The role of the integrated stress response pathway on amino acid regulation of murine lactation

Essential amino acids are the substrate for milk protein synthesis in the mammary gland. However, some amino acids play a supplementary role by signaling on cellular pathways that regulate lactation. The integrated stress response pathway, through GCN2, senses unacetylated tRNAs and correspondingly inhibits protein translation initiation. We hypothesize that a GCN2 depleted mouse would not respond to protein restriction, it would maintain the level of milk production of a non-restricted mouse, thus utilizing dietary protein more efficiently. In this experiment, we used a genetic model in which GCN2 is deleted in mammary epithelial cells during lactation. Then, we restricted dietary protein in both, wild type and GCN2 knockout dams. We determined that GCN2 seems to be a minor factor on lactation with regard to protein output in a murine model.

Introduction

The dairy industry in the US is projected to grow to one trillion dollars by 2024 and currently makes up 1% of the Gross Domestic Product (GDP) of the US (Shahbandeh, 2020). However, despite the dairy industry's impact on the economy, it is an ailing industry with profit margins continually shrinking. Their largest expenditure is feed, which makes nutrient efficiency for milk protein produced key to the sustainability in the future. Increasing dry matter intake dry matter intake may increase overall milk protein yield, but the efficiency of dairy cattle will decrease when given more feed (Huhtanen & Hristov, 2009). Proving again that an increase in dry matter intake may not increase milk protein yield enough to justify spending more on feed.

Dairy cattle only utilize 25% of nitrogen to make animal products (Oenema, 2006). The other 55-95% is eliminated in the body as waste via urine or manure. As proteins are composed of nitrogen rich amino acids (AAs), they make up most of these losses. Supplying an animal with an excess of AAs which are not critical for formation of milk proteins will cause the AAs to be oxidized into carbon dioxide and ammonium waste products. These products are closely linked to an increase in urea synthesis as a means of eliminating the ammonia radical formed by AA breakdown (Schutz, 2011). Surplus nitrogen in the form of ammonium can also be excreted in feces. Nitrifying bacteria found in the soil can convert ammonia into nitrites and nitrates, which can have unintended negative effects on the environment.

When nitrogen is excreted in the animal's feces, it can runoff into waterways by rain. This is problematic as the nitrates that are formed via nitrifying bacteria can cause an increase in algal blooms which can drastically change the marine environment. This happens by a decrease of oxygen in water, blockage of sunlight necessary for underwater plants, and an accumulation of toxic microalgae which can cause death to a multitude of marine life (Kibet et al., 2016,

Anderson et al., 2012). The algal blooms can further diminish oxygen availability upon death as the decomposition of algae by microbes uses up even more of the oxygen in water (Diaz & Rosenberg, 2008).

Human health can also be negatively affected by means of groundwater pollution caused from excess nitrogen consumption in dairy cattle. Since manure contains nitrogen at high concentrations, it is often used as fertilizer in all parts of the world. Ammonium is again the main source found in manure and ends up being converted to nitrate in the soil. These nitrates can go on to contaminate groundwater and lead to birth defects, cancer, enlarged thyroid glands, and hypertension (Gao et al., 2012). To make matters worse, the excess nitrogen in fertilizer only lowers the efficiency of the fertilizers while allowing more nitrogen waste losses to the environment.

Air pollution is also a factor to consider as nitrogen excreted in urea is prone to volatilization. The result is formation of ammonia which is a gaseous molecule capable of forming atmospheric aerosols that can linger in the air for weeks at a time. This makes ammonia directly and indirectly one of the largest contributors to ambient air pollution (Zhu et al., 2015). Ambient air pollution is currently a top ten risk factor contributing to mortality rates worldwide (Lim, 2013). This should raise cause for alarm as ammonia pollution is currently not well regulated worldwide and the levels of ammonia present in the air are only just starting to be monitored.

To prevent further nitrogen pollution to our environment, there needs to be a greater understanding of the regulatory pathways in mammary cells that influence milk protein yield beyond a simple substrate effect. As evidenced above, supplying dairy cattle with abundant

nitrogen in the diet is inefficient and not viable for environmental or economic reasons. However, reducing dietary nitrogen without any nutritional strategy and sacrificing production losses is not an option either. Further investigation must be performed to find ways of promoting a high milk protein yield should the dairy industry wish to continue to be profitable and sustainable in the future.

Research has found ways to increase milk yields in lactating dairy cows through interventions such as recombinant bovine somatotropin (rbST) treatment, which has shown to vastly improve milk yields and increase efficiency by increasing synthesis of milk with a normal composition, uptake of nutrients used in milk synthesis, activity per secretory mammary cell, and an improvement in blood flow to mammary tissue (Bauman, 1999). Despite the benefits for the industry and society as a whole, organizations have pushed back heavily against treating cows with rbST using claims that rbST causes increases in insulin-like growth factor 1 (IGF-1) in humans who consume dairy products from cows treated with rbST. This is untrue, however, as it is known that rbST has no significant effect on either micro or macro-composition of milk from cows with rbST and there was no significant concentration increase of IGF-1 in milk (Collier & Bauman, 2014).

Even though peptide hormones like rbST offer promising efficiency increases in dairy cattle across the world, it seems unlikely that it will ever become mainstream until misconceptions against peptide hormones are changed in the greater public. An alternative approach to increasing milk production without instigating controversy is to use natural feed ingredients to modify nutrient efficiency for milk production. Amino acids, the molecules that make up all proteins, also play a role in signaling in a variety of cellular pathways that regulate protein synthesis.

One way that AAs act as regulators for protein synthesis is via the mechanistic target of rapamycin complex 1 (mTORC1). The mTORC1 kinase is responsible for detecting nutrient availability and is stimulated by growth factors, insulin, and AAs (Bar-Peled & Sabatini, 2014, Saxton & Sabatini, 2017). When presented with these factors, mTORC1 promotes protein synthesis mainly by phosphorylation of p70S6 kinase 1 and the eIF4 binding protein (Saxton & Sabatini, 2017, Laplante & Sabatini, 2009). In this way, it is a positive regulator of protein synthesis and part of its activation is dependent on AAs available for use in protein synthesis. mTORC1 is distinguished for the role it plays in protein synthesis with regards to lactation. However, much less is known about the role that inhibitory pathways play with regard to protein synthesis during lactation.

One inhibitory pathway of protein synthesis in mammals is the general control non-depressible 2 kinase (GCN2). This experiment is primarily looking at GCN2 to determine if optimizing a diet to provide enough AAs to prevent GCN2 activation is a viable approach to both increasing the efficiency of milk protein synthesis and increasing total MPYs. Some research in bovine mammary epithelial cells has shown that deprivation of certain essential amino acids (EAAs) can be enough to stimulate GCN2 activation and thus results in a decrease in protein synthesis within the bovine mammary epithelial cells (Edick et al., 2020). However, little has been done with regard to in vivo models using GCN2 to study the effects on lactation and protein synthesis. This experiment will be using a murine model to determine the effects of GCN2 on lactation when AAs are limited.

GCN2 is an inhibitory pathway of protein synthesis and is conserved in all eukaryotic cells (Berlanga et al., 2001). It comprises one of four known components of the integrated stress response (ISR). The ISR provides eukaryotic cells a way to severely limit protein synthesis at a translational level

when exposed to a host of stresses (Donnelly et al., 2013, Pakos-Zebrucka et al., 2016). GCN2 in specific is responsible for sensing AA availability. GCN2 is activated by an increase in uncharged tRNAs but is also responsible for detecting AA imbalance (Bröer & Bröer, 2017, Gallinetti et al., 2012).

The other systems that make up the rest of the ISR are PKR-like ER kinase (PERK), protein kinase double-stranded RNA-dependent (PKR), and heme-regulated inhibitor (HRI). PERK is activated by a buildup of misfolded proteins in the endoplasmic reticulum (ER) of cells (Romine & Wiseman, 2019). The reduction in protein synthesis gives the ER time to refold and dispose of proteins trapped in the ER (Donnelly et al., 2013). PKR plays a role in promoting apoptosis of cells when exposed to a viral infection but can also be activated in response to oxidative stress, ER stress, along with cytokine signaling and growth factors (Cheshire et al., 2021, Nakamura et al., 2010). HRI activation can be linked to stresses such as proteasome inhibition and arsenite exposure (Yerlikaya et al., 2008, Lu et al., 2001).

These systems all act to phosphorylate the alpha subunit of eukaryotic initiation factor 2 at the serine 51 residue (eIF2α), which is offered as a rate-limiting step for mRNA translation in eukaryotes (Kimball, 1999). eIF2α phosphorylation decreases total global protein translation, but selectively increases translation and expression of the ATF4 transcription factor (Harding et al., 2004). ATF4 plays several key functions in the ISR. The first is to upregulate expression of regulated in development and DNA damage response 1 (REDD1), which is a protein that is responsible for mTORC1 inhibition via a TSC1/TSC2-dependent mechanism (Deegan et al., 2012). This then causes the induction of autophagy in cells. ATF4 is also responsible for the import of AAs into cells as *Atf4*−/− cells have shown a marked decrease in their ability to import AAs (Harding et al., 2004). It can also play a role in transactivating expression of C/EBP homology protein (CHOP). Together, ATF4 and CHOP induce expression of Tribbles 3 (TRB3), a negative regulator of Akt. Since Akt is a stimulator for mTORC1 activity, the TRB3

upregulation can lead to decreased mTORC1 activity and can further promote autophagy induction (Hayat, 2015).

As eIF2α phosphorylation leads to a severe decline in global protein synthesis, this is a potentially important regulator in lactation from an inhibitory viewpoint. Certain EAA and conditionally EAA have been shown to be more important in stimulation of GCN2 when their levels are low. In a bovine mammary epithelial cell model, Arg and Lys independent deprivation were shown to have statistically significant higher levels of eIF2α phosphorylation than Leu, which was close to the control levels of eIF2α phosphorylation. Similarly, the deprivation of all three showed the greatest levels of eIF2α phosphorylation (Edick et al., 2020). Using a low crude protein diet and GCN2 knockout model, it will be possible to examine the effects that GCN2 has on milk protein synthesis levels when constitutively expressed under low protein diet conditions compared to a control in a murine model. We hypothesized that GCN2 constitutive activation with a low protein diet would simulate, or closely follow the lactation results of a wild type mouse with a high protein diet.

Materials & Methods

Animals and experimental design

Eif2ak4 $^{lox/lox}$ mice, which have a lox P sequence insertion in the gene that encodes for the GCN2 protein, were obtained from Dudley Lamming and crossed with mice containing whey acidic protein (WAP)-Cre recombinase from Laura Hernandez. Mice were backcrossed 10 times to the C57BL/6j background prior to breeding for use as experimental animals. Mice for use in experiments were then obtained from colony breeder mice in a pairing of a wild type (WT) GCN2 female (*Eif2ak4* $^{flox/flox}$ *WAP-Cre*$^{-/-}$) and a knock-out (KO) GCN2 male (*Eif2ak4* $^{flox/flox}$

WAP-Cre$^{-/+}$). After reaching sexual maturity, the progeny from the breeders were genotyped by Transnetyx to determine if the animal was a GCN2 KO or WT.

Mice were then set up in mating pairs of opposite genotypes. The first litter was sacrificed on the fifth day after parturition one. Experimental mating pairs were left to breed during this time, and the male was removed when the dam was pregnant with her second litter. Pregnancy weights were taken throughout the duration of mating, up until the dam had given birth with her second litter.

On second parturition, lactation day 0 (LD0), the dam and pups were combined in a new cage while still on a standard diet. On LD1, litters were standardized to n=5, and the mice were assigned a low protein diet (LP) of 9% crude protein (Envigo TD171019) or a high protein (HP) 18% crude protein (Envigo TD171020).

The weights of the dam and pups were taken each day from LD1 to LD13. Any pup mortality was recorded and accounted for in analysis. The amount of feed consumed was measured every other day starting LD2, and the amount of feed put back into the cage was recorded. This amount was always 30 grams or more of diet. On LD9 and LD10, the dam's milk output was measured via a weigh-suckle-weigh (WSW) method (Weaver et al., 2016). The whole litter was weighed and put into a ventilated box, which was placed in their home cage to prevent suckling while still allowing visual contact. After a 4-hour separation, the litter was weighed again, and left to nurse with the dam for 45 minutes. The final weights of the litter were measured after this time and recorded.

Sample collection

On LD11, the dams underwent a hand milking to determine the rate of alpha S1 casein synthesis (Arriola Apelo et al., 2014). This started with a two-hour separation of pups, followed by a 30 minute recombination, similar to the WSW method used on LD9 and LD10. After 30 minutes, the pups were placed back into a ventilated box and a $^{2}H_{5}$-Phe isotope injection was given intraperitoneally (IP) at an amount of 0.26 mg per gram of dam weight. Tail bleedings were taken at time 0 (T0) before the injection and at T5, T15, T30, T60, T120, T240 minutes post injection. At 240 minutes after injection, the dam was placed under an isoflurane anesthetic following the final tail bleeding. 200 μL of oxytocin was injected intramuscular after under anesthetic, and the dam was placed on a warming pad with a continuous airflow of oxygen and 2% isoflurane. Milk was squeezed out and collected with a capillary tube for placement into a 1.5mL microcentrifuge tube. The casein was precipitated using 1:1 amounts of a 1 M acetic acid, sodium acetate mix in 1:1 proportions (pH of ~4.6). The resulting solution was vortexed and incubated while shaking at 37°C for 30 minutes. After 30 minutes the casein was spun down at 4000 rcf for 10 minutes at 4°C. Supernatant was ejected following spin down, and a casein pellet was left at the bottom. A washing buffer of 0.15 M acetic acid, sodium acetate mix in 1:1 proportions (pH of ~4.6) was then added at a volume of 1mL and vortexed before spinning down at 12000 rcf for 10 minutes at 4°C. The supernatant was again ejected. This step was repeated once more for a total of two spin downs with the washing solution. The casein then underwent a further two spin downs with acetone instead of a washing buffer, using the same guidelines as with the washing buffer. After the final spin down the acetone was ejected and the pellet was left to air dry before being stored at -80°C until analysis.

LD13 was the final day of the experiment and included a two-hour glucose fast, a shortened WSW, and tissue collection. A tail bleeding was taken before removal of food to

determine fed glucose levels. The pups were then separated for 1 hour for the shortened WSW. After the hour passed, litter weights were taken, and the pups were returned to the dam for 45 minutes. Following the 45 minutes, litter weights were taken. After the two-hour fast, a terminal submandibular bleeding was taken, and fasted glucose was measured. The mouse was confirmed dead via cervical dislocation after blood flow slowed. Tissues were collected in the following order each time. The fourth and third mammary glands of the dam, her liver, quadriceps, visceral adipose, kidneys, heart, lungs, brain, and DNA tail snip. The pup livers were collected following a cervical decapitation which was performed immediately prior to liver collection for each pup. All tissues were placed in liquid nitrogen during collection. They were then transferred to dry ice where they were organized, before being stored in a -80°C freezer.

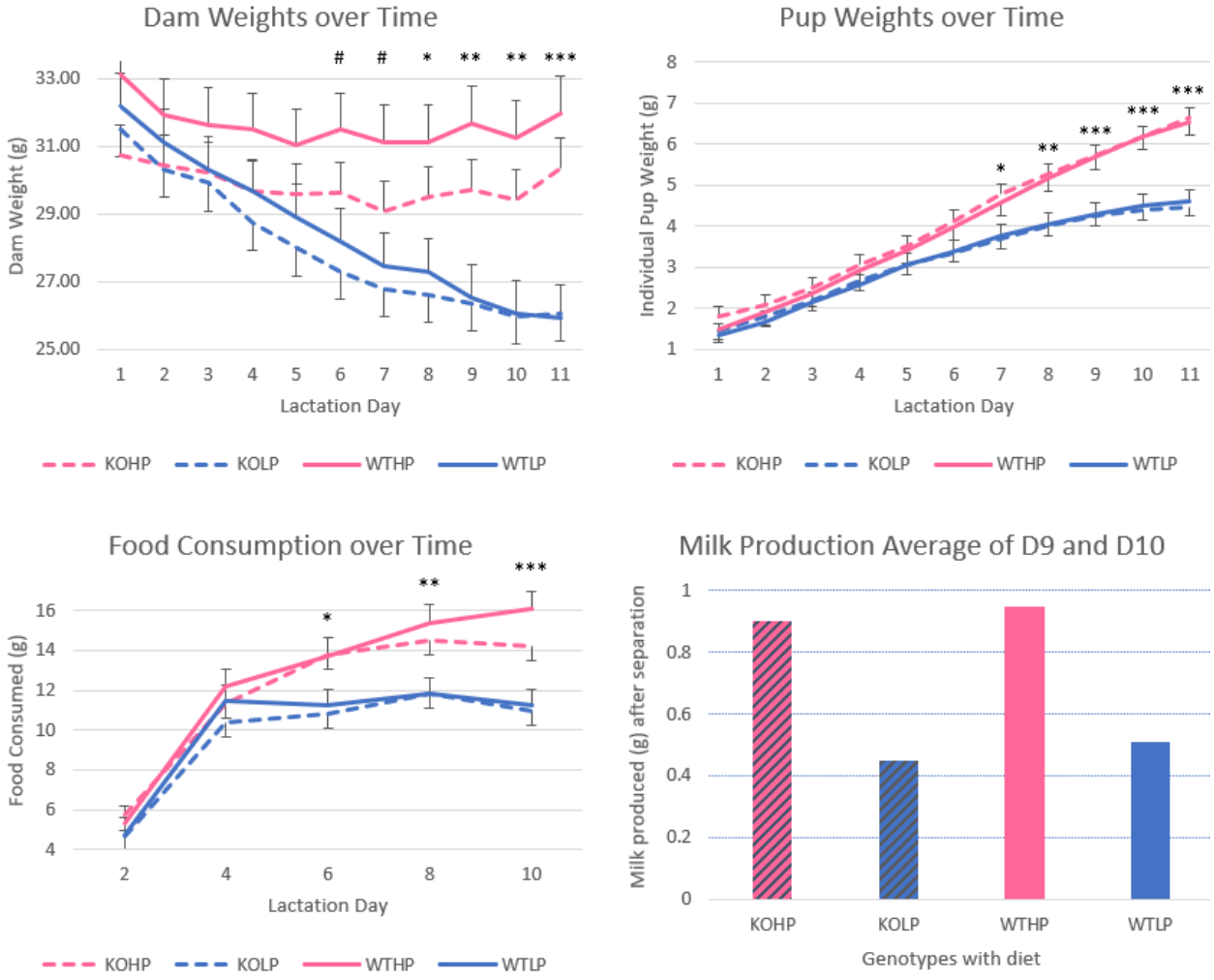
Statistical analysis

All data analysis was performed in RStudio. Live animal data was analyzed using ANOVA with repeated measures. Significance levels were set at $P \leq 0.05$, with trends at $0.05 < P \leq 0.10$. All live animal data was analyzed up to LD11, as the hand milking was strenuous on the mouse and impacted live animal data past this point.

Results

We found no significant interactions from the genotype of the mouse on the corresponding dam weight. There was a trend that started LD6 ($P = 0.09$, Fig. 1) between the different diets that continued until LD8 when it became significant ($P = 0.03$). Significance only increased with time, becoming $P < 0.01$ by LD9. Dams with LP diets also showed continual weight loss throughout the experiment whereas dams with HP diets started to stabilize in weight around LD6.

Standardized individual pup weight also showed no effect based on genotype. Diet interactions became significant on LD7 ($P = 0.03$, Fig. 2) and continued becoming increasingly significant throughout the experiment, reaching a value of $P < 0.001$ by LD9. Pup growth in LP diets appeared to begin plateauing at the end of the experiment while HP litters still showed strong growth. Food consumption showed no effect based upon dam genotype as well. Diet interactions became significant on LD6 ($P = 0.01$, Fig. 3), and reached $P < 0.001$ by LD10. This became more significant throughout the duration of the experiment.



*Significance levels 0.05, 0.01, 0.001, correspond to *, **, ***. Trends ($P < 0.10$) designated as #. All significance noted is due to a diet effect. Milk production p-values not indicated.*

Discussion

Our results showed that there seemed to be little interaction between genotype and dietary protein level in GCN2 KO mice relative to GCN2 WT. In fact, the KOLP treatment showed poorer lactation results than the WTLP. This is not at all what was expected as we hypothesized that the KOLP would perform better than the WTLP, and would mimic, or closely follow the results of the WTHP treatment.

Our results did show that the diets used were sufficient at creating a statistically significant effect. This validates that the protein concentrations used were correct if we wished to have GCN2 activation in the WTLP mice. Additionally, 18% crude protein is seen as standard for a mouse supporting a litter of up to seven pups (National Research Council Subcommittee on Laboratory Animal Nutrition, 1995). Since our LP diet was 9% crude protein, a 50% decrease from a standard diet, it is reasonable to assume that protein concentrations that low would be sufficient for GCN2 activation if we are to assume GCN2 is an important regulator of protein synthesis during lactation.

A possible explanation for why GCN2 appeared to have no effect during lactation could be that stimulation of mTORC1 is far more important when it comes to regulation of protein synthesis than GCN2. For example, our LPKO should be incapable of expressing GCN2 in mammary epithelial cells even though expression would normally be high in a WT mouse. However, because mTORC1 isn't being stimulated when AA levels are low, it may be in this way that the GCN2 KO mice appeared to show no genotype effect, but still showed a significant diet effect. It is also possible that the LP diet was still stimulating mTORC1, albeit at a less efficient rate, but that the separation between diets was due to a simple substrate availability

effect. It makes sense that less circulating AAs would lead to less opportunities to synthesize proteins necessary for pup growth.

These are both possible explanations for why a GCN2 genetic model did not show any changes during lactation. Western Blots of mammary tissue may prove insightful into distinguishing which, if either, was the reason why. Lower levels of mTORC1 downstream targets from Western Blots in LP mice versus HP mice would show that mTORC1 was likely not being stimulated sufficiently. This could mean that mTORC1 stimulation is more important than GCN2 inactivation from a milk protein efficiency perspective. However, close to normal levels in both treatment groups could show strong evidence towards the diet being regulated more via a substrate effect.

To ensure that the mice were the proper genotype, and that the KO was occuring in mammary cells, it would also be important to look at eIF2α phosphorylation levels when compared to various other tissues. If there was no eIF2α phosphorylation in LPKO mice in mammary tissue, but eIF2α phosphorylation in LPWT mice mammary tissue, it is as an indicator that the GCN2 KO was working. However, if the KO was affecting other tissues than just the mammary gland it would show that the genetic model was not working as intended.

Overall, the results proved to be the opposite of what we hypothesized. Regardless of genotype, the diet was what controlled the overall lactation results. While unexpected, it is still important to determine the role, if any, GCN2 has on lactation. A further study using the same genetic model, but with protein levels sufficient to stimulate mTORC1, and different enough to promote GCN2 activation could be used to determine if there were any differences in growth when compared to downstream target levels of each kinase. Should results show the same

findings as in this experiment, it could prove that GCN2 is not an important regulator of milk protein synthesis during lactation.

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