Final Scientific Report

Cover Page

BARD Project Number: IS-3837-06-R

Date of Submission of the report: May, 2010

Project Title: Translational Mechanisms Governing Milk Protein Levels and Composition

Investigators

Principal Investigator (PI):
Barash, Itamar                                      ARO, the Volcani Center

Co-Principal Investigator (Co-PI):
Rhoads, Robert E.                               Louisiana State University Health Sciences Center

Institutions

Keywords not appearing in the title and in order of importance. Avoid abbreviations.

Abbreviations commonly used in the report: AA- amino acids; BRCAA- branched chain AA; 4E-BP1 – eIF4E binding protein; His- Histidine; IRS-1 insulin responsive protein 1; Lys- Lysine; mTOR – mammalian target of rapamycin; PI3K- phosphatidylinositol-3-kinase; S6K1- p70S6 kinase; Thr- Threonine

Budget: I S: $ 155,000      US: $ 155,000      Total: $ 310,000

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Signature                          Signature
Principal Investigator                Authorizing Official, Principal Institution

Appendix G6a
Publication Summary (numbers)

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*The data is not yet sufficient for an additional manuscript in respective journal.

Postdoctoral Training: List the names of all postdocs who received more than 50% of their funding by the grant.

Ewa Grudzien-Nogalska,

Cooperation Summary (numbers)

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Description Cooperation:

The collaboration between the laboratories during the current BARD grant period (2006-2009) has consisted mainly of e-mail discussions, but in the previous BARD grant period (2000-2003), the U.S. collaborator made one trip to the laboratory of the Israeli collaborator, and the Israeli collaborator made two trips to the laboratory of the U.S collaborator.

Patent Summary (numbers)

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Abstract

Original objectives:
The long-term goal of the research is to achieve higher protein content in the milk of ruminants by modulating the translational apparatus of the mammary gland genetically, nutritionally, or pharmacologically. The short-term objectives are to obtain a better understanding of 1) the role of amino acids (AA) as regulators of translation in bovine and mouse mammary epithelial cells and 2) the mechanism responsible for the synergistic enhancement of milk-protein mRNA polyadenylation by insulin and prolactin.

Background of the topic:
In many cell types and tissues, individual AA affect a signaling pathway which parallels the insulin pathway to modulate rates and levels of protein synthesis. Diverse nutritional and hormonal conditions are funneled to mTOR, a multidomain serine/threonine kinase that regulates a number of components in the initiation and elongation stages of translation. The mechanism by which AA signal mTOR is largely unknown. During the current grant period, we have studied the effect of essential AA on mechanisms involved in protein synthesis in differentiated mammary epithelial cells cultured under lactogenic conditions. We also studied lactogenic hormone regulation of milk protein synthesis in differentiated mammary epithelial cells. In the first BARD grant (2000-03), we discovered a novel mechanism for mRNA-specific hormone-regulated translation, namely, that the combination of insulin plus prolactin causes cytoplasmic polyadenylation of milk protein mRNAs, which leads to their efficient translation. In the current BARD grant, we have pursued the signaling pathways of this novel hormone action.

Major conclusions/solutions/achievements:
The positive and negative signaling from AA to the mTOR pathway, combined with modulation of insulin sensitization, mediates the synthesis rates of total and specific milk proteins in mammary epithelial cells. The current in vitro study revealed cryptic negative effects of Lys, His, and Thr on cellular mechanisms regulating translation initiation and protein synthesis in mammary epithelial cells that could not be detected by conventional in vivo analyses. We also showed that a signaling pathway involving Jak2 and Stat5, previously shown to lead from the prolactin receptor to transcription of milk protein genes, is also used for cytoplasmic polyadenylation of milk protein mRNAs, thereby stabilizing these mRNAs and activating them for translation.

Implications:
In vivo, plasma AA levels are affected by nutritional and hormonal effects as well as by conditions of exercise and stress. The amplitude in plasma AA levels resembles that applied in the current in vitro study. Thus, by changing plasma AA levels in the epithelial cell microenvironment or by sensitizing the mTOR pathway to their presence, it should be possible to modulate the rate of milk protein synthesis. Furthermore, knowledge that phosphorylation of Stat5 is required for enhanced milk protein synthesis in response to lactogenic opens the possibility for pharmacologic approaches to increase the phosphorylation of Stat5 and, thereby, milk protein production.
Achievements:

Significance of main scientific achievements or innovations

The current study contributes important and novel information to the relatively unexplored field of translation regulation in mammary biology. It shows that translation initiation and the mTOR pathway mediate essential mammary gland functions and also that mammary epithelial cells can be an important model for studying regulation of protein synthesis.

1. Negative Effects of the Amino Acids Lys, His, and Thr on S6K1 Phosphorylation in Mammary Epithelial Cells. Looking for a diverse role of individual AA on translation initiation, we have divided the essential AA into three groups according to their effect on the phosphorylation of the translation marker S6K1 in mouse and bovine epithelial cells. We showed, for the first time, that the mammary epithelial cells are subjected 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 (i.e. β-casein) 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 observed severe decrease in total protein synthesis and specific milk protein synthesis in particular. AA concentrations in the culture medium were comparable to those in plasma. Plasma AA levels are subject to nutritional and hormonal effects, and the changes in protein synthesis caused by elevated levels of the three inhibitory AA may reflect the effect of altered physiological conditions. Importantly, AA exist in three pools: plasma, intracellular, and protein-bound. 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. Thus, the current in vitro study in mammary epithelial cells was able to reveal cryptic negative effects of Lys, His, and Thr on cellular mechanisms regulating translation initiation and protein synthesis that were not observed by conventional in vivo analyses. In unpublished data, we also confirmed the
involvement of the class III PI3 kinase vps34 in mediating AA signaling to mTOR. Our data suggest that it may be possible to achieve elevated S6K activity and protein synthesis in differentiated mammary epithelial cells cultured in DMEM/F12 medium by manipulating AA composition.

2. Regulation of translation by cytoplasmic polyadenylation of specific mRNAs. In the first three-year BARD grant period (2000-03), we demonstrated that insulin plus prolactin synergistically increases the rate of β-casein mRNA translation in mouse mammary epithelial cells (CID-9). This effect is mediated through mRNA stabilization by rapid cytoplasmic poly(A) elongation upon treatment with both hormones. Inhibitors of the PI3K, mTOR and MAPK pathways blocked insulin-stimulated total protein and β-casein synthesis but not the synergistic stimulation. Additionally, our studies suggested that phosphorylation of 4E-BP1, known to be stimulated by prolactin, was not responsible for the synergistic increase in milk protein synthesis since mTOR and PI3K inhibitors which prevent 4E-BP1 phosphorylation were not able to abolish the stimulation of β-casein translation or poly(A) elongation upon treatment with combination of hormones. By contrast, an inhibitor of polyadenylation, the chain terminator cordycepin, inhibited synthesis of β-casein in the presence of insulin and prolactin, which strongly suggested that the increase in translational efficiency of β-casein mRNA was due to cytoplasmic polyadenylation. Additionally, we demonstrated that both hormones must be withdrawn overnight and than added back to observe the synergistic stimulation of β-casein synthesis. This suggested that insulin and prolactin cooperate in the same pathway, rather than activate separate pathways, to stimulate cytoplasmic polyadenylation, which in turn leads to increase in milk protein mRNA stability and translation.

In the current BARD grant period, we investigated the signaling pathways for the synergistic stimulation of β-casein mRNA stability and translation by prolactin and insulin. Prolactin binds to the prolactin receptor, which in turn activates Jak2 kinase, a protein associated with the cytoplasmic domain of the prolactin receptor. The signal transducer and activator of transcription (Stat5) is then recruited to the receptor and phosphorylated by Jak2. This causes its dimerization and translocation to the nucleus where it can activate transcription of genes involved in alveolar morphogenesis, including the milk protein β-casein. Prolactin receptor can also activate other
signaling cascades like Ras-MAPK, Akt, and PKC pathway. Whereas earlier studies concentrated on transcriptional regulation via Jak2-Stat5 signaling pathway, we focused on mRNA stabilization and translation of milk protein. We demonstrated that synergistic stimulation of β-casein synthesis by insulin and prolactin is significantly inhibited by the presence of either Jak2 or Stat5 inhibitors, indicating that the at least some of the same signaling components that regulate transcription of milk protein genes also regulate translation of milk protein mRNA. Another measure of polyadenylation is mRNA stability. We showed that β-casein mRNA is destabilized upon hormone withdrawal but becomes stable again upon addition of insulin and prolactin, but not either hormone alone. We found that this stabilization is prevented by both Jak2 and Stat5 inhibitors. These results suggest a new cytoplasmic role for Stat5 besides its being a transcription factor.

**Agricultural and/or economic impacts of the research findings**
Our results suggests that nutritional or cellular conditions should be sought *in vivo* to lower the levels of Lys, His and Thr in the plasma in order to obtain higher levels of milk protein secretion. Also, the new cytoplasmic role of Stat5 in promoting specific milk protein synthesis suggests that pharmacological approaches might be sought to activate Stat5 in order to boost milk protein synthesis.

**Details of cooperation**
The collaboration between the labs during the current BARD grant period (2006-2009) has consisted mainly of e-mail discussions, but in the previous BARD grant period (2000-2003), the U.S. collaborator made one trip to the laboratory of the Israeli collaborator, and the Israeli collaborator made two trips to the laboratory of the U.S collaborator.

**List of Publications**
Appendix:

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Manuscripts:


Unpublished data

**AA regulation of protein synthesis.** The role of the class III PI3 kinase Vps34 in mediating the AA effect was studied in mouse and bovine mammary epithelial cells. CID-9 cells were stably transfected with a newly prepared construct composed of the β-lactoglobulin (BLG) regulatory sequences linked to the hVPS34 coding region. To this end, our analyses show difference in the dose response curves that measure the effect Leu on pS6K1 in wild type and transfected cells. This supports previous reports suggesting that Vps34 is mediator of AA signaling and is located upstream of mTOR. It also provides an additional site for manipulating AA effect on cellular protein synthesis.

**Lactogenic hormone regulation of milk protein synthesis.** We investigated the signaling pathways activated in the presence of lactogenic hormones that leads to cytoplasmic polyadenylation of milk protein mRNAs. Two signaling components downstream of the prolactin receptor known to mediate the transcriptional effects of prolactin are the kinase Jak2 and the transcription factor Stat5. When the latter is phosphorylated by Jak2, it activates transcription of milk protein genes. We showed that these two signaling molecules are also involved in specific cytoplasmic polyadenylation of milk protein mRNAs. Previously we directly measured the length of poly(A) on β-casein mRNA as a function of hormonal status. In the current work, we measured two consequences of cytoplasmic polyadenylation: stabilization of β-casein mRNA against rapid *in vivo* degradation and enhanced synthesis of β-casein. Fig. 1 shows that β-casein mRNA decays rapidly when only insulin is present (compare column 2 to column 1). Prolactin partially stabilizes the mRNA (column 3), but insulin plus prolactin stabilize it completely (column 4) over the 6-h period of the experiment. Note that these changes in β-casein mRNA levels are not due to transcriptional effects since the transcription inhibitor actinomycin D was present. The synergistic effect of insulin plus prolactin on mRNA stability is prevented by both a Stat5 inhibitor (column 5) and a Jak2 inhibitor (column 6). The Stat5 inhibitor, N’-((4-oxo-4H-chromen-3-
yl)methylene)nicotinohydrazide, binds to the SH2 domain of Stat5 and prevents from being recruited to Jak2, where it is phosphorylated. The Jak2 inhibitor, AG 490, blocks its kinase activity.

The requirement for Stat5 phosphorylation for cytoplasmic polyadenylation of β-casein mRNA was shown using a different read-out of polyadenylation, namely, the translation of β-casein mRNA to make 35S-labeled β-casein in a 30-min pulse labeling experiment (Fig. 2). An increase in the rate of β-casein synthesis occurs in the presence of insulin plus prolactin (compare column 2 to column 1), but both the Stat5 inhibitor (column 3) and the Jak2 inhibitor (column 4) prevent the increase.

The effect of lactogenic hormones on polyadenylation, as manifested in both mRNA stability (Fig. 1) and translational activation (Fig. 2), is mediated through Stat5 phosphorylation (Fig. 3). There is little phosphorylated Stat5 after hormone withdrawal (Fig. 3A, lane 1), but within 5 min of hormone addition, there is robust phosphorylation of Stat5 (lane 2). Phosphorylation is prevented by increasing concentrations of the Stat5 inhibitor (lanes 5-7) as well as by the Jak2 inhibitor (Fig. 3B, compare lane 1 to lane 4).

These results indicate that the Jak2-Stat5 pathway, previously known to mediate the transcriptional effects of prolactin, also mediates cytoplasmic polyadenylation, which results in both greater stability of milk protein mRNAs and greatly enhanced translation. To our knowledge, this is the first cytoplasmic role for Stat5. This finding opens the possibility of pharmacological approaches that would increase Stat5 phosphorylation and thereby, milk protein synthesis.
Figure 1. Inhibitors of the olactin signaling pathway destabilize β-casein mRNA in the cytoplasm. CID 9 cells were allowed to differentiate in the presence of 5 µg/ml insulin and 0.1 µg/ml prolactin for 5 d after transfer to Matrigel-coated dishes. Cells were deprived of hormones for 15 h (column 1), and then either 5 µg/ml insulin (column 2), 0.5 µg/ml prolactin (column 3), or both (column 4) were added back followed by incubation for 6 h. Actinomycin D was added to the cell culture medium of all samples 1 h before hormone addition and was present during hormone treatments at the concentrations of 5 µg/ml and 1 µg/ml, respectively. Inhibitors of either Stat5 (column 5) or Jak2 (column 6) were added at the concentrations of 200 and 50 µM, respectively, 1 h before hormone addition and were present during hormone treatment at the concentrations 50 and 25 µM, respectively. Cells were lysed and RNA extracted. Relative β-casein mRNA levels were determined by real-time PCR. The level of β-casein mRNA in cells deprived of hormones for 15 h (column 1) was set as 1.0.

Figure 2. Inhibitors of prolactin signaling pathway abolish the synergistic stimulation of β-casein synthesis by insulin and prolactin. CID 9 cells were allowed to differentiate and deprived of hormones for 15 h as in Figure 1. Hormones were added back simultaneously with 50 µCi/ml [35S]Met. Radioactivity incorporated into β-casein protein was measured 30 min later by immunoprecipitation. The Stat5 inhibitor (column 3) and Jak2 inhibitor (column 4) were added to the culture medium 30 min before hormone addition at concentrations of 50 and 25 µM, respectively.
**Figure 3. The effect of Stat5 and Jak2 inhibitors on Stat5 phosphorylation.** CID 9 cells were allowed to differentiate, deprived of hormones, and the indicated hormones restored as in Figure 1. Cells were lysed at the indicated times and protein extracts subjected to SDS-PAGE on 8% gels followed by western blot analysis to detect phospho-Stat5 (*upper blots*) and total Stat5 (*lower blots*). *Panel A*, effects of the Stat5 inhibitor. *Panel B*, effects of the Jak2 inhibitor.