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Journal of Nutritional Biochemistry 59 (2018) 114–122

Journal of Nutritional Biochemistry

Paracellular calcium flux across Caco-2 cell monolayers: Effects of individual amino acids☆

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Received 30 January 2018; received in revised form 26 April 2018; accepted 27 April 2018

Abstract

High-protein diets are generally considered beneficial for calcium (Ca) economy and bone health. Improved intestinal Ca absorption efficiency may be one mechanism by which higher-protein diets affect Ca homeostasis and bone health. The signaling pathways and individual amino acids (AA) responsible for this effect have not been fully elucidated and may involve the transcellular pathway, paracellular pathway or a combination. The primary aim of this study was to investigate whether a mixture of AA and/or functionally distinct individual AA directly affect paracellular Ca absorption across an intestinal epithelial cell model (Caco-2 Bbe). Using Ussing chambers, we examined the effect of six treatments — vehicle (Veh), 80 mM raffinose (Raf; positive control), 2× mixed AA(2×AA, twice the concentration in standard growth media), the branched-chain amino acid leucine (2–10 mM Leu), the aromatic amino acid phenylalanine (2–10 mM Phe) and the dibasic amino acid lysine (2–10 mM Lys) — on Ca flux. Leu (5 mM) increased Ca flux by 38% (+122 nmol Ca/cm^2/h, $P<.001$) as compared to Veh, while 10 mM Phe reduced Ca flux. No other differences were observed. Leu increased Ca flux through cellular redistribution of the Ca permissive channel Cldn-2 to the tight junction membrane ($P<.05$). Inhibition of mTORC1 signaling did not abrogate the effect of Leu on Cldn-2 localization, indicating a non-mTORC1-dependent signaling pathway is involved. These data indicate that Leu may improve Ca absorption in a cell model, potentially contributing to the observed benefits of higher-protein diets on bone health in humans.
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Keywords: Calcium absorption; Amino acids; Dietary protein; Intestinal cell; Caco-2

1. Introduction

High protein intake is well known to increase urinary calcium (Ca) excretion; it is estimated that for every 1-g increase in protein intake, approximately 1 mg of Ca is excreted in the urine [1]. Initially, the hypercalciuric response to high protein intake was thought to be due to increased bone resorption. Presumably, this hypercalciuria resulted from obligatory carbonate release from the skeleton to buffer the fixed acid load caused by metabolism of sulfur-containing amino acids, termed the "acid-ash hypothesis" [2]. However, over the last 2 decades, Ca absorption and kinetic studies using dual-stable isotope methodology determined that the hypercalciuria is due to increased intestinal Ca absorption rather than bone resorption [4–7].

Limited evidence suggests that dietary amino acids may differentially affect Ca absorption and/or renal excretion [8–10]. For example, a recent study determined that, when added to a low-protein (0.7 g/kg) diet, aromatic amino acids and dibasic amino acids had different effects on Ca absorption in young women assessed using dual-stable Ca isotopes with dibasic amino acids resulting in higher Ca absorption [10]. In another study, aromatic amino acids increased urinary Ca excretion as compared to branched-chain amino acids [11]. The direct effect of these three different amino acid classes — aromatic, di-basic and branched chain — on intestinal cell Ca handling has not been evaluated.

The mechanisms through which amino acids or dietary protein increases intestinal Ca absorption are only partially defined. Ca is absorbed in the intestine through two distinct pathways: a transcellular, facilitated diffusion pathway and a paracellular pathway, both of which are up-regulated by vitamin D. The transcellular pathway occurs through a three-step process whereby luminal Ca is taken up by the cell through apical Ca channels TRPV5/6, bound by calbindin D-9K in the cytosol and extruded out the basolateral membrane primarily through the Na-Ca ATPase. This pathway is up-regulated by vitamin D and exhibits saturable kinetics with the Km of TRPV6 at 0.44 mM [12,13]. In contrast, paracelluar Ca absorption occurs as Ca diffuses

☆ This work was funded by Army RDT&E. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations.
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https://doi.org/10.1016/j.jnutbio.2018.04.017
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down a concentration gradient from the intestinal lumen, through tight junctions, and into circulation. Paracellular Ca flux is facilitated by Ca-permissive pores formed by members of the claudin (Cldn) family of transmembrane tight junction proteins, specifically Cldn-2 and Cldn-12 [14], which are also vitamin D responsive [15]. While paracellular Ca absorption occurs along the length of the intestine, the transcellular pathway occurs primarily in the proximal small intestine. It is estimated that the paracellular pathway is responsible for the majority of Ca absorption when Ca intake is adequate. Thus, the mechanism by which dietary protein and/or amino acids increase Ca absorption may be mediated through the transcellular, facilitated diffusion pathway and/or the paracellular pathway. Characterization of the pathways and identification of specific amino acids involved in the salient effect of dietary protein on Ca absorption could aid in future development of nutritional interventions to enhance Ca absorption efficiency.

A role for the transcellular pathway in mediating Ca absorption in response to dietary protein has been demonstrated in rodents [3]. When rats consumed high levels of dietary protein, there was an increase in Ca absorption and retention. Further, this study demonstrated increased Ca uptake into duodenal brush border membrane vesicles isolated from rats acclimatized to the high-protein diet, thus supporting a role for the transcellular pathway in the effect of protein on Ca absorption [3]. As part of that study, a microarray performed on duodenal mucosa from rats consuming a low- or high-protein diet for 1 week [16] revealed a 2.6-fold increase in mRNA expression of Cldn-2 in rats consuming the high-protein diet, suggesting that the paracellular pathway may also be modulated by dietary protein (unpublished data) [16]. However, the effect of dietary protein on paracellular Ca uptake was not examined in that study. In addition, whether specific amino acids directly affect paracellular Ca absorption has not been evaluated.

Given the lack of mechanistic information regarding amino acids and their effects on Ca absorption, the primary aim of the current study was to investigate whether a mixture of amino acids and/or functionally distinct individual amino acids (aromatic, dibasic or branched chain) directly affect paracellular Ca absorption across intestinal epithelial cells. The secondary aim was to examine the effects of these bioactive amino acids on mRNA and protein expression of Ca-permissive tight junction proteins.

2. Materials and methods

2.1. Cell culture

Caco2 BBe cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in 75-cm^2 flasks at 37°C with a 5% CO_2:95% O_2 atmosphere and >95% humidity. Cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM; ATCC) supplemented with 10% fetal bovine serum (ATCC), 1% penicillin–streptomycin, 1% HEPES buffer, 1% L-glutamine and 1% nonessential amino acids. Medium was changed three times per week, and cells were passaged when 90% confluent, approximately one to two times per week. All media reagents were purchased from Gibco (Grand Island, NY, USA). In order to confirm that results could be replicated, at least two iterations were performed for each experiment.

For Ca flux experiments, cells were seeded on collagen-coated 1.1- cm^2 Snapwell inserts (Corning) at a density of 6.6×10^4 cells/cm^2. For protein and mRNA experiments, cells were seeded on collagen-coated 4.67-cm^2 Transwell inserts (Corning) at the same density. Experiments were carried out 21–23 days postseeding to ensure differentiation into a small intestinal phenotype, which was confirmed by a time course evaluation of mRNA expression of the small intestinal enzymes alkaline phosphatase and sucrase isomaltase as well as transepithelial electrical resistance (TER; Supplemental Fig. 1).

2.2. Ussing chamber flux studies

At 21–23 days postseeding, Snapwell inserts were mounted in Ussing chambers (Physiologic Instruments; Fig. 1C) and bathed on the mucosal and serosal sides with modified Ringer's solution (mmol/L): NaCl, 120; KCl, 4.2; $MgCl_2$, 0.3; $MgSO_4$, 0.4; $NaHCO_3$, 25; NaH_2PO_4, 0.4; Na_2HPO_4, 0.5; glycine, 1; L-glutamine, 200; glucose, 25; all chemicals were purchased from Sigma. The mucosal solution contained 10 mmol/L Ca and the serosal solution contained 1.3 mmol/L Ca in order to impose a Ca gradient similar to postprandial conditions in the human gastrointestinal tract [17]. All other treatments were added in equal concentrations to both the mucosal and serosal solutions. Experimental treatments were as follows: Ringer's solution alone (vehicle); 80 mM raffinose (positive control, Raf); 2× mixed amino acids (2×AA, defined as twice the concentration in the typical Gibco MEM growth medium); 2, 5 or 10 mM phenylalanine (Phe); 2, 5 or 10 mM leucine (Leu); and 2, 5 or 10 mM lysine (Lys). For comparison, the 2×AA treatment contained 0.8 mM of each Leu and Lys and 0.4 mM Phe in addition to other essential and nonessential amino acids (see Supplemental Table for complete listing of amino acids). The 2-, 5- and 10-mM amino acid concentrations were chosen based on total free amino acids (4 mM) in human plasma 1 h after a protein-rich meal and the total concentration of free amino acids in the jejunal lumen after a protein-rich meal (29 mM, with a range of <1 mM to 6 mM depending on the individual amino acid) [18]. Thus, the range of concentrations used in the current study encompasses the physiological range observed *in vivo* after high protein consumption. The concentration of raffinose was previously reported to increase paracellular Ca flux across Caco-2 cell monolayers [19]. Ringer's solutions were prewarmed to 37°C and adjusted with 5% carbon dioxide/95% oxygen gas to pH 7.4. A circulating water bath and continuous gassing maintained these conditions throughout the duration of the experiment. After mounting the Snapwell inserts, chambers were allowed to equilibrate for 30 min followed by the addition of the stable Ca isotope salt $^{44}CaCl_2$ (Trace Sciences) to the mucosal chambers (bath side) at a 20:1 tracer-to-tracee ratio. The $^{44}CaCl_2$ was reconstituted in 0.5 M HCl, and the addition of $^{44}CaCl_2$ was considered time point 0. Spontaneous potential difference (PD) was measured using electrodes in KCl- and agar-filled tips that were inserted into the serosal and mucosal sides of the chambers. The PD was evaluated through the electrodes using a voltage clamp that was corrected for blank Snapwell resistance. The voltage clamp was kept in the open-circuit condition and was only clamped when electrical readings were being collected for 3-min periods at times 0, 30, 60 and 90 min (T_0, T_{30}, T_{60} and T_{90}). Vehicle and positive control (Raf) treatments were included in every iteration resulting in a greater number of replicates for these groups. In order to confirm that cells responded equivalently to the treatments over the range of passage number used (65–90), flux experiments for each condition were conducted on cells from multiple passages.

Samples were collected from the serosal chambers prior to T_0 to determine background ^{44}Ca concentrations and at T_{30}, T_{60} and T_{90} and replaced with equal volumes of modified Ringer's solution. Samples were collected from the mucosal chambers at T_0 (B_{Pre}) and T_{90} (B_{Post}) to determine actual ^{44}Ca concentrations. Samples were stored at −20°C until shipping to USDA Grand Forks Human Nutrition Research Center for isotopic analysis.

2.3. Isotopic analysis

Samples were diluted 1:10 with 2% HNO_3 in double-deionized H_2O, and isotope prevalence was determined by ICP-MS using a Perkin Elmer ELAN DRCII and Nexion 350D ICPMS (Perkin Elmer) set to detect 42, 43, 44 and 48 atomic mass units. The minimum limit of detection was 0.27, 0.21, 0.13 and 0.28 μg/ml, respectively. Quality control included the use of a continuing calibration blank and calibration verification with a frequency of every 10 samples. Validation was accomplished using a NIST-Certified Standard, Trace Elements in Natural Water lot# 1640a (National Institute of Standards and Technology), which was analyzed at the beginning, middle and end of every batch of samples.

2.4. ^{44}Ca flux calculations

Flux of ^{44}Ca isotope was calculated by adapting a previously reported model [20]. Briefly, unidirectional ^{44}Ca flux (J_{ms}) was determined at T_{30}, T_{60} and T_{90} using the following equation:

$$J_{ms} = \frac{V \times (S_2 - (S_1 \times DF))}{SA \times cm^2 \times T},$$

where V is hemichamber volume, S_2 and S_1 are the measured concentrations of ^{44}Ca at the current and preceding time points, respectively, DF is the dilution factor to account for loss of ^{44}Ca with sampling, cm^2 is the surface area of the Snapwell insert and T is the time interval between S_1 and S_2. The SA variable represents ^{44}Ca specific activity and was calculated by dividing the average ^{44}Ca concentration from the T_0 and T_{90} mucosal samples by the total Ca content as determined by ICP-MS. The J_{ms} values from each time point were averaged to get an overall mean flux value for each chamber which was used for analysis.

2.5. RNA isolation and real-time polymerase chain reaction (PCR) analysis

Cell monolayers on Transwell inserts were washed twice with phosphate-buffered saline (PBS) and scraped in lysis buffer (Qiagen). An RNeasy Mini Kit (Qiagen) was then used to isolate RNA from the resulting lysate, and quantitative PCR (qPCR) was conducted as previously reported [21]. Briefly, isolated RNA was quantified using a Nanodrop 2000 (ThermoFisher), and 50 ng was reverse transcribed to cDNA using a High-Capacity cDNA Synthesis Kit (Life Technologies). Target mRNA levels were then determined by TaqMan real-time qPCR on an ABI StepOne rtPCR System (Applied Biosystems) using Taqman Fast Advanced Master Mix (Life Technologies) and Taqman assays for the following targets: beta-actin (Hs01060665_g1), Cldn-2 (Hs00252666_s1)

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