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Plasma Homocysteine Concentrations in Novel Microminipigs

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Abstract. *A novel microminipig has been recently developed for use in biomedical research. In the present study, age- and sex-related differences, as well as 24-h fluctuations in plasma total homocysteine concentrations (tHcy), were investigated in these microminipigs. tHcy (mean±SD) was 10.2±3.4 μM and significantly correlated with age. By contrast, neither the differences in tHcy between sexes nor the 24-h fluctuations in tHcy after feeding were significant. The kinetics of plasma tHcy after intravenous injection of reduced Hcy showed that its levels peaked within 5 min post-injection, as did the levels of tHcy. These results suggested that reduced Hcy is rapidly oxidized or metabolized. The half-lives of reduced Hcy, tHcy, and reduced cysteine in the blood were 47, 71, and 141 min, respectively. In conclusion, there was a significantly positive correlation between age and plasma tHcy in microminipigs. After intravenous injection of reduced Hcy, plasma tHcy quickly returned to pre-injection levels.*

Homocysteine is produced from methionine. During its metabolism, homocysteine is re-methylated to methionine or converted to cysteine (1). Disturbances in methionine metabolism lead to altered methylation, leading to epigenetic changes in gene expression (2, 3). Hyperhomocysteinemia, defined as plasma homocysteine >30 μM, interferes with methionine metabolism and induces global DNA hypomethylation (2). In addition, hyperhomocysteinemia stimulates inflammation itself (4, 5) and has been implicated in atherosclerosis and dementia (6). Total homocysteine is composed of a protein-bound fraction, a free oxidized fraction, and a free reduced form (7, 8). Chambers and co-workers demonstrated that the reduced form of homocysteine is closely associated with vascular endothelial dysfunction (9).

Swine have several physiological and anatomical similarities to humans (10) and are an appropriate animal model in biomedical research. They also provide an alternative to monkeys and dogs, in efforts to respond to animal welfare concerns and to minimize the use of these animals. Several minipig strains have been developed but controlling their weight and body size is difficult. Recently, a novel microminipig was introduced (11, 12). Its small size and low body weight (at maturity, <25 kg) favor its use as a reliable and easily-manageable experimental animal (13-16).

Several studies determined plasma homocysteine concentration in swine (17, 18) but none of them examined sex- and age-related changes in plasma homocysteine levels and its pharmacokinetics in the blood. In the present work we examined plasma homocysteine levels in microminipigs. Our results provide fundamental reference information for these animals on age- and sex-related differences in homocysteine and on the pharmacokinetics of homocysteine in the blood.

Materials and Methods

Animals and blood collection. To investigate the age-related changes of plasma total homocysteine concentrations in microminipigs (Fuji Micra inc., Fujimomiya, Shizuoka, Japan), we obtained plasma samples of 87 male (mean±SD: 15.9±10.25 months) and 44 female (mean±SD: 16.9±8.4 months) healthy microminipigs. To examine daily fluctuations of total homocysteine, we used three male microminipigs that, 13 months old. Feeding time was at 9 in the morning, and blood collections were performed prefeeding and at 1, 2, 4, 6, 8, 12, and 24 h after feeding (3% of bodyweight). For a pharmacokinetics study of

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homocysteine, we obtained four female microminipigs. Age and bodyweight of these microminipigs were 29, 32, 33 and 34 months and 12.2, 10.8, 11.7 and 11.6 kg, respectively. All microminipigs were maintained in breeding rooms, with temperature maintained at 24±3˚C and relative humidity at 50±20%, with a 12-h light/dark cycle. Tap water was available *ad libitum*. Food (Kodakara 73; Marubeni Nisshin Feed Inc., Chuo-Ku, Tokyo, Japan) was supplied twice a day (3% of bodyweight/day). The use of animals in these experiments complied with all relevant guidelines set by the Kagoshima University. This animal experiment was approved by Kagosima University Committee of Animal Experimentation (A11035).

Pharmacokinetics test. A polyurethane tube was inserted and located in the sinus *venarum cavarum*. We used this tube for blood collection and administration of homocysteine. We prepared a DL-homocysteine solution (1 M WAKO, Osaka, Osaka, Japan), which was dissolved in phosphate-buffered saline (pH 7.4), and administered immediately to each microminipig *via* polyurethane catheter (17 μmol/kg of body weight). Blood collections were performed before administration, and at 5, 15, 30, 60, 120, 180, 240 min, and one day after administration. The blood samples were placed into ethylene diamine tetra-acetic acid (EDTA) 2 K tubes (NIPRO, Osaka, Osaka, Japan) and centrifuged immediately for 15 min at 4˚C and 1,500 × *g*. Plasma samples were rapidly stored at −20˚C.

Measurement of homocysteine, cysteine and methionine concentrations. For measurement of total plasma homocysteine, and total cysteine, we referred to previous studies (19, 20). In measurement of reduced homocysteine and reduced cysteine, the preparation was the same as the method as above excepting including a reducing step using Tris [2-carboxyethyl] phosphine. The quantitative analysis of methionine was as described by Moriyama *et al*. (21). Plasma concentration (Cp) profiles for homocysteine were analyzed by fitting the following biexponential equation with the nonlinear least-squares method (22): $Cp=Ae^{(-\alpha t)} + Be^{(-\beta t)}$. The elimination rate constant (k_e) and half-life ($t_{1/2}$) were calculated using the following equations: $k_e=\alpha\beta\ (A+B)\times(A\beta+B\alpha)^{-1}$; $t_{1/2}=0.693\times k_e^{-1}$.

Statistics. Data were analyzed by using the Pearson correlation coefficient or one-way ANOVA followed by Dunnett's *t*-test. All analyses were performed using the Statistical Package for Social Science ver. 19.0 (SPSS, Chicago, IL, USA). A value of $p<0.05$ was considered statistically significant.

Results and Discussion

Total homocysteine concentration in microminipigs. In the present study, 87 male and 44 female healthy microminipigs were investigated. Mean ±SD plasma total homocysteine concentration in male and female microminipigs were 14.67±5.73 μM and 14.63±3.79 μM, respectively, and did not significantly differ ($p=0.97$). In human epidemiological studies, total homocysteine concentration is significantly higher in males compared to females (23-25) but these studies were carried-out on middle-aged or elderly people. The female hormone estradiol is closely related to plasma total homocysteine (26). One possible cause for the discrepancy between humans and microminipigs is that the latter did not reach middle age in this experiment. On the other hand, a significant positive correlation was recognized between age and plasma total homocysteine concentrations, with a Pearson correlation coefficient of 0.574 ($p<0.01$) (Figure 1). In human studies, aging is described as one of the important factors underlying elevated plasma total homocysteine concentrations (24, 27). For purposes of comparison of total homocysteine among pigs, we determined the mean plasma homocysteine concentration in 40 young (<8 months old) microminipigs to be 10.16±3.42 μM (95% confidence interval=9.07 to 11.2 μM, max: 17.75 μM, min: 4.74 μM). The total homocysteine concentration in Pietrain pigs (4.5 months old, n=8) and Göttingen pigs (10 months old, n=16) were 10.9±2.1 μM and 4.9±1.0 μM, respectively (17, 18).

Three microminipigs were used for evaluation of plasma total homocysteine concentrations for 24 h after feeding. Total homocysteine concentrations at pre-feeding, and 1, 2, 4, 6, 8, 12, and 24 h after feeding in three microminipigs were 8.91, 8.92, 8.55, 7.81, 7.22, 7.25, 7.63, and 7.62 μM; 7.39, 8.55, 7.16, 6.76, 5.87, 5.97, 6.45, and 6.62 μM; and 9.53, 9.61, 10.19, 10.60, 10.60, 10.87, 11.82, and 9.36 μM, respectively. We could not find a consistent tendency of daily fluctuations of plasma total homocysteine concentrations in these microminipigs.

Pharmacokinetics test. The metabolism and pharmacokinetics of homocysteine were determined by measuring the concentrations of total and reduced homocysteine, and total cysteine after intravenous injection of DL-homocysteine in four microminipigs. The results showed a rapid (time-to-maximum concentration <5 min) and significant ($p<0.05$ *vs*. before administration) increase in plasma total homocysteine from 10.53 to 72.33 μM (Figure 2A). Plasma reduced homocysteine was maximal within 5 min ($p<0.05$ *vs*. before administration), increasing from 2.88 to 11.54 μM (Figure 2B), whereas plasma total cysteine did not change significantly. In all four microminipigs, a tendency towards an increase in the average plasma reduced cysteine concentration, from 45.09 to 63.23 μM, 5 min after DL-homocysteine administration was noted, but none of the changes were significant (Figure 2C). The plasma half-lives of total homocysteine, reduced homocysteine, and reduced cysteine were calculated using a two-compartment model and determined to be 70.7, 46.9, and 141.0 min, respectively. Reduced DL-homocysteine in the circulation was rapidly oxidized to homocysteine-albumin, -cysteine, or -homocysteine through the formation of a disulfide bond (9). Because the cysteine concentration also increased after DL-homocysteine injections, homocysteine is probably metabolized to cysteine by an enzymatic pathway, although the increase in cysteine concentration was lower than that of total homocysteine. This can be explained by the metabolism of homocysteine to methionine or its excretion *via* the kidney (1).

In this study, we found a significantly positive correlation between age and plasma total homocysteine concentration in

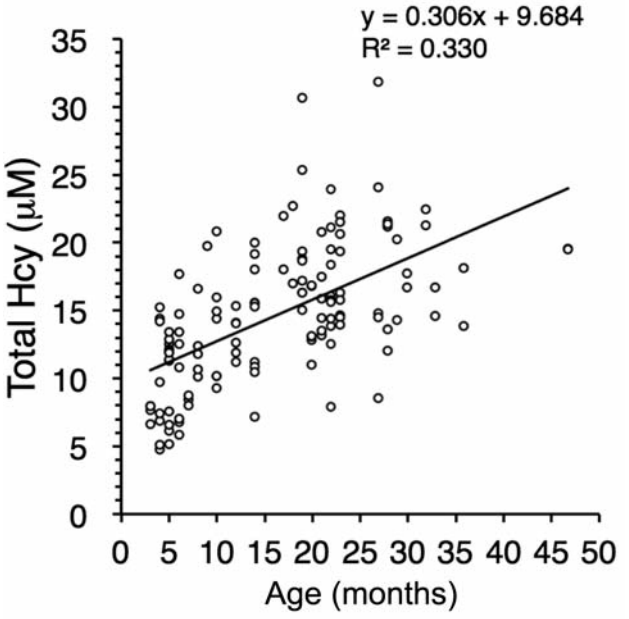

Figure 1. *Plasma total homocysteine (Hcy) concentrations in 87 male and 44 female microminipigs. The blood samples from each microminipig were de-proteinized in trichloroacetic acid, conjugated with 0.3% (w/v) 4-fluoro-7-sulfobenzofurazan (Dojindo, Kumamoto, Japan), and then applied to ultra-fast liquid chromatography (Shimadzu, Kyoto, Japan). There was a significant positive correlation between age and plasma total Hcy concentrations (Pearson correlation coefficient=0.574, p<0.01).*

microminipigs, whereas there were no significant changes related to either sex or feeding. Our results also showed a transient elevation of total and reduced plasma homocysteine concentrations after the intravenous injection of DL-homocysteine, followed by a quick return to pre-injection levels. Kawaguchi *et al*. measured the major hematological and serum biochemical parameters in microminipigs and reported values very similar to those determined in Göttingen and Yucatan minipigs (17, 18). Based on our findings, homocysteine metabolism in microminipig is likely to be similar to that in other pig strains.

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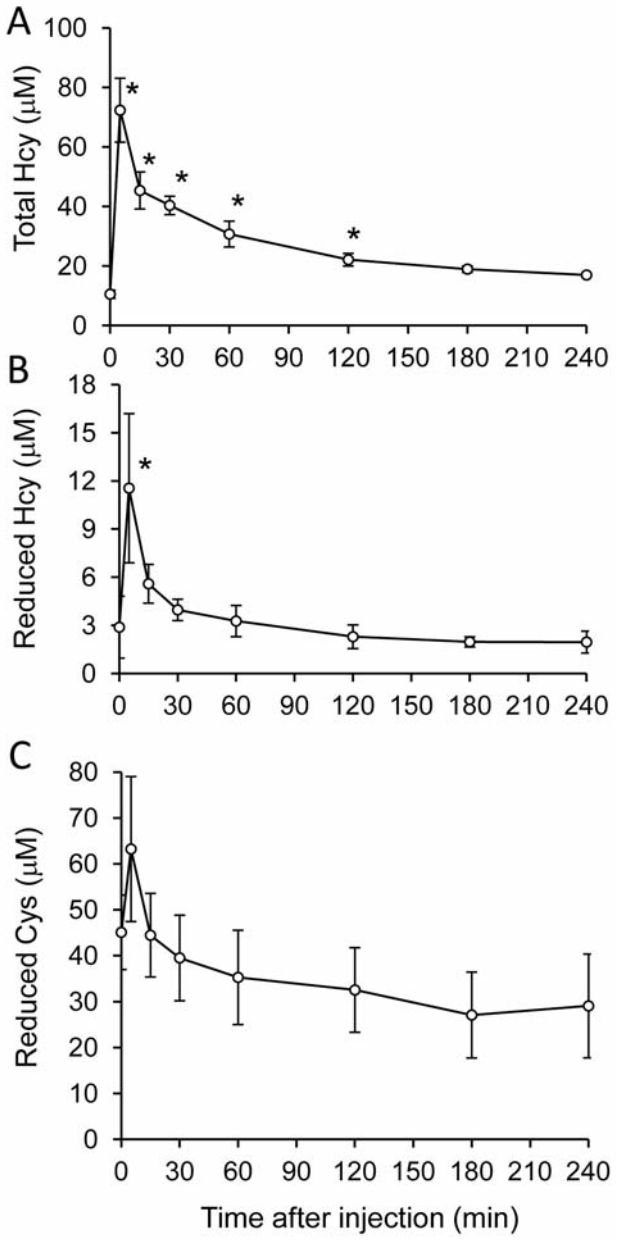

Figure 2. *Time course of plasma homocysteine (Hcy) concentrations after intravenous injection. Four female microminipigs were intravenously injected with freshly-prepared DL-homocysteine (17 μmol/kg of body weight; Wako, Osaka, Japan). Blood was collected at the indicated times and immediately frozen until total Hcy (A), reduced Hcy (B), and reduced cysteine (Cys) (C) were measured. Values are expressed as the mean±SD. *Significantly different from the pre-administration value (p<0.05).*

References

1 Friedman AN, Bostom AG, Selhub J, Levey AS and Rosenberg IH: The kidney and homocysteine metabolism. J Am Soc Nephrol *12*: 2181-2189, 2001.
2 Ingrosso D and Perna AF: Epigenetics in hyperhomocysteinemic states. A special focus on uremia. Biochim Biophys Acta *1790*: 892-899, 2009.
3 Kaelin WG Jr. and McKnight SL: Influence of metabolism on epigenetics and disease. Cell *153*: 56-69, 2013.
4 Wu N, Siow YL and O K: Induction of hepatic cyclooxygenase-2 by hyperhomocysteinemia *via* nuclear factor-kappa B activation. Am J Physiol Regul Integr Comp Physiol *297*: R1086-1094, 2009.

5 da Cunha AA, Ferreira AG and Wyse AT: Increased inflammatory markers in brain and blood of rats subjected to acute homocysteine administration. Metab Brain Dis *25*: 199-206, 2010.
6 Schalinske KL and Smazal AL: Homocysteine imbalance: a pathological metabolic marker. Adv Nutr *3*: 755-762, 2012.
7 Sengupta S, Chen H, Togawa T, DiBello PM, Majors AK, Büdy B, Ketterer ME and Jacobsen DW: Albumin thiolate anion is an intermediate in the formation of albumin-S-S-homocysteine. J Biol Chem *276*: 30111-30117, 2001.
8 Ueland PM: Homocysteine species as component of plasma redox thiol status. Clin Chem *41*: 340-342, 1995.
9 Chambers JC, Ueland PM, Wright M, Doré CJ, Refsum H and Kooner JS: Investigation of relationship between reduced, oxidized, and protein-bound homocysteine and vascular endothelial function in healthy human subjects. Circ Res *89*: 187-192, 2001.
10 Kawaguchi H, Miyoshi N, Miura N, Fujiki M, Horiuchi M, Izumi Y, Miyajima H, Nagata R, Misumi K, Takeuchi T, Tanimoto A and Yoshida H: Microminipig, a non-rodent experimental animal optimized for life science research: novel atherosclerosis model induced by high fat and cholesterol diet. J Pharmacol Sci *115*: 115-121, 2011.
11 Kaneko N, Itoh K, Sugiyama A and Izumi Y: Microminipig, a non-rodent experimental animal optimized for life science research: preface. J Pharmacol Sci *115*: 112-114, 2011.
12 Miyoshi N, Horiuchi M, Inokuchi Y, Miyamoto Y, Miura N, Tokunaga S, Fujiki M, Izumi Y, Miyajima H, Nagata R, Misumi K, Takeuchi T, Tanimoto A, Yasuda N, Yoshida H and Kawaguchi H: Novel microminipig model of atherosclerosis by high fat and high cholesterol diet, established in Japan. In Vivo *24*: 671-680, 2010.
13 Kawaguchi H, Yamada T, Miura N, Takahashi Y, Yoshikawa T, Izumi H, Kawarasaki T, Miyoshi N and Tanimoto A: Reference values of hematological and biochemical parameters for the world smallest microminpigs. J Vet Med Sci *74*: 933-936, 2012.
14 Kawaguchi H, Yamada T, Miura N, Noguchi M, Izumi H, Miyoshi N and Tanimoto A: Sex differences of serum lipid profile in novel microminipigs. In Vivo *27*: 617-621, 2013.
15 Akioka K, Kawaguchi H, Kitajima S, Miura N, Noguchi M, Horiuchi M, Miyoshi N and Tanimoto A: Investigation of necessity of sodium cholate and minimal required amount of cholesterol for dietary induction of atherosclerosis in microminipigs. In Vivo *28*: 81-90, 2014.
16 Kawaguchi H, Yamada T, Miura N, Ayaori M, Uto-Kondo H, Ikegawa M, Noguchi M, Wang KY, Izumi H and Tanimoto A: Rapid development of atherosclerosis in the world's smallest microminipig fed a high-fat/high-cholesterol diet. J Atheroscler Thromb 2013. in printing.
17 Rolland PH, Friggi A, Barlatiler A, Piquet P, Latille V, Faye MM, Guillou J, Charpiot P, Bodard H, Ghiringhelli O, Calaf R, Luccioni R and Garçon D: Hyperhomocysteinemia-induced vascular damage in the minipig. Captopril-hydrochlorothiazide combination prevents elastic alterations. Circulation *91*: 1161-1174, 1995.
18 Ambrosi P, Rolland PH, Bodard H, Barlatier A, Charpiot P, Guisgand G, Friggi A, Ghiringhelli O, Habib G, Bouvenot G, Garçon D and Luccioni R: Effects of folate supplementation in hyperhomocysteinemic pigs. J Am Coll Cardiol *34*: 274-279, 1999.
19 Fiskerstrand T, Refsum H, Kvalheim G and Ueland PM: Homocysteine and other thiols in plasma and urine: automated determination and sample stability. Clin Chem *39*: 263-271, 1993.
20 Refsum H, Ueland PM and Svardal AM: Fully automated fluorescence assay for determining total homocysteine in plasma. Clin Chem *35*: 1921-1927, 1989.
21 Moriyama M, Makiyama I, Shiota M, Uesugi K, Kannan Y, Ohta M, Kimura K and Sugano T: Decreased ureagenesis from alanine, but not from ammonia and glutamine, in the perfused rat liver after partial hepatectomy. Hepatology *23*: 1584-90, 1996.
22 Yamaoka K, Tanigawara Y, Nakagawa T and Uno T: A pharmacokinetic analysis program (multi) for microcomputer. J Pharmacobiodyn *4*: 879-885, 1981.
23 Seshadri S, Beiser A, Selhub J, Jacques PF, Rosenberg IH, D'Agostino RB, Wilson PW and Wolf PA: Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med *346*: 476-483, 2002.
24 Molero AE, Altimari CC, Duran DA, Garcia E, Pino-Ramirez G and Maestre GE: Total plasma homocysteine values among elderly subjects: Findings from the Maracaibo Aging Study. Clin Biochem *39*: 1007-1015, 2006.
25 Anan F, Maski T, Umeno Y, Yonemochi H, Eshima N, Saikawa T and Yoshimatsu H: Correlations between homocysteine levels and atherosclerosis in Japanese type 2 diabetic patients. Metabolism *56*: 1390-1395, 2007.
26 Mijatovic V, Kenemans P, Jakobs C, van Baal WM, Peters-Muller ER, and van der Mooren MJ: A randomized controlled study of the effects of 17 beta-estradiol-dydrogesterone on plasma homocysteine in postmenopausal women. Obstet Gynecol *91*: 432-436, 1998.
27 Nakazato M. Maeda T, Takamura N, Wada M, Yamasaki H, Johnston KE and Tamura T: Relation of body mass index to blood folate and total homocysteine concentrations in Japanese adults. Eur J Nutr *50*: 581-585, 2011.

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