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Methionine, leucine, isoleucine, or threonine effects on mammary cell signaling and pup growth in lactating mice

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ABSTRACT

Two studies were undertaken to assess the effects of individual essential AA supplementation of a protein-deficient diet on lactational performance in mice using litter growth rates as a response variable. The first study was designed to establish a dietary protein response curve, and the second to determine the effects of Leu, Ile, Met, and Thr supplementation of a protein-deficient diet on lactational performance. In both studies, dams were fed test diets from parturition through d 17 of lactation, when the studies ended. Mammary tissue was collected on d 17 from mice on the second experiment and analyzed for mammalian target of rapamycin (mTOR) pathway signaling. Supplementation with Ile, Leu, or Met independently increased litter weight gain by 11, 9, and 10%, respectively, as compared with the protein-deficient diet. These responses were supported by independent phosphorylation responses for mTOR and eIF4E binding protein 1 (4eBP1). Supplementation of Ile, Leu, and Met increased phosphorylation of mTOR by 55, 34, and 47%, respectively, as compared with the protein-deficient diet. Phosphorylation of 4eBP1 increased in response to Ile and Met supplementation by 60 and 40%, respectively. Supplementation of Ile and Met increased phosphorylation of Akt/protein kinase B (Akt) by 41 and 59%, respectively. This work demonstrated that milk production responds nonlinearly to protein supply, and milk production and the mTOR pathway responded independently to supplementation of individual AA. The former demonstrates that a linear breakpoint model is an inappropriate description of the responses, and the latter demonstrates that no single factor limits AA for lactation. Incorporation of a multiple-limiting AA concept and nonlinear responses into milk protein response models will help improve milk yield predictions and allow derivation of diets that will increase postabsorptive N efficiency and reduce N excretion by lactating animals.
Key words: single amino acid, litter growth rates, cell signaling, mice

INTRODUCTION

It has been reported that the utilization of N for milk production by dairy cows was approximately 25%, with the remainder lost in urine and feces (Spears et al., 2003; Nadeau et al., 2007). This N loss is a significant economic loss for dairy producers and a critical source of environmental pollution (Hanigan et al., 1998). Considering the significant correlation between N intake and N excretion, in particular urinary N (Kebreab et al., 2001), it is very likely that dairy cows are fed protein above their true needs due to poor definitions of AA requirements. Dietary AA formulation is based on the single-limiting AA theory laid out by Mitchell and Block (1946) according to von Liebeg (1863). The single-limiting AA concept assumes that transfer of AA from the gut lumen to milk protein occurs at a constant efficiency until requirements are met and at 0 efficiency thereafter (a linear, breakpoint model); thus, the process is substrate limited with no adaptability. However, it has been demonstrated that AA not only serve as substrates for protein synthesis, but also regulate translation initiation and elongation via one or more cell signaling pathways (Shah et al., 2000; Anthony et al., 2002; Appuhamy et al., 2011a, 2012). Such regulation coupled with variable mammary AA transport activity confers variable AA use efficiency, which violates the fixed efficiency assumption that is critical to the framework proposed by Mitchell and Block (1946).

The integrated stress response and mammalian target of rapamycin (**mTOR**) pathways control translation initiation and elongation rates, and these pathways are affected by specific AA. The former regulates translation initiation through recruitment of the initiator

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Met-transfer RNA to 40S ribosomal subunits. This process is mediated by eukaryotic initiation factor 2 (**eIF2**), which can be phosphorylated at Ser51 resulting in inhibition of its function (Kimball and Jefferson, 2006). It has been demonstrated that AA mediate phosphorylation of eIF2α through the general control non-repressible kinase (**GCN2**) in mouse embryonic stem cells (Harding et al., 2000). Total EAA starvation in culture increased eIF2α phosphorylation in bovine mammary cells (Appuhamy et al., 2011a); however, the absence of individual EAA had only numerical effects on eIF2α phosphorylation (Appuhamy et al., 2012).

Amino acid availability also regulates the mTOR signaling pathway, which controls rates of translational initiation by repressing the inhibitory activity of eIF4E binding protein 1 (**4eBP1**), increasing rates of elongation by stimulating eukaryotic elongation factor 2 (**eEF2**), and possibly by enhancing ribosomal activity through activation of ribosomal protein S6 (Arriola Apelo et al., 2014). Removing Leu from medium had significant inhibitory effects on mTOR signaling (Kimball, 2001). Similarly elevated Leu was found to stimulate mTOR signaling in muscle cells (Escobar et al., 2006; Avruch et al., 2009). In bovine mammary tissue, deprivation of all AA or removing Leu or Ile individually affected phosphorylation of the downstream mTOR protein ribosomal protein S6 kinase 1 (**S6K1**) and casein fractional synthesis rates (**FSR**; Appuhamy et al., 2012). Removing Met also decreased phosphorylation of mTOR and casein FSR. Eukaryotic elongation factor 2, also thought to be under the control of mTOR, inhibits elongation when phosphorylated on Thr56 (Redpath et al., 1996). It has been suggested that eEF2 may be a limiting factor in milk protein synthesis (Christophersen et al., 2002). Removing all EAA from the medium increased eEF2 phosphorylation in bovine mammary tissue (Appuhamy et al., 2012), and individual EAA (Leu, Ile, Met, Thr) removal had differing effects on signaling proteins and casein FSR of mammary tissue in vitro (Appuhamy et al., 2012).

These responses are supported by inducible AA transport rates (Bequette et al., 2001), which serve to maintain the supply of each AA as their use is altered. These integrative, multifactorial responses are difficult to reconcile with a linear, breakpoint model, and the concept of a single-limiting AA and strict order of limitation as laid out by Mitchell and Block (1946). Thus, we hypothesized that supplementation of a protein deficient diet with individual EAA would independently improve lactational performance via the effects of EAA on cell signaling responses, and the responses would be inconsistent with the concept of a single-limiting AA theory. The objective of the present study was to investigate the independent effects of supplementation of a protein deficient diet with Leu, Ile, Met, and Thr on lactational performance in vivo.

MATERIALS AND METHODS

Animals and Experimental Design

Two studies were undertaken. The first study was designed to assess lactational responses to dietary protein, and the second study was designed to determine the independent effects of Leu, Ile, Met, and Thr supplementation of a protein deficient diet on lactational performance.

All animal work was approved by the Animal Welfare and Health Committee of Shandong Agricultural University. Pregnant mice were purchased from the Shandong University (Jinan, China) Laboratory Animal Center at 15 d of pregnancy. Mice were housed in a vivarium under controlled conditions. The system was set to maintain conditions between 22 and 24°C at 55 to 60% humidity with 10 h of light and 14 h of darkness throughout the study. Whereas this was achieved in the second study, the facility controls did not consistently maintain these conditions in the first study due to poor temperature between 18 and 20°C.

In both studies, dams were fed the test diets from parturition through the end of the study. Each dam was housed in an individual cage. Feed offered and orts were measured and recorded daily during the second study. At birth, pups were weighed and randomly culled to a total of 8 pups per litter to ensure a consistent litter size for each dam. Litter weight was measured at end of the study and used to calculate weight gain, which was the response variable. Some infanticide and maternal or pup death occurred. Litters experiencing death of the dam [n = 8 (1st); 14 (2nd)] were excluded from the analyses. Litters experiencing infanticide [n = 12 (1st); 9 (2nd)] were included in results, as infanticide represented nutrient recycling to the dam. In this case, the initial weight of consumed offspring was included in the calculations. The occurrence of this recycling likely introduced some error, as the efficiency of use of the recycled N would not be equal to dietary N; however, there was no simple way to correct for this effect and the net result would have been under-prediction of net nutrient supply to the dam, which represents a conservative estimate of the responses.

In the first experiment, modified American Institute of Nutrition (**AIN**)-76a purified diets, containing 6, 9, 12, 15, 18, 21, 24, or 27% protein, were fed for 17 d to lactating mice (n = 10 litters/treatment) to establish a dietary protein response curve. All diets were isocaloric (Trophic Animal Feed High-tech Co. Ltd., Nantong, China). Diet composition is described in Table 1.