Role of the Transcription Factor ATF4 in the Anabolic Actions of Insulin and the Anti-anabolic Actions of Glucocorticoids*  

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In most mammalian cells, insulin and glucocorticoids promote anabolism and catabolism, respectively. Whereas the opposing effects of insulin and glucocorticoids on catabolic gene expression have been explained at the molecular level, comparatively little is known about how these hormones alter anabolic gene expression. These studies identify ATF4 as an anabolic transcription factor that is repressed by glucocorticoids and induced by insulin. Insulin-mediated induction of ATF4 required the mammalian target of rapamycin complex 1, was required for the activation of a genetic program for the cellular uptake of essential amino acids and the synthesis of nonessential amino acids and aminoacyl-tRNAs, and was coupled to the repression of Foxo-dependent genes needed for protein and lipid catabolism. These results suggest that ATF4 plays a central role in hormonal regulation of amino acid and protein anabolism by coupling amino acid uptake and synthesis, as well as the generation of charged tRNAs, to mammalian target of rapamycin complex 1-mediated mRNA translation.

Insulin levels rise after feeding, promoting anabolic processes (such as the uptake of amino acids, glucose, and lipid and the synthesis of protein, glycogen, and lipid), while inhibiting catabolic processes (such as protein breakdown, gluconeogenesis, glycogenolysis, lipolysis, fatty acid oxidation, and ketogenesis) (1). In contrast to insulin, glucocorticoids inhibit anabolism while promoting catabolism (2). These effects of glucocorticoids are required for human survival during times of prolonged fasting or stress, when protein and lipid are mobilized from peripheral tissues and utilized for gluconeogenesis, fatty acid oxidation, and ketogenesis. Human health requires that the opposing metabolic effects of insulin and glucocorticoids remain in net balance, yet a number of pathologic conditions tip the balance toward catabolism, resulting in atrophy of peripheral tissues. These conditions include starvation, glucocorticoid excess (Cushing syndrome), absolute or relative insulin deficiency (as in uncontrolled type 1 or type 2 diabetes mellitus), critical illnesses (such as burns, trauma, or sepsis), or chronic illnesses (such as uremia, cancer, AIDS, or tuberculosis). Indeed, atrophy of skin, muscle, and bone are predictable side effects when supraphysiologic doses of glucocorticoids are used clinically to suppress the immune response.

Insulin and glucocorticoids induce many of their metabolic effects by altering the expression of key genes needed for anabolic and catabolic processes. Transcriptional control of catabolic gene expression by these hormones has been extensively studied, leading to a generalized molecular model that explains how catabolic genes are induced by glucocorticoids and repressed by insulin. Under fasting conditions, the presence of glucocorticoids coupled with lower levels of insulin permits the glucocorticoid receptor (GR) 2 and Foxo transcription factors to bind cis-acting DNA regulatory elements present in genes encoding key proteins needed for protein catabolism (atrogin-1 and muscle-specific RING finger protein 1 (MuRF1)), lipid catabolism (pyruvate dehydrogenase kinase-4 (PDK4)), and gluconeogenesis (phosphoenolpyruvate carboxykinase (PEPCK)). All of these catabolic genes require the combined presence of the GR and Foxo proteins to become transcriptionally active (3–8). Conversely, after feeding, high levels of insulin initiate a signal transduction cascade that includes activation of the protein kinase Akt (9). Akt phosphorylates the Foxo proteins, causing them to release from their DNA-binding sites and translocate out of the nucleus (10), thus de-activating the catabolic genes. Importantly, under physiologic conditions, insulin has a dominant effect, overriding glucocorticoids to turn off catabolism (11). Conversely, when insulin levels are low, the continued presence of glucocorticoids provides the default setting of metabolism (toward catabolism), maintaining homeostasis in the absence of feeding, which may occur with unpredictable frequency.

In contrast to our current understanding of how catabolic genes are regulated by glucocorticoids and insulin, relatively little is known of how these hormones regulate anabolic gene expression. This is particularly true of anabolic genes that might augment insulin-mediated protein synthesis or might be repressed during glucocorticoid-mediated protein catabolism. The anabolic genes encoding amino acid biosynthetic enzymes, amino acid transporters, and aminoacyl-tRNA synthetases are known to be activated by ATF4 (also known as CREB2), a basic

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2 The abbreviations used are: GR, glucocorticoid receptor; PDK4, pyruvate dehydrogenase kinase, isoenzyme 4; Glul, glutamate-ammonia ligase (glutamine synthase); MEF, mouse embryonic fibroblast; IGF-1, insulin-like growth factor-1; FBS, fetal bovine serum albumin; PBS, phosphate-buffered saline; siRNA, small interfering RNA; RNAi, RNA interference; qPCR, quantitative PCR; DMEM, Dulbecco’s modified Eagle’s medium; IGF-1, insulin-like growth factor-1.
leucine zipper transcription factor that is increased when cultured cells are deprived of amino acids or subjected to endoplasmic reticulum stress (12–15). The effects of glucocorticoids and insulin on ATF4 and these genes are currently not known, although mice lacking ATF4 are >50% smaller than normal in size and have a variety of developmental defects, which is consistent with a deficiency in protein anabolism (16–18). The studies presented here had two principal goals: first, to examine whether glucocorticoid- and insulin-mediated effects on catalytic gene expression might be accompanied by reciprocal effects on anabolic gene expression; and second, to begin to understand the molecular pathways used by glucocorticoids and insulin to regulate genes involved in amino acid and protein anabolism.

**EXPERIMENTAL PROCEDURES**

**Materials and Buffers**—Dexamethasone, insulin (from bovine pancreas), rapamycin, and mouse recombinant insulin-like growth factor-1 (IGF-1) were obtained from Sigma. [3H]Leucine was obtained from American Radiolabeled Chemicals (ARC). The following buffers were used. Buffer A contains 50 mM Heps-KOH, pH 7.4, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 5 mM sodium EDTA, 5 mM sodium EGTA, and protease inhibitor mixture (25 μg/ml N-acetyl-leucinal-leucinal-norleucinal, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 200 μM phenylmethylsulfonyl fluoride). Buffer B contains 10 mM Tris-HCl, pH 7.6, 100 mM NaCl 1% (w/v) SDS, and protease inhibitor mixture. Buffer C contains 20 mM Heps-KOH, pH 7.6, 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, and protease inhibitor mixture. Buffer D contains 250 mM Tris-HCl, pH 6.8, 10% SDS, 25% glycerol, 0.2% (w/v) bromphenol blue, and 5% (w/v) 2-mercaptoethanol.

**Cell Culture**—Tissue culture medium A is Dulbecco’s modified Eagle’s medium (DMEM) containing 1 g/liter glucose (Mediatech 10-014) plus 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 10% (v/v) fetal bovine serum (FBS). Medium B is DMEM containing 4.5 g/liter glucose (Mediatech 10-013) plus 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 10% FBS. Medium C is DMEM containing 4.5 g/liter glucose (ATCC 30-2002) plus 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 10% FBS. Mouse L cells (D9 strain (19)) were kindly provided by Dr. Stuart Kornfeld (Washington University, St. Louis, MO) and were maintained in monolayer in medium A at 37 °C and an atmosphere of 8–9% CO₂. MEF-1 cells (20) were maintained in monolayer in medium B at 37 °C and an atmosphere of 8–9% CO₂. With the exception of experiments using RNA interference (as described below), mouse L cells and MEF cells were set up for experiments in medium A or medium B, respectively, at a density of 7 × 10⁵ cells/100-mm dish. Two days later, cells were washed twice with phosphate-buffered saline (PBS) and then subjected to the incubation protocols described in the figure legends. C2C12 mouse myoblasts (ATCC) were maintained in medium C, set up for experiments at a density of 7 × 10⁴ cells/100-mm dish, and induced to differentiate into myotubes by replacing 10% FBS with 2% horse serum, as described (3). After 4 days in differentiation medium, myotubes were washed twice with PBS and then subjected to the incubation protocols described in the figure legends.

**RNA Interference**—Mouse L cells were set up at a density of 1.5 × 10⁶ cells/60-mm dish on day 0. On day 1, cells were washed with PBS, re-fed with medium A lacking penicillin and streptomycin (3 ml/dish), followed by the addition of a mixture containing 1 ml of OptiMEM (Invitrogen), 10 μl Lipofectamine 2000 reagent (Invitrogen), and 200 nm siRNA duplex (to give a final siRNA concentration of 50 nM). Control cells were transfected with siCONTROL nontargeting siRNA (Dharmacon). The siRNA duplex targeting ATF4 contained the following nucleotide sequence (5’ to 3’), sense GAGCAUUCUUAG-UUUAGUU and antisense CUAAACUAAAGGAUGCU-CUU. On day 2 (24 h after transfection), cells were re-fed with serum-free medium A and then subjected to varying conditions of incubation as described in the figure legends. Following incubation, the cells from two identically treated dishes were pooled and harvested for total cellular protein extracts; the cells from three identically treated dishes were pooled and harvested for total cellular RNA, followed by qPCR analysis; or the cells from two identically treated dishes were harvested for analysis of intracellular free amino acids.

**Analysis of Cellular mRNAs Using Oligonucleotide Microarrays**—Following incubation, the cells from three identically treated 100-mm dishes were pooled, and total cellular RNA was harvested using STAT-60 reagent (Tel-Test), according to the manufacturer’s protocol. The RNA was then subjected to microarray analysis using established protocols (21), GeneChip mouse genome 430 2.0 microarrays, and a GeneChip 3000 scanner (Affymetrix). Data were analyzed using GeneChip operating software (version 1.1.1, from Affymetrix), and the final results represent the average fold change in mRNA levels obtained from three independent experiments.

**Analysis of Cellular mRNAs by qPCR**—Total cellular mRNA was isolated using the RNAeasy kit (Qiagen), according to the manufacturer’s protocol. In experiments using C2C12 myotubes, total cellular RNA was first extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions prior to purification on RNAeasy columns. cDNA synthesis, qPCR, and data analysis were performed as described previously (22). All qPCRs were performed in triplicate, and the cycle threshold values were averaged to give the final results. mRNA encoding 36B4 was used as the invariant control in all studies. qPCR primer sequences may be found in the Supplemental Material.

**Analysis of Cell Number and Viability**—Following incubation, mouse L cells were washed with PBS, lifted from 100-mm dishes using 2 ml of trypsin/EDTA solution (Mediatech 25-052), followed by the immediate addition of 18 ml of medium A containing 1% FBS. An aliquot of resuspended cells was then stained with trypan blue and counted using a hemocytometer.

**Metabolic Labeling**—[3H]Leucine (120 Ci/mmol; 20 μCi/dish) was added directly to the cell culture medium, after which one dish from each experimental condition was placed at 4 °C (to measure background incorporation of [3H]leucine), whereas three dishes from each condition were placed back in the 37 °C incubator. Following incubation, cells were washed...
twice with PBS and then harvested, and an aliquot of cells was subjected to precipitation using 10% (w/v) trichloroacetic acid in the presence of 0.45% salmon sperm DNA. Trichloroacetic acid precipitates were then applied to glass fiber filters set upon a vacuum manifold. Filters were sequentially washed with 10% trichloroacetic acid, 5% trichloroacetic acid, and 95% ethanol and then placed in scintillation mixture for measurement of acid-insoluble radioactivity. The final results were obtained by averaging the counts obtained from the three dishes in each condition that were incubated at 37 °C, then subtracting the background count obtained from the dish incubated at 4 °C, and then normalizing the counts to the total milligrams of protein/dish under each condition.

Preparation of Nuclear Extracts—Because ATF4 has an extremely short half-life (30–60 min) secondary to proteosomal degradation (23), the cells were treated with the proteosome inhibitor N-acetyl-leucinal-leucinal-norleucinal (25 μg/ml), for the final 1 h of incubation, as is routinely done in studies of proteins with rapid turnover, such as sterol regulatory element-binding proteins (24). Following incubation, the cells from three identically treated 100-mm dishes of cells were washed once with cold PBS, scraped, pooled into 50-ml tubes on ice, and then centrifuged at 1000 g for 5 min. Cell pellets were resuspended in 1.2 ml of Buffer A and then passed through a 22-gauge needle 22 times, followed by centrifugation at 1000 g for 5 min. The 1000 g pellet was resuspended in 250 μl of Buffer C, rotated at 4 °C for 1 h, and then centrifuged at 20,000 g for 20 min to remove insoluble material. The protein concentration was then measured using the BCA kit (Pierce), after which a 25-μl aliquot was mixed with 0.25 volume of Buffer D and heated for 5 min at 95 °C. SC-200 (Santa Cruz Biotechnology), which recognizes the COOH terminus of mouse ATF4.

Measurement of Intracellular Free Amino Acids—Following incubation, cells were washed three times with cold PBS and scraped into 10% (v/v) methanol (150 μl/60-mm dish). Lysates from two identically treated dishes of cells were pooled, and protein concentrations were determined using the BCA method, and free (nonhydrolyzed) amino acid concentrations were determined using cation exchange chromatography (Hitachi L-8800 amino acid analyzer).

RESULTS

Glucocorticoids Induce Mouse L Cell Fibroblasts to Enter a Metabolically Quiescent, Long Lived State That Is Reversed by Insulin—Mouse L cell fibroblasts were used to study the metabolic effects of glucocorticoids and insulin. These immortalized cells contain high levels of endogenous GR and resemble nontransformed peripheral mammalian cells in their capacity to slow their rate of anabolism in response to glucocorticoids (25). As shown in Fig. 1A, dexamethasone dramatically prolonged cell survival when mouse L cells were cultured in the absence of serum (which was omitted to prevent interference with any subsequent studies using insulin). In this experiment, cells were incubated in the absence or presence of dexamethasone and re-fed with fresh culture medium every 2 weeks. After various times of incubation, dishes of cells from each group were harvested for analysis of cell number and viability. In the absence of dexamethasone, cells initially proliferated but then died within 10 days of incubation. In contrast, if dexamethasone was present, the number of cells per dish did not increase, and many cells
remained viable for >85 days of incubation. These effects of dexamethasone were reversible, as removal of dexamethasone from the cell culture medium after 15 days of incubation allowed the cells to initially proliferate, but ultimately led to cell death. The promotion of cell survival under these conditions was a specific effect of GR agonists (including corticosterone), which were arbitrarily set at 1. Cort, corticosterone; Prog, progesterone. B, mouse L cells were incubated for 48 h in the absence or presence of dexamethasone (100 nM) and/or 5% fetal bovine serum, as indicated. Total cellular RNA was analyzed by qPCR, and transcript levels were normalized to the transcript levels in cells incubated in the absence of dexamethasone and serum, which were arbitrarily set at 1. Asns, asparagine synthetase; Psat1, phosphoserine aminotransferase 1; Dex, dexamethasone. C, mouse L cells were incubated for varying times in serum-free medium in the absence or presence of dexamethasone (100 nM), as indicated. Total cellular RNA was analyzed by qPCR, and transcript levels in the presence of dexamethasone were normalized to the transcript levels in cells incubated in the absence of dexamethasone and harvested at the same time point, which were arbitrarily set at 1.

To test the idea that dexamethasone and insulin might have opposing effects on metabolism under serum-free conditions, mouse L cells were incubated in the absence or presence of dexamethasone for 48 h, followed by an additional 6 h of incubation in the absence or presence of insulin, which was directly added to the cell culture medium in the continued presence of dexamethasone. Dexamethasone decreased global protein synthesis (as assessed by the incorporation of [3H]leucine into trichloroacetic acid-insoluble cellular protein), and under these conditions, insulin induced a 4-fold increase in protein synthesis (Fig. 1B). These data indicate that GR activation causes serum-starved mouse L cells to enter a metabolically quiescent, long lived state. Under these conditions, insulin overrides the effects of glucocorticoids on both cell survival and protein synthesis.

To test the hypothesis that glucocorticoids and insulin might have opposing effects on metabolic gene expression, global gene expression was examined using oligonucleotide microarrays. Serum-starved L cells were incubated for 54 h in the absence or presence of dexamethasone, or 54 h in the presence of dexamethasone with insulin also present for the final 6 h of incubation. To identify mRNA transcripts that may have important roles in metabolism, the analysis focused on transcripts that were subject to opposing regulation by dexamethasone and insulin. Fig. 1C describes mRNA transcripts that were either repressed ≥50% by dexamethasone and then induced ≥2-fold by insulin (class I transcripts, which consisted of 54 mRNAs) or induced ≥2-fold by dexamethasone and then repressed ≥50% by insulin (class II transcripts, which consisted of 82 mRNAs). The complete microarray data has been deposited in GEO (Gene Expression Omnibus), and the complete list of class I and II transcripts is provided in supplemental Table 1.

Many of the class I transcripts encoded proteins with clear roles in anabolism, including 4 amino acid transporters, 5 amino acid biosynthetic enzymes, 4 aminoacyl-tRNA synthetases, as well as 12 proteins required for lipid synthesis or uptake. In contrast, several of the class II transcripts encoded proteins with established roles in catabolism and the cellular response to starvation, including Atrogin-1, MuRF1, and PDK4, as well as the mRNA encoding glutamine synthetase (Glul). Thus, dexamethasone induced catabolic gene expression, while suppressing anabolic gene expression; and insulin overcame the catabolic effects of dexamethasone, shifting the balance back toward anabolic gene expression. The 13 class I transcripts encoding amino acid transporters, amino acid biosynthetic enzymes, or aminoacyl-tRNA synthetases were particularly interesting, as this group of mRNAs was not previously known to be regulated by insulin or glucocorticoids.

Glucocorticoids Repress Expression of ATF4 and mRNAs Involved in Amino Acid and Protein Anabolism—Of the 13 class I transcripts involved in amino acid and protein anabolism, 7 are known to be induced through the actions of ATF4 when cells are deprived of amino acids (12). Moreover, all of the genes encoding these 13 class I transcripts contained potential ATF-binding sites (shown in supplemental Table 2). This suggested the hypothesis that ATF4 might be required for expression of these mRNAs and thus that glucocorticoids might exert an inhibitory effect on ATF4. To test this hypothesis, mRNA levels were measured using quantitative real time RT-PCR, and...
ATF4 protein levels were analyzed by immunoblot. Fig. 2A shows that dexamethasone repressed levels of two representative class I transcripts (encoding asparagine synthetase (Asns) and phosphoserine aminotransferase 1 (Psat1)) and also repressed levels of ATF4 mRNA and protein. The effect of dexamethasone occurred at low concentrations, approximating a repressed levels of ATF4 mRNA and protein. The effect of dexamethasone on ATF4 mRNA and ATF4-dependent mRNAs was slow, requiring 48 h for maximal effect, and was temporally dissociated from the induction of PDK4 mRNA, which was maximal within 12–24 h of dexamethasone treatment (Fig. 2B). Taken together, these data indicate that GR activation suppresses ATF4 and ATF4-dependent mRNAs and induces catabolic mRNAs, provided that anabolic stimuli (such as insulin or serum) are not present.

**ATF4 Is Required for the Expression of mRNAs Involved in Amino Acid and Protein Anabolism**—To determine whether ATF4 might be required for the expression of mRNAs involved in amino acid and protein anabolism, RNAi was used to knockdown ATF4 expression. In cells that were incubated in serum-free medium lacking dexamethasone, an siRNA targeting the coding region of ATF4 mRNA mimicked the effect of dexamethasone, reducing levels of ATF4 mRNA and protein as well as 13 mRNA transcripts encoding amino acid biosynthetic enzymes, amino acid transporters, or aminoacyl-tRNA synthetases (Fig. 3A). In contrast, the siRNA targeting ATF4 did not significantly alter levels of control transcripts (desmin and mammalian target of rapamycin (mTOR)), mRNAs encoding PDK4 and Glul (class II transcripts), mRNA encoding glutamine dehydrogenase (Glud1) (another amino acid biosynthetic enzyme), or mRNAs encoding the low density lipoprotein receptor (LDL-R) and hydroxymethylglutaryl-CoA reductase (HMGCGR) (two representative class I transcripts involved in lipid anabolism). Similar results were obtained...
using an siRNA targeting the 5′-untranslated region of the ATF4 mRNA transcript (not shown). siRNA was also used to test the role of a closely related basic leucine zipper transcription factor, ATF5, which was encoded by a class I transcript and has been shown to activate a reporter construct containing the asparagine synthetase gene promoter (26). The siRNA targeting ATF5 produced >80% knockdown of ATF5 mRNA, but there was no effect on either ATF4 or ATF4-dependent mRNAs (not shown). These data indicate that ATF4 is required for expression of mRNAs involved in amino acid and protein anabolism and that glucocorticoids repress these mRNAs at least in part by decreasing ATF4 expression.

In mouse L cells, the following eight amino acids are nonessential: Glu, Gln, Asp, Ala, Ser, Gly, and Pro (27). The ATF4-dependent transcripts encode seven enzymes (Asns, Psat1, Phgdh, Psph, Shmt2, Mthfd2, and Pycr1) necessary for the synthesis of four of these nonessential amino acids (Asn, Ser, Gly, and Pro) (Fig. 3B). The ATF4-dependent transcripts also encode three amino acid transporters (Slc1a4, Slc7a1, Slc7a5) sufficient for the uptake of the 12 essential amino acids (28) (Fig. 3B). This suggested the hypothesis that ATF4 expression might be required to maintain intracellular levels of Asn, Ser, Gly, Pro, and the 12 essential amino acids. To test this idea, ATF4 expression was reduced using RNAi, and intracellular free amino acids were measured using ion exchange chromatography. This technique measures levels of 19 amino acids (excluding Trp), although Asn is not distinguished from Asp, and Gln is not distinguished from Glu.

Fig. 3C shows that the siRNA targeting ATF4 reduced levels of Gly, Ser, and Pro, as well as levels of 10 essential amino acids. The levels of Cys were too low to be accurately measured. Levels of Ala, and the combined levels of Asn and Asp and of Gln and Glu were not significantly altered. The net effect of ATF4 repression was to reduce levels of total and essential amino acids by 21 and 30%, respectively (Fig. 3C, right panel). Taken together, these data indicate that glucocorticoids repress ATF4 expression, which is required for the expression of anabolic mRNAs needed to maintain intracellular levels of most amino acids.

**Insulin Overrides the Repressive Effect of Glucocorticoids on ATF4 and Downstream mRNAs**—The microarray studies indicated that 13 mRNAs involved in amino acid and protein anabolism were not only repressed by dexamethasone but were also induced when insulin was added to dexamethasone-treated cells (Fig. 1C). This suggested the hypothesis that insulin might override the repressive effect of dexamethasone on ATF4, thereby increasing levels of these mRNAs. In support of this hypothesis, insulin increased levels of both ATF4 mRNA and protein in dexamethasone-treated cells (Fig. 4A). In the absence of dexamethasone, insulin had little effect on ATF4 mRNA or protein, presumably because levels were already high (Fig. 4A).

Fig. 4B shows a time course study examining the effect of insulin in dexamethasone-treated L cells. At early time points (2–4 h), insulin decreased PDK4 mRNA but increased ATF4 mRNA and global protein synthesis. This was followed by an increase in ATF4-dependent mRNAs (encoding asparaginate synthetase and Psat1), which began to rise after 4–6 h of insulin treatment. Although protein synthesis and ATF4 mRNA levels remained relatively stable over the next 18 h of insulin treatment, levels of ATF4-dependent mRNAs continued to accumulate. The effects of insulin on ATF4 and ATF4-dependent mRNAs occurred at low physiologic concentrations of insulin (Fig. 4C). IGF-1 had an identical effect on ATF4 mRNA and protein levels (not shown). The EC50 for both insulin and IGF-1 was <3 nm, suggesting that their effects were mediated through the insulin and IGF-1 receptors, respectively. siRNA was used to determine whether insulin-mediated induction of these mRNAs required ATF4 (Fig. 4D). The siRNA targeting ATF4 did not alter levels of control transcripts or prevent insulin-mediated repression of PDK4 mRNA, but it blocked the insulin-mediated increase in mRNAs involved in amino acid and protein anabolism. Thus, these data suggest that insulin increases levels of these anabolic mRNAs by increasing ATF4 expression.

**mTORC1 Mediates Effect of Insulin on ATF4 and Downstream mRNAs**—The positive effect of insulin on protein synthesis requires the mammalian target of rapamycin complex 1 (mTORC1), a ubiquitously expressed protein kinase complex (29, 30). Rapamycin, a highly specific chemical inhibitor of mTORC1 (31), was used to test whether mTORC1 might be part of a signaling pathway that links insulin to ATF4. Fig. 5A shows that rapamycin reduced levels of ATF4 mRNA and protein and ATF4-dependent mRNAs in serum-starved mouse L cells, thus mimicking the effect of glucocorticoids. Moreover, in dexamethasone-treated L cells, rapamycin blocked the insulin-mediated increase in ATF4 mRNA, ATF4 protein, and ATF4-dependent mRNAs (Fig. 5B). These data indicate that activity of mTORC1 is required for high levels of expression of ATF4 and ATF4-dependent mRNAs.

Mouse embryonic fibroblasts (MEF cells) and C2C12 mouse myotubes were used to test whether ATF4 might be a downstream target of mTORC1 in other cell lines. When MEF cells are deprived of serum, mTORC1 is less active (32), and under these conditions, only a small amount of ATF4 protein was present (Fig. 6A). The addition of serum, which is known to increase mTORC1 activity (32), increased levels of ATF4 mRNA and protein, as well as levels of several representative mRNA transcripts that were found to be ATF4-dependent in mouse L cells. The serum-dependent increases in ATF4 and downstream transcripts were blocked by rapamycin, indicating a dependence on mTORC1 activity.

In contrast to mouse L cells and MEF cells, C2C12 myotubes represent a differentiated, nonproliferating cell type that is used as a model of skeletal muscle. In C2C12 myotubes, insulin/IGF-1 signaling induces protein synthesis and myotube hypertrophy, and these effects are blocked by rapamycin (3–4, 33). Fig. 6B shows that insulin increased levels of nuclear ATF4 protein in myotubes, and the effect was blocked by rapamycin. Fig. 6C shows the effect of insulin/IGF-1 signaling on mRNA levels. IGF-1 increased levels of mRNAs encoding ATF4 and all of the mRNAs that were ATF4-dependent in mouse L cells. This effect was also blocked by rapamycin. Taken together, these data suggest that the expression of ATF4 and ATF4-dependent mRNAs may be a generalized effect of mTORC1 activation.

**Regulation of ATF4 by Glucocorticoids and Insulin**
DISCUSSION

These studies identify ATF4 as an anabolic transcription factor that is subject to opposing regulation by glucocorticoids and insulin. The finding that ATF4 is required for expression of mRNAs encoding amino acid biosynthetic enzymes, amino acid transporters, and aminoacyl-tRNA synthetases is not surprising. It is well established that levels of many of these mRNAs are increased in a cell autonomous manner when cells are deprived of amino acids and that ATF4 is required for this increase (12–15). However, it was not previously known that ATF4 and the downstream mRNAs comprise an anabolic program regulated by hormones that mediate the mammalian responses to fasting and feeding (glucocorticoids and insulin, respectively). This suggests an important role for ATF4 in anabolism and, more specifically, in protein synthesis and cell growth.

The data are consistent with the model shown in Fig. 7. Under catabolic conditions, the presence of glucocorticoids and the absence of insulin signaling reduce ATF4 expression. Because ATF4 is required for the expression of mRNAs encoding amino acid transporters, amino acid biosynthetic enzymes, and aminoacyl-tRNA synthetases, levels of these mRNAs fall. Conversely, under anabolic conditions, insulin signaling overrides the effect of glucocorticoids, thereby increasing levels of ATF4 and the downstream mRNAs. Thus, insulin and glucocorticoids regulate anabolic mRNAs for amino acid and protein anabolism through combined effects on the GR and ATF4. This contrasts with the mechanism by which catabolic mRNAs are regulated, through effects of insulin and glucocorticoids on the GR and Foxo proteins (3–8).

Importantly, the effect of insulin on ATF4 occurs in the continued presence of glucocorticoids, which is consistent with the opposing yet interdependent yin-yang relationship of glucocorticoids and insulin; by promoting catabolism and inhibiting anabolism, physiologic concentrations of glucocorticoids prime cells for insulin, which dominantly reverses the effects of glucocorticoids on metabolic gene expression (11). This priming effect of glucocorticoids is also seen in vivo. A common

FIGURE 4. Effect of insulin on ATF4 and downstream mRNAs. A, mouse L cells were incubated in serum-free medium in the absence or presence of dexamethasone (Dex) (100 nM) for 48 h and then for an additional 6 h in the absence or presence of insulin (100 nM). Following incubation, nuclear protein extracts were analyzed by immunoblot using the anti-ATF4 polyclonal antibody, and total cellular RNA was analyzed by qPCR. ATF4 mRNA levels were normalized to the ATF4 mRNA level obtained in the absence of dexamethasone and insulin, which was arbitrarily set at 1. B, mouse L cells were preincubated for 48 h in serum-free medium containing dexamethasone (100 nM) and then for varying times in the absence or presence of insulin (100 nM), as indicated. Total cellular RNA was analyzed by qPCR, and transcript levels in the presence of insulin were normalized to the transcript levels in cells incubated in the absence of insulin and harvested at the same time point, which were arbitrarily set at 1. Parallel dishes of identically treated cells received [3H]leucine for the final 2 h of incubation. Following incubation, these dishes of cells were harvested for measurement of [3H]leucine contained in acid-insoluble cellular fractions, which was normalized in the same way that mRNA levels were normalized. C, cells were preincubated for 48 h in serum-free medium containing dexamethasone (100 nM) and then for an additional 6 h in the presence of varying concentrations of insulin, as indicated. Following incubation, total cellular protein extracts were analyzed by immunoblot using the anti-ATF4 polyclonal antibody, and total cellular RNA was analyzed by qPCR. Transcript levels were normalized to the transcript levels in cells incubated in the absence of insulin, which were arbitrarily set at 1. D, mouse L cells were transfected with the indicated siRNA duplex, then incubated for 48 h in serum-free medium in the presence of dexamethasone (100 nM), followed by an additional 8 h of incubation in the absence or presence of insulin (100 nM), as indicated. Total cellular protein extracts were analyzed by immunoblot using the anti-ATF4 polyclonal antibody, and total cellular RNA was analyzed by qPCR. Transcript levels were normalized to the transcript levels in control cells (nontransfected cells incubated in the absence of insulin).
FIGURE 5. Effect of rapamycin on ATF4 and downstream mRNAs. A, mouse L cells were preincubated for 48 h in serum-free medium and then for an additional 8 h in the absence of rapamycin (100 nM), as indicated. Total cellular protein extracts were analyzed by immunoblot using the anti-ATF4 polyclonal antibody, and total cellular RNA was analyzed by qPCR. Transcript levels were normalized to the transcript levels in cells incubated in the absence of insulin (100 nM) and/or rapamycin (100 nM), as indicated. Total cellular protein extracts were analyzed by immunoblot using the anti-ATF4 polyclonal antibody, and total cellular RNA was analyzed by qPCR. Transcript levels were normalized to the transcript levels in cells incubated in the absence of rapamycin, which were arbitrarily set at 1.

FIGURE 6. Regulation of ATF4 and downstream mRNAs by mTORC1 in other cell types. A, MEF-1 cells were preincubated in serum-free medium for 24 h and then for an additional 6 h in the absence or presence of 10% fetal bovine serum and/or rapamycin (20 nM), as indicated. Total cellular protein extracts were analyzed by immunoblot using the anti-ATF4 polyclonal antibody, and total cellular RNA was analyzed by qPCR. Transcript levels were normalized to the transcript levels in cells incubated in the absence of serum and rapamycin, which were arbitrarily set at 1. B, mouse C2C12 myotubes were preincubated in serum-free medium for 24 h and then for an additional 24 h in the absence or presence of rapamycin (100 nM) and/or insulin (100 nM), as indicated. Total cellular protein extracts were analyzed by immunoblot using the anti-ATF4 polyclonal antibody. C, C2C12 myotubes were preincubated in serum-free medium for 24 h and then for an additional 24 h in the absence or presence of rapamycin (100 nM) and/or IGF-1 (10 nM), as indicated. Total cellular RNA was analyzed by qPCR. Transcript levels were normalized to the transcript levels in cells incubated in the absence of IGF-1 and rapamycin, which were arbitrarily set at 1.

FIGURE 7. Model describing the regulation of ATF4 by glucocorticoids and insulin. A.A., amino acids.

practice in the study of insulin action in whole animals is to first subject the animals to a period of fasting prior to re-feeding, which then induces an overshoot of insulin secretion and action (34). However, this overshoot response to re-feeding is absent not only in insulin-deficient animals but also in animals that lack glucocorticoids. In adrenalectomized animals, the overshoot response is restored by the administration of exogenous glucocorticoids (34).

One of the functional consequences of ATF4 expression is to maintain intracellular amino acid levels. Intracellular amino acid levels are tightly regulated through a complex interplay of several processes, including the synthesis of nonessential amino acids; the import, export, and degradation of essential and nonessential amino acids; and protein synthesis and degradation. The ATF4-dependent mRNAs identified through the current RNAi experiments in mouse L cells encode three amino acid transporters that together are sufficient for the cellular uptake of all essential amino acids and the seven enzymes involved in the synthesis of the nonessential amino acids Asp, Gly, Ser, and Pro. Thus, the ATF4-dependent mRNAs appear to directly regulate only 16 of the 20 amino acids. This suggests the interesting possibility that regulation of the remaining four amino acids (Gln, Glu, Asp, and Ala) is uncoupled from the regulation of all of the other amino acids. Two additional pieces of data support this hypothesis. First, reduction of ATF4 expression specifically reduced free intracellular levels of Ser, Gly, Pro and the essential amino acids, with no discernable regulation of all of the other amino acids. Two additional pieces of data support this hypothesis. First, reduction of ATF4 expression specifically reduced free intracellular levels of Ser, Pro and the essential amino acids, with no discernable regulation of all of the other amino acids. Second, the mRNA encoding one amino acid biosynthetic enzyme, Glul, was increased by glucocorticoids and repressed by insulin, and thus was reciprocally related to expression of the ATF4-dependent enzymes.

Why might Glul be up-regulated by glucocorticoids and down-regulated by insulin? Glul plays at least two key roles in catabolism, when peripheral cells break down protein and amino acids as a source of energy. First, by conjugating NH4+ to Gln, Glul removes free NH4+ that is generated when amino acids are catabolized. Second, Glul generates Gln, which is then exported into the circulation and taken up in the liver or kidney. In liver and kidney, the amino groups of Gln contribute to the formation of urea or urinary NH4+, respectively, whereas the
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carbon skeletons of Glu may be used as an energy source or to generate glucose via gluconeogenesis. Ala plays a similar role under catabolic conditions. On the other hand, perhaps the ATF4-dependent amino acids (Asn, Gly, Ser, Pro, and the 12 essential amino acids) are kept at a lower level under catabolic conditions to limit protein synthesis and cell growth.

The model in Fig. 7 also shows that the effect of insulin on ATF4 is mediated through mTORC1, a protein kinase complex with a ubiquitous and essential role in protein synthesis and cell growth. The molecular pathways downstream of mTORC1 are just beginning to be defined (29, 30). To date, the best characterized downstream substrates of mTORC1 are p70 S6 kinase 1 and the tumor-suppressor 4EBP1 (eukaryotic translation initiation factor 4E-binding protein 1); phosphorylation of these two proteins by mTORC1 rapidly stimulates ribosomal translation of mRNAs (29, 30). But other downstream targets certainly exist, and the current data suggest that ATF4 is one of them. This idea is also supported by previous microarray analysis of interleukin-stimulated lymphocytes, in which the mTORC1 inhibitor rapamycin repressed expression of mRNAs encoding amino acid transporters, amino acid biosynthetic enzymes, and aminoacyl-tRNA synthetases (35). Thus, the existing data suggest that these mRNAs (and likely ATF4) are generalized downstream targets of mTORC1. Regulation of ATF4 allows mTORC1 to couple the uptake and synthesis of amino acids and synthesis of charged tRNAs (mediated through ATF4) to global mRNA translation (mediated through regulation of p70 S6 kinase 1 and 4EBP1).

It is perhaps important to note that insulin (and thus mTORC1 activation) increased protein synthesis and ATF4 mRNA prior to the induction of ATF4-dependent mRNAs. By increasing ATF4 expression, mTORC1 sets in motion a series of events that presumably allow cells to acquire and generate amino acids and aminoacyl-tRNAs needed for continued protein synthesis and cell growth.

An unresolved question is how mTORC1 activity increases levels of ATF4 protein. The current data indicate that at least part of the mTORC1 effect on ATF4 is mediated at the mRNA level, either through stabilization or increased transcription of ATF4 mRNA. mTORC1 might also have a post-transcriptional effect on ATF4, which inhibit global, nonspecific mRNA translation but paradoxically promote ATF4 mRNA translation via an internal ribosomal entry site in the ATF4 transcript.

In these current studies, insulin increased ATF4 expression in association with an increase in global protein synthesis. Because elF2α kinases increase ATF4 but decrease global protein synthesis, this finding may suggest that elF2α kinases and mTORC1 increase ATF4 protein levels by different mechanisms. However, it also leaves open the possibility that mTORC1 activity, by increasing global protein synthesis, might induce a state of relative amino acid deprivation or endoplasmic reticulum stress within cells, which then activates the elF2α kinase-dependent pathway for ATF4 mRNA translation. Consistent with this idea, amino acid deprivation, like insulin, not only increases ATF4 translation but also increases ATF4 mRNA levels (39).

The mechanism by which glucocorticoids decrease ATF4 mRNA and protein levels is also not yet clear. The simplest interpretation of the current data is that glucocorticoids inhibit mTORC1 activity, a conjecture that is supported by the recent finding that glucocorticoids induce expression of Redd-1, a protein known to inhibit mTORC1 activity (40).

Although the current data indicate that ATF4 is required for expression of anabolic mRNAs encoding amino acid transporters, amino acid biosynthetic enzymes, and aminoacyl-tRNA synthetases, it is not yet known if ATF4 alone is sufficient. For example, the induction of Slc7a1 mRNA under conditions of amino acid deprivation appears to require a heterodimeric complex of ATF4 and C/EBPβ (another basic leucine zipper family member) (41). C/EBPβ also plays an important role in the induction of the asparagine synthetase gene under conditions of amino acid deprivation (42). It will be important to determine whether C/EBPβ or another factor (or insulin-mediated event) might also be required for the effect of ATF4 on anabolic mRNAs.

The current studies have focused on how hormones regulate ATF4 and mRNAs for amino acid and protein anabolism. However, the mouse L cell system might also be useful for the study of other pathways and processes that are regulated by glucocorticoids and insulin. For instance, in mouse L cells, hormonal control of metabolic processes is closely linked to cell survival. Physiologic concentrations of glucocorticoids dramatically increased cell survival, while inducing transcriptional changes that are suggestive of a fasting response (induction of catabolic genes and repression of anabolic genes). Moreover, all of these effects of glucocorticoids on cell survival and metabolic gene expression in mouse L cells were blocked by insulin signaling, which also has the effect of preventing longevity in whole animals (43). Although the effect of glucocorticoids on cell survival was unexpected, it is perhaps not surprising in retrospect. Because of their anti-anabolic and catabolic effects, glucocorticoids are essential for mammalian survival when caloric intake is limited, and are thus 2–3-fold elevated when mammal are subjected to caloric restriction, an intervention that induces metabolic quiescence and longevity by an undetermined mechanism (44–46). This raises the question of whether glucocorticoids might mediate at least some of the life span-promoting effects of caloric restriction in mammals, and further studies focused on this possibility might help us understand the relationships between caloric intake, hormones, metabolism, and life span.

Acknowledgments—I thank Sharon Malmberg, Phil Karp, Tammy Nesselhauf, Jeffrey Blumberg, Debbie Morgan, Lisa Beatty, and Norma Anderson for excellent technical assistance; Drs. Michael Brown, Joseph Goldstein, Daniel Foster, Guoxen Chen, Yuri Bashmakov, Michael McPhaul, and David Price for helpful discussions; Drs. Michael Welsh, Peter Snyder, Chris Benson, Jennifer Stern, David Motto, Andrew Norris, and Jay Horton for helpful discussions and critical review of the manuscript; and the Molecular Analysis Core Facility at the University of Iowa for analysis of amino acid levels.
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