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Quantitation of urinary 3-methylhistidine excretion in growing dogs as an index of *in vivo* skeletal muscle catabolism

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Abstract

Purpose: To quantitate urinary 3-methylhistidine (3-mh) excretion as an index of *in vivo* muscle catabolism in dogs fed diets containing either normal or high protein levels.

Methods: Twelve male, 5-month-old Beagle dogs were housed individually in metabolism cages and fed a non-meat, purified diet. They were divided into two diet groups of six dogs each, receiving 22.6% (NP) or 41.1% (HP) DM crude protein, respectively. Three dogs from each group received an intravenous injection of 385 ± 29 kBq [^{14}C] 3-mh · HCl. Urine and feces were collected daily until radioactivity returned to background levels (17 days). Urinary 3-mh was measured using an amino acid analyzer and percentage of bound 3-mh was estimated via acid hydrolysis.

Results: Results are reported as means ± SEM. 3-mh recovery in urine and feces of dogs were 263 ± 28 kBq and 50.7 ± 2.2 kBq and 327 ± 45 kBq and 25.9 ± 25.9 kBq for the NP and HP groups, respectively. The total cumulative 3-mh recoveries for the NP and HP groups were 81.8% ± 2.8 and 91.4% ± 2.7, respectively. Bound 3-mh accounted for 2.1 to 4.8% of urinary ^{14}C-3-mh.

Conclusions: Growing Beagle dogs excrete a higher percentage of 3-mh in feces (13.5% vs. 6.7%) when consuming the NP versus the HP diet. It appears that some of the ^{14}C was lost in CO_2 and/or re-circulated in the body, as reported for sheep and pigs. We conclude that urinary 3-mh does not appear to be a quantitative index of *in vivo* muscle catabolism in growing dogs.

Keywords: 3-methylhistidine; Muscle turnover; Growing dogs; Canine; Quantitation

1. Introduction

Skeletal muscle approximates 40% of total body protein and constitutes the largest single tissue in the body of mammals. Skeletal muscle turnover is thus a quantitatively important component of total body protein metabolism [1]. 3-methylhistidine (3-mh) was identified in human urine in 1954 [2] and found to be present in skeletal muscle soon thereafter [3]. 3-mh is formed by post-translational methylation of histidine in actin and myosin. As the muscle protein is catabolized, 3-mh is released and not re-incorporated into protein, but primarily excreted unchanged in the urine. During the last 30 years, 3-mh excretion in urine has been shown to reflect skeletal muscle catabolism in humans and in several animal species [1–11]. Urinary 3-mh excretion provides an assessment of skeletal muscle breakdown in species in which no further metabolism or retention of 3-mh occurs, such as humans [4,8,12,13–16], cats [1], rabbits [1], and cattle [6]. In rats, 3-mh is partially acetylated before excretion, but this 3-mh can also be measured after acid hydrolysis [9]. In some animals, such as sheep [5], goats [3], and pigs [7], 3-mh is retained in the body as β-alanyl-3-methylhistidine for variable lengths of time before excretion, and therefore, urinary excretion does not accurately reflect muscle protein catabolism in these species.

The extent of urinary 3-mh excretion has not been reported in the dog. Therefore, the first objective of this study was to determine if dietary protein level had an effect on muscle protein turnover as measured by urinary 3-mh excretion in growing dogs receiving a normal (~25%) or high

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Table 1
Composition of the purified diets

Ingredients	Normal protein[x] (g/kg)	High protein[y] (g/kg)
Casein[a]	125	250
Soybean protein[b]	125	250
Cornstarch[c]	332	167
Sucrose[d]	166	84
Mineral mixture [18]	50	50
Vitamin mixture [18]	5	5
L-methionine[e]	3	0
Choline chloride (70%)[f]	4.3	4.3
Animal tallow[g]	100	90
Hydrogenated beef tallow[h]	30	40
Safflower oil[i]	30	30
Cellufil[j]	30	30
Total dry matter	1000	1000

[a] New Zealand Milk Products, Santa Rosa, CA.
[b] Archer Daniels Midland Co., Decatur IL.
[c] National Starch and Chemical Corp., Bridgewater, NJ.
[d] Westco Products, Sacramento, CA.
[e] Ajimomoto Co. Inc., Tokyo, Japan.
[f] Dupont, Highland, IL.
[g] Florin Tallow, Dixon, CA.
[h] A.C. Humko, Memphis, TN.
[i] Ventura Foods, City of Industry, CA.
[j] Amersham, Cleveland, OH.
[x] Normal protein diet, mean protein = 22.6% by analysis.
[y] High protein diet, mean protein = 41.1% by analysis.

(~50%) protein diet. The second objective was to quantitate urinary excretion of an intravenously administered dose of radiolabeled 3-mh in order to assess the extent of urinary excretion in dogs.

2. Materials and methods

In experiment 1A, twelve male Beagle dogs were housed individually in metabolism cages. The dogs averaged 5 months of age and 5.5 kilograms in weight at the start of this experiment. The dogs were maintained according to the Guide for the Care and Use of Laboratory Animals (NRC 1985) and the Animal Welfare Act. The dogs were adapted over a 20 day period to the cages and to a room temperature of 20°C ± 2°C, with a light cycle of 12 hours. The dogs were fed *ad libitum* a non-meat, purified diet (Table 1) based on casein (New Zealand Milk Products, Santa Rosa, CA) and soybean protein (Archer Daniels Midland Co., Decatur, IL). The dogs were divided into two diet groups of six dogs each, one group fed the diet containing 25% protein (NP) and the other 50% protein (HP) by formulation. The diets provided 22.6% ± 1.17 (SEM) and 41.1% ± 1.31 (SEM) crude protein, respectively, by dry matter (DM) analysis. The analyzed protein levels are reported as means from multiple analyses of three batches of each diet. Fresh water was available at all times. The dogs were weighed twice weekly, and their food intake was measured daily. Urine was collected daily for 17 days. Urine was collected into plastic bottles that were attached to the drain of the cage floor pan and contained 1 mL of 6 N HCl to prevent microbial growth in the urine. The bottles were changed daily, 24-hour urine volumes were recorded, and a 50 mL aliquot frozen at −20°C until analyzed. Feces from each dog were collected daily for 17 days from the cage walls and floor by scraper, weighed, placed into plastic containers and frozen at −20°C until analyzed.

In experiment 1B, three dogs from each diet group in experiment 1A received single intravenous injections of 385 ± 29 kBq [^{14}C] 3-mh · HCl (3-methyl ^{14}C, Amersham, Arlington Heights, IL) in order to quantitate the urinary and fecal excretion of 3-methyl histidine. The total amount of radiolabeled 3-mh, which contained 2020 kBq/mL of radioactivity, was determined by using an analytical balance to weigh the individual injection syringes before and after drawing up the dose for each dog. The radioisotope was >99% free of impurities, based on analysis by automatic amino acid analyzer (Beckman 7300, Fullerton, CA)-flow liquid scintillation system (Flo-One® Radiomatic, Beta series A-200, Meriden, CT). Urine and feces were collected daily from the injected dogs until radioactivity returned to background levels (17 days). Urine collection was performed as in experiment 1A. Radioactivity of each urine sample was measured by combining 1 mL of urine with 9 mL of scintillation fluid (Ultima Gold, Packard Biosciences, Meriden, CT), then ^{14}C counted in a liquid scintillation analyzer (Packard 2000CA Liquid Scintillation Analyzer, Downers Grove, IL). Quench correction was determined by using an external standard. An aliquot of daily urine from all dogs was assayed using an automatic amino acid analyzer for the determination of total 3-mh excretion. An aliquot of daily urine from the injected dogs (n = 6) was assayed on an automatic amino acid analyzer-flow liquid scintillation system to determine which amino acids were radioactive. This latter chromatography was performed after concentrating the samples using a 1.5 cm column of cation exchange resin (Dowex AG50W-X8, Biorad, Richmond, CA) and elution with 6 N HCl. This concentration process was necessary in order to exceed the detection minimum of the liquid scintillation flow system. The percentage of conjugated 3-mh (N-acetyl-3-mh or other conjugates) was determined by measuring 3-mh concentration before and after hydrolysis by heating with 6 N HCl for 24 hour at 120°C.

Feces from each dog (n = 6 per diet group) were collected daily for 17 days and stored as for experiment 1A. Feces were thawed overnight, then homogenized (Polytron homogenizer, Brinkman Instruments, Westbury, NY). A 100 mg aliquot of the homogenate was placed in a Teflon-capped glass counting vial and decolorized by successive addition of 0.2 mL perchloric acid (11.7 mol/L) and 0.4 mL of hydrogen peroxide (8.8 mol/L) followed by incubation at 70°C for 3 hours. The resulting digest was suspended in 10 mL scintillation fluid for radioactivity determination as for the urine samples.

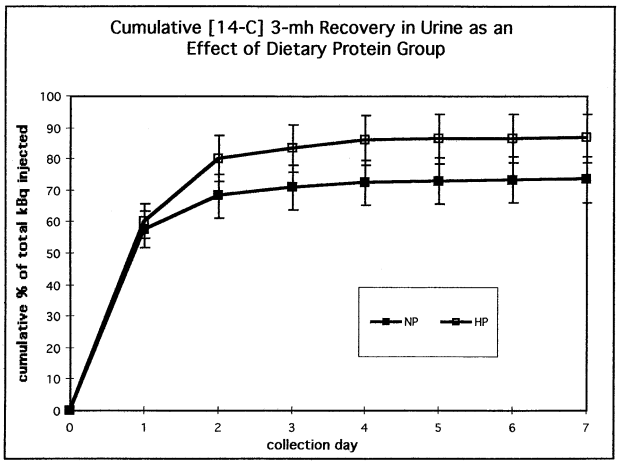

Fig. 1. Effect of dietary protein concentration on recovery of radiolabeled 3-mh in the urine of dogs. (Means ± SEM of the dogs within each diet group, $n = 3$/group). (■) = normal protein group (NP); (□) = high protein group (HP).

Results are reported as means in the text and as means ± standard error of the mean (SEM) in the tables, except for body weight and food intake measurements which are reported as group averages. Results of radioisotope recovery in experiment 1B were analyzed for statistical significance between groups by analysis of variance (ANOVA) with two groups. A value of $p < 0.05$ was accepted for significant differences.

3. Results

The recovery of the radiolabeled 3-mh in the urine of the dogs was 84.7% (326 kBq) for the HP diet group and 68.3% (263 kBq) for the NP diet group. In both groups, hydrolysis of urine increased 3-mh in the range of 2.1 to 4.8%. Radioanalysis of amino acids in the urine samples showed that the radioactivity was confined to 3-methylhistidine. Cumulative urinary recoveries of the radiolabel are shown in Fig. 1. There was a greater variability of urinary recovery of the label among dogs in the HP group (SD = 16.8%) compared to dogs in the NP group (SD = 7.7%).

The total volume of urine excreted during the collection period was 2.5 L and 1.8 L for the HP and NP groups, respectively ($n = 6$ dogs per group). The urinary 3-mh concentration was 657 nmol/mL for the dogs fed the HP diet and 814 nmol/mL for the dogs fed the NP diet. The daily amount of 3-mh excreted was 11.2 μmol/kg/d and 9.8 μmol/kg/d, for the HP and NP groups respectively. Results of urine and fecal radiolabel recovery are shown in Table 2. The total recovery of the radiolabeled 3-mh in the feces was 6.7% for the dogs fed the HP diet and 13.5% for the dogs fed the NP diet ($n = 3$ per diet group). The difference in total fecal recovery of the labeled 3-mh was significant between the diet groups ($p = 0.02$). The total percentage

Table 2
^{14}C-3-methylhistidine recovery in urine and feces of growing dogs fed the normal protein (NP) or high protein (HP) diet.

	Group	
	NP[a]	HP[b]
Total injected ^{14}C-3-methyl histidine (kBq)	385 ± 29	385 ± 29
Urine		
^{14}C-3-mh total excreted (kBq)	263 ± 29	326 ± 44
^{14}C-3-mh (% of injected label)	68.3 ± 2.8	84.7 ± 4.1
3-mh concentration (nmol/mL)	814 ± 14.9	656 ± 14.4
Total urine excretion (mL)	1830 ± 27.5	2580 ± 20.7
Feces		
^{14}C-3-mh (kBq)	52 ± 3.7*	26 ± 2.6
^{14}C-3-mh (% of injected label)	13.5 ± 0.5*	6.7 ± 1.2
Total recovered		
^{14}C-3-mh (kBq)	315 ± 33	352 ± 37
^{14}C-3-mh (% of injected label)	81.8 ± 2.8	91.4 ± 2.7

Values are expressed as means ± SEM.

Per diet group, $n = 3$, except $n = 6$ for urine 3-mh concentration and total urine excretion.

[a] NP = normal protein diet group.

[b] HP = high protein diet group.

* $p < 0.05$.

of intravenously injected [^{14}C] 3-methylhistidine recovered from urine and feces from the dogs was 91.4% and 81.8% for the HP and NP groups, respectively. This difference was not statistically significant ($p = 0.15$).

The total study length, including acclimation to the diets and cages and the radiolabel injection and collection, was six weeks. At the end of this period, the average age of the dogs was 6½ months and all dogs had gained over 3 kg of body weight. (Table 3) This represents an average increase in body weight of 63% (from 5.4 to 8.8 kg, $n = 6$) for the HP group and 60% (from 5.5 to 8.8 kg, $n = 6$) for the NP group. The weight gain difference between the groups was not significant. Overall, mean food intake for each group was greater than 270 grams per day during all weeks of this study except for the week immediately following the radiolabel injection (HP mean = 309 g/d and NP mean = 194 g/d). Thus, with the exception of that one week, there was no significant difference in food intake between the groups.

4. Discussion

Recovery of the injected radiolabeled 3-methylhistidine from these growing Beagle dogs was 91.4% and 81.8% from the HP and NP groups, respectively. Recoveries were variable among dogs within each group as well. Incomplete recovery of the radiolabel could be the result of incomplete collection of urine and feces and/or loss of the ^{14}C label in expired CO_2. Although incomplete collection of urine and feces is possible due to the size of the cage floor pan and the woven construction of the screen over the pan, recovery method from all cages was the same and does not explain

Table 3
Body weight and food intake of dogs fed the normal protein (NP) or high protein (HP) diet.

Time during study	Body weight (kg)		Food intake (g)	
	NP	HP	NP	HP
Acclimation period	5.5 ± 1.3	5.4 ± 0.9	277 ± 76	353 ± 37
Week after isotope injection	6.9 ± 1.2	7.3 ± 0.7	190 ± 27*	309 ± 59
End of collection period	8.8 ± 1.4	8.8 ± 0.8	334 ± 39	375 ± 46

Values are reported as group means ± SEM, $n = 6$ per diet group.
See text for study schedule and group descriptions.
*$p < 0.05$.

the difference in label recovery between the groups. Expired CO_2 was not collected since it was anticipated to be less than 1% of the ^{14}C recovered [1,5,8].

The differing percentage of radiolabel recovery between the two groups of dogs could be the result of different rates of urinary excretion and/or 3-mh entry into the intestinal tract via bile, pancreatic, or other secretions, followed by metabolism by intestinal microbes. In this study, the dogs fed the HP diet excreted a greater volume of urine and a higher percentage of the injected 3-mh than did the dogs fed the NP diet. The total urinary excretion of the label was 84.7% compared to 68.3%, respectively. The quantity of protein ingested by the HP dogs per day ranged from 127 to 154 g compared to the range of 43 to 75 g per day for the NP dogs. Excess nitrogen in the diet would have been excreted in the urine, causing a diuretic effect and contributing to the more rapid clearance of the 3-mh label in this group. It appears that the lower urine volume of the NP group resulted in a lower efficiency of excretion of 3-mh in the urine, longer retention of the label in the body fluids and tissues, and more 3-mh being excreted in the feces. The longer retention would allow more time for entry of the ^{14}C-3-mh into the lumen of the gastrointestinal tract and for oxidation to CO_2. Entry of the 3-mh into the intestinal tract through biliary, pancreatic, and other secretions would provide an opportunity for microbes to metabolize 3-mh and also increase excretion of the label in the feces of the dogs in the NP group.

Additional influences on radiolabel recovery in this study could be: (1) age of the dogs; (2) acetylation or conjugation of the 3-mh; and (3) re-circulation of the label in body tissues. From work in sheep which showed that quantitative urinary recovery of 3-mh increases as the animals mature from 4 weeks to 7 years old [4], age may play a role in urinary 3-mh excretion. Blazer-Yost and Jezyk [17] suggest that changes in canine urinary amino acid excretion during the first six months of life are associated with progressive development of the kidney. Bound 3-mh ranged from 2.1 to 4.8%, and may be the result of acetylation or other conjugation of 3-mh in the dog, or simply be the result of additive experimental error of performing two assays on each sample. Studies done in sheep [5] and in pigs [6] suggest re-circulation of the 3-mh as β-alanyl-3-methyl histidine. If this occurs in the dog, it may have contributed to the incomplete recovery of the isotope from the dogs.

It might be suggested that the lack of complete recovery of radiolabel precludes an evaluation of the effect of dietary protein in these dogs. However, since the total recovery of ^{14}C-3-mh in both groups was greater than 80%, the greater quantity of 3-mh in the urine of the HP group indicates that a higher protein intake did result in a greater rate of protein turnover. We conclude that urinary 3-mh does not appear to be a true quantitative index of *in vivo* muscle catabolism in growing dogs. Further studies should include using adult dogs and collecting expired CO_2.

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