ATF4 is a transcription factor that induces a genetic program for amino acid synthesis and amino acid uptake. Previous work demonstrated that ATF4 expression is increased either by insulin or by the general amino acid control (GAAC) response, an evolutionarily ancient pathway that is activated when eukaryotic cells are deprived of amino acids. It is not known whether insulin and the GAAC pathway increase ATF4 expression by the same or different mechanisms. In these studies, we demonstrate that insulin-mediated ATF4 expression occurs as part of a coordinated anabolic program that does not require an essential component of the GAAC pathway, the protein kinase GCN2. Moreover, insulin and the GAAC pathway have an additive effect on expression of ATF4 and downstream mRNAs for amino acid synthesis and uptake. These data suggest that the GAAC pathway may facilitate insulin-mediated anabolism when exogenous amino acids are limiting. We conclude that insulin signaling and the GAAC response comprise two distinct yet complimentary pathways to ATF4 expression, allowing anabolism to be finely tuned to amino acid availability.

In mammals, changes in nutrient availability induce changes in levels of metabolic hormones, which in turn orchestrate the metabolism of trillions of cells, allowing them to cooperate and function as a single organism (1, 2). Conversely, in unicellular organisms, metabolism is largely governed by nutrient-sensing pathways, which sense changes in nutrient availability and generate an adaptive, cell autonomous metabolic response (3). Interestingly, many nutrient-sensing pathways of unicellular eukaryotes have been retained in higher organisms such as mammals; one such nutrient-sensing pathway that is highly conserved is the general amino acid control (GAAC) pathway, which senses amino acid deficiencies and restores intracellular amino acid levels (4). The GAAC pathway was originally described in yeast and was later found to be present in mammalian cells (4–11).

Several molecular aspects of the GAAC pathway are well defined (4); amino acid deprivation increases levels of uncharged tRNAs, which bind to and activate the kinase GCN2. GCN2 phosphorylates the eukaryotic translation initiation factor 2α (eIF2α), thereby inhibiting global protein synthesis but increasing synthesis of a few specific proteins. One such protein that is increased under these conditions is a basic leucine zipper transcription factor that activates a large panel of genes encoding amino acid transporters and amino acid biosynthetic enzymes. In yeast, this transcription factor is referred to as GCN4 (4); the mammalian counterpart is activating transcription factor 4 (ATF4, also known as CREB2 (5–11)). In addition to stimulating ATF4 mRNA translation, amino acid deprivation increases levels of GCN4 or ATF4 mRNA (4, 6). The end result of the GAAC pathway is activation of genes that correct amino acid deficiencies and restore homeostasis.

ATF4 is also subject to opposing regulation by hormones that play a central role in regulating mammalian metabolism (12). Glucocorticoids (catabolic hormones that are required for mammalian survival during prolonged fasting or stress) decrease levels of ATF4 mRNA and protein, leading to the repression of ATF4-dependent mRNAs encoding amino acid transporters and amino acid biosynthetic enzymes. Glucocorticoid-mediated repression of ATF4 is coupled with inhibition of global mRNA translation and protein synthesis and the activation of genes for protein catabolism. Conversely, insulin (an anabolic hormone) overcomes all of these effects of glucocorticoids. Thus, insulin increases levels of ATF4 mRNA, ATF4 protein, ATF4-dependent mRNAs, and global protein synthesis, and insulin represses genes for protein catabolism. Taken together, these data suggest that ATF4 mediates a portion of the coordinated anabolic response to insulin and that ATF4 is repressed as part of the coordinated catabolic response to glucocorticoids. Indeed, the in vivo role of ATF4 in mammalian anabolism has been established through studies of ATF4-deficient mice, which exhibit pleiotropic growth defects (13–16), at least some of which are rescued by a high protein diet (17).

These considerations led us to ask the following question. What is the relationship between evolutionarily ancient nutrient-sensing pathways and the hormonal signaling pathways of multicellular organisms? With regards to ATF4, one possibility is that glucocorticoids and insulin regulate the GAAC pathway to control ATF4 and genes needed for amino acid synthesis and uptake. For example, insulin-mediated protein synthesis might induce a state of amino acid depletion within cells, thereby acti-
Regulation of ATF4 by Insulin and Amino Acid Deprivation

**A** **INCUBATION PROTOCOL**

| SERUM-STARE | +/− DEX | ADD INSULIN | OR HISTIDINOL | 48 h | 6 h | ASSAY |

**B** **ATF4 IMMUNOBLOT**

| DEX | INSULIN | HISTIDINOL | LANE | 1 | 2 | 3 | 4 |

**C** **mRNA EXPRESSION IN DEX-TREATED CELLS**

| mRNA LEVEL (RELATIVE TO DEX ALONE) | ATF | Psat1 | Asns | Mthfd2 | Slc7a5 | Ldl-R | Hmgcoar | Atrogin-1 | PDK4 | ACC | FAS | Protein & Lipid Catabolism |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EFFECT OF INSULIN | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| EFFECT OF HISTIDINOL | + | + | + | + | + | + | + | + | + | + | + | + |

**TABLE 1.** Histidinol overcomes glucocorticoid-mediated repression of ATF4 and ATF4-dependent mRNAs. A, incubation protocol to examine the effects of dexamethasone (Dex), insulin, and histidinol. B, mouse L cells were preincubated for 48 h in medium C in the absence or presence of dexamethasone (10 μM) and then for an additional 6 h in the absence or presence of insulin (100 nM) or histidinol (2 mM), as indicated. In this and all other experiments, histidinol or insulin was added directly to the existing cell culture medium (in the continued presence of dexamethasone). Following incubation, nuclear protein extracts were analyzed by immunoblot using the anti-ATF4 polyclonal antibody. The data shown are representative of three independent experiments. C, mouse L cells were preincubated for 48 h in medium C containing dexamethasone (10 μM) and then for an additional 6 h in the absence or presence of insulin (100 nM) or histidinol (2 mM), as indicated. Following incubation, total cellular RNA was analyzed by qPCR, and transcript levels were normalized to the transcription levels in cells incubated in the absence of histidinol and insulin, which were set at 1. The representative mRNAs involved in amino acid synthesis or uptake encode, from right to left: ATF4; asparagine synthetase (Asns); phosphoserine aminotransferase 1 (Psat1); methylenetetrahydrofolate dehydrogenase (NAD+ dependent); methylenetetrahydrofolate cyclohydrolase (Mthfd2); the Slc1a4, solute carrier family 1, member 4 (also known as ASCT1) (Sli1a4); the solute carrier family 7, member 1 (also known as CAT-1) (Sli7a1); and the solute carrier family 7, member 3 (also known as LAT-1) (Sli7a3). The representative mRNAs involved in protein or lipid catabolism encode, from right to left: atrogin-1 and pyruvate dehydrogenase kinase, isoenzyme 4 (PDK4). B and C, the data shown are representative of three independent experiments.

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Regulation of ATF4 by Insulin and Amino Acid Deprivation

**RESULTS**

**Activation of the GAAC Pathway by Histidinol Overcomes Glucocorticoid-mediated Repression of ATF4**—Mouse L fibroblasts are an immortalized cell line with a previously defined response to glucocorticoids and insulin (12, 26). When mouse L cells are incubated in serum-free medium containing glucocorticoids, they enter a metabolically quiescent state where expression of ATF4 and ATF4 target genes is repressed. Glucocorticoid-mediated repression of ATF4 is coupled with repression of...
Regulation of ATF4 by Insulin and Amino Acid Deprivation

ATF4-independent anabolic genes for lipid synthesis and uptake and the induction of catabolic genes for protein and lipid breakdown (12). Under these conditions, a physiologic concentration of insulin dominantly overrides the effects of glucocorticoids, thus repressing catabolic genes and inducing expression of ATF4 and both ATF4-dependent and ATF4-independent anabolic genes (12).

To test the hypothesis that activation of the GAAC pathway might overcome the repressive effect of glucocorticoids on ATF4, we used histidinol, an amino alcohol that competitively inhibits histidinyl-tRNA synthetase, thereby increasing levels of uncharged tRNAHis and activating GCN2 (7, 27). Our incubation protocol is shown in Fig. 1A; mouse L cells were incubated in the absence or presence of dexamethasone for 48 h followed by an additional 6-h incubation in the absence or presence of either insulin or histidinol, which was directly added to the cell culture medium in the continued presence of dexamethasone.

In the absence of dexamethasone, ATF4 was expressed at a high level (Fig. 1B, lane 1), and neither insulin (12) nor histidinol (supplemental Fig. 1) increased levels of ATF4 or ATF4-dependent mRNAs. Dexamethasone reduced expression of ATF4 (Fig. 1B, lane 2), and under these conditions, histidinol, like insulin, increased ATF4 protein levels (Fig. 1B, lanes 3 and 4). In contrast to insulin (Fig. 1C, left side), histidinol did not increase levels of mRNAs involved in lipid synthesis and uptake nor decrease levels of mRNAs involved in protein or lipid breakdown (Fig. 1C, right side). Rather, histidinol specifically increased the mRNA encoding ATF4, which was followed by an increase in ATF4-dependent mRNAs encoding amino acid biosynthetic enzymes and amino acid transporters (Fig. 2A).

FIGURE 3. Effect of dexamethasone on intracellular free amino acids, pre-tRNAs and global protein synthesis. A–C, mouse L cells were incubated for 48 h in medium C containing dexamethasone (Dex; 10 nM). A, following incubation, free amino acids in cell lysates were measured using ion exchange chromatography and then normalized to the total protein concentration in each sample and normalized to the level in cells incubated in the absence of dexamethasone, which were set at 1. The data shown represent the sum of all measured species of amino acids. Dexamethasone reduced the pooled levels of essential amino acids by 77% and nonessential amino acids by 16% (p ≤ 0.03). Levels of individual species of amino acids are found in supplemental Fig. 2. B, following incubation, pre-tRNA levels were analyzed by qPCR and normalized to the 18S mRNA to determine the levels of all species of pre-tRNAs in cells incubated in the absence of dexamethasone, which were set at 1. C, [3H]leucine was present for the final 2 h of incubation. Following incubation, the amount of [3H]leucine contained in acid-insoluble cellular fractions was measured, normalized to the total protein concentration in each sample, and then normalized to the level in cells incubated in the absence of dexamethasone, which was set at 1. A–C, data represent the average values obtained from at least three independent experiments. Error bars represent S.E., and asterisks indicate a significant change (p < 0.04 by unpaired, two-tailed t test) in cells treated with dexamethasone.
Insulin Coordinately Increases Intracellular Amino Acids and tRNA Synthesis—Insulin opposes the repressive effects of glucocorticoids on protein synthesis (2) and on ATF4 and ATF4-dependent mRNAs (12) (Fig. 1). Thus, we hypothesized that insulin might also overcome the repressive effect of glucocorticoids on levels of intracellular amino acids and pre-tRNAs. Fig. 4, A–C, shows that insulin coordinately increased levels of free amino acids, pre-tRNAs, and global protein synthesis in dexamethasone-treated cells. In contrast, histidinol increased levels of amino acids (Fig. 4A) but did not increase levels of pre-tRNAs or protein synthesis (Fig. 4, B and C). These data are consistent with the notion that insulin has a generalized anabolic effect that coordinately increases ATF4, amino acids, tRNAs, and protein synthesis, whereas the GAAC pathway increases ATF4 and inhibits global protein synthesis to restore intracellular amino acid levels.

Insulin Increases ATF4 Expression Independently of the GACC Pathway—We reasoned that if insulin and histidinol utilized the same pathway to increase ATF4, then insulin should not increase ATF4 in the presence of a maximal dose of histidinol. On the other hand, if insulin and histidinol increased ATF4 by different mechanisms, then the two compounds might have an additive or synergistic effect. Fig. 5, A and B, shows that insulin and histidinol had an additive effect on ATF4 mRNA levels and synergistic effects on levels of ATF4-dependent mRNAs. Moreover, as shown in Fig. 5C, silencing of GCN2 did not prevent the effect of insulin (top panel) but limited the combined effects of insulin and histidinol on ATF4 mRNA and ATF4-dependent mRNAs (bottom panel). These data suggest that there are two distinct pathways to ATF4 expression: a nutrient-sensing pathway mediated by GCN2 and an anabolic, hormonal signaling pathway that is controlled by insulin.

DISCUSSION

Insulin inhibits several effects of glucocorticoids that comprise an integrated response to starvation or stress (2, 28). Thus, insulin has a broad anabolic effect, repressing catabolic genes; inducing genes for lipid anabolism; stimulating global mRNA translation; inducing genes encoding tRNAs; and increasing expression of ATF4. Through ATF4, insulin induces genes for amino acid synthesis and uptake, thereby increasing levels of intracellular amino acids.

ATF4 is also regulated by the GAAC pathway, an evolutionarily ancient nutrient-sensing mechanism found in unicellular organisms that do not rely on hormones such as insulin to regulate metabolism. The GAAC pathway has a more limited effect than the insulin signaling pathway. Like insulin, the GAAC pathway overcomes the repressive effect of glucocorticoids on ATF4 mRNA and protein levels, thereby increasing intracellular amino acids. In contrast to insulin, the GAAC pathway requires GCN2 and does not repress catabolic gene expression, induce genes for lipid anabolism or tRNAs, or stimulate protein synthesis. Indeed, through mechanisms that are ATF4-independent, the GAAC pathway reduces global protein synthesis as part of the adaptive response to amino acid deprivation (4), and interestingly, leads to the repression of hepatic lipogenesis as well (29).

Importantly, the insulin signaling pathway increases ATF4 expression independently of the GAAC pathway. We hypothesize that these two distinct pathways coexist in mammals so that anabolism can be finely tuned to the amino acid content of the diet (Fig. 6). In our simplified model, mammalian nutrient intake is represented as one of two possibilities: a diet high in amino acids or low in amino acids. Either of these diets will stimulate insulin secretion and thus insulin signaling.

Insulin signaling coordinately increases synthesis of uncharged tRNAs and ATF4. ATF4 increases the synthesis of nonessential amino acids and the capacity of the cell to take up both nonessential and essential amino acids. However, cellular
uptake of amino acids also requires that amino acids are present in the extracellular environment.

If the diet contains a high level of amino acids, then the combination of insulin signaling, dietary amino acids, and insulin-mediated ATF4 expression will increase intracellular amino acids. Under these conditions, uncharged tRNAs will tend to be converted into charged tRNAs, the GAAC pathway (which is activated by uncharged tRNAs) will tend to remain inactive, and high levels of charged tRNAs will promote anabolism.

On the other hand, if the diet contains a low level of amino acids, intracellular amino acid levels will fall, leading to a rise in uncharged tRNA levels and activation of the GAAC pathway (4). Co-activation of the insulin signaling and GAAC pathways would enhance the expression of ATF4 and downstream amino acid transporters and amino acid biosynthetic enzymes. We hypothesize that the combined additions of insulin and histidinol mimic this scenario.

If the combined effects of the GAAC and insulin signaling pathways are sufficient to increase intracellular amino acids, then uncharged tRNAs would be converted to charged tRNAs, the GAAC pathway would be inactivated, and anabolism would proceed. Alternatively, if the intracellular amino acid deficiency cannot be overcome, then persistent GAAC pathway activity would inhibit anabolism (4, 29).

In summary, our data suggest that there are two biochemically distinct pathways to ATF4 expression, and we hypothesize that the GAAC pathway is active to varying degrees, depending on the amino acids that are available from the diet. The evolutionary conservation of ATF4 and the development of complex regulatory mechanisms to control ATF4 are consistent with the critical role of amino acids in cellular function. Our data suggest that in mammals, ATF4 is under dual control: by the ancient, cell autonomous GAAC pathway; and by signaling pathways that evolved later and brought key anabolic transcription factors such as ATF4 under control of metabolic hormones such as glucocorticoids and insulin.

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