMG-CoA synthase is the key regulated enzyme in the synthesis of ketone bodies (Hegardt 1999, Challis *et al.* 2001). Although the liver is generally considered to be the site of ketone-body synthesis, it has been estimated that skeletal muscle may be responsible for as much as 25% of ketone-body production in poorly controlled diabetes (Nosadini *et al.* 1985, Bailey *et al.* 1990), and therefore increased expression of this transcript following corticosteroid administration is also consistent with insulin resistance.

The last group of transcripts consists of four that may be skeletal muscle in origin, vascular endothelial in origin, or both. All four showed enhanced expression and are clearly associated with insulin resistance. The first is kininase II (EC 3·4·15·1), which converts angiotensin I to angiotensin II. Kininase II, also known as angiotensin-converting enzyme (ACE), is generally thought of as an ectoenzyme bound to vascular endothelial cells (Sun *et al.* 1994). However, it has been reported that skeletal muscle membranes also contain this enzyme (Dragovic *et al.* 1996). Regardless of the origin of this transcript, inhibitors of this enzyme have been shown to improve glucose uptake into skeletal muscles (Bottermann & Classen 1991, Dietze *et al.* 1996). Thus the enhanced expression of kininase II would contribute to insulin resistance. The second is ET-1, which is a potent vasoconstrictor. Synthesis of ET-1 has been reported in several organs such as heart, lung, and skeletal muscle suggesting that it may also play a role as a local regulator of blood flow (Sakurai *et al.* 1991, Guo *et al.* 1998). Elevated levels of ET-1 are also associated with insulin resistance (Ottosson-Seeberger *et al.* 1997, Idris *et al.* 2001), reduced blood flow to the musculature and thus reduced glucose disposal (Santure *et al.* 2002). The third is AT1A. This receptor mediates the vasoconstrictor

activity of angiotensin II, and blocking AT1A improves glucose disposal (Hoenack & Roesen 1996, Brown *et al.* 2002). Interestingly, gene therapy with AT1A antisense DNA has been shown to improve insulin sensitivity in the high-fructose-fed rat model for glucose intolerance (Katovich *et al.* 2001). The last transcript in this group is PAI-1, which binds and inactivates the plasminogen activator urokinase, a major control point in the regulation of fibrinolysis. Elevated levels of PAI-1 are common in insulin-resistant patients and are a high risk factor for infarction (Peltonen *et al.* 1995, Thogersen *et al.* 1998, Shirakami *et al.* 2002).

In the present report we have used microarrays to profile the changes in gene expression that occur in rat skeletal muscle and which relate to insulin resistance following a single dose of MPL. In contrast to chronic treatment, temporal profiling along with the use of microarrays provides a broad 'motion picture' of the development and decay of components of corticosteroid-induced insulin resistance. This picture portrays multiple points of direct interference with the insulin signaling cascade with the reduced expression of IRS-1 and perhaps ERK3. It also portrays multiple points of interference with insulin signaling through the enhanced expression of PTP1B, LAR, and perhaps kininase II, AT1A and p38 MAPK. There are direct expression changes that lead to the reduction in the use of glucose by skeletal muscle with the decrease in mGDPH and the increase in PDK4. Similarly, the temporal profile illustrates the shift of skeletal muscle towards fat for energy production that includes reductions in the expression of GPAT, SCD2, and FAT along with enhanced expression of UCP3 and HMG-CoA synthase. The results also provide insight into the reduced blood flow to skeletal muscle that impairs glucose disposal and contributes to the hypertension that develops with chronic corticosteroid use. Increases in kininase II, ET-1, AT1A, and PAI-1 provide a comprehensive picture of reduced muscle blood flow and the foundation for infarction. Finally, the temporal profile provides a perspective on the complex transcriptional regulation responsible for these changes in gene expression. In addition to the effects of corticosteroids on gene expression mediated by the activated glucocorticoid receptor interacting with glucocorticoid-responsive elements there is the decrease in two transcription factors (SREBP-1 and RXRγ), both of which enhance the expression of genes that increase insulin sensitivity. There were also increases in PPARδ, C/EBPβ nuclear factor interleukin 6 (NFIL6), C/EBPδ (NFIL6β), and IL6R1, all of which enhance the expression of genes promoting the use of fat. The combination of IL6R1 along with the two NFIL6 transcription factors suggests that corticosteroid-induced insulin resistance may involve enhancing the sensitivity of skeletal muscle to other systemic factors that promote insulin resistance. By using a single bolus dose of MPL in ADX animals we were able to determine the duration of the transcriptional responses. The observation that some responses lasted as long as 30–40 h suggests that repeated dosing with daily divided or daily dosing regimens may lead to accumulating effects.

Besides the pharmacological use of corticosteroids there are other conditions that involve insulin resistance. Cachexia, sepsis, and trauma all result in insulin resistance with glucocorticoids clearly involved in these processes (Martinez-Riquelme & Allison 2003). In addition, type 2 diabetes mellitus, which has diffuse genetic and environmental (diet and exercise) origins, is a syndrome of insulin resistance. Observations indicating that insulin resistance is part of the metabolic syndrome stemming from prenatal corticosteroid exposure suggest a role for the hypothalmic/pituitary/adrenal axis, in at least some cases of type 2 diabetes (Challis *et al.* 2001, Bertram & Hanson 2002, Newnham *et al.* 2002).

Insulin resistance, regardless of cause, involves a disruption of the systemic balance in fuel utilization between carbohydrates and fats. Because skeletal muscle is responsible for 75–80% of the insulin-mediated disposal of glucose, all types of insulin resistance must involve this tissue. Although corticosteroid-induced insulin resistance is not a perfect model for all insulin-resistant states, it does provide an insightful picture of the polygenic expression changes that can be involved in the expression of insulin resistance. It is of interest that the numerous and diverse transcriptional alterations are produced by MPL with time frames that temporally correspond to the transient hyperglycemia and hyperinsulinemia. Further explorations of the relationships between systemic manifestations of insulin resistance and transcriptional alterations in skeletal muscle and other tissues are of importance to understanding and perhaps identifying additional drug targets for this pathology with polygenic origins. Understanding the temporal cascade of events may also aid in designing dosing regimens that minimize this adverse consequence of the pharmacological use of corticosteroids.

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