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Negative Effects of the Amino Acids Lys, His, and Thr on S6K1 Phosphorylation in Mammary Epithelial Cells

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ABSTRACT

The role of essential amino acids (AA) on protein synthesis via the mTOR pathway was studied in murine mammary epithelial cells cultured under lactogenic conditions. Leu, Ile, and Val increased S6K1 phosphorylation compared to that measured in AA-deprived cells. Trp, Phe, and Met had no effect. Surprisingly, Lys, His, and Thr inhibited S6K1 phosphorylation in both murine and bovine mammary cells. Thr exhibited the most potent inhibition, being the only amino acid that competed with Leu's positive role. In non-deprived cells, there was no observable effect of Lys, His, or Thr on S6K1 phosphorylation at concentrations up to five times those in the medium. However, their addition as a mix revealed a synergistic negative effect. Supplementation of Lys, His, and Thr abrogated mTOR Ser 2448 phosphorylation, with no effect on Akt Ser 473—an mTORC2 target. This confirms specific mTORC1 regulation of S6K1 phosphorylation. The individual supplementation of Lys, His, and Thr maintained a low level of IRS-1 phosphorylation, which was dose-dependently increased by their combined addition. Thus, in parallel to inhibiting S6K1 activity, these AA may act synergistically to activate an additional kinase, phosphorylating IRS-1 via an S6K1-independent pathway. In cultures supplemented by Lys, His, and Thr, cellular protein synthesis decreased by up to 65%. A more pronounced effect was observed on β-casein synthesis. These findings indicate that positive and negative signaling from AA to the mTOR pathway, combined with modulation of insulin sensitization, mediate the synthesis rates of total and specific milk proteins in mammary epithelial cells. J. Cell. Biochem. 105: 1038–1047, 2008.

KEY WORDS: MAMMARY GLAND; β-CASEIN; TRANSLATION; 4E-BP1; S6K1; IRS-1; mTOR

In many cell types and tissues, individual amino acids (AA) affect a signaling pathway which parallels the insulin's downstream cascade to modulate rates and levels of protein synthesis. The diverse nutritional and hormonal effects are funneled to mTOR, a multidomain serine/threonine kinase that regulates a number of components in the initiation and elongation stages of translation. During evolution, mTOR became a center that merges the insulin-controlled IRS-phosphoinositide 3-kinase (PI3K) pathway, which encompasses TSC1, TSC2, and Rheb signaling, with specific nutrient-sensitive signaling that involves activation of a distinct class 3 PI3K [Nobukuni et al., 2005; Gulati and Thomas, 2007]. mTOR participates in two distinct multiprotein complexes: mTOR complex 1 (mTORC1), which is sensitive to the antifungal metabolite rapamycin, and mTORC2 which, under most conditions, is not [Loewith et al., 2002; Wullschleger et al., 2006]. mTORC2 regulates the actin cytoskeleton through Akt and PKC, while mTORC1 exclusively mediates cellular "nutrient-sensing." The mechanism by which AA signal mTORC1 and its downstream targets is largely unknown. However, manipulation of AA levels has been shown to affect its interaction with the associated protein raptor, which may serve as part of the regulatory process [Kim et al., 2002]. mTOR phosphorylates and activates 4E-BP1, a regulatory protein that binds to the mRNA cap-binding protein eIF4E: when 4E-BP1 is unphosphorylated, it inhibits cap-dependent translation by competitively blocking the binding of eIF4G to eIF4E [Gingras et al., 1999; Harris and Lawrence, 2003].

The ribosomal protein S6 kinase (S6K1) is another target of mTOR. Phosphorylation on Thr 389 activates S6K1, which then phosphorylates the ribosomal protein S6. This was thought to lead to the translation of 5′TOP mRNAs, which encode components of the translation apparatus and account for 15–20% of the total cellular mRNA. Recent findings, however, have put this assumption into question, suggesting that translation of 5′TOP mRNAs does not depend on S6K1 activity or phosphorylation [Pende et al., 2004; Ruvinsky et al., 2005; Wullschleger et al., 2006]. Nevertheless, the concept of exploiting S6K1 phosphorylation status as an indicator of

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the cell's inclination to shut down translation upon AA deficiency has been previously raised and validated [Hara et al., 1998; Moshel et al., 2006].

S6K1 activity has been reported to affect IRS-1 in a negative-feedback loop, by first inducing its phosphorylation and then inhibiting its function [Um et al., 2004; Tremblay et al., 2007]. Obesity-induced insulin resistance has been associated with elevated S6K1 activity, and $S6K1^{-/-}$ mice showed decreased phosphorylation of IRS-1 on Ser 307 and Ser 636/639 [Um et al., 2004]. In fact, IRS proteins contain more than 70 Ser/Thr residues that are potential targets for phosphorylation by multiple factors, transducing the inhibition of IRS activity in a multitude of different ways [Zick, 2005]. Thus, S6K1-mediated IRS-1 activity may enable the cell to control its anabolic functions under inconstant conditions of nutrient supply and additional stimuli.

AA-activated signaling towards protein translation through mTOR has been studied in various tissues and cell lines, including adipose tissue [Lynch et al., 2002; Um et al., 2004; Zhang et al., 2007], liver [Reiter et al., 2004], intestine [Corl et al., 2008], heart [Crozier et al., 2005; Vary and Lynch, 2006], and pancreas [Xu et al., 1998]. To this end, the muscle and muscle cells have been the most investigated targets for the AA-signaling effect on protein synthesis [Talvas et al., 2006; Deldicque et al., 2008; Stipanuk, 2007]. In those studies, branched-chain AA (BCAA), particularly Leu, were shown to induce translation initiation and eventually, protein synthesis. There is much less information on the contribution of other specific AA to this signaling pathway: Arg increases S6K1 phosphorylation in intestinal epithelial cells from rat [Ban et al., 2004] and piglet [Corl et al., 2008], as well as protein synthesis in the latter. Gln also increases translation initiation in rat intestinal epithelial cells [Nakajo et al., 2005] and confluent L6 cells [Iresjo et al., 2005], but was reported to reduce S6K1 and 4E-BP1 phosphorylation in muscle C_2C_{12} cells. The inhibitory effect of Gln on S6K1 phosphorylation could not overcome the stimulatory effect of Leu and did not result in altered levels of protein synthesis [Deldicque et al., 2008].

During lactation, epithelial cells in the mammary gland synthesize large amounts of proteins within relatively short intervals. In models of differentiated murine and bovine mammary epithelial cell lines stimulated for the production of milk proteins by lactogenic hormones, we were recently able to pinpoint translation as an individual process that mediates AA's, and particularly Leu's, stimulatory effects on total and specific milk-protein synthesis [Moshel et al., 2006]. In these cultures, AA deprivation or selective deprivation of Leu resulted in dephosphorylation of 4E-BP1 and S6K1 on Thr 389 but not on Ser 411. Consequently, there was a negative, protein-specific effect on β-lactoglobulin synthesis. Addition of Leu to 6-h AA-deprived cells specifically stimulated BLG synthesis from pre-existing mRNA, rapidly reversing the non-phosphorylated status of S6K1 and 4E-BP1. To search for additional stimuli and putative balances in the cultured mammary epithelial system that might regulate the translation and synthesis of proteins, we analyzed the role of other essential AA. Three of these, His, Lys, and Thr, were found to exert a negative effect on S6K1 and 4E-BP1 phosphorylation. As a mix they synergistically inhibited the synthesis of total proteins and of the specific milk protein β-casein. The underlying mechanism appears to involve suppression of mTORC1 activity and modulation of IRS-1 phosphorylation.

MATERIALS AND METHODS

CHEMICALS AND ANTIBODIES

Rapamycin and LY294002, obtained from Cell Signaling (Beverly, MA), were dissolved in DMSO and used at a final concentration of 100 ng/ml and 15 μg/ml, respectively. Antibodies to p4E-BP1, S6K1, pS6K1, eIF4E, mTOR, pmTOR, pAKT, IRS-1, and pIRS-1 were obtained from Cell Signaling. HRP-labeled anti-rabbit secondary antibodies were obtained from Zymed Laboratories (San Francisco, CA).

CELL CULTURE

Murine CID-9 mammary epithelial cells were grown in DMEM:F12 (Biological Industries, Beit Haemek, Israel) containing 5% (w/v) heat-inactivated fetal calf serum (FCS, Biological Industries), insulin (bovine, 5 μg/ml, Sigma, St. Louis, MO), gentamicin (50 μg/ml, Sigma) and combined antibiotics (penicillin, 100 U/ml and streptomycin, 0.1 mg/ml, Biological Industries). L-1 cells, a cloned cell line derived from lactating bovine mammary gland [German and Barash, 2002], were grown in DMEM:F12 containing 5% FCS, insulin (bovine, 5 μg/ml), hydrocortisone (1 μg/ml, Sigma), gentamicin (50 μg/ml) and combined antibiotics.

For extracellular matrix (ECM)-dependent activation, cells were plated on Matrigel (Collaborative Bio-Medical Products, Bedford, MA) in a six-well dish (0.6×10^6 cells/well) or 12-well dish (0.3×10^6 cells/well). The Matrigel was allowed to dry prior to plating. After 24 h, the medium was replaced with DMEM:F12 containing insulin (5 μg/ml), hydrocortisone (1 μg/ml) and prolactin (ovine, 3 μg/ml) without FCS.

AA depletion was achieved by incubating the cells in Earle's Balanced Salts solution containing insulin (5 μg/ml), hydrocortisone (1 μg/ml) and prolactin (ovine, 3 μg/ml).

Individual AA were purchased from Biological Industries. The effect of essential AA on the relevant parameters was studied by supplementing them to the cultures at quantities related to their levels in the DMEM:F12 medium. Changes in pH were monitored and buffered. The AA concentrations in this medium (in mM) were: Leu 0.451, His 0.451, Lys 0625, Thr 0.449, Cys 0.198, Ile 0.416, Arg 0.848, Met 0.116, Phe 0.215, Trp 0.04, Tyr 0.214, Val 0.452, Gln 2.5. When supplemented as a mix, selected AA were dissolved together and added according to these values.

IMMUNOBLOT ANALYSIS

Cells were lysed in 300 μl/well (6-well dishes) or 150 μl/well (12-well dishes) of ice-cold buffer (50 mM Tris pH 7.4, 150 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 0.5% w/v NP-40, 1 mM PMSF, 1 μg/ml aprotinin, 1 mM Na_3Vo_4), for 30 min. Extracted proteins were fractionated by SDS–PAGE and transferred to nitrocellulose membranes. Equal amounts of loaded protein were confirmed by staining the blot with Ponceau S (Sigma). Membranes were blocked and reacted with the appropriate antibodies (diluted 1:500) at 4°C overnight. This was followed by incubation for 1 h with goat anti-rabbit antibody diluted 1:3,000. Signals were

generated with Super Signal, West Pico Chemilumnescent Substrate (Pierce, Rockford, IL) according to the manufacturer's protocol. Signals in the linear range of development were quantified using the software Gel-Pro Analyzer, ver.3.2 (Media Cybernetics, Bethesda, MD) and expressed relative to the value of control AA-deprived cells.

METABOLIC LABELING

CID-9 cells were cultured on Matrigel in 35-mm dishes and labeled for 1 h with 1 ml DMEM containing 100 μCi/ml [^{35}S]Met as previously described [Moshel et al., 2006]. Briefly, total incorporation of [^{35}S]Met into TCA-precipitable protein was measured in 10-μl aliquots pipetted onto a nitrocellulose membrane. To determine de novo β-casein synthesis, aliquots of cell lysate, containing equal amounts of TCA-precipitable protein, were pretreated with Protein A-Sepharose beads (Pharmacia, Uppsala, Sweden), then reacted with anti-β-casein antibodies diluted 1:500 [Barash et al., 1995] and precipitated with Protein A Sepharose. Proteins were separated by SDS–PAGE, and the gels were exposed to XAR Kodak film following fluorography. AA-deprived cells were labeled in Earle's Balanced Salts solution containing Met (17.24 g/ml, representing its level in DMEM:F12), so as to maintain equal Met pools in control and deprived cells. Insulin, prolactin, and hydrocortisone were present during labeling [Moshel et al., 2006].

RESULTS

THE ROLE OF ESSENTIAL AA IN S6K1 PHOSPHORYLATION

The murine CID-9 mammary epithelial cells were derived from continuously proliferating, non-transformed mammary epithelial cell populations that lack tumorigenic properties [Danielson et al., 1984]. To achieve lactogenic conditions, these cells were differentiated on Matrigel for 5 days in the presence of the lactogenic hormones insulin, hydrocortisone and prolactin. Consequently, proliferation ceased, the cells aggregated into mammospheres and synthesized milk proteins.

To study their role in S6K1 phosphorylation, essential AA were supplemented individually for 10 min to 6-h AA-deprived CID-9 cells, and S6K1 phosphorylation on Thr 389 was determined by immunoblot analysis (Fig. 1). The 6-h deprivation period was selected in a prior study (not shown) to allow the detection of both positive and negative effects of AA manipulation on S6K1 phosphorylation. The indicated amounts of AA added to the medium to generate the dose-responsive effect are given relative to their amounts in the DMEM:F12 medium, the latter reflecting the biological requirements of the cell culture.

Figure 1 classifies the essential AA into three groups according to their effect on S6K1 phosphorylation. Leu, Ile, and Val (the BCAA) had a stimulatory effect on phosphorylation relative to that measured in the AA-deprived cells. At as low as 0.04 times their levels in the DMEM:F12 medium, Leu and Val were already inducing S6K1 phosphorylation. A higher level, 0.2 times its medium concentration, was needed for Ile to exert its positive effects. Trp, Phe, and Met did not affect S6K1 phosphorylation at concentrations of up to 25-fold their level in the medium. In contrast, when added

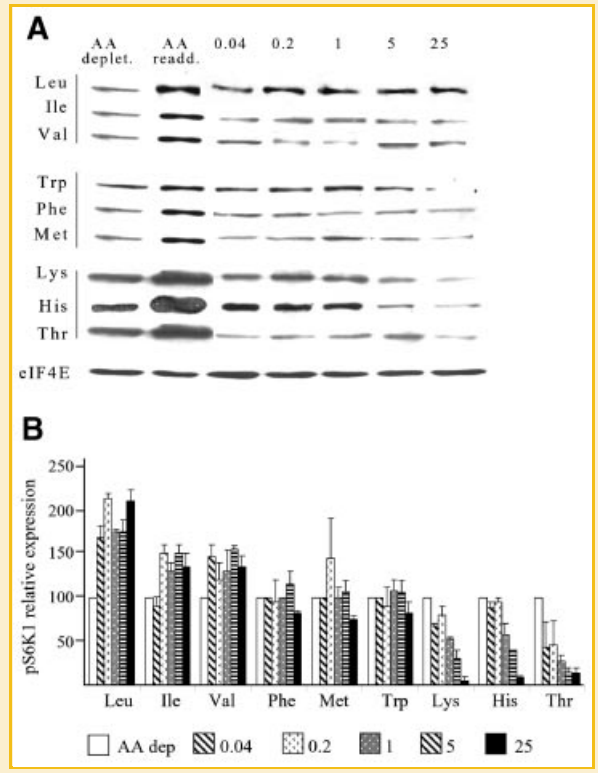

Fig. 1. Essential AA have diverse effects on S6K1 phosphorylation in CID-9 mammary epithelial cells. Differentiated, hormonally treated CID-9 cells were incubated in DMEM:F12 medium lacking AA for 6 h. Each of the AA was supplemented individually for 10 min at the indicated concentrations. A: S6K1 phosphorylation (Thr 389) and eIF4E levels were determined by immunoblot analysis. Numbers above the lanes refer to the level of the supplemented AA relative to that in the DMEM:F12 medium. B: Densitometric analysis of signals obtained from the S6K1 immunoblot. Signals in the linear range of development were quantified as described in Materials and Methods Section. In each analysis, values from the AA-supplemented cells were related to their AA-deprived controls. Average values ± SEM from three to seven independent analyses are presented. No effect was observed for the supplementation of each of the AA on eIF4F levels, as exemplified here by the addition of Thr. Deplet., depleted; Ly, LY294002; Rap, rapamycin; readd., re-addition.

individually, the three AA Lys, His, and Thr had a negative effect on S6K1 phosphorylation. Thr exhibited the most potent effect, causing a decrease in S6K1 phosphorylation to about 50% of its control level at the relatively low concentration of X0.04. His and Lys reduced S6K1 phosphorylation at concentrations equal to or above their levels in the medium. Cellular eIF4E levels were determined for each AA addition. eIF4E expression was not affected by their addition at concentrations of up to ×25, confirming the analysis of comparable amounts of total proteins in the various samples.

The negative effects of Lys, His, and Thr on S6K1 phosphorylation were confirmed in the L-1 bovine mammary cell line [German and Barash, 2002] (Fig. 2). In contrast to the increased phosphorylation observed after Leu addition, the three AA, added separately, decreased S6K1 phosphorylation at concentrations that were one to fivefold (X1–X5) those in the medium. Added as a mix, these AA completely abolished S6K1 phosphorylation at the latter concentration. L-1 cell eIF4E levels were not affected by supplementation

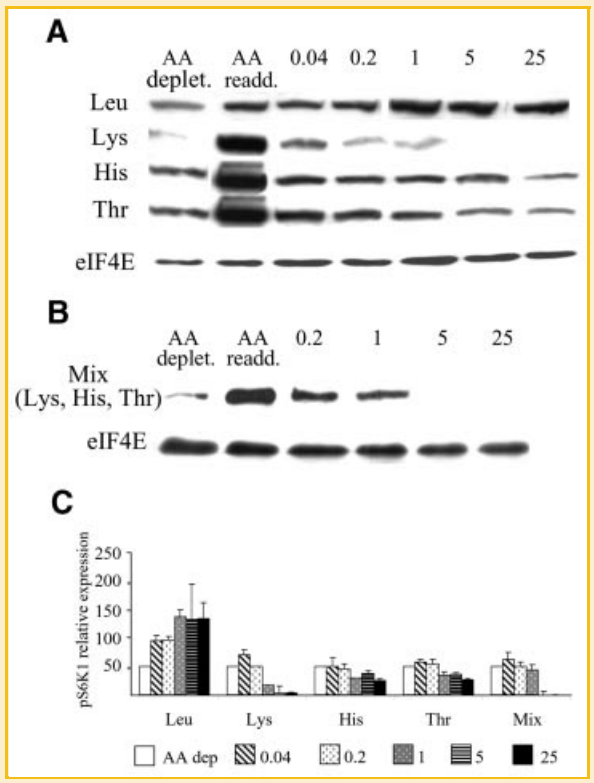

Fig. 2. Thr, His, and Lys inhibit S6K1 phosphorylation in bovine mammary L-1 cell cultures. Differentiated, hormonally treated L-1 cells were incubated in medium lacking AA for 6 h. Thr, His, and Lys were supplemented individually (A), or as a mix (B), and S6K1 phosphorylation was determined by immunoblot analysis. Numbers above the lanes refer to the level of the supplemented AA relative to that in the DMEM:F12 medium. The mix of Thr, His, and Lys contained amounts of these AA that were similar to those in the DMEM:F12 medium (1X) or the indicated fractions or multiples thereof. C: Densitometric analysis of signals obtained from the S6K1 immunoblot. Signals in the linear range of development were quantified as described in Materials and Methods Section. In each analysis, values from the AA-supplemented cells were related to their AA-deprived controls. Average values ± SEM from three independent analyses are presented. No effect was observed for the supplementation of each of the AA or their mix on eIF4F levels, as exemplified by the addition of Thr. Deplet., depleted; Ly, LY294002; Rap, rapamycin; readd., re-addition.

with Leu, or by the addition of Lys, His, or Thr, individually or as a mix.

Following their analysis in the AA-deprived cells, Lys, His, and Thr were further tested for their effect on S6K1 and 4E-BP1 phosphorylation in an environment that supports translation. These AA were supplemented to cultured CID-9 cells together with Leu at a concentration of X5 (Fig. 3). Thr overcame Leu's stimulatory effect, reducing S6K1 phosphorylation by 45 ± 8% below that in the Leu-treated cells at one-fifth of the medium's level of Leu (i.e., ThrX1). Phosphorylated S6K1 signals were hardly detected at the higher concentrations of Thr. A decrease in 4E-BP1 phosphorylation, as reflected by reciprocal shift from the highly phosphorylated γ subunit to the partly phosphorylated β subunit, was also detected at the latter concentrations No effects on S6K1 phosphorylation were observed following the addition of Lys or His together with Leu, but a decrease in 4E-BP1 phosphorylation status was noted when these AA were supplemented to the cultures at levels equal to that of Leu (i.e., X5). The negative effect of the individual AA, Thr, His and Lys, on both S6K1 and 4E-BP1 phosphorylation was confirmed in the absence of Leu.

To complete the analyses and test the effects of Lys, His, and Thr on S6K1 phosphorylation in intact cultures, experiments were performed in differentiated CID-9 cells, maintained continuously in DMEM:F12 medium (Fig. 4). None of these AA, at up to X5 concentration, affected S6K1 phosphorylation. However, a decrease in S6K1 phosphorylation was noted when they were added together as a mix at their levels in the medium (X1). Complete abrogation of S6K1 phosphorylation, resembling the negative effect of rapamycin, was noted at higher (X5 and X25) concentrations.

THE INHIBITORY EFFECTS OF Lys, His, AND Thr ON S6K1 PHOSPHORYLATION INVOLVE THE mTOR PATHWAY

It is widely accepted that the stimulatory effect of nutrients involves mTOR activation. Phosphorylation of mTOR on Ser 2448 modulates the effects of both insulin and AA, and controls protein synthesis. However, differences in the responses of various cell types to this effect have been reported [Nave et al., 1999; Du et al., 2007; Vary and Lynch, 2007]. To address whether the inhibitory effects of Lys, His and Thr on S6K1 and 4E-BP1 phosphorylation are mediated via this pathway, mTOR levels and activity (phosphorylation on Ser 2448) were analyzed in the CID-9 cells.

mTOR levels were not affected by supplementation of the individual AA at X5 concentration to AA-deprived cells (Fig. 5). In contrast, mTOR phosphorylation on Ser 2448 was completely inhibited when Lys, His, and Thr were added, either individually or as a mix.

Experiments demonstrated in Figure 6 confirmed a positive role for total AA or Leu, and an inhibitory effect for Lys, His, and Thr, added individually or as a mix, on both S6K1 and 4E-BP1 phosphorylation, as compared to signals obtained in the AA-depleted cells. Under these conditions, comparably low IRS-1 phosphorylation levels, just above the limit of detection, were measured in the AA-depleted CID-9 cells and those treated with Lys, His, or Thr. An increase in IRS-1 phosphorylation was observed after the addition of total AA or Leu to the AA-deprived cells. Surprisingly, a dose-dependent increase in IRS-1 phosphorylation was also repeatedly observed when the cultures were supplemented with a mix of Lys, His, and Thr, reaching the level of IRS-1 phosphorylation induced by total AA at X5 concentration. As shown in Figure 6B, the effects of the mix on IRS-1 and S6K1 phosphorylation were inversely correlated.

The kinase Akt/PKB is a well-characterized effector of PI3K, which is downstream in the IRS-1 pathway [Sarbassov et al., 2005]. Akt/PKB is phosphorylated on Thr 308 of the activation loop by PDK. Akt/PKB is also phosphorylated on Ser 473 of the hydrophobic motif [Alessi et al., 1996]. The rictor-mTOR complex (mTORC2) has been suggested to be the illusive kinase for this important phosphorylation in 3T3-L1 adipocytes [Hresko and Mueckler, 2005].

To analyze the putative involvement of mTORC2 in AA regulation of translation initiation, Akt phosphorylation on Ser 473 was determined in the 6-h AA-deprived CID-9 cells. Within a 10-min time frame—which is both necessary and sufficient for the

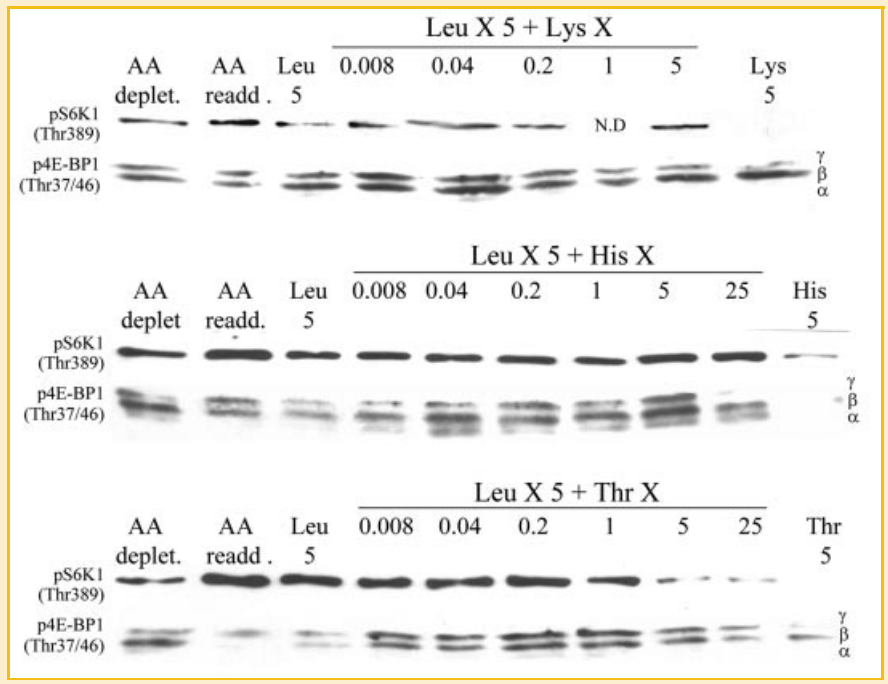

Fig. 3. Thr, but not His or Lys, prevents the stimulatory effect of Leu on S6K1 phosphorylation. Differentiated, hormonally treated CID-9 cells were incubated in medium lacking AA for 6 h. Each of the indicated AA were supplemented individually to the cultures for 10 min together with Leu at the indicated concentrations (relative to their level in the DMEM:F12 medium). Leu was added at fivefold its concentration in the medium. Representative figure of three independent analyses is presented. Deplet., depleted; Ly, LY294002; Rap, rapamycin; readd., re-addition.

activation of mTOR, and the phosphorylation of S6K1 and 4E-BP1 by Leu or total AA, and for inhibition of their phosphorylation by His, Lys, and Thr—no effect could be seen for the various AA on Akt/PKB phosphorylation. Nor was any effect observed on phosphorylated Akt by the addition of rapamycin, ruling out the putative negative-feedback inhibition of Akt by mTOR reported previously in the human rhabdomyosarcoma cell lines Rh30 and RD [Wan et al., 2007]. In contrast, addition of LY294002 to the cultures completely eliminated Akt phosphorylation on Ser 473. This effect has also been observed for wortmannin [Alessi et al., 1996] and indicates PI3K-dependent Akt activity [Manning and Cantley, 2007].

TOTAL PROTEIN SYNTHESIS AND β-CASEIN TRANSLATION ARE REDUCED IN CULTURES TREATED WITH Thr, His, AND Lys

To study the effect of His, Lys, and Thr on the translation and synthesis of total proteins and β-casein in the CID-9 cell cultures, we first established a time frame for de novo protein synthesis under conditions of AA depletion. Cells were cultured in serum-free medium for 5 days on Matrigel in the presence of insulin, prolactin and hydrocortisone. The cells stopped proliferating, differentiated into mammospheres and began producing milk proteins. Then, the medium was replaced with one lacking AA for various periods of time. During the last hour of incubation, the cells were labeled with ^{35}[S]Met. Hormones were present during the AA depletion and labeling periods. Figure 7 (inset) demonstrates a gradual decrease in de novo protein synthesis during the 48 h of AA depletion. Based on these data and a previous report [Moshel et al., 2006], an 18-h AA-deprivation period was selected to allow detection of the putative effects, both negative and positive, of AA supplementation. During the AA supplementation, cells were treated with 5 μg/ml actinomycin D, an inhibitor of DNA-dependent RNA synthesis, to prevent transcription and thereby restrict translation to the pre-existing mRNA [Moshel et al., 2006]. In contrast to the rapid response of protein phosphorylation to AA stimulation, a longer time was needed for incorporation of [^{35}S]Met into the newly synthesized proteins. A 2 h period was previously selected for these analyses [Moshel et al., 2006].

As shown in Figure 7A, there was no significant ($P < 0.05$) effect on total protein synthesis by the addition of Leu, Thr, His, or Lys at X5 concentration for 2 h to the AA-depleted cultures. However, addition of these AA as a mix caused a dose-dependent decrease of 25–65% in total protein synthesis when supplemented at concentrations of X5–X50, respectively (Fig. 7A). No significant ($P < 0.05$) difference between the effect of Leu and that of 100 ng/ml rapamycin was observed.

The effect of Lys, His, and Thr on β-casein synthesis was measured by immunoprecipitation of equal amounts of TCA-precipitable proteins from the AA-deprived and supplemented cells (Fig. 7B). The combined addition of these AA as a mix at concentrations of X25 or X50 reduced β-casein translation and synthesis compared to the AA-depleted cells and even the rapamycin-treated ones. To confirm that this negative effect did not result from decreased cell viability, differentiated CID-9 cells were treated with the Lys, His, Thr mix at concentrations of up to X25 for 2 h. The cells were harvested and re-cultured in growth medium containing 5% FCS and insulin for 2 days (Fig. 8). No significant ($P < 0.05$) difference between the number of untreated cells and those supplemented with the mix was observed.

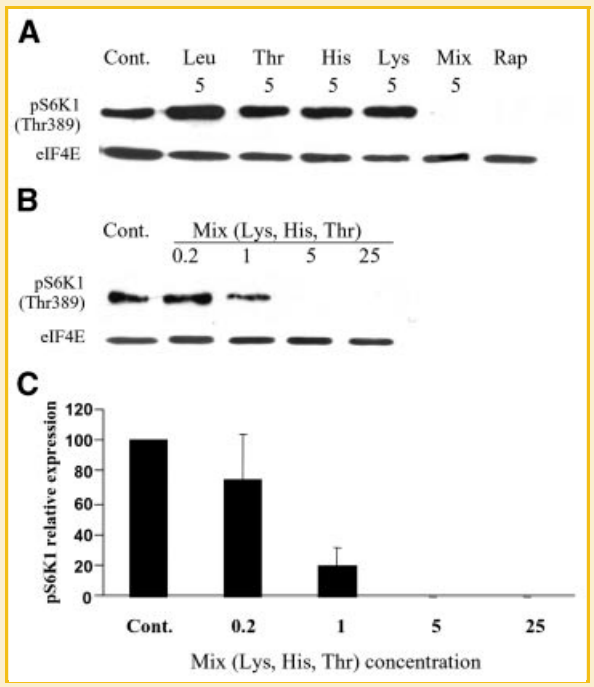

Fig. 4. The combined effect of Thr, His, and Lys on S6K1 phosphorylation is revealed in intact, non-AA-deprived cultures. Differentiated, hormonally treated CID-9 cells were supplemented with Thr, His, and Lys individually (A) or as a mix (B), and S6K1 phosphorylation was analyzed by immunoblot. C: Densitometric analysis of signals obtained from the S6K1 immunoblot presented in (B). Signals in the linear range of development were quantified as described in Materials and Methods Section. In each analysis, values from the AA-supplemented cells were related to their AA-deprived controls. Average values ± SEM from three independent analyses are presented. Cont.: control, differentiated cells cultured in hormonally supplemented DMEM:F12 medium. The mix of Thr, His, and Lys contained amounts of these AA that were similar to those in the DMEM:F12 medium or the indicated fractions or multiples thereof. Deplet., depleted; Ly, LY294002; Rap, rapamycin; readd., re-addition.

DISCUSSION

The effect of essential AA on mechanisms involved in protein translation and synthesis was studied here in differentiated mammary epithelial cells cultured under lactogenic conditions.

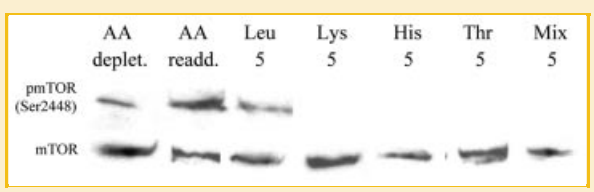

Fig. 5. Thr, His, and Lys inhibit mTOR phosphorylation. Differentiated, hormonally treated CID-9 cells were incubated in medium lacking AA for 6 h. Thr, His, and Lys were supplemented individually or as a mix to the cultures for 10 min and mTOR levels and phosphorylation were analyzed by immunoblot assay. Numbers above the lanes refer to the concentrations of these AA relative to their concentrations in the DMEM:F12 medium. Representative figure of three independent analyses is presented. Deplet., depleted; Ly, LY294002; Rap, rapamycin; readd., re-addition.

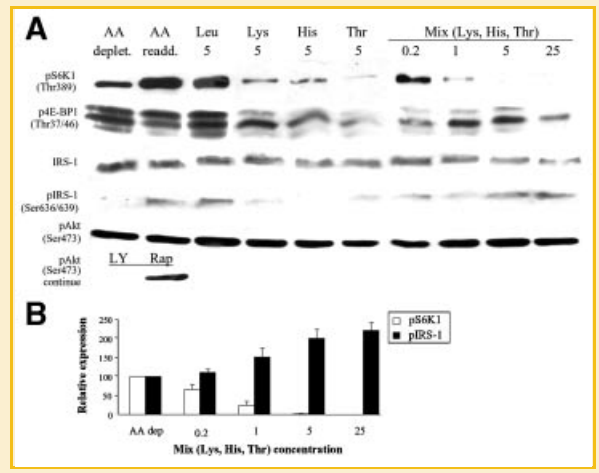

Fig. 6. Diverse effects of Thr, His, and Lys, added separately or in combination, on IRS-1 phosphorylation, and their lack of influence on Akt activity. Thr, His, and Lys were supplemented individually or as a mix for 10 min to the differentiated cultures of CID-9 cells. A: The effect on the indicated proteins was measured by immunoblot analysis. Numbers above the lanes refer to the concentrations of these AA relative to their concentrations in the DMEM:F12 medium. The mix of Thr, His, and Lys contained amounts of these AA that were similar to those in the DMEM:F12 medium or the indicated fractions or multiples thereof. B: Densitometric analysis of the immunoblot signals marking the opposite effects of the mix on S6K1 and IRS-1 phosphorylation. Signals in the linear range of development were quantified as described in Materials and Methods Section. In each analysis, values from the AA-supplemented cells were related to their AA-deprived controls. Average values ± SEM from three to four independent analyses are presented. Deplet., depleted; Ly, LY294002; Rap, rapamycin; readd., re-addition.

The cells were organized into mammospheres and maintained synthesis and secretion of milk proteins.

According to their role in S6K1 phosphorylation, the essential AA were classified into three groups: the BCAA, which increased S6K1 phosphorylation; Trp, Phe, and Met, which did not affect S6K1 phosphorylation, and His, Lys, and Thr, which had a negative impact, resulting in lower S6K1 phosphorylation than that measured in the AA-depleted and non-depleted cells.

The BCAA Leu, Ile, and Val are the most abundant of the essential AA. In addition to being indispensable protein building blocks, these AA act as nutritional regulators of protein synthesis [Kimball and Jefferson, 2006]. Of the three BCAA, Leu is the most effective and its specific and stimulatory role on translation initiation and protein synthesis has been established in several organs and cell types, including mammary epithelial cells ([Moshel et al., 2006] and the current manuscript). A scarcity of information on the roles of the other AA in regulating the mechanism of translation, especially in reproductive organs, impairs our understanding of the control of cellular protein synthesis. Thus, the current study focused on the newly discovered negative signaling effect of Lys, His and Thr on mTOR-mediated translational mechanisms in differentiated mammary epithelial cells.

Despite their common role in S6K1 and 4E-BP1 phosphorylation, Lys, His, and Thr do not share obvious structural or molecular characteristics that could account for their comparable negative effects. In the murine CID-9 cells, Thr appeared to be the most potent

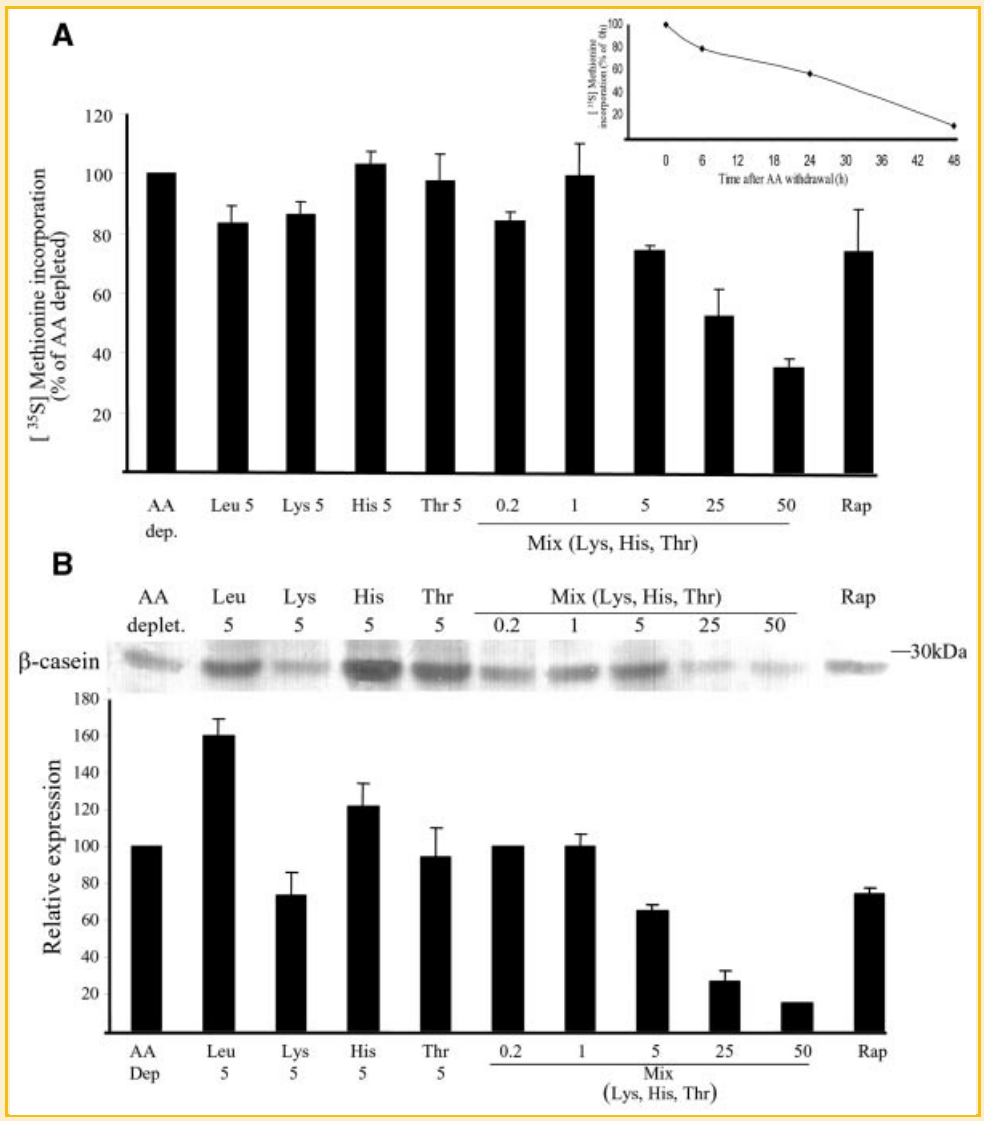

Fig. 7. Protein synthesis in mammary epithelial cells is inhibited by the combined addition of His, Lys, and Thr. A: Differentiated, hormonally treated CID-9 cells were incubated in DMEM:F12 medium lacking the total set of AA for 18 h. The indicated AA were added for the last 2 h. [^{35}S]Met was added to the medium for the final hour. Its incorporation into TCA-precipitable proteins was measured in cell lysates and presented as mean ± SEM of three independent analyses. Inset: AA withdrawal for 18 h results in a 45% decrease in [^{35}S]Met incorporation. B: De-novo β-casein synthesis was measured by immunoprecipitation in aliquots containing equal amounts of TCA-precipitable protein as described in Materials and Methods Section. Samples were subjected to SDS–PAGE followed by autoradiography. Signals in the linear range of development were quantified as described in Materials and Methods Section. In each analysis, values from the AA-supplemented cells were related to their AA-deprived controls. Average values ± SEM from four independent analyses are presented. Numbers on the X-axis in (A) and above the lanes or below the bars in (B) refer to the concentrations of these AA relative to their concentrations in the DMEM:F12 medium. The mix of Thr, His, and Lys contained amounts of these AA that were similar to those in the DMEM:F12 medium or the indicated fractions or multiples thereof. Deplet., depleted; Ly, LY294002; Rap, rapamycin; readd., re-addition.

amino acid, capable of overcoming Leu's positive role. Synergy between the negative effects of the three AA was also noted: phosphorylation of S6K1 was still detectable in cells treated with each of the individual AA at up to 25-fold higher concentrations than in the DMEM:F12 medium. However, as a mix, these AA completely eliminated S6K1 phosphorylation, at a concentration of X5.

The pathway via which Lys, His, and Thr affect S6K1 and 4E-BP1 phosphorylation involves inhibition of mTOR activity. mTOR phosphorylation on Ser 2448 was completely abrogated by the individual supplementation of each of the three AA at X5 concentration. Interestingly, under these conditions, phosphorylated S6K1 and 4E-BP1 could still be detected. This discrepancy could be explained by residual mTOR activity that was not detected by the immunoblot analysis but may have resulted in limited phosphorylation of these downstream effectors. However, we cannot exclude the possibility that phosphorylation of mTOR on Ser 2448 is important but not absolutely vital for controlling its activity, and that other sites may compensate for its inactivation in mammary epithelial cells [Peterson et al., 2000].

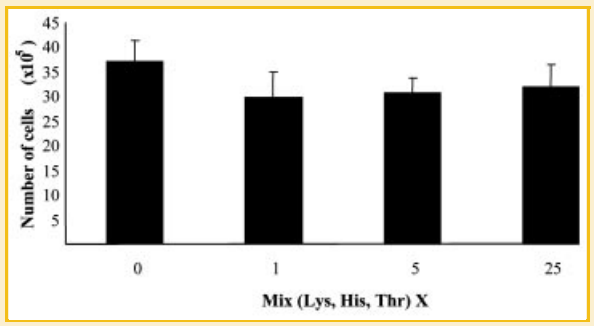

Fig. 8. Cell viability is not affected by the combined addition of Lys, His, and Thr. Differentiated, hormonally treated CID-9 cell cultures were deprived of the total set of AA for 18 h and then supplemented with a mix of Lys, Hys, and Thr at the indicated concentrations for 2 h. The cells were then released from culture by trypsin and re-cultured for 2 days in proliferating DMEM:F12 medium containing insulin (5 μg/ml) and 5% FCS. The cells were counted at the end of the incubation period and results are presented as mean ± SEM of three independent analyses. The mix of Thr, His, and Lys contained amounts of these AA that were similar to those in the DMEM:F12 medium (1X) or the indicated multiples thereof (5X, 25X). Deplet., depleted; Ly, LY294002; Rap, rapamycin; readd., re-addition.

The signaling pathway that emerges from the accumulated data on the negative effects of Lys, His, and Thr is illustrated in Figure 9. The competition with Leu and the inhibition of mTOR phosphorylation suggest that His, Lys, and Thr exert their negative effects on S6K1 and 4E-BP1 phosphorylation via the specific AA pathway which signals to mTOR, possibly by inhibiting the class 3 PI3K, hVps34 [Byfield et al., 2005; Nobukuni et al., 2005]. Their effect does not involve mTORC2, as reflected by the lack of change in phosphorylation of Akt on Ser 473. The suppression of S6K1 phosphorylation exerted by the individual supplementation of these AA prevents S6K1's inductive effect on IRS-1 phosphorylation as part of the feedback loop – a prior step to the inhibition of its activity [Um et al., 2004; Zick, 2005]. Surprisingly, a dose-dependent increase in IRS-1 phosphorylation repeatedly coincided with the addition of the inhibitory AA as a mix. Thus, in parallel to decreasing S6K1 activity, these AA may act synergistically to activate an additional kinase, phosphorylating IRS-1 via an S6K1-independent pathway. A candidate for this activity is the c-Jun NH2-terminal kinase-1 (JNK-1), which has been reported to respond to AA-induced metabolic stress and affect IRS-1 activity [Aubel et al., 2001; Hirosumi et al., 2002]. It has been shown that limited variations in the supply of several AA to mammalian cells can modulate JNK-1 activity. However the 4-h time range needed for these AA to induce the JNK-1 response sheds some doubt on this theory.

While the identification of the putative kinase mediating the S6K1-independent effect on IRS-1 phosphorylation needs to be further elucidated, the data suggest that a mix of Lys, His and Thr can inhibit and desensitize the AA- and insulin-mediated signaling towards protein synthesis, respectively. An apparent sharp decrease of about 65% in the synthesis of total proteins occurred within 2 h after supplementation of the mix at X50 concentration, indicating a cellular inclination to shut down anabolic activities. In contrast, only a 25% decrease in the rate of protein synthesis was noted in cells treated with rapamycin, which selectively inhibits the

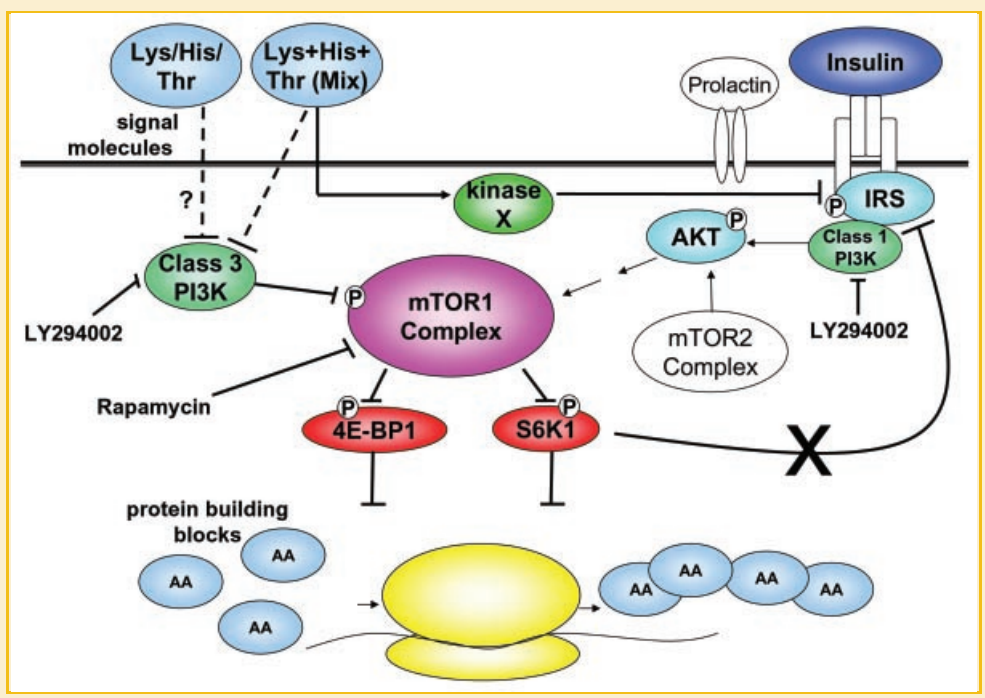

Fig. 9. Proposed model for the synergistic inhibitory effect of Lys, Hys, and Thr, acting individually or as a mix, on translational mechanisms and protein synthesis in mammary epithelial cells. The effect of Leu is not indicated. We suggest that this effect is conveyed via mTOR and S6K1 phosphorylation, leading to increased IRS-1 phosphorylation and inactivation. Deplet., depleted; Ly, LY294002; Rap, rapamycin; readd., re-addition.

AA-induced pathway in the same culture system. The more severe effect of the AA mix relative to that of rapamycin was also reflected in the synthesis of β-casein, which was inhibited by the addition of increasing levels of the inhibitory AA mix.

Indeed, it has only recently become apparent that translation initiation mediates essential mammary gland functions and that the mammary epithelial cells are an important model for studying regulation of protein synthesis. The synergistic activity of insulin and prolactin in the induction of β-casein synthesis by elongating its poly A mRNA tail [Choi et al., 2004] is a remarkable example of this type of control. Here, we show for the first time that the mammary epithelial cells are subject to the inhibitory signaling of three essential AA—His Lys and Thr, which decrease S6K1 and 4E-BP1 phosphorylation via the mTOR pathway. At physiological levels, these individual AA were necessary and sufficient to generate the negative signals with respect to translation initiation. However, repression of protein synthesis could be achieved only by the combined addition of higher levels of the three. Added as a mix, these AA signaled the phosphorylation of IRS-1—a prerequisite for the inhibition of insulin's downstream effectors. Together with the inhibition of AA signaling, this may have generated the apparent severe decrease in total protein synthesis, and of the specific milk protein β-casein in particular.

In vivo, plasma AA levels are subject to nutritional and hormonal effects, as well as to conditions of exercise and stress [Cynober, 2002]. For example, in lactating dairy cows, plasma lysine increased by 67% after fish meal feeding [Metcalf et al., 1994]. Prolonged exercise caused a decrease of about 40% in the plasma level of BCAA in physically fit men [Refsum et al., 1979]. This amplitude in plasma AA levels may serve to pinpoint the in vitro variations in AA levels which are sufficient to affect S6K1 and 4E-BP1 phosphorylation within a physiological range. Indeed, AA levels in the culture medium and in the plasma of rats cover a comparable concentrations [Garlick and Grant, 1988]. Enhanced uptake of AA under specific physiological conditions [Mackle et al., 2000] may also render the higher amounts of the AA mix, which affect protein production, physiologically relevant. Importantly, AA may be located in three pools: plasma, intracellular and protein-bound [Cynober, 2002]. The large number of cellular AA transporters with different and overlapping properties and the changes in blood flow and hormonal effects make it difficult to link AA levels in individual compartments with cellular metabolism [Bequette et al., 1998]. The current in-vitro study reveals cryptic negative effects of Lys, His, and Thr on cellular mechanisms regulating translation initiation and protein synthesis in the mammary epithelial cells, which were not exposed to conventional in vivo analyses.

Finally, the mix of His, Lys, and Thr may uniquely extend the growing list of mTOR inhibitors which are currently based mainly on rapamycin analogs [Chen, 2007]. As a pharmacological agent, this mix could be tested for the treatment of immunological and malignant diseases.

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