J Food Sci Nutr
Vol 10, p 40~45 (2005)

J Food Science and Nutrition

Measurement of Age-Related Changes in Bone Matrix Using ^2H_2O Labeling

Jeongae Lee[1] and Yoo Kyeong Kim[2†]

[1]Bioanalysis & Biotransformation Research Center, Korea Institute of Science & Technology, Seoul 136-791, Korea
[2]Department of Home Economics Education, Kyungpook National University, Daegu 702-701, Korea

Abstract

Age-related changes in bone metabolism are well established by biochemical markers of bone matrix in serum and urine, but analysis of the residual bone matrix, which is still turning over, has not been investigated. In the present study, we measured *in vivo* rates of bone protein synthesis using a precursor-product method based on the exchange of ^2H from ^2H_2O into amino acids. Four percent ^2H_2O was administered to mice in drinking water after intraperitonial (i.p) bolus injection of 99.9% ^2H_2O. Mice were divided into the two groups: growing young mice were administered 4% ^2H_2O for 12 weeks after an i.p bolus injection at 5 week of age, whereas weight stable adult mice started drinking 4% ^2H_2O 8 weeks later than the growing group and continued 4% ^2H_2O drinking for 8 weeks. Mass isotopomer abundance in alanine from bone protein was analyzed by gas chromatography/mass spectrometry. Body ^2H_2O enrichments were in the range of 1.88~2.41% over the labeling period. The fractional synthesis rates (ks) of bone protein were 2.000 ±0.071%/d for growing mice and 0.243±0.014%/d for adult mice. These results demonstrate that the bone protein synthesis rate decreases with age and present direct evidence of age-related changes in bone protein synthesis.

Key words: age, bone matrix, ^2H_2O, fractional synthesis rate

INTRODUCTION

Age-related changes in bonemetabolism have been extensively investigated (1-6). These findings have been established by measuring bone mineral density (BMD) and the levels of biochemical markers, circulating by-products of bone metabolism in serum and urine. However, few studies have investigated the residual bone protein turnover mainly owing to difficulties involved in measuring it directly.

The use of isotope tracers of amino acids as incorporated markers during protein synthesis is an attractive method to investigate the dynamic nature of bone remodeling. However, major problems remain in defining the labeling of the true precursor for protein synthesis, i.e. the aminoacyl- or iminoacyl-tRNA, due to the complex subcellular organization of amino acid pools (7-9). Use of surrogate amino acid (AA) pools, such as plasma AA, or of a flooding dose approach, in which a large bolus dose of labeled amino acid is given to label all free blood and tissue amino acid pools to the same extent, have been employed to attain equilibration of labels in the precursor pool; however, the methods still suffer from practical limitations (10). Another problem derives from the very slow turnover rate of bone protein, which leads to label recycling and thus the underestimating of the turnover rate (9-12).

A precursor-product approach has practical advantages to overcome problems in defining a true precursor pool of protein synthesis if isotopic enrichment (I.E.) of the biosynthetic precursor pool is held relatively constant during the labeling period (13). However, continuous long-term administration of a labeled substrate to maintain relatively constant I.E. in the biosynthetic precursor pool may be unfeasible for slow turnover proteins as the cost of maintaining constant I.E. over a long time period can be prohibitive.

Since Ussing (14) introduced the use of ^2H_2O, a stable isotope of water, in animals to measure rates of protein renewal in 1941, ^2H_2O has been used as a tracer to measure the synthesis rates of fatty acids (15-17), cholesterol (18), glucose (19-21), and DNA (17,22,23). Recently, *in vivo* rates of protein synthesis have been measured in rodents (11,24) and humans (25) following ^2H_2O ingestion based on the incorporation of ^2H from ^2H_2O into the C-H bonds of the amino acids through specific, enzyme-catalyzed reactions (11,25). ^2H_2O can enter into C-H bonds of free AA's in the cell only through the reactions of intermediary metabolism, but ^2H_2O cannot enter into AA's that are present in peptide bonds or that

†Corresponding author. E-mail: yookim@wmail.knu.ac.kr
Phone: +82-53-950-5929, Fax: +82-53-950-5924

are bound to transfer RNA. The presence of ^{2}H-label in C-H bonds of protein-bound AA after ^2H_2O administration therefore means that the protein was assembled from AA that were in the free form during the period of ^2H_2O exposure - i.e. that the protein is newly synthesized. In addition, essential AA's may incorporate a single hydrogen atom from body water into the α-carbon C-H bond, through rapidly reversible transamination reactions. Non-essential AA's, e.g. alanine, contain a larger number of metabolically exchangeable C-H bonds and are therefore expected to exhibit higher I.E. values per molecule from ^2H_2O (11).

^2H_2O labeling provides many advantages allowing continuous long-term labeling to attain constant isotopic enrichment of the precursor pool in terms of reproducible incorporation of ^{2}H from ^2H_2O into the C-H bonds of amino acids, the constancy of body ^2H_2O enrichments over time without toxicities or side-effects, and the ease of oral ^2H_2O administration combined with the relatively low cost of ^2H_2O.

In the present study, we measured the *in vivo* rate of bone protein synthesis through ^{2}H enrichment of the alanine pool during ^2H_2O exposure to investigate age-related changes in bone metabolism in mice.

MATERIALS AND METHODS

Materials

^2H_2O was purchased from Cambridge Isotope Labs (Andover, MA, USA). Chemicals were purchased from Sigma, Inc. (St. Louis, MO, USA), unless otherwise stated.

Animals

Five-week-old female BC57Blk/6ksj mice (10∼15 g, Jackson Laboratories, Bar Harbor, ME, USA) were housed five per cage at 22∼24°C in an air-conditioned chamber with a 12 hour light-dark cycle. Diet (Purina® rodent chow) and water were provided *ad-libitum*. All experimental procedures were performed in accordance with the NIH guidelines for the care and use of laboratory animals.

^2H_2O administration protocol

The ^2H_2O labeling protocol (22,26) consisted of an initial intraperitoneal (i.p) injection of 99.9% ^2H_2O to achieve ≈2.5% body water enrichment (assuming the total body water to be 60% of the body weight) followed by administration of 4% ^2H_2O in drinking water for the duration of study. Mice were divided into the following two groups: growing young mice were administered with 4% ^2H_2O for 12 weeks after i.p bolus injection at age of 5 weeks, whereas weight stable adult mice started 4% ^2H_2O drinking 8 weeks later than the growing young group and continued 4% ^2H_2O drinking for 8 weeks. Mice (n =5 per each time point) were sacrificed every two weeks over the labeling period by cervical dislocation. Serum was collected longitudinally toestablish the time course of body ^2H_2O enrichments.

Isolation and hydrolysis of bone protein

The rear left femur was collected and was dissected free of soft tissue and distal ends. Bone marrow and trabecular bone were removed using a needle with a sharp cutting surface. After washing 3 times with water, the bone was splintered and powdered under liquid N_2 in a Spex mill and defatted with chloroform:methanol (1:1, v:v). After drying, the powdered bone was subjected to acid hydrolysis in 6 N HCl (110°C, 24 hr). The hydrolyzed AA were dried under N_2 gas and derivatized to *N*-acetyl, *N*-methyl ester for analysis by gas chromatography/mass spectrometry (GC/MS) (13).

Measurement of ^2H_2O enrichment of body water

The ^2H_2O enrichment of body water was measured by a GC/MS on a ThermoFinnigan Trace gas chromatography interfaced to a ThermoFinnigan PolarisQ ion trap mass spectrometer (ThermoFinnigan, Austin, Texas, USA). Briefly, the hydrogen atoms from the water were chemically transferred to acetylene by reaction with calcium carbide in a sealed vial. Acetylene gas was then derivatized by injection into another sealed vial containing 0.5 mL Br_2 (0.1 mM) dissolved in CCl_4 (22,26). The resulting tetrabromoethane was dissolved in CCl_4 and was analyzed by GC/MS, using a DB-1701 column (15 m × 0.25 mm × 0.25 μm; J&W, Folsom, CA, USA) with methane chemical ionization. The temperature programwas as follows: initial temperature: 60°C, held for 1 min; the first program rate: 50°C/min to 100°C, held for 3 min, the second program rate: 50°C/min to 220°C, held for 2.5 min. The ions at m/z 265 and 266 were analyzed using selected ion monitoring. The carrier gas was helium at a column flow of 0.8 mL/min at 80°C. The splitless injection mode was used.

Measurement of isotope abundance of alanine

The mass isotopomer abundance of alanine was analyzed as the *N*-acetyl, *N*-methyl ester derivative (13). The alanine derivative was analyzed at m/z 146 (M0) and 147 (M1) by methane chemical ionization mode using a Rtx®-5MS (Restek, Bellefonte, PA, USA, crossbond® 5% diphenyl-95% dimethyl polysiloxane) capillary column (30 m × 0.25 mm I.D. × 0.25 μm film thickness) at the following temperature program: initial temperature: 80°C, held for 1 min; program rate: 20°C/min to 280°C, held for 3 min. The carrier gas was helium at

a column flow of 0.8 mL/min at 80°C. The splitless injection mode was used.

The fractional synthesis or replacement rate (ks) of bone protein was determined by application of the continuous labeling, precursor-product formula (7,13,27).

$$k_s(d^{-1}) = -\left[\ln\left(1 - \frac{AA(protein)_t(I.E.)}{A^{\infty}(I.E.)}\right)\right] / time(d),$$

where AA(protein)$_t$ represents the enrichment as atom percent excess of the protein-bound AA measured at time t and A^{∞} represents the enrichment of amino acid at the asymptotic or plateau value possible under the labeling conditions present. The average body ^2H_2O enrichment was used to calculate theoretical asymptotic enrichment in alanine (A^{∞}) using standard mass isotopomer distribution analysis (MIDA) formulae established by Hellerstein et al. (11,27).

RESULTS

Body weight gain

The body weights of growing young mice gradually increased from 16.69±0.71 g to 24.64±0.97 g over the 12 week-labeling period (Fig. 1), which indicates that the administration of 4% ^2H_2O in drinking water did not cause any physiological alterations. For the adult mice, body weights remained relatively stable from 21.91± 0.93 g to 22.83±0.54 g for the subsequent 8 weeks of labeling.

Body water enrichment attained

Body ^2H_2O enrichments in mice were measured by GC/MS and were in the range of 1.88~2.41% over the labeling period in both groups, indicating that the body pool of ^2H_2O precursor maintained stable (Fig. 2). The differences in ^2H_2O enrichment between drinking water and body water resulted from dilution with metabolic-water produced endogenously by fuel oxidation as well as residual moisture in the dry chow.

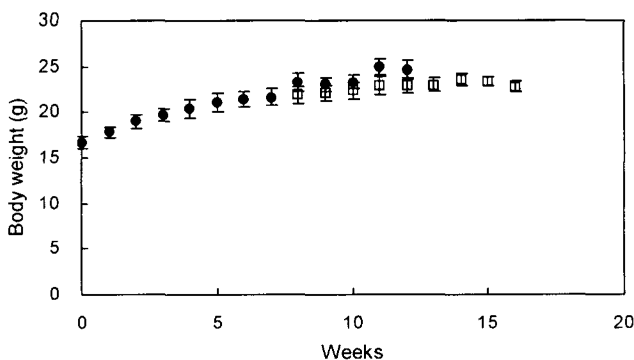

Fig. 1. Body weights of mice maintained on 4% ^2H_2O in drinking water after baseline priming bolus. Values represent mean±SE (n=5 per time point). ●: growing young mice group, □: adult mice group.

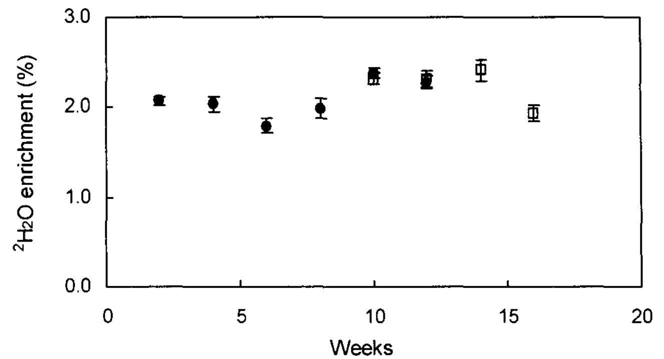

Fig. 2. Time course of body ^2H_2O enrichments in mice maintained on 4% ^2H_2O in drinking water after baseline priming bolus. Values represent mean±SE (n=5 per time point). ●: growing young mice group, □: adult mice group.

Bone protein synthesis rate

Fig. 3 shows the ^2H enrichments of alanine released on the hydrolysis of the femur bone. Fig. 4 depicts the linear regression line from the natural logarithms of the differences between the enrichment of alanine at plateau possible under the labeling conditions present and the mean enrichment of alanine released from bone. The fractional synthesis rates (ks), estimated from the slopes of the lines of Fig. 3 and 4 during labeling periods, were 2.000±0.071%/d for growing young mice and 0.243± 0.014%/d for adult mice. After 8 weeks of labeling, 63.7% of bone protein was replaced with new bone protein in growing young mice. Aging decreased the newly synthesized fraction of bone protein to 24.6% in adult mice.

DISCUSSION

In the present study, we focused on the changes in bone protein to investigate the effects of aging on bone turnover. Previous studies (1-6) have demonstrated bone loss with age mainly by monitoring changes in BMD

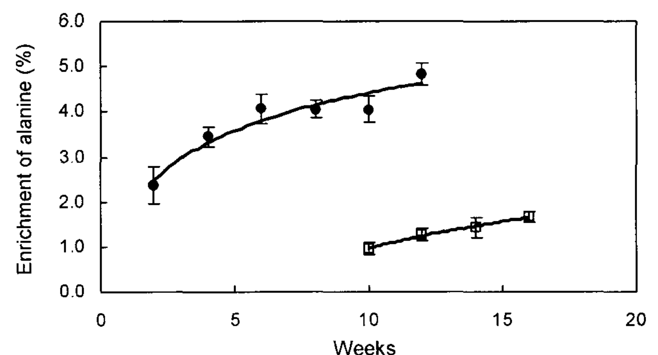

Fig. 3. Label incorporation curves into alanine isolated from bone protein in mice. Values represent mean±SE (n=5 per time point). ●: growing young mice group, □: adult mice group.

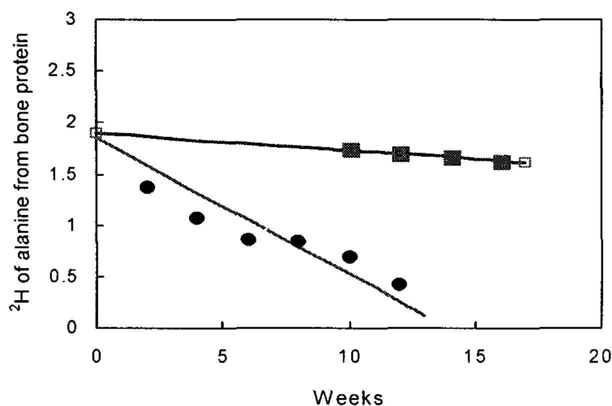

Fig. 4. Natural logarithms of the differences between the enrichment of alanine at plateau possible under the labeling conditions present and the mean enrichment of alanine released from bone. The fractional synthesis rate is the negative of the slope of the linear regression line fitted to the data. Values represent mean ± SE (n=5 per time point). ●: growing young mice group, ■: adult mice group.

and circulating levels of biochemical markers. However, these indicators have important limitations in reflecting the life-long bone remodeling process. BMD represents current skeletal status and requires sequential measurements 12 to 24 months apart to identify significant bone loss in humans (28-30). Biochemical markers have clinical advantages in monitoring bone metabolism and treatment efficacy (31-35). However, thus far biochemical markers have not been of much clinical value due to low sensitivity and specificity to bone (32,34). Moreover, few biochemical markers are available for animal studies and thereby changes in animal bone have been primarily measured by BMD, bone volume, and histomorphometry (4,6,36).

Some researchers have used an isotope-labeling approach to measure kinetic changes of physiologically important proteins with age. Balagopal et al. (37) examined the effects of aging on *in vivo* synthesis of skeletal muscle myosin heavy chain and sarcoplasmic protein in humans using a primed continuous infusion of ^{13}C-leucine. A decline in the synthesis rate of mixed muscle protein and whole body protein was observed from young to middle age with no further change with advancing age. An age-related decline of myosin heavy chain synthesis rate was also observed with progressive decline occurring from young, through middle, to old age, which was also found in mitochondrial protein kinetics in human-skeletal muscle (38). However, sarcoplasmic protein synthesis did not decline with age. These observations suggest that each protein has a specific kinetic pattern with advancing age.

Numerous studies have measured the synthesis rate of collagen from lung (39-42), heart (42,43), skeletal muscle (44), skin (44,45), and bone (46-48) in rodents (42, 43,45,48) and humans (40,46,47) with isotope labeled tracers. However, the kinetics of bone protein still remains unclear owing to some practical constraints for reliable kinetic measurement on bone protein, which has a very slow turnover rate (10). Recently, a new method using ^2H_2O as a tracer has been developed to measure rates of protein synthesis and successfully applied to bone protein, skeletal muscle, and cardiac muscle in rats (11), and plasma albumin in humans (25). The unique features of ^2H_2O labeling, i.e. high reproducibility, safety, and low cost, permit full exploitation of the precursor-product relationship, because constant labeling is feasible for $>4\sim5$ half-lives of almost any protein of interest. Thus, variable dilution within tRNA-AA pools can be overcome and thereby the errors arising from extrapolation with an alternative precursor pool may decline.

Babraj et al. (46) measured *in vivo* collagen synthesis rate in human bone with a flooding dose method using ^{13}C-labeled proline. Although they observed differences in the isotope incorporation rate in bone collagen by sequential extracts, they failed to find an aging effect on bone collagen and concluded that the difference in bone between young and elderly people represents a chemical or physical rather than biological phenomenon.

However, our results clearly demonstrate a dynamic process of bone remodeling. Old bone matrix was continually replaced with newly synthesized protein. Bone matrix protein was replaced exponentially in growing young mice and fractional replacement occurred in a linear pattern in adult mice. To our knowledge, the current results provide clear evidence supporting age-related changes in bone matrix.

ACKNOWLEDGEMENTS

This research was supported by Kyungpook National University Research Fund, 2002.

REFERENCES

1. Chan GK, Duque G. 2002. Age-related bone loss: old bone, new facts. *Gerontology* 48: 62-71.
2. Raisz LG, Seeman E. 2001. Causes of age-related bone loss and bone fragility: an alternative view. *J Bone Miner Res* 16: 1948-1952.
3. Wang X, Shen X, Li X, Agrawal CM. 2002. Age-related changes in the collagen network and toughness of bone. *Bone* 31: 1-7.
4. Banu J, Wang L, Kalu DN. 2002. Age-related changes in bone mineral content and density in intact male F344 rats. *Bone* 30: 125-130.
5. Bailey AJ, Sims TJ, Ebbesen EN, Mansell JP, Thomsen JS, Mosekilde L. 1999. Age-related changes in the biochemical properties of human cancellous bone collagen: relationship to bone strength. *Calcif Tissue Int* 65: 203-

210.
6. Takeuchi Y, Watanabe S, Ishii G, Takeda S, Nakayama K, Fukumoto S, Kaneta Y, Inoue D, Matsumoto T, Harigaya K, Fujita T. 2002. Interleukin-11 as a stimulatory factor for bone formation prevents bone loss with advancing age in mice. *J Biol Chem* 277: 49011-49018.
7. Waterlow JC, Garlick PJ, Millward DJ. 1978. *Protein turnover in mammalian tissues and in the whole body.* 2nd ed. North Holland, Amsterdam.
8. Khairallah EA, Mortimore GE. 1976. Assessment of protein turnover in perfused rat liver. Evidence for amino acid compartmentation from differential labeling of free and tRNA-bound valine. *J Biol Chem* 251: 1375-1384.
9. Hellerstein M. 2000. Methods for measuring polymerisation biosynthesis: three general solutions to the problem of the "true precursor". *Diabetes Nutr Metab* 13: 46-60.
10. Rennie MJ. 1999. An introduction to the use of tracers in nutrition and metabolism. *Proc Nutr Soc* 58: 935-944.
11. Hellerstein MK, Neese RA, Kim YK, Schade-Serin V, Collins M. 2002. Measurement of synthesis rates of slow-turnover proteins from ^2H_2O incorporation into non-essential amino acids (NEAA) and application of mass isotopomer distribution analysis (MIDA). *FASEB J* 66: A256.
12. Jackson SH, Heininger JA. 1973. A reassessment of the collagen reutilization theory by an isotope ratio method. *Clin Chim Acta* 46: 153-160.
13. Wolfe RR. 1992. Protein synthesis and breakdown. In *Radioactive and stable isotope tracers in medicine.* A John Wiley & Sons, Inc, New York. p 377-416.
14. Ussing HH. 1941. The rate of protein renewal in mice and rats studied by means of heavy hydrogen. *Acta Physiol Scand* 2: 209-221.
15. Leitch CA, Jones PJ. 1991. Measurement of triglyceride synthesis in humans using deuterium oxide and isotope ratio mass spectrometry. *Biol Mass Spectrom* 20: 392-396.
16. Leitch CA, Jones PJ. 1993. Measurement of human lipogenesis using deuterium incorporation. *J Lipid Res* 34: 157-163.
17. Strawford A, Antelo F, Christiansen M, Hellerstein MK. 2004. Adipose tissue triglyceride turnover, *de novo* lipogenesis, and cell proliferation in humans measured with ^2H_2O. *Am J Physiol Endocrinol Metab* 286: E577-588.
18. Jones PJ, Scanu AM, Schoeller DA. 1988. Plasma cholesterol synthesis using deuterated water in humans: effect of short-term food restriction. *J Lab Clin Med* 111: 627-633.
19. Saadatian M, Peroni M, Diraison M, Beylot M. 2000. *In vivo* measurement of gluconeogenesis in animals and humans with deuterated water: a simplified method. *Diabetes Metab* 26: 202-209.
20. Perdigoto R, Furtado AL, Porto A, Rodrigues TB, Geraldes CF, Jones JG. 2003. Sources of glucose production in cirrhosis by ^2H_2O ingestion and ^2H NMR analysis of plasma glucose. *Biochim Biophys Acta* 1637: 156-163.
21. Landau BR, Wahren J, Chandramouli V, Schumann WC, Ekberg K, Kalhan SC. 1995. Use of ^2H_2O for estimating rates of gluconeogenesis. Application to the fasted state. *J Clin Invest* 95: 172-178.
22. Neese RA, Misell LM, Turner S, Chu A, Kim J, Cesar D, Hoh R, Antelo F, Strawford A, McCune JM, Christiansen M, Hellerstein MK. 2002. Measurement *in vivo* of proliferation rates of slow turnover cells by ^2H_2O labeling of the deoxyribose moiety of DNA. *Proc Natl Acad Sci USA* 99: 15345-15350.
23. Collins ML, Eng S, Hoh R, Hellerstein MK. 2003. Measurement of mitochondrial DNA synthesis *in vivo* using a stable isotope-mass spectrometric technique. *J Appl Physiol* 94: 2203-2211.
24. Previs SF, McCabe BJ, Chandramouli V, Landau BR. 2002. Estimation of protein turnover *in vivo* using D_2O. *Diabetes* 50: A301.
25. Previs SF, Fatica R, Chandramouli V, Alexander JC, Brunengraber H, Landau BR. 2004. Quantifying rates of protein synthesis in humans by use of ^2H_2O: application to patients with end-stage renal disease. *Am J Physiol Endocrinol Metab* 286: E665-672.
26. Neese RA, Siler SQ, Cesar D, Antelo F, Lee D, Misell L, Patel K, Tehrani S, Shah P, Hellerstein MK. 2001. Advances in the stable isotope-mass spectrometric measurement of DNA synthesis and cell proliferation. *Analytic Biochem* 298: 189-195.
27. Hellerstein MK, Neese RA. 1999. Mass isotopomer distribution analysis at eight years: theoretical, analytic and experimental considerations. *Am J Physiol* 276: E1146-1162.
28. Lenchik L, Kiebzak GM, Blunt B. 2002. What is the role of serial bone mineral density measurements in patient management? *J Clin Densitom* 5: S29-38.
29. Westhoff C. 2002. Bone mineral density and DMPA. *J Reprod Med* 47: 795-799.
30. Berger A. 2002. Bone mineral density scans. *BMJ* 325: 484.
31. Seregni E, Martinetti A, Ferrari L, Bombardieri E. 2001. Clinical utility of biochemical marker of bone remodelling in patients with bone metastases of solid tumors. *Q J Nucl Med* 45: 7-17.
32. Dessauer A. 1997. Analytical requirements for biochemical bone marker assays. *Scand J Clin Lab Invest* 227: 84-89.
33. Dogan E, Posaci C. 2002. Monitoring hormone replacement therapy by biochemical markers of bone metabolism in menopausal women. *Postgrad Med J* 78: 727-731.
34. Seibel MJ. 2002. Nutrition and molecular markers of bone remodeling. *Curr Opin Clin Nutr Metab Care* 5: 525-531.
35. Woitge HW, Seibel MJ. 2001. Biochemical markers to survey bone turnover. *Rheum Dis Clin North Am* 27: 49-80.
36. Advani S, LaFrancis D, Bogdanovic E, Taxel P, Raisz LG, Kream BE. 1997. Dexamethasone suppresses *in vivo* levels of bone collagen synthesis in neonatal mice. *Bone* 20: 41-46.
37. Balagopal P, Rooyackers OE, Adey DB, Ades PA, Nair KS. 1997. Effects of aging on *in vivo* synthesis of skeletal muscle myosin heavy-chain and sarcoplasmic protein in humans. *Am J Physiol* 273: 790-800.
38. Rooyackers OE, Adey DB, Ades PA, Nair KS. 1996. Effect of age on *in vivo* rates of mitochondrial protein synthesis in human skeletal muscle. *Proc Natl Acad Sci USA* 93: 15364-15369.
39. Bradley K, McConnell-Breul S, Crystal RG. 1975. Collagen in the human lung. Quantitation of rates of synthesis and partial characterization of composition. *J Clin Invest* 55: 543-550.
40. Hildebran JN, Airhart J, Stirewalt WS, Low RB. 1981. Prolyl-tRNA-based rates of protein and collagen synthesis in human lung fibroblasts. *Biochem J* 198: 249-258.
41. Kehrer JP, Lee YC, Solem SM. 1986. Comparison of *in vitro* and *in vivo* rates of collagen synthesis in normal and damaged lung tissue. *Exp Lung Res* 10: 187-201.
42. Nakamoto T, Porter JR. 1984. Malnutrition and oxygen at high pressure: influence on collagen synthesis in lung

and heart of the newborn rat. *Aviat Space Environ Med* 55: 528-533.
43. Murray JC, Parkins CS. 1988. Collagen synthesis in CBA mouse heart after total thoracic irradiation. *Radiother Oncol* 13: 137-143.
44. Laurent GJ. 1982. Rates of collagen synthesis in lung, skin and muscle obtained *in vivo* by a simplified method using [^{3}H]proline. *Biochem J* 206: 535-544.
45. Molnar JA, Alpert N, Burke JF, Young VR. 1986. Synthesis and degradation rates of collagens *in vivo* in whole skin of rats, studied with $^{18}O_2$ labelling. *Biochem J* 240: 431-435.
46. Babraj J, Cuthbertson DJ, Rickhuss P, Meier-Augenstein W, Smith K, Bohe J, Wolfe RR, Gibson JN, Adams C, Rennie MJ. 2002. Sequential extracts of human bone show differing collagen synthetic rates. *Biochem Soc Trans* 30: 61-65.
47. McNurlan MA, Garlick PJ, Frost RA, Decristofaro KA, Lang CH, Steigbigel RT, Fuhrer J, Gelato M. 1998. Albumin synthesis and bone collagen formation in human immunodeficiency virus-positive subjects: differential effects of growth hormone administration. *J Clin Endocrinol Metab* 83: 3050-3055.

(Received December 7, 2004; Accepted January 31, 2005)