to sex hormones in obese men and women undergoing weight loss therapy. *J Clin Endocrinol Metab* 1994; **78**: 1515–1520.

24 Saad F, Gooren L, Haider A *et al.* Effects of testosterone gel followed by parenteral testosterone undecanoate on sexual dysfunction and on features of the metabolic syndrome. *Andrologia* 2008; **40**: 44–48.

25 Laaksonen DE, Niskanen L, Punnonen K *et al.* The metabolic syndrome and smoking in relation to hypogonadism in middle-aged men: a prospective cohort study. *J Clin Endocrinol Metab* 2005; **90**: 712–719.

26 Vermeulen A. Androgen replacement therapy in the aging male. A critical evaluation. *J Clin Endocrinol Metab* 2001; **86**: 2380–2390.

27 Ferrucci L, Maggio M, Bandinelli S *et al.* Low testosterone levels and the risk of anemia in older men and women. *Arch Intern Med* 2006; **166**: 1380–1388.

28 Perretta M, Valladares L, Garrido F *et al.* Hormonal control of gene expression: differential activation of rat bone marrow RNA polymerases by erythropoietin and testosterone. *Arch Biol Med Exp (Santiago)* 1979; **12**: 309–318.

29 Fried W, Gurney CW. The erythropoietic-stimulating effects of androgens. *Ann N Y Acad Sci* 1968; **149**: 356–365.

30 Rishpon-Meyerstein N, Kilbridge T, Simone J *et al.* The effect of testosterone on erythropoietin levels in anemic patients. *Blood* 1968; **31**: 453–460.

31 Naets JP, Wittek M. The mechanism of action of androgens on erythropoiesis. *Ann N Y Acad Sci* 1968; **149**: 366–376.

32 Naets JP, Wittek M. Erythropoiesis in anephric man. *Lancet* 1968; **1**: 941–943.

33 Demirbag R, Yilmaz R, Ulucay A *et al.* The inverse relationship between thoracic aortic intima media thickness and testosterone level. *Endocr Res* 2005; **31**: 335–344.

34 Nishiyama T, Ishizaki F, Anraku T *et al.* The influence of androgen deprivation therapy on metabolism in patients with prostate cancer. *J Clin Endocrinol Metab* 2005; **90**: 657–660.

35 Yahyaoui R, Esteva I, Haro-Mora JJ *et al.* Effect of long-term administration of cross-sex hormone therapy on serum and urinary uric acid in transsexual persons. *J Clin Endocrinol Metab* 2008; **93**: 2230–2233.

36 Pizzichini M, Leoncini R, Vannoni D, Marinello E, Pagani R. The influence of testosterone on purine nucleotide metabolism in rat liver. *Life Sci* 1995; **57**: 2127–2135.

37 Adamopoulos D, Vlassopoulos C, Seitanides B *et al.* The relationship of sex steroids to uric acid levels in plasma and urine. *Acta Endocrinol (Copenh)* 1977; **85**: 198–208.

38 Fox IH. Metabolic basis for disorders of purine nucleotide degradation. *Metabolism* 1981; **30**: 616–634.

39 Fabregat I, Revilla E, Machado A. Short-term control of the pentose phosphate cycle by insulin could be modulated by NADPH/NADP ratio in rat adipocytes and hepatocytes. *Biochem Biophys Res Commun* 1987; **146**: 920–925.

40 Facchini F, Chen YD, Hollenbeck CB *et al.* Relationship between resistance to insulin-mediated glucose uptake, urinary uric acid clearance, and plasma uric acid concentration. *JAMA* 1991; **266**: 3008–3011.

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Review

SIRT1/eNOS Axis as a Potential Target against Vascular Senescence, Dysfunction and Atherosclerosis

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Sir2 (silent information regulator-2), an NAD$^+$-dependent histone deacetylase, is highly conserved in organisms ranging from archaea to humans. Yeast Sir2 is responsible for silencing at repeated DNA sequences in mating-type loci, telomeres and rDNA, and plays critical roles in DNA repair, stress resistance and longevity.
The phenomenon of human aging is known to be a critical cardiovascular risk factor. Senescence of endothelial cells has been proposed to be involved in vascular dysfunction and atherogenesis. Recent studies have demonstrated that mammalian *Sirt1* NAD$^+$-dependent protein deacetylase, the closest homologue of Sir2, regulates vascular angiogenesis, homeostasis and senescence. This review focuses on SIRT1 as a potential therapeutic target against atherosclerosis.

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Key words; SIRT1, Vascular senescence, Dysfunction, Atherosclerosis

Introduction

Recent studies demonstrate that cellular senescence is involved in various pathological conditions, such as atherosclerosis. In this review, we discuss the potential protective effect of SIRT1 on vascular endothelial cells.

Sirtuins

During the last decade, aging research has progressed through the use of lower organism models, such as the budding yeast *Saccharomyces cerevisiae* and the nematode *Caenorhabditis elegans*. In *S. cerevisiae*, the Sir2 (silent information regulator-2) family of genes governs budding exhaustion and replicative life span[1, 2]; deletions of Sir2 shorten life span and an extra copy of this gene increases life span. In addition to promoting longevity, the activity of Sir2 is enhanced by caloric restriction (CR), which extends life span in diverse species. Sir2 has been identified as an NAD$^+$-dependent histone deacetylase and is responsible for maintenance of chromatin silencing and genome stability[3]. Sir2 genes are conserved during evolution, and seven homologs of sirtuins (*Sirt1-7*) have been cloned in mammals. Mammalian sirtuins have diverse cellular localizations, modify multiple substrates, and affect cellular functions. SIRT1 is localized in the cytoplasm and nucleus, and SIRT6 and SIRT7 are localized in the nucleus. SIRT3, SIRT4 and SIRT5 reside in the mitochondria and SIRT2 is localized in the cytoplasm. SIRT1, SIRT2, SIRT3 and SIRT5 are NAD$^+$-dependent deacetylases, whereas SIRT4 and SIRT6 are primarily mono-ADP-ribosyl transferases.

SIRT1, the closest homologue of Sir2, targets a wide range of transcriptional regulators, including p53, PML (promyelocytic leukemia protein), FoxO (forkhead box O), NF-κB (nuclear factor κB) and PPAR-γ (peroxisome proliferators-activated receptor-γ)[4-8]. Like yeast Sir2, SIRT1 regulates the cell cycle, senescence, apoptosis and metabolism, and might act as a longevity factor in mammals.

Endothelial Senescence Induces Vascular Dysfunction and Atherosclerosis

The phenomenon of human aging is known to

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be a critical cardiovascular risk factor. Minamino *et al.* proposed that the senescence of endothelial cells is involved in endothelial dysfunction and atherogenesis[9]. Histological study of human atherosclerotic lesions has demonstrated the existence of vascular cells that exhibit the morphological features of senescence[10].

Moreover, it has been reported that angiogenesis becomes impaired with advancing age[11] and that aging reduces the antithrombogenic properties of the endothelium[12]. These senescent changes of vascular structure and function have been suggested to result in the increased risk of atherosclerotic cardiovascular disease in the elderly.

According to the free-radical theory, reactive oxygen species (ROS) may be potential candidates responsible for vascular dysfunction and atherosclerosis[13], and upon the production of high levels of ROS, the redox balance is disturbed and cells shift into a state of oxidative stress, which subsequently leads to endothelial dysfunction and senescence with shortening of telomeres[14]. Endothelial NO synthase (eNOS) activity is reduced in human senescent endothelial cells, accompanied by a reduction of nitric oxide (NO) production. Endothelial-derived NO regulates vascular relaxation and has athero-protective effects[15]. Intriguingly, endothelial NO can protect against a state of oxidative stress, and activation of eNOS and subsequent production of NO delay endothelial cellular senescence[16, 17].

SIRT1 Plays a Critical Role in Endothelial Homeostasis

SIRT1 likely plays a critical role in endothelial homeostasis by regulating endothelial nitric oxide synthase (eNOS). A recent study showed that levels of cGMP and eNOS are elevated in tissues of calorie-restricted mice, and production of NO by CR increases SIRT1 expression. The induction of SIRT1 expression is blunted in eNOS-deficient mice, and eNOS has been implicated in regulation of the expression of SIRT1[18].

It has been reported that SIRT1 promotes endothelial-dependent vasodilation by targeting eNOS for deacetylation, leading to enhanced NO production[19]. Intriguingly, SIRT1 has been shown to directly bind to eNOS, which is deacetylated at lysines 496 and 506 in the calmodulin-binding domain and posttranscriptionally leads to activation of eNOS. Inhibition of SIRT1 by a deacetylase-defective mutant SIRT1 decreases NO bioavailability and inhibits endothelium-dependent vasorelaxation. Consistent with these results, we showed that SIRT1 inhibition by sirtinol or RNAi-mediated knock down induced a premature senescent phenotype in human endothelial cells. Conversely, overexpression of SIRT1 prevented oxidative stress-induced endothelial senescence[20]. Intriguingly, a micro RNA (miR-217) was recently identified and miR-217 induced endothelial senescence through direct inhibition of SIRT1[21].

In addition to endothelial protection, SIRT1 regulates the angiogenic activity of endothelial cells. It has been reported by Potente *et al.* that SIRT1 deacetylase activity plays a critical role in the angiogenesis of endothelial cells[22]. Knockdown of SIRT1, but not SIRT2-7, was uniquely associated with loss of sprouting angiogenesis *in vitro*. Moreover, *Sirt1* mutant mice, which have genetic deletion of SIRT1 activity in the endothelium postnatally, have impaired formation of new vessels in response to angiogenic signals such as ischemic stress.

Cilostazol Inhibits Oxidative Stress-Induced Premature Senescence via Up-Regulation of SIRT1 in Human Endothelial Cells

A PDE3 inhibitor, cilostazol, is used as a vasodilating anti-platelet drug for treating intermittent claudication, and in preclinical studies was shown to have a protective effect on endothelial cells by increasing eNOS activity[23]. Cilostazol increases intracellular cAMP content accordingly and activates protein kinase A (PKA) and PI3K/Akt signaling[24]. We found that treatment with cilostazol inhibited the senescent phenotype. Cilostazol increased eNOS activity, expression of eNOS and the phosphorylation of eNOS at Ser^{1177} in parallel with the phosphrylation of Akt at Ser^{473}. These results suggest that the protective effect against a senescent phenotype may be attributable to an increase in NO via eNOS activation by cilostazol[25].

To explore the mechanism by which cilostazol prevents endothelial senescence, we considered that an increase in NO production could promote the longevity gene, SIRT1. We found that cilostazol significantly increased SIRT1 mRNA and protein in a concentration-dependent manner. In contrast, SIRT1 inhibition abrogated the effect of cilostazol on specific senescent changes. Although NO is known to be involved in reducing oxidative stress and the progression of atherosclerosis, we suggest that the NO-mediated prevention of senescence is attributable to SIRT1 function (**Fig. 1**). These findings implicate the eNOS-NO-SIRT1 axis as one of the fundamental determinants of endothelial senescence, and the role of SIRT1 as a driver of cellular stress resistance and longevity is note-

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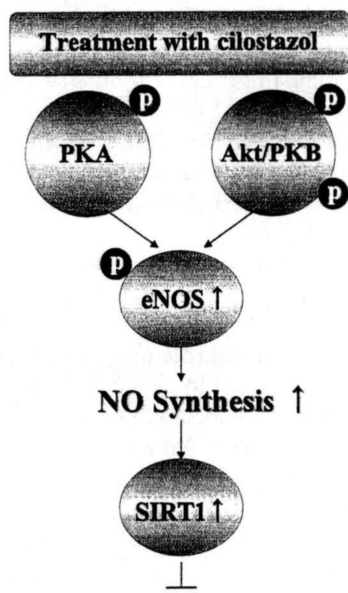

Fig. 1. Signaling pathway of inhibition of endothelial senescence, dysfunction and atherosclerosis by cilostazol treatment.

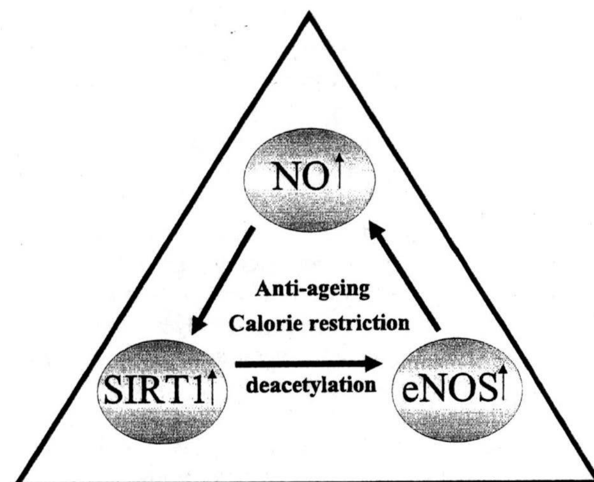

Fig. 2. Possible mechanisms of prevention of vascular senescence, dysfunction and atherosclerosis.

worthy in the context of its expression profile (**Fig. 2**).

In addition to these results, we found that drugs utilized for drug-eluting stents (DES), including paclitaxel and limus family members (e.g. sirolimus, everolimus), inhibit the growth of endothelial cells and lead to endothelial senescence caused by delayed re-endothelialization[26]. We showed that the development of endothelial senescence induced by sirolimus and everolimus is SIRT1-dependent, whereas paclitaxel acts through a SIRT1-independent pathway. Because the effects of sirolimus and everolimus involve SIRT1 modulation, cilostazol reverses sirolimus- or everolimus-induced senescence. Our results could have the interesting clinical implication that triple anti-platelet therapy may have more beneficial effects on endothelial senescence than standard dual therapy with sirolimus- or everolimus-eluting stents.

Activation of SIRT1, a Potential Therapeutic Target against Atherosclerosis

CR extends life span in diverse species. A recent study by Colman *et al.* at Wisconsin National Primate Center (WNPRC) reported that calorie-restricted Rhesus macaques showed a lower incidence of age-related diseases, such as cancer, diabetes, and cardiovascular disease, and lower age-related mortality[27]. Resveratrol, a CR mimetic, is a polyphenolic activator of SIRT1. Resveratrol increases mitochondrial biogenesis in endothelial cells via the activation of eNOS and SIRT1[28]. Resveratrol has also been shown to increase the expression of eNOS, and a combination of resveratrol with an HMG-CoA reductase inhibitor (statin) increased the activation of eNOS, resulting in increased functional recovery in a model of acute myocardial infarction[29]. Therefore, we suggest that increased NO bioavailability by other pharmaceutical products, such as statins, or agents with phytoestrogenic properties, such as resveratrol, may exert a protective effect against endothelial senescence via up-regulation of eNOS and SIRT1, and this possibility deserves further investigation. Our results and the findings by other laboratories indicate that micromolar levels of resveratrol are sufficient to exert vasculoprotective effects[26, 30]. Considering that each gram of fresh grape skin contains 50–100 μg resveratrol and it is found mainly in high-quality red wine at a concentration of 20 to 60 μmol/L as previously reported[31], it becomes apparent that effective concentrations are unlikely to be reached in plasma *in vivo.* However, resveratrol is a lipophilic substance, and exhibits higher bioavailability and slower clearance, and has been shown to accumulate in tissues such as the heart, liver, and kidney[32]. By daily consumption of grapes, berries, red wine or dietary supplements containing resveratrol, an effective concentration of resveratrol may be achievable *in vivo.* Recently, novel small molecule activators of Sirt1 (SRTs), even 1000-fold more potent than resveratol, have been identified (Sirtris Pharmaceuticals Inc., Boston, USA)[33]. SRTs induce many of the beneficial metabolic changes observed with CR/resveratrol treat-

ment[34]. We propose that using these chemical agents to activate SIRT1 may be a new attractive therapy for protection against vascular senescence, dysfunction and atherosclerosis.

Conclusions

SIRT1 is likely to play an important role in the prevention of human cardiovascular disease, including atherosclerosis. There is some evidence that SIRT1 interacts with the vascular eNOS/NO system. Just as the French paradox stands out as an excellent example of a reduced incidence of cardiovascular disease, activation of SIRT1 may have a beneficial effect on vascular senescence, dysfunction and atherosclerosis.

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References

1) Guarente L: Sir2 links chromatin silencing, metabolism, and aging. Genes Dev, 2000; 14: 1021-1026
2) Sinclair D, Mills K, Guarente L: Aging in *Saccharomyces cerevisiae*. Annu Rev Microbiol, 1998; 52: 533-560
3) Braunstein M, Rose AB, Holmes SG, Allis CD, Broach JR: Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. Genes Dev, 1993; 7: 592-604
4) Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L, Weinberg RA: hSIR2 (SIRT1) functions as an NAD-dependent p53 deacetylase. Cell, 2001; 107: 149-159
5) Langley E, Pearson M, Faretta M, Bauer UM, Frye RA, Minucci S, Pelicci PG, Kouzarides T: Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. EMBO J, 2002; 21: 2383-2396
6) Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW, Greenberg ME: Stress-dependent regulation of FOXO transcription factors by the SIRT1deacetylase. Science, 2004; 303: 2011-2015
7) Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, Mayo MW: Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J, 2004; 23: 2369-2380
8) Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, Machado De Oliveira R, Leid M, McBurney MW, Guarente L: Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. Nature, 2004; 429: 771-776
9) Minamino T, Miyauci H, Yoshida T, Ishida Y, Yoshida H, Komuro I: Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction. Circulation, 2002; 105: 1541-1544
10) Burrig KF: The endothelium of advanced arteriosclerotic plaques in humans. Arterioscler Thromb, 1991; 11: 1678-1689
11) Rivard A, Fabre JE, Silver M, Chen D, Murohara T, Kearney M, Magner M, Asahara T, Isner JM: Age-dependent impairment of angiogenesis. Circulation, 1999; 99: 111-120
12) Schneiderman J, Sawdey MS, Keeton MR, Bordin GM, Bernstein EF, Dilley RB, Loskutoff DJ: Increased type 1 plasminogen activator inhibitor gene expression in atherosclerotic human arteries. Proc Natl Acad Sci U S A, 1992; 89: 6998-7002
13) Shi Y, Camici GG, Lüscher TF: Cardiovascular determinants of life span. Pflugers Arch, 2009 Sep 12; in press
14) Kurz DJ, Decary S, Hong Y, Trivier E, Akhmedov A, Erusalimsky JD: Chronic oxidative stress compromises telomere integrity and accelerates the onset of senescence in human endothelial cells. J Cell Sci, 2004; 117: 2417-2426
15) Oemar BS, Tschudi MR, Godoy N, Brovkovich V, Malinski T, Lüscher TF. Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis. Circulation, 1998; 97: 2494-2498
16) Hayashi T, Matsui-Hirai H, Miyazaki-Akita A, Fukatsu A, Funami J, Ding QF, Kamalanathan S, Hattori Y, Ignarro LJ, Iguchi A: Endothelial cellular senescence is inhibited by nitric oxide: implications in atherosclerosis associated with menopause and diabetes. Proc Natl Acad Sci U S A, 2006; 103: 17018-17023
17) Vasa M, Breitschopf K, Zeiher AM, Dimmeler S: Nitric oxide activates telomerase and delays endothelial cell senescence. Circ Res, 2000; 87: 540-542
18) Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, Falcone S, Valerio A, Cantoni O, Clementi E, Moncada S, Carruba MO: Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science, 2005; 310: 314-317
19) Mattagajasingh I, Kim CS, Naqvi A, Yamamori T, Hoffman TA, Jung SB, DeRicco J, Kasuno K, Irani K: SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. Proc Natl Acad Sci U S A, 2007; 104: 14855-14860
20) Ota H, Akishita M, Eto M, Iijima K, Kaneki M, Ouchi Y: Sirt1 modulates premature senescence-like phenotype in human endothelial cells. J Mol Cell Cardiol, 2007; 43: 571-579
21) Menghini R, Casagrande V, Cardellini M, Martelli E, Terrinoni A, Amati F, Vasa-Nicotera M, Ippoliti A, Novelli G, Melino G, Lauro R, Federici M: MicroRNA 217 Modulates Endothelial Cell Senescence via Silent Information Regulator 1. Circulation, 2009 Sep 28, in press
22) Potente M, Ghaeni L, Baldessari D, Mostoslavsky R, Rossig L, Dequiedt F, Haendeler J, Mione M, Dejana E, Alt FW, Zeiher AM, Dimmeler S: SIRT1 controls endothelial angiogenic functions during vascular growth. Genes Dev, 2007; 21: 2644-2658
23) Kambayashi J, Liu Y, Sun B, Shakur Y, Yoshitake M, Czer-

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Accepted for publication: November 12, 2009
published online: March 9, 2010

wiec F: Cilostazol as a unique antithrombotic agent. Curr Pharm Des, 2003; 9: 2289-2302
24) Hashimoto A, Miyakoda G, Hirose Y, Mori T: Activation of endothelial nitric oxide synthase by cilostazol via a cAMP/protein kinase A- and phosphatidylinositol 3-kinase/Akt-dependent mechanism. Atherosclerosis, 2006; 189: 350-357
25) Ota H, Eto M, Kano MR, Ogawa S, Iijima K, Akishita M, Ouchi Y: Cilostazol inhibits oxidative stress-induced premature senescence via upregulation of Sirt1 in human endothelial cells. Arterioscler Thromb Vasc Biol, 2008; 28: 1634-1639
26) Ota H, Eto M, Ako J, Ogawa S, Iijima K, Akishita M, Ouchi Y: Sirolimus and everolimus induce endothelial cellular senescence via sirtuin 1 down-regulation: therapeutic implication of cilostazol after drug-eluting stent implantation. J Am Coll Cardiol, 2009; 53: 2298-2305
27) Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, Allison DB, Cruzen C, Simmons HA, Kemnitz JW, Weindruch R: Caloric restriction delays disease onset and mortality in rhesus monkeys. Science, 2009; 325: 201-204
28) Csiszar A, Labinskyy N, Pinto JT, Ballabh P, Zhang H, Losonczy G, Pearson K, de Cabo R, Pacher P, Zhang C, Ungvari Z: Resveratrol induces mitochondrial biogenesis in endothelial cells. Am J Physiol Heart Circ Physiol, 2009; 297: H13-20
29) Penumathsa SV, Thirunavukkarasu M, Koneru S, Juhasz B, Zhan L, Pant R, Menon VP, Otani H, Maulik N: Statin and resveratrol in combination induces cardioprotection against myocardial infarction in hypercholesterolemic rat. J Mol Cell Cardiol, 2007; 42: 508-516
30) Ungvari Z, Orosz Z, Rivera A, Labinskyy N, Xiangmin Z, Olson S, Podlutsky A, Csiszar A: Resveratrol increases vascular oxidative stress resistance. Am J Physiol Heart Circ Physiol, 2007; 292: H2417-2424
31) Wallerath T, Deckert G, Ternes T, Anderson H, Li H, Witte K, Förstermann U: Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. Circulation, 2002; 106: 1652-1658
32) Bertelli A, Bertelli AA, Gozzini A, Giovannini L: Plasma and tissue resveratrol concentrations and pharmacological activity. Drugs Exp Clin Res, 1998; 24: 133-138
33) Milne JC, Lambert PD, Schenk S, Carney DP, Smith JJ, Gagne DJ, Jin L, Boss O, Perni RB, Vu CB, Bemis JE, Xie R, Disch JS, Ng PY, Nunes JJ, Lynch AV, Yang H, Galonek H, Israelian K, Choy W, Iffland A, Lavu S, Medvedik O, Sinclair DA,Olefsky JM, Jirousek MR, Elliott PJ, Westphal CH: Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. Nature, 2007; 450: 712-716
34) Feige JN, Lagouge M, Canto C, Strehle A, Houten SM, Milne JC, Lambert PD, Mataki C, Elliott PJ, Auwerx J: Specific SIRT1 activation mimics low energy levels and protects against diet-induced metabolic disorders by enhancing fat oxidation. Cell Metab, 2008; 8: 347-358

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ORIGINAL ARTICLE: EPIDEMIOLOGY, CLINICAL PRACTICE AND HEALTH

Association of plasma sex hormone levels with functional decline in elderly men and women

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Aim: We aimed to determine whether plasma sex hormone levels are associated with activities of daily living (ADL), cognition, depression and vitality in elderly individuals with functional decline.

Methods: Two hundred and eight consecutive persons 70 years or older (108 men and 100 women; mean ± standard deviation, 81 ± 7 years) with a chronic stable condition, receiving long-term care at a long-term care facilities located in Nagano Prefecture, Japan, were enrolled. Plasma total testosterone, free testosterone (only in men), dehydroepiandrosterone (DHEA), DHEA sulfate (DHEA-S) and estradiol levels were determined in the morning after an overnight fast. Comprehensive geriatric assessment was performed including basic ADL by Barthel Index, instrumental ADL, cognitive function by Hasegawa Dementia Scale – Revised, mood by Geriatric Depression Scale and ADL-related vitality by Vitality Index.

Results: Simple regression analysis showed that, in men, plasma total and free testosterone levels were associated with basic ADL ($R = 0.292$ and $R = 0.282$), instrumental ADL ($R = 0.261$ and $R = 0.408$), cognitive function ($R = 0.393$ and $R = 0.553$) and vitality ($R = 0.246$ and $R = 0.396$), while DHEA(-S) was associated with cognitive function, and estradiol with cognitive function as well as vitality. In women, the only significant correlation was between DHEA(-S) and basic ADL. Adjustment for age and nutritional markers did not influence the associations of plasma sex hormone levels with functional scores except for that of free testosterone with Barthel Index.

Conclusion: These results suggest that sex hormones have sex-specific associations with physical and neuropsychiatric functions in elderly individuals, and that endogenous testosterone is related to global function in elderly men.

Keywords: activities of daily living (ADL), comprehensive geriatric assessment, dehydroepiandrosterone sulfate, estradiol, testosterone.

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Introduction

In addition to the abrupt reduction in estrogen production in women during the menopause, both men and women experience an age-associated decrease in the levels of androgens.[1–3] Physical and neuropsychiatric

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function also declines with age; however, the association of sex hormones with functional decline is not fully understood. One nursing home study found that a higher total testosterone (T) level was associated with better activities of daily living (ADL) performance such as transferring and eating among frail elderly men, while estrone and dehydroepiandrosterone (DHEA) levels were inversely related to ADL in women.[4] Although several observational studies examining the relationship between endogenous androgen and cognitive function in elderly men have also been published,[5–8] most surveys have investigated only a few aspects of functions rather than the whole spectrum and have been carried out based on community samples of white people in Western countries. In addition, many studies are restricted to one sex and few have focused on frail or disabled elderly individuals.

Thus, additional data are needed to elucidate the relationship between plasma hormone levels and functional status in elderly individuals with functional decline to better understand the application of hormone replacement therapy to bring about the most beneficial effects. In our preliminary study in a small sample of frail elderly men, a higher plasma T level was associated with higher functional scores.[9] To extend this pilot study, we included a larger sample of elderly men and women with functional decline, and evaluated whether sex hormone levels, including DHEA sulfate (DHEA-S) and estradiol, are associated with functioning on the basis of comprehensive geriatric assessment.

Methods

Study design and participants

In this cross-sectional observational study, 208 consecutive persons aged 70 years or older (108 men aged 70–95 years and 100 women aged 70–93 years; mean ± standard deviation, 82 ± 7 and 81 ± 6 years, respectively) who attended health service facilities for the elderly (facilities that provide nursing care and rehabilitation services to elderly people with disability, "Mahoroba-no-Sato") located in Nagano Prefecture, Japan, were enrolled. The participants were in a chronic stable condition and receiving Long-term Care Insurance either for facility admission or day-care service. The principal exclusion criteria were malnutrition (serum albumin, <3.5 mg/dL), extremely low ADL status (Barthel Index,[10] <50), malignancy, acute inflammation (fever, white blood cell count of >10 000/mL, or other signs of infection within 4 weeks before enrollment), severe anemia (blood hemoglobin, <10.0 g/dL) and overt endocrine diseases because these diseases may affect both plasma sex hormone levels and functions. The following information was collected from medical history charts or by interviewer-administered questionnaire; past medical history, present diagnosis of any disease, medication and nutritional intake. Comorbid conditions included in the current analysis were hypertension, chronic heart disease (angina, myocardial infarction, congestive heart failure, arrhythmia), stroke, osteoarthropathy (arthritis, rheumatism, osteoporosis, history of fractures) and diabetes mellitus. We also obtained data on anti-androgenic treatment or intake of glucocorticoids, opiates or hormone supplements which could affect plasma hormone levels, but no subject was taking any of these. The institutional review board of Kikyogahara Hospital approved the study protocol, and all participants or their families gave written informed consent.

Hormone measurements

Blood samples were obtained from the participants in the morning after an overnight fast, and plasma hormone levels, in addition to blood cell counts and blood chemical parameters, were determined by a commercial laboratory (Health Sciences Research Institute, Yokohama, Japan). Free-T, DHEA-S and DHEA were assayed using sensitive radioimmunoassays. Total-T and estradiol were assayed using chemiluminescence immunoassays with minimum detection limits of 7 ng/dL (0.2 nmol/L) and 4 pg/mL (14.7 pmol/L), respectively. The intra-assay coefficients of variation for these measurements were less than 5%.

Functional and anthropometric measurements

Trained nurses and physical therapists visited the participants at the health service facilities and performed comprehensive geriatric assessments. Basic ADL was assessed by Barthel Index,[10] instrumental ADL (IADL) by Lawton and Brody's IADL,[11] cognitive function by Hasegawa Dementia Scale – Revised (HDS-R, 30-point scale),[12] mood by Geriatric Depression Scale (GDS, 15 items)[13] and ADL-related vitality by Vitality Index (10-point scale).[14] In the current study, three items (food preparation, household tasks and laundering) were removed from the original version of Lawton and Brody's IADL scale to assess men; thus, IADL scale ranged 0–5 points in men and 0–8 in women. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters.

Statistical analysis

Data were analyzed using SPSS statistical software (version 11.0). Data were compared between men and women using the Student's *t*-test for continuous variables and χ^2-tests for categorical variables. Pearson's simple correlation coefficients were determined by plasma sex hormone levels, age and functional

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measures. Standardized regression coefficients from multivariate linear regression analysis of functional measurements in relation to age, nutritional markers and plasma hormone levels were determined. An unpaired Student's t-test was used for the differences in hormone levels and functional status according to associated diseases. $P < 0.05$ was considered statistically significant.

Results

The characteristics of the study subjects are presented in Table 1. Sex differences were found in the levels of hemoglobin and total cholesterol, and also in the percentage of subjects with heart disease and stroke. On average, subjects showed mild-to-moderate functional decline, and scores of Barthel Index, HDS-R and Vitality Index were higher in women than in men. Plasma level of total-T in male cohorts was lower than that reported in healthy elderly men,[15] but comparable to those in frail elderly men.[4] All plasma hormone levels were significantly higher in men than in women.

In simple regression analysis, age was negatively correlated with most of the functional scores except for instrumental ADL and GDS in men and GDS in women (data not shown). Because an age-associated decline of plasma sex hormone levels[1–3] and an influence of nutritional status on hormone levels[16,17] have been reported, we analyzed the correlations between hormone levels, age and BMI (Table 2). However, only free-T in men was significantly associated with age, and only total-T in women was correlated with BMI. Because DHEA, testosterone and estradiol have precursor-metabolite relationships in the steroid-hormone biosynthesis cascade, we evaluated the correlations between each of the plasma hormone levels (Table 2). Some, but not all, plasma sex hormone levels showed significant correlations in both sexes.

Table 1 Distribution of variables in study subjects

	Men	Women
No. of subjects	108	100
Age, years	82 ± 7 (70–95)	81 ± 6 (70–93)
Nutritional parameters		
Body mass index, kg/m²	21.8 ± 3.3 (15.1–29.0)	22.9 ± 3.8 (16.0–33.6)
Hemoglobin, g/dL	13.7 ± 1.7 (10.4–18.7)	12.8 ± 1.3 (10.0–15.6)**
Albumin, g/dL	4.2 ± 0.3 (3.5–4.9)	4.2 ± 0.3 (3.5–4.9)
Total cholesterol, mg/dL	181 ± 32 (119–273)	205 ± 33 (126–288)**
Chronic diseases		
Hypertension, *n* (%)	31 (28.7)	36 (36.0)
Heart disease, *n* (%)	9 (8.3)	19 (19.0)*
Stroke, No. (%)	35 (32.4)	19 (19.0)*
Osteoarthropathy, *n* (%)	23 (21.3)	31 (31.0)
Diabetes mellitus, *n* (%)	10 (9.3)	14 (14.0)
Functional parameters		
Barthel Index	84 ± 17 (50–100)	93 ± 9 (60–100)**
Instrumental ADL†	2.6 ± 2.0 (0–5)	5.9 ± 2.3 (0–8)
HDS-R	19 ± 7 (2–30)	23 ± 6 (5–30)**
Vitality Index	9.2 ± 1.1 (5–10)	9.7 ± 0.6 (6–10)**
GDS	5.6 ± 3.2 (0–13)	5.4 ± 3.0 (0–13)
Hormones		
Total testosterone, nmol/L	14.8 ± 5.8 (2.5–30.5)	1.3 ± 0.6 (0.2–2.9)**
Free testosterone, pmol/L	22.2 ± 8.7 (3.1–43.4)	
DHEA-S, μmol/L	1.75 ± 1.18 (0.26–5.47)	1.34 ± 0.54 (0.38–2.70)**
DHEA, nmol/L	7.63 ± 3.82 (2.43–25.7)	4.86 ± 2.08 (1.04–11.1)**
Estradiol, pmol/L	109.4 ± 48.1 (14.7–228.0)	59.5 ± 38.9 (14.7–206.7)**

Probability values of chronic diseases were compared between men and women by χ^2-test; *$P < 0.05$. Age, nutritional parameters, functional parameters (except for instrumental ADL) and hormone measurements were compared between men and women by Student's t-test; **$P < 0.001$. Values except those for chronic diseases are shown as mean ± standard deviation (range). †Lawton and Brody's instrumental ADL scale ranges 0–5 points in men and 0–8 in women, respectively. ADL, activities of daily living; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; GDS, Geriatric Depression Scale; HDS-R, Hasegawa Dementia Scale – Revised.

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Table 2 Correlation between plasma sex hormone levels, age and body mass index

	Age	BMI	Total-T	Free-T	DHEA-S	DHEA	Estradiol
Men							
Age	–	0.035	−0.121	−0310**	−0.254	−0.111	−0.047
BMI		–	0.006	0.026	−0.177	−0.151	−0.055
Total-T			–	0.672***	0.043	0.075	0.476***
Free-T				–	0.468***	0.392**	0.414***
DHEA-S					–	0.382**	0.342*
DHEA						–	0.084
Estradiol							–
Women							
Age	–	−0.187	0.079		−0.062	0.017	−0.028
BMI		–	0.320*		0.121	−0.070	0.040
Total-T			–		0.202*	0.355**	0.162
DHEA-S					–	0.561***	0.131
DHEA						–	0.097
Estradiol							–

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. All data are presented as Pearson correlation coefficients. BMI, body mass index; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; Free-T, free testosterone; Total-T, total testosterone.

We also assessed whether plasma hormone levels were different between individuals with or without chronic diseases including hypertension, heart disease, cerebrovascular disease, osteoarthropathy and diabetes mellitus, using a Student's *t*-test, but there were no significant differences in hormone levels according to these conditions (data not shown). On the other hand, a significant difference was observed in Barthel Index scores between subjects with and without cerebrovascular disease in men (77 ± 16 vs 86 ± 16, $P < 0.01$).

The associations between plasma hormone levels and functional scores were evaluated. As shown in Table 3, in men, plasma total-T and free-T levels were positively correlated with functional scores except for GDS. DHEA(-S) and estradiol were positively correlated with cognitive function, and DHEA and estradiol were associated with Vitality Index as well. In contrast, in women, a significant correlation was observed only between DHEA(-S) and Barthel Index.

Multiple regression analysis revealed that the associations between sex hormones and functions were independent of age and BMI except that the associations between free-T and Barthel Index in addition to DHEA and vitality were not significant after adjustment. The statistical results were similar when serum albumin or total cholesterol was entered into the regression model instead of BMI (data not shown).

Because all measured sex hormones were associated with HDS-R in men, we entered free-T, DHEA-S and estradiol into the regression model as covariates in addition to age and BMI (Table 4). Free-T remained a significant determinant of HDS-R, while DHEA-S and estradiol did not hold a significant association with HDS-R. When a stepwise model (forward selection) was used to test for the determinants for HDS-R with the same five covariates, the *P*-value for the regression was minimized when only free-T was chosen as a variable ($R^2 = 0.227$, overall *P*-value for the regression <0.05).

Discussion

The present study demonstrated that men with higher plasma T levels had better ADL, cognitive function and vitality. Also, a higher estradiol level was related to better cognitive function as well as vitality, and a higher DHEA(-S) level was related to better cognitive function. In women, DHEA(-S) level was related to higher basic ADL, but T and estradiol levels showed no correlation with functional scores. The positive associations between sex hormones and functional scores were independent of age and nutritional status, suggesting that plasma sex hormone levels, especially that of testosterone in men, are independently related to functional status in elderly individuals.

Concerning cognitive function, our findings are consistent with the results of the previous observational studies examining the relationship between endogenous androgen and cognitive function in elderly men.[5,7,8] Several interventional studies have shown an improvement in spatial cognition and working memory after treatment with T, suggesting that T might have a beneficial effect on cognitive function.[18–21] Also, DHEA(-S), the most abundant circulating steroid in both sexes and the biosynthetic precursor of T, has been shown to have neurotrophic and neuronal remodeling activity.[22,23]

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Table 3 Linear regression model of hormone levels on functional scores unadjusted and adjusted for age, and age and body mass index

	Total-T	Free-T	DHEA-S	DHEA	Estradiol
Men					
Unadjusted					
Barthel Index	0.292**	0.282**	0.094	−0.058	0.110
Instrumental ADL	0.261*	0.408**	0.239	0.140	0.129
HDS-R	0.393***	0.553***	0.390*	0.393**	0.266*
Vitality Index	0.246*	0.396***	0.210	0.297*	0.291*
GDS	−0.103	−0.097	−0.181	−0.027	−0.060
Adjusted for age					
Barthel Index	0.250**	0.183	0.044	−0.077	0.107
Instrumental ADL	0.255*	0.402***	0.216	0.137	0.124
HDS-R	0.366***	0.488***	0.317*	0.361**	0.243*
Vitality Index	0.218*	0.348***	0.160	0.176	0.288*
GDS	−0.068	−0.065	−0.146	−0.024	−0.005
Adjusted for age and BMI					
Barthel Index	0.281**	0.112	0.101	0.109	0.114
Instrumental ADL	0.229*	0.414**	0.314*	0.400*	0.053
HDS-R	0.340**	0.443**	0.329*	0.480**	0.236*
Vitality Index	0.285*	0.321*	0.140	0.227	0.292*
GDS	−0.067	−0.015	−0.013	0.002	0.079
Women					
Unadjusted					
Barthel Index	0.085		0.280*	0.293*	−0.068
Instrumental ADL	−0.050		0.071	0.171	0.071
HDS-R	−0.051		0.080	−0.034	0.121
Vitality Index	−0.076		0.167	0.091	0.043
GDS	0.004		−0.087	−0.014	0.052
Adjusted for age					
Barthel Index	0.120		0.225*	0.288*	−0.068
Instrumental ADL	−0.003		0.041	0.166	0.052
HDS-R	−0.028		0.038	−0.037	0.120
Vitality Index	−0.060		0.140	0.089	0.043
GDS	−0.008		−0.066	−0.012	0.052
Adjusted for age and BMI					
Barthel Index	0.142		0.269*	0.221*	0.035
Instrumental ADL	−0.067		0.092	0.178	0.046
HDS-R	−0.110		0.045	−0.051	0.106
Vitality Index	−0.103		0.137	0.043	0.104
GDS	0.063		−0.020	0.038	0.056

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data are presented as standardized regression coefficients. ADL, activities of daily living; BMI, body mass index; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; Free-T, free testosterone; GDS, Geriatric Depression Scale – 15 items; HDS-R, Hasegawa Dementia Scale-Revised; Total-T, total testosterone.

In addition, our recent study showed that a low plasma T level is related to endothelial dysfunction in middle-aged men,[24] suggesting a mechanistic link between T and cerebrovascular function.

With respect to mood, although some large scale epidemiological studies[25,26] failed to show a clear correlation between T and depression in middle-aged men, another study has shown that low T levels are associated with depression in healthy elderly men.[27] The reason is unknown but it might be due simply to the cohort difference between community-dwelling healthy men and frail elderly men, or to the low reliability of GDS in demented people.[14] In the current study, in men, estradiol was also associated with cognitive function and vitality. However, multiple regression analysis with both free-T and estradiol as covariates suggested that estradiol is merely a marker as a metabolite of androgens and does not exert a direct action on neuropsychiatric

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Table 4 Multiple regression analysis on cognitive function with free-T, DHEA-S and estradiol as covariates in addition to age and BMI in men

	HDS-R β	*P*
Age	−0.346	0.087
BMI	0.091	0.649
Free-T	0.466	0.030
DHEA-S	0.011	0.964
Estradiol	−0.213	0.321

β, standardized regression coefficient; BMI, body mass index; DHEA-S, dehydroepiandrosterone sulfate; Free-T, free testosterone; HDS-R, Hasegawa Dementia Scale – Revised.

function in men consistent with the results of cross-sectional studies.[5,7,8]

Because T has anabolic effects on muscle and may improve cognition, our findings on the association of T with ADL are not surprising. While several observational studies have demonstrated the correlation of endogenous testosterone with muscle mass and strength[28–31] and physical performance[4,32] in older men, interventional surveys have provided mixed findings[33–38] and the studies using healthy men have found only increased muscle mass and strength but not improved physical function.[35–38] In addition, results of studies investigated the correlation between endogenous testosterone and fall risks are inconsistent.[30,32] Future interventional studies enrolling frail and/or disabled elderly men might clarify the causal relationship between testosterone and frailty. Although the correlation between sex hormones and physical function or ADL in women is contradictory across studies,[39–43] our findings are consistent with one report showing that the plasma level of DHEA(-S) is related to basic ADL in middle-aged to elderly women.[39]

The explanations for the sex difference in the correlation between hormones and function could be due to sex differences in hormone secretion and metabolism.[41,44,45] In fact, plasma estradiol level in women was approximately half of that in men, and distributed in a narrow range (52% cases fell into a range of 14.7–53.1 pmol/L), providing a possible explanation for no association of estrogen levels with functioning in women. Measurement of active forms of estrogens such as free or bioavailable estradiol, although the assays are not available in Japan, might show some significant correlations with functional levels; however, most of the previous studies investigating the relationship between endogenous estrogen levels and physical performance or cognitive function in postmenopausal women, including one study that measured bioavailable estradiol levels, found negative results.[46–50] Accordingly, in the ranges of circulating endogenous hormone levels, estradiol may not be related to functional levels in older women. On the other hand, information on the sex-specific distribution of steroid hormone receptors is limited. Recently, Bezdickova *et al.* reported that nuclear androgen receptor staining was observed in the mammillary body, precentral gyrus and hippocampus in the human male brain but not in the female brain.[51] The sex difference in the correlation between hormones and functions should be further determined based on the ligand–receptor relationship.

The limitations of our study should be acknowledged. First, we cannot exclude an influence of the associated diseases or the comorbid condition on our results, although no significant differences were observed in hormone levels or functional status in subjects with or without chronic diseases, except that the Barthel Index was significantly lower in male subjects with cerebrovascular disease. Second, only free-T was measured as the active form of T by radioimmunoassays instead of bioavailable or calculated free-T, because sex hormone-binding globulin and direct assays of bioavailable T were not available. Finally, it should be recognized that our results were obtained from a cross-sectional study and do not provide direct evidence of a causal relationship; therefore, it is possible that high sex hormone levels were the result of enhanced physical or mental health.

In summary, our cross-sectional survey revealed that sex hormones have sex-specific relationships with physical and neuropsychiatric function in elderly individuals. In men, endogenous androgen is independently associated with ADL, cognitive function and vitality. Although it has been reported that testosterone or DHEA supplementation in healthy elderly men did not affect physical or cognitive function,[37,38] our findings suggest that elderly men with functional decline could be a better target for androgen replacement to improve physical and cognitive function.

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References

1 Feldman HA, Longcope C, Derby CA *et al.* Age trends in the level of serum testosterone and other hormones in middle-aged men: longitudinal results from the Massachusetts Male Aging Study. *J Clin Endocrinol Metab* 2002; **87**: 589–598.

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2 Harman SM, Metter EJ, Tobin JD *et al.* Longitudinal effects of aging on serum total and free testosterone levels in healthy men: Baltimore Longitudinal Study of Aging. *J Clin Endocrinol Metab* 2001; **86**: 724–731.
3 Davison SL, Bell R, Donath S *et al.* Androgen levels in adult females: changes with age, menopause, and oophorectomy. *J Clin Endocrinol Metab* 2005; **90**: 3847–3853.
4 Breuer B, Trungold S, Martucci C *et al.* Relationships of sex hormone levels to dependence in activities of daily living in the frail elderly. *Maturitas* 2001; **39**: 147–159.
5 Yaffe K, Lui LY, Zmuda J, Cauley J. Sex hormones and cognitive function in older men. *J Am Geriatr Soc* 2002; **50**: 707–712.
6 Moffat SD, Zonderman AB, Metter EJ *et al.* Longitudinal assessment of serum free testosterone concentration predicts memory performance and cognitive status in elderly men. *J Clin Endocrinol Metab* 2002; **87**: 5001–5007.
7 Barrett-Connor E, Goodman-Gruen D, Patay B. Endogenous sex hormones and cognitive function in older men. *J Clin Endocrinol Metab* 1999; **84**: 3681–3685.
8 Muller M, Aleman A, Grobbee DE *et al.* Endogenous sex hormone levels and cognitive function in aging men. *Neurology* 2005; **64**: 866–871.
9 Akishita M, Yamada S, Nishiya H *et al.* Testosterone and comprehensive geriatric assessment in frail elderly men (Letter). *J Am Geriatr Soc* 2003; **51**: 1324–1326.
10 Mahoney FI, Barthel DW. Functional evaluation: Barthel Index. *Md State Med J* 1965; **14**: 61–65.
11 Lawton MP, Brody EM. Assessment of older people, self-maintaining and instrumental activities of daily living. *Gerontologist* 1969; **9**: 179–186.
12 Kato S, Shimogaki M, Onodera H *et al.* Revised Hasegawa Dementia Scale (HDS-R). *Jpn J Geriatr Psychiatr* 1991; **2**: 1339–1347.
13 Yesavage JA. Geriatric Depression Scale. *Psychopharmacol Bull* 1988; **24**: 709–711.
14 Toba K, Nakai R, Akishita M *et al.* Vitality Index as a useful tool to assess elderly with dementia. *Geriatr Gerontol Int* 2002; **2**: 23–29.
15 Leifke E, Gorenoi V, Wichers C *et al.* Age-related changes of serum sex hormones, insulin-like growth factor-1 and sex-hormone binding globulin levels in men: cross-sectional data from a healthy male cohort. *Clin Endocrinol* 2000; **53**: 689–695.
16 Muller M, den Tonkelaar I, Thijssen JH *et al.* Endogenous sex hormones in men aged 40–80 years. *Eur J Endocrinol* 2003; **149**: 583–589.
17 Travison TG, Araujo AB, Kupelian V *et al.* The relative contributions of aging, health, and lifestyle factors to serum testosterone decline in men. *J Clin Endocrinl Metab* 2007; **92**: 549–555.
18 Cherrier MM, Asthana S, Plymate S *et al.* Testosterone supplementation improves spatial and verbal memory in healthy older men. *Neurology* 2001; **57**: 80–88.
19 Cherrier MM, Matsumoto AM, Amory JK *et al.* Testosterone improves spatial memory in men with Alzheimer disease and mild cognitive impairment. *Neurology* 2005; **64**: 2063–2068.
20 Janowsky JS. The role of androgens in cognition and brain aging in men. *Neuroscience* 2006; **138**: 1015–1020.
21 Moffat SD. Effects of testosterone on cognitive and brain aging in elderly men. *Ann N Y Acad Sci* 2005; **1055**: 80–92.
22 Compagnone NA, Mellon SH. Dehydroepiandrosterone: a potential signaling molecule for neocortical organization during development. *Proc Natl Acad Sci USA* 1998; **95**: 4678–4683.
23 Mao X, Barger SW. Neuroprotection by dehydroepiandrosterone-sulfate: role of an NF kappa B-like factor. *Neuroreport* 1998; **9**: 759–763.
24 Akishita M, Hashimoto M, Ohike Y *et al.* Low testosterone level is an independent determinant of endothelial dysfunction in men. *Hypertens Res* 2007; **30**: 1029–1034.
25 Araujo AB, Durante R, Feldman HA *et al.* The relationship between depressive symptoms and male erectile dysfunction: cross-sectional results from the Massachusetts Male Aging Study. *Psychosom Med* 1998; **60**: 458–465.
26 Mazur A. Biosocial models of deviant behavior among male army veterans. *Biol Psychol* 1995; **41**: 271–293.
27 Barrett-Connor E, Von Mühlen DG, Kritz-Silverstein D. Bioavailable testosterone and depressed mood in older men: the Rancho Bernardo Study. *J Clin Endocrinol Metab* 1999; **84**: 573–577.
28 Roy TA, Blackman MR, Harman SM *et al.* Interrelationships of serum testosterone and free testosterone index with FFM and strength in aging men. *Am J Physiol Endocrinol Metab* 2002; **283**: E284–E294.
29 van den Beld AW, de Jong FH, Grobbee DE *et al.* Measures of bioavailable serum testosterone and estradiol and their relationships with muscle strength, bone density, and body composition in elderly men. *J Clin Endocrinol Metab* 2000; **85**: 3276–3282.
30 Schaap LA, Pluijm SM, Smit JH *et al.* The association of sex hormone levels with poor mobility, low muscle strength and incidence of falls among older men and women. *Clin Endocrinol (Oxford)* 2005; **63**: 152–160.
31 Araujo AB, Travison TG, Bhasin S *et al.* Association between testosterone and estradiol and age-related decline in physical function in a diverse sample of men. *J Am Geriatr Soc* 2008; **56**: 2000–2008.
32 Orwoll E, Lambert LC, Marshall LM *et al.* Endogenous testosterone levels, physical performance, and fall risk in older men. *Arch Intern Med* 2006; **166**: 2124–2131.
33 Page ST, Amory JK, Bowman FD *et al.* Exogenous testosterone (T) alone or with finasteride increases physical performance, grip strength, and lean body mass in older men with low serum T. *J Clin Endocrinol Metab* 2005; **90**: 1502–1510.
34 Sih R, Morley JE, Kaiser FE *et al.* Testosterone replacement in older hypogonadal men: a 12-month randomized controlled trial. *J Clin Endocrinol Metab* 1997; **82**: 1661–1667.
35 Snyder PJ, Peachey H, Hannoush P *et al.* Effect of testosterone treatment on body composition and muscle strength in men over 65 years of age. *J Clin Endocrinol Metab* 1999; **84**: 2647–2653.
36 Storer TW, Woodhouse L, Magliano L *et al.* Changes in muscle mass, muscle strength, and power but not physical function are related to testosterone dose in healthy older men. *J Am Geriatr Soc* 2008; **56**: 1991–1999.
37 Nair KS, Rizza RA, O'Brien P *et al.* DHEA in elderly women and DHEA or testosterone in elderly men. *N Engl J Med* 2006; **355**: 1647–1659.
38 Emmelot-Vonk MH, Verhaar HJ, Nakhai Pour HR *et al.* Effect of testosterone supplementation on functional mobility, cognition, and other parameters in older men: a randomized controlled trial. *JAMA* 2008; **299**: 39–52.
39 Haren MT, Malmstrom TK, Banks WA *et al.* Lower serum DHEAS levels are associated with a higher degree of physical disability and depressive symptoms in middle-aged to older African American women. *Maturitas* 2007; **57**: 347–360.
40 Goldman N, Glei DA. Sex differences in the relationship between DHEAS and health. *Exp Gerontol* 2007; **42**: 979–987.

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41 Mazat L, Lafont S, Berr C *et al.* Prospective measurements of dehydroepiandrosterone sulfate in a cohort of elderly subjects: relationship to gender, subjective health, smoking habits, and 10-year mortality. *Proc Natl Acad Sci USA* 2001; **98**: 8145–8150.

42 Berr C, Lafont S, Debuire B *et al.* Relationships of dehydroepiandrosterone sulfate in the elderly with functional, psychological, and mental status, and short-term mortality: a French community-based study. *Proc Natl Acad Sci USA* 1996; **93**: 13410–13415.

43 Yanase T, Muta K, Nawata H. Serum concentrations of dehydroepiandrosterone sulfate (DHEA-S) in oldest old Japanese women correlate with cognitive activity rather than activities of daily living. *Geriatr Gerontol Int* 2006; **6**: 194–198.

44 Nafziger AN, Herrington DM, Bush TL. Dehydroepiandrosterone and dehydroepiandrosterone sulfate: their relation to cardiovascular disease. *Epidemiol Rev* 1991; **13**: 267–293.

45 Trivedi DP, Khaw KT. Dehydroepiandrosterone sulfate and mortality in elderly men and women. *J Clin Endocrinol Metab* 2001; **86**: 4171–4177.

46 Lebrun CE, van der Schouw YT, de Jong FH *et al.* Relations between body composition, functional and hormonal parameters and quality of life in healthy postmenopausal women. *Maturitas* 2006; **55**: 82–92.

47 Cunningham CJ, Sinnott M, Denihan A *et al.* Endogenous sex hormone levels in postmenopausal women with Alzheimer's disease. *J Clin Endocrinol Metab* 2001; **86**: 1099–1103.

48 Luetters C, Huang MH, Seeman T *et al.* Menopause transition stage and endogenous estradiol and follicle-stimulating hormone levels are not related to cognitive performance: cross-sectional results from the study of women's health across the nation (SWAN). *J Womens Health (Larchmt)* 2007; **16**: 331–344.

49 Yaffe K, Grady D, Pressman A, Cummings S. Serum estrogen levels, cognitive performance, and risk of cognitive decline in older community women. *J Am Geriatr Soc* 1998; **46**: 816–821.

50 Barrett-Connor E, Goodman-Gruen D. Cognitive function and endogenous sex hormones in older women. *J Am Geriatr Soc* 1999; **47**: 1289–1293.

51 Bezdickova M, Molikova R, Bebarova L *et al.* Distribution of nuclear receptors for steroid hormones in the human brain: a preliminary study. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 2007; **151**: 69–71.

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Effects of long-term dipeptidyl peptidase-IV inhibition on body composition and glucose tolerance in high fat diet-fed mice

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Gastric inhibitory polypeptide (GIP)

ABSTRACT

Aim: Glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are major incretins associated with body weight regulation. Dipeptidyl peptidase-IV (DPP-IV) inhibitor increases plasma active GLP-1 and GIP. However, the magnitude of the effects of enhanced GLP-1 and GIP signaling by long-term DPP-IV inhibition on body weight and insulin secretion has not been determined. In this study, we compared the effects of long-term DPP-IV inhibition on body composition and insulin secretion of high fat diet (HFD)-fed wild-type (WT) and GLP-1R knockout ($GLP\text{-}1R^{-/-}$) mice.
Main methods: HFD-fed WT and $GLP\text{-}1R^{-/-}$ mice were treated with or without DPP-IV inhibitor by drinking water. Food and water intake and body weight were measured during 8 weeks of study. CT-based body composition analysis, Oral glucose tolerance test (OGTT), batch incubation study for insulin secretion and quantitative RT-PCR for expression of incretin receptors in isolated islets were performed at the end of study.
Key findings: DPP-IV inhibitor had no effect on food and water intake and body weight, but increased body fat mass in $GLP\text{-}1R^{-/-}$ mice. DPP-IV inhibitor-treated WT and $GLP\text{-}1R^{-/-}$ mice both showed increased insulin secretion in OGTT. In isolated islets of DPP-IV inhibitor-treated WT and $GLP\text{-}1R^{-/-}$ mice, glucose-induced insulin secretion was increased and insulin secretion in response to GLP-1 or GIP was preserved, without downregulation of incretin receptor expression.
Significance: Long-term DPP-IV inhibition may maintain body composition through counteracting effects of GLP-1 and GIP while improving glucose tolerance by increasing glucose-induced insulin secretion through the synergistic effects of GLP-1 and GIP.

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Introduction

Oral glucose administration leads to much greater insulin release than the equivalent intravenous glucose challenge. Gut hormonal substances released in response to glucose include the incretins glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide /glucose-dependent insulinotropic peptide (GIP), which are responsible for ~50% of postprandial insulin release. GLP-1 and GIP potentiate glucose-induced insulin secretion from pancreatic β-cells by binding their respective receptors and subsequently increasing the intracellular cAMP concentration. In addition to their action on the enteroinsular axis, GLP-1 inhibits glucagon secretion (Komatsu et al. 1989), delays gastric emptying (Willms et al. 1996), decreases body weight through suppression of appetite (Turton et al. 1996), and suppresses β-cell apoptosis (Toyoda et al. 2008), while GIP enhances energy storage in adipocytes (Miyawaki et al. 2002) and calcium accumulation in bone (Tsukiyama et al. 2006). Thus, the incretins are associated with various systems of metabolic homeostasis, including that of both glucose and body weight.

However, the effects of GLP-1 and GIP are limited by their short half-life of a few minutes, which is primarily due to the action of dipeptidyl peptidase-IV (DPP-IV). DPP-IV is an enzyme distributed throughout the body including plasma and the endothelial lining of several organs, and cleaves two amino acids of biologically active peptides including GLP-1 and GIP by recognizing proline or alanine in the second N-terminal amino acid. The resulting N-terminal-truncated forms of GLP-1 and GIP are devoid of bioactivity. Since DPP-IV-deficient rodents show improved glucose tolerance and increased insulin secretion with elevated plasma active GLP-1 levels after oral glucose loading (Marguet et al. 2000; Nagakura et al. 2001), DPP-IV inhibitor and DPP-IV-resistant GLP-1

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receptor agonist are potential targets for the treatment of type 2 diabetes mellitus as a new class of antidiabetic agent. GLP-1 receptor agonist both increases insulin secretion and improves glucose tolerance and decreases body weight in rodents and humans (Szayna et al. 2000; Buse et al. 2004). DPP-IV inhibitor also increases insulin secretion and improves glucose tolerance, but its effect on body weight is controversial (Pospisilik et al. 2002; Lamont and Drucker 2008; Reimer et al. 2002; Ahrén et al. 2002). It is reported that DPP-IV inhibitor do not increase insulin secretion after glucose loading in GLP-1 receptor (GLP-1R)/GIP receptor (GIPR) double knockout (DIRKO) mice, indicating that both GLP-1 and GIP are critically involved in the insulinotropic action of long-term DPP-IV inhibition (Flock et al. 2007). However, the magnitude of the effects of enhanced GLP-1 and GIP signaling by long-term DPP-IV inhibition on body weight and insulin secretion has not been determined.

In the present study, we investigated the long-term effects of DPP-IV inhibition on body composition and insulin secretion using high fat diet (HFD)-fed wild-type (WT) and GLP-1R knockout ($GLP\text{-}1R^{-/-}$) mice.

Materials and methods

Animals

Mice (C57BL/6 background) were housed under a light/dark cycle of 12 h with free access to food and water. As ingestion of a meal rich in fat is a strong stimulus of incretin signaling (Harada et al. 2008), male WT and $GLP\text{-}1R^{-/-}$ mice were fed a high fat diet (45% fat, 20% protein and 35% carbohydrate by energy) from 7 weeks of age. Groups of treated HFD-fed WT and $GLP\text{-}1R^{-/-}$ mice received DPP-IV inhibitor in drinking water (0.5% W/V), while groups of untreated HFD-fed WT and $GLP\text{-}1R^{-/-}$ mice received drinking water without DPP-IV inhibitor. All the $GLP\text{-}1R^{-/-}$ mice were genotyped by Southern blot analysis. The DPP-IV inhibitor, provided by Taisho Pharmaceutical Co., Ltd., showed an inhibitory action on DPP-IV enzymatic activity against substrate H-Gly-Pro-7-amino-4-methyl coumarin (Gly-Pro-AMC) with IC_{50} (half maximal inhibitory concentration) of 0.0046 uM (Fukushima et al. 2008), while its IC_{50} on DPP-8 and DPP-9 were only 1.34 uM and 0.527 uM, respectively (unpublished data). Throughout the 8 weeks of study, water and food intake and body weight were measured once every 3 days. All mice care and procedures were approved by the Animal Care Committee of Kyoto University.

CT-based body composition analysis

The WT and $GLP\text{-}1R^{-/-}$ mice treated with or without DPP-IV inhibitor for 8 weeks were anesthetized and scanned along the body axis using LaTheta (LCT-100M) experimental animal CT system (Aloka, Tokyo, Japan). Contiguous 1-mm slice images of the whole abdominal cavity were used for quantitative assessment using LaTheta software (version 1.00). Weights of total fat mass, which comprises visceral fat mass and subcutaneous fat mass, and lean mass were quantitatively evaluated.

Oral glucose tolerance test (OGTT)

The WT and $GLP\text{-}1R^{-/-}$ mice treated with or without DPP-IV inhibitor for 8 weeks were fasted for 16 h and administered glucose (2 g/kg weight body) orally. Blood was collected from the orbital sinus of the mice at the indicated times (0, 15, 30, 60 and 120 min after glucose loading). Blood glucose levels were measured by the enzyme-electrode method. Plasma insulin levels were measured using an ELISA kit (Shibayagi, Gunma, Japan).

Measurement of plasma active GLP-1 levels and DPP-IV activity

For measurement of active GLP-1 levels, blood collected at 15 min after oral glucose loading was mixed with 2% EDTA· 4Na and 1% DPP-IV inhibitor (Linco Research, St Charles, MO). Active GLP-1 levels in plasma obtained by centrifugation (2000× g, 10 min, 4 °C) were measured using an active GLP-1 (7-36) ELISA kit (Linco Research).

Plasma DPP-IV activity was measured using a published method (Fukushima et al. 2008). In brief, 12.5 µl of plasma in duplicate was incubated with 37.5 µl of substrate cocktail (66.7 µM Gly-Pro-AMC, 25 mM HEPES, 140 mM NaCl, 26.6 mM $MgCl_2$, and 1% (w/v) BSA, pH 7.8) in the dark at room temperature for 5 min. The reaction was stopped by addition of 50 µl of 25% (v/v) acetic acid. Fluorescence was measured using a spectrofluorometer at excitation 360 nm/emission 465 nm. A standard curve was drawn using free AMC in standard buffer (25 mM HEPES, 140 mM NaCl, 20 mM $MgCl_2$, 1% (w/v) BSA, pH 7.8). DPP-IV activity (mU) is shown as the AMC (µM) generated in 1 ml plasma for 1 min of reaction time.

Measurement of insulin secretion in isolated islets

Islets were isolated from mice and preincubated at 37 °C for 30 min in 20 ml of Krebs-Ringer bicarbonate buffer (KRBB; 120 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.4 mM $CaCl_2$, 20 mM $NaHCO_3$) supplemented with 10 mM HEPES and 0.2% (w/v) BSA and gassed with a mixture of 95% O_2 and 5% CO_2 (KRBB medium) containing 2.8 mM glucose. 10 size-matched islets collected in each tube were incubated at 37 °C for 30 min in 700 µl of KRBB medium containing 2.8 mM or 11.1 mM glucose with or without incretin peptides (100 nM human GLP-1 or 100 nM human GIP (Peptide Institute, Inc. Osaka, Japan)). Islets were then pelleted by centrifugation (9000× g, 2 min, 4 °C) and aliquots of the buffer were sampled. The amount of immunoreactive insulin was determined by radioimmunoassay (RIA). To determine insulin content, islets were homogenized in 400 µl acid-ethanol (37% HCl in 75% ethanol, 15:1000 (v/v)) and extracted at 4 °C overnight. The acidic extracts were dried by vacuum, reconstituted, and subjected to insulin measurement.

Measurement of mRNA expression of GLP-1R and GIPR in isolated islets

Measurement of mRNA expression of GLP-1R and GIPR was performed by quantitative RT-PCR as described previously (Harada et al. 2008). Briefly, total RNA was extracted from isolated islets with RNeasy mini kit (Qiagen, Valencia, CA) and treated with DNase (Qiagen). First strand cDNA was synthesized by SuperScript™ II Reverse Transcriptase system (Invitrogens, Grand Island, NY) according to manufacturer's instructions. SYBER Green PCR Master Mix (Applied Biosystems) was prepared for the PCR run. The PCR included 2 min at 50 °C and 10 min at 90 °C, followed by 50 cycles at 95 °C for 15 s and at 60 °C for 1 minute. The sequences of GLP-1R primers were 5′-CAACCGGACCTTTGATGACTA-3′ and 5′-GCTGTGCAGAACCGGTACAC-3′; the sequences of GIPR primers were 5′-CCTCCACTGGTCCCTACAC-3′ and 5′-GATAAACACCCTCCACCAGTAG-3′; the sequences of GAPDH primers were 5′-AAATGGTGAAGGTCGGTGTG-3′ and 5′-TCGTTGATGGCAACAATCTC-3′.

Statistical analyses

Data are expressed as means ± SE. Statistical analyses were performed by ANOVA and unpaired student's t test. P values < 0.05 were considered significant.

Results

Body weight and body composition of DPP-IV inhibitor-treated HFD-fed mice

Water intake, food intake, and body weight of HFD-fed WT and $GLP\text{-}1R^{-/-}$ mice with or without DPP-IV inhibitor administration were measured. In WT mice, water and food intake in DPP-IV

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inhibitor-treated and untreated mice were similar during the 8 weeks of the study (Fig. 1A). In $GLP\text{-}1R^{-/-}$ mice, water and food intake in DPP-IV inhibitor-treated and untreated mice also were similar (Fig. 1A). A significant difference in body weight between DPP-IV inhibitor-untreated WT and $GLP\text{-}1R^{-/-}$ mice appeared from the 36th day (30.9 ± 1.3 g vs. 27.0 ± 0.6 g, $P<0.05$) (Fig. 1B). Body weight of WT mice and $GLP\text{-}1R^{-/-}$ mice was unaffected by DPP-IV inhibitor treatment during the 8 weeks of the study. To measure the effect of DPP-IV inhibitor on body composition, CT-based analysis was performed (Fig. 1C). In WT mice, there was no significant difference in body fat ratio between DPP-IV inhibitor-treated and untreated mice. However, the body fat ratio of DPP-IV inhibitor-treated $GLP\text{-}1R^{-/-}$ mice was significantly increased compared with that of untreated $GLP\text{-}1R^{-/-}$ mice (44.13 ± 1.55 vs. 32.60 ± 3.50, $P<0.05$).

OGTT of DPP-IV inhibitor-treated mice

In OGTT, blood glucose levels at 30 and 60 min were significantly lower in DPP-IV inhibitor-treated WT and $GLP\text{-}1R^{-/-}$ mice compared to those in untreated WT and $GLP\text{-}1R^{-/-}$ mice, respectively (Fig. 2A). In WT mice, the plasma insulin level of DPP-IV inhibitor-treated mice was 2.3 times higher at 15 min than that of untreated control mice ($P<0.05$), while in $GLP\text{-}1R^{-/-}$ mice, the plasma insulin levels of DPP-IV inhibitor-treated mice were 1.6 and 1.4 times higher at 15 and 30 min than those of untreated control mice, respectively ($P<0.05$) (Fig. 2B). In addition, the plasma insulin level of DPP-IV inhibitor-treated WT mice was 1.6 times higher at 15 min compared with that of DPP-IV inhibitor-treated $GLP\text{-}1R^{-/-}$ mice ($P<0.05$) (Fig. 2B).

We also measured plasma DPP-IV activity and active GLP-1 levels in WT and $GLP\text{-}1R^{-/-}$ mice at 15 min by OGTT. 75–80% of plasma DPP-IV activity in both untreated WT and $GLP\text{-}1R^{-/-}$ mice was inhibited by DPP-IV inhibitor treatment (Fig. 2C). Plasma levels of active GLP-1 were significantly elevated in DPP-IV inhibitor-treated WT and $GLP\text{-}1R^{-/-}$ mice compared to those in the respective untreated mice (Fig. 2D).

Insulin secretion and incretin receptor expression of islets isolated from DPP-IV inhibitor-treated mice

To determine insulin secretion in response to glucose and GLP-1 and GIP, batch incubation experiments were performed using islets isolated from WT and $GLP\text{-}1R^{-/-}$ mice after 8 weeks of treatment (Fig. 3A). In islets of WT mice, insulin secretion in response to 2.8 mM glucose was similar in DPP-IV inhibitor-treated and untreated mice. However, insulin secretion in response to 11.1 mM glucose, 11.1 mM glucose with GLP-1, and 11.1 mM glucose with GIP was significantly higher in DPP-IV inhibitor-treated mice than those in untreated mice. In addition, both GLP-1 and GIP augmented insulin secretion in the presence of 11.1 mM glucose in both DPP-IV inhibitor-treated and untreated mice. In islets of $GLP\text{-}1R^{-/-}$ mice, as in those of WT mice, insulin secretion in response to 2.8 mM glucose was similar in DPP-IV inhibitor-treated and untreated mice, and insulin secretion in response to 11.1 mM glucose, 11.1 mM glucose with GLP-1, and 11.1 mM glucose with GIP was significantly higher in DPP-IV inhibitor-treated mice than those in untreated mice. However, in $GLP\text{-}1R^{-/-}$ mice, potentiation of insulin secretion by incretin in the presence of 11.1 mM glucose was observed only by GIP and not by GLP-1 in both DPP-IV inhibitor-treated and untreated mice. Insulin content was similar among all groups of mice (data not shown).

To determine the effect of DPP-IV inhibitor treatment on the mRNA expression of GLP-1R and GIPR in islets, we performed quantitative RT-PCR after 8 weeks of study (Fig. 3B). The mRNA expression levels of GLP-1R and GIPR in DPP-IV inhibitor-treated and untreated WT mice were similar, as were total mRNA expression levels of GIPR in DPP-IV inhibitor-treated and untreated $GLP\text{-}1R^{-/-}$ mice.

Discussion

In the present study, we evaluated body composition and glucose control in the absence of the GLP-1 signaling using $GLP\text{-}1R^{-/-}$ mice treated with DPP-IV inhibitor for 8 weeks to clarify GLP-1 and GIP action under long-term DPP-IV inhibition.

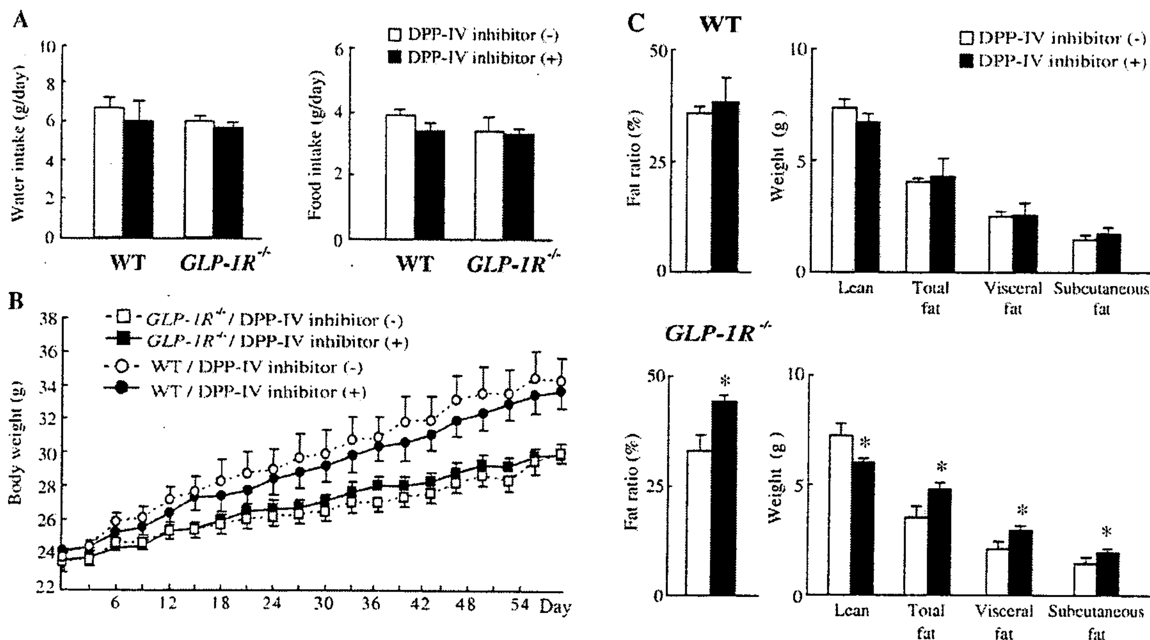

Fig. 1. Water and food intake, body weight and CT-based body composition analysis. (A) Water intake (left) and food intake (right) of WT and $GLP\text{-}1R^{-/-}$ mice treated with (filled) or without (open) DPP-IV inhibitor at the last week of study (average of 1 day). (B) Body weight change of WT (circle) and $GLP\text{-}1R^{-/-}$ (square) mice treated with (filled) or without (open) DPP-IV inhibitor. (C) CT-based body composition analysis of WT (upper) and $GLP\text{-}1R^{-/-}$ (lower) mice treated with (filled) or without (open) DPP-IV inhibitor. Fat ratio calculated as: total fat/(lean + total fat) × 100. Values are means ± SE. *$P<0.05$ vs. untreated mice ($n=5$–6).

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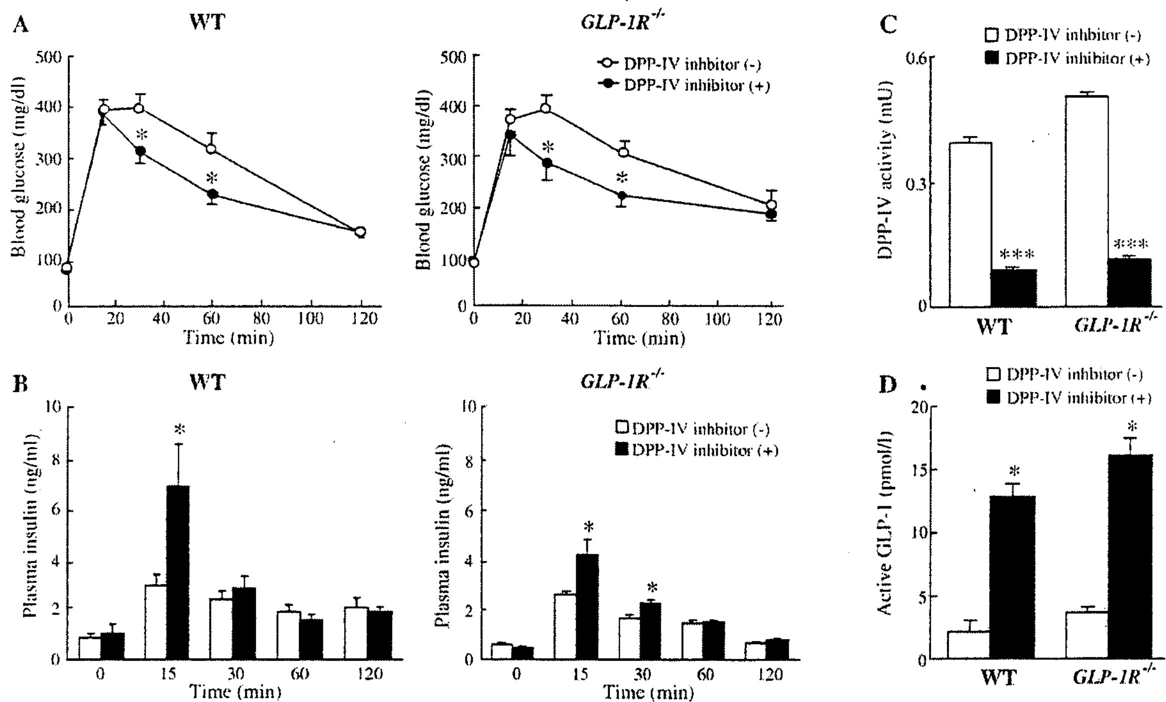

Fig. 2. OGTT. Blood glucose levels (A) and plasma insulin levels (B) of WT (left) and $GLP\text{-}1R^{-/-}$ (right) mice treated with (filled) or without (open) DPP-IV inhibitor after 8 weeks of study. Plasma DPP-IV activity (C) and plasma levels of active GLP-1 (D) at 15 min for WT and $GLP\text{-}1R^{-/-}$ mice treated with (filled) or without (open) DPP-IV inhibitor. Values are means ± SE. $^{*}P<0.05$, $^{***}P<0.001$ vs. untreated mice ($n=5$–6).

HFD-fed DPP-IV-deficient rodents exhibit reduced food intake and resistance to development of obesity with elevated active GLP-1 levels (Yasuda et al. 2002; Conarello et al. 2003), and DPP-IV inhibitor has been shown to reduce body weight in some previous studies using rodent models (Pospisilik et al. 2002; Lamont and Drucker 2008). In the present study, no alteration in body weight was found after 8 weeks of DPP-IV inhibitor treatment either in HFD-fed WT or $GLP\text{-}1R^{-/-}$ mice, although 75–80% of plasma DPP-IV activity was inhibited and plasma active GLP-1 levels were significantly elevated after oral glucose loading. However, CT-based body composition analysis revealed that DPP-IV inhibitor treatment increased body fat mass in $GLP\text{-}1R^{-/-}$ mice but not in WT mice. DPP-IV is well known to be involved in inactivation of both GLP-1 and GIP, and plasma active GIP levels are elevated by treatment of DPP-IV inhibitor (Deacon et al. 2001). The receptor for GIP, differently from that for GLP-1, is expressed in adipocytes, and GIP directly facilitates energy accumulation in adipose tissue (Miyawaki et al. 2002; Naitoh et al. 2008). Our results suggest that fat accumulation is potentiated by fat-augmenting factors including GIP in the absence of the GLP-1 signaling under long-term DPP-IV inhibition. Thus, the lack of change in body weight and fat mass in DPP-IV inhibitor-treated WT mice may be due to the counteracting effects of enhanced GLP-1 and GIP signaling. In addition, $GLP\text{-}1R^{-/-}$ mice showed less body weight gain compared to that of WT mice, consistent with the previous report on $GLP\text{-}1R^{-/-}$

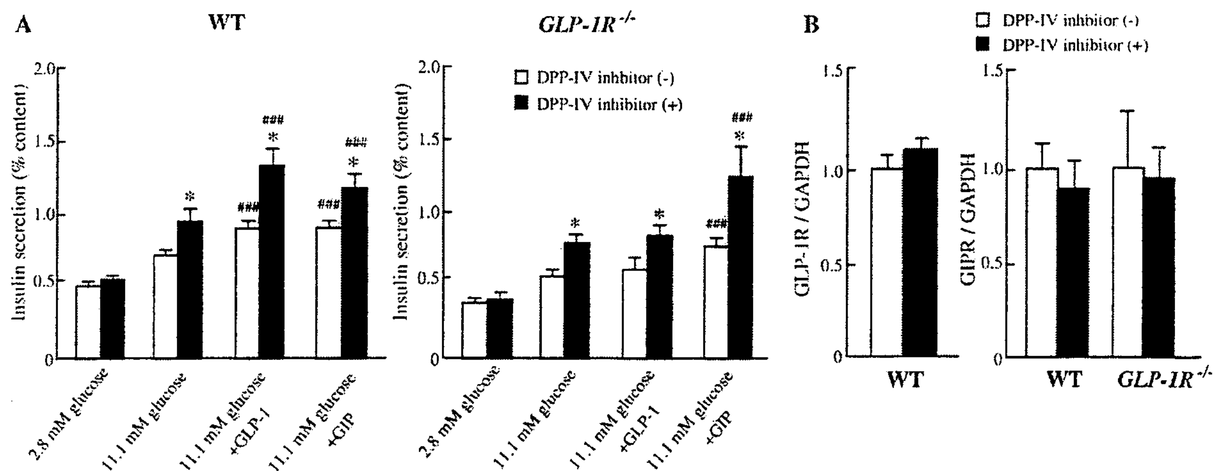

Fig. 3. Functional study of isolated islets. (A) Insulin secretion from islets isolated from WT (left) and $GLP\text{-}1R^{-/-}$ (right) mice treated with (filled) or without (open) DPP-IV inhibitor after 8 weeks of study. Values are means ± SE. $^{*}P<0.05$ vs. untreated mice. $^{\#\#\#}P<0.05$ vs. 11.1 mM glucose. (B) The mRNA expression of GLP-1R and GIPR in islets isolated from WT and $GLP\text{-}1R^{-/-}$ mice treated with (filled) or without (open) DPP-IV inhibitor. GLP-1R and GIPR mRNA levels were corrected for GAPDH mRNA levels, respectively. Data of DPP-IV inhibitor-treated mice is shown relative to untreated mice. Values are means ± SE. ($n=5$–8).

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mice showing reduced body weight gain compared to that of WT mice, possibly due to enhanced locomotor activity and increased energy expenditure (Hansotia et al. 2007).

GLP-1 and GIP both are clearly involved in the effects of long-term DPP-IV inhibition on improved glucose tolerance, as DPP-IV inhibitor fails to increase insulin secretion and decrease plasma glucose after oral glucose loading in DIRKO mice (Flock et al. 2007). Our comparison in the present study of *GLP-1R*$^{-/-}$ mice and WT mice enables us to estimate the magnitude of the effects of enhanced GLP-1 and GIP by long-term DPP-IV inhibition on insulin secretion separately. Improved glucose tolerance and increased plasma insulin with elevated active GLP-1 levels were found in both DPP-IV inhibitor-treated WT and *GLP-1R*$^{-/-}$ mice by OGTT, indicating that GIP contributes to the insulinotropic effects of long-term DPP-IV inhibition in *GLP-1R*$^{-/-}$ mice. Moreover, blood insulin at 15 min in DPP-IV inhibitor-treated *GLP-1R*$^{-/-}$ mice was about half of that in DPP-IV inhibitor-treated WT mice. These results confirm that GLP-1 and GIP are important mediators of the insulinotropic effects of long-term DPP-IV inhibition. In addition, insulin secretion from islets in response to 11.1 mM glucose was increased in DPP-IV inhibitor-treated WT and *GLP-1R*$^{-/-}$ mice, indicating that glucose sensitivity of insulin secretion is augmented by long-term DPP-IV inhibitor administration unrelated to the GLP-1 signaling. However, the mechanism is not known. A recent report found that the glucose sensitivity of insulin secretion in isolated islets of mice improved after GLP-1 receptor agonist treatment due to augmented cAMP-induced activation of protein kinase A (PKA) through the GLP-1 receptor (Winzell and Ahrén 2008). It also was reported that activated PKA due to GLP-1 signaling increased expression of transcription factor pancreatic-duodenum homeobox-1 (PDX-1), translocation of PDX-1 from cytoplasm to nucleus, and phosphorylation of glucose transporter type 2 (GLUT2) in β-cells (Wang et al. 2001; Thorens et al. 1996). Thus, the increased glucose sensitivity of insulin secretion in islets unrelated to the GLP-1 signaling may be the result of augmented GIP signaling due long-term DPP-IV inhibition through similar mechanisms. Further study is required to clarify the augmentation of glucose sensitivity of islets after long-term DPP-IV inhibitor administration.

In addition to the plasma active incretin level, the expression of incretin receptors in islets also influences their insulinotropic effect (Lynn et al. 2001; Xu et al. 2007). Indeed, it has been reported that continuous GLP-1 stimulation results in desensitization of GLP-1R, which can subsequently reduce insulin secretion in response to GLP-1 in insulin-secreting cell lines (Widmann et al. 1996; Green et al. 2005). However, the expression of GLP-1R and GIPR in islets did not change in DPP-IV inhibitor-treated mice in the present study. Furthermore, insulin secretion in response to incretins was maintained in the islets of DPP-IV inhibitor-treated mice, demonstrating that sensitivity of the incretin receptors did not decrease even after 8 weeks of continuous incretin stimulation. These results suggest that the action of DPP-IV inhibitor in glucose control is preserved during long-term DPP-IV inhibitor administration.

Conclusion

Long-term DPP-IV inhibition does not alter body composition, possibly due to the counteracting effects of enhanced GLP-1 and GIP, but does improve glucose tolerance through the synergistic insulinotropic effects of enhanced GLP-1 and GIP, as well as by improved glucose responsiveness in pancreatic islets.

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References

Ahrén B, Simonsson E, Larsson H, Landin-Olsson M, Torgeirsson H, Jansson PA, Sandqvist M, Båvenholm P, Efendic S, Eriksson JW, Dickinson S, Holmes D. Inhibition of dipeptidyl peptidase IV improves metabolic control over a 4-week study period in type 2 diabetes. Diabetes Care 25 (5), 869–875, 2002.

Buse JB, Henry RR, Han J, Kim DD, Fineman MS, Baron AD. Exenatide-113 Clinical Study Group. Effects of Exenatide (Exendin-4) on glycemic control over 30 weeks in sulfonylurea-treated patients with type 2 diabetes. Diabetes Care 27 (11), 2628–2635, 2004.

Conarello SL, Li Z, Ronan J, Roy RS, Zhu L, Jiang G, Liu F, Woods J, Zycband E, Moller DE, Thornberry NA, Zhang BB. Mice lacking dipeptidyl peptidase IV are protected against obesity and insulin resistance. Proceedings of the National Academy of Sciences of the United States of America 100 (11), 6825–6830, 2003.

Deacon CF, Danielsen P, Klarskov L, Olesen M, Holst JJ. Dipeptidyl peptidase IV inhibition reduces the degradation and clearance of GIP and potentiates its insulinotropic and antihyperglycemic effects in anesthetized pigs. Diabetes 50 (7), 1588–1597, 2001.

Flock G, Baggio LL, Longuet C, Drucker DJ. Incretin receptors for glucagon-like peptide 1 and glucose-dependent insulinotropic polypeptide are essential for the sustained metabolic actions of Vildagliptin in mice. Diabetes 56 (12), 3006–3013, 2007.

Fukushima H, Hiratate A, Takahashi M, Mikami A, Saito-Hori M, Munetomo E, Kitano K, Chonan S, Saito H, Suzuki A, Takaoka Y, Yamamoto K. Synthesis and structure-activity relationships of potent 4-fluoro-2-cyanopyrrolidine dipeptidyl peptidase IV inhibitors. Bioorganic & Medicinal Chemistry 16 (7), 4093–4106, 2008.

Green BD, Liu HK, McCluskey JT, Duffy NA, O'Harte FP, McClenaghan NH, Flatt PR. Function of a long-term, GLP-1-treated, insulin-secreting cell line is improved by preventing DPP IV-mediated degradation of GLP-1. Diabetes, Obesity & Metabolism 7 (5), 563–569, 2005.

Hansotia T, Maida A, Flock G, Yamada Y, Tsukiyama K, Seino Y, Drucker DJ. Extrapancreatic incretin receptors modulate glucose homeostasis, body weight, and energy expenditure. The Journal of Clinical Investigation 117 (1), 143–152, 2007.

Harada N, Yamada Y, Tsukiyama K, Yamada C, Nakamura Y, Mukai E, Hamasaki A, Liu X, Toyoda K, Seino Y, Inagaki N. A novel gastric inhibitory polypeptide (GIP) receptor splice variant influences GIP sensitivity of pancreatic {beta}-cells in obese mice. American Journal of Physiology. Endocrinology and Metabolism 294 (1), E61–E68, 2008.

Komatsu R, Matsuyama T, Namba M, Watanabe N, Itoh H, Kono N, Tarui S. Glucagonostatic and insulinotropic action of glucagon-like peptide-1-(7-36) amide. Diabetes 38 (7), 902–905, 1989.

Lamont BJ, Drucker DJ. Differential antidiabetic efficacy of incretin agonists versus DPP-4 inhibition in high fat-fed mice. Diabetes 57 (1), 190–198, 2008.

Lynn FC, Pamir N, Ng EH, McIntosh CH, Kieffer TJ, Pederson RA. Defective glucose-dependent insulinotropic polypeptide receptor expression in diabetic fatty zucker rats. Diabetes 50 (5), 1004–1011, 2001.

Marguet D, Baggio L, Kobayashi T, Bernard AM, Pierres M, Nielsen PF, Ribel U, Watanabe T, Drucker DJ, Wagtmann N. Enhanced insulin secretion and improved glucose tolerance in mice lacking CD26. Proceedings of the National Academy of Sciences of the United States of America 97 (12), 6874–6879, 2000.

Miyawaki K, Yamada Y, Ban N, Ihara Y, Tsukiyama K, Zhou H, Fujimoto S, Oku A, Tsuda K, Toyokuni S, Hiai H, Mizunoya W, Fushiki T, Holst JJ, Makino M, Tashita A, Kobara Y, Tsubamoto Y, Jinnouchi T, Jomori T, Seino Y. Inhibition of gastric inhibitory polypeptide signaling prevents obesity. Nature Medicine 8 (7), 738–742, 2002.

Nagakura T, Yasuda N, Yamazaki K, Ikuta H, Yoshikawa S, Asano O, Tanaka I. Improved glucose tolerance via enhanced glucose-dependent insulin secretion in dipeptidyl peptidase IV-deficient Fischer rats. Biochemical and Biophysical Research Communications 284 (2), 501–506, 2001.

Naitoh R, Miyawaki K, Harada N, Mizunoya W, Toyoda K, Fushiki T, Yamada Y, Seino Y, Inagaki N. Inhibition of GIP signaling modulates adiponectin levels under high-fat diet in mice. Biochemical and Biophysical Research Communications 376 (1), 21–25, 2008.

Pospisilik JA, Stafford SG, Demuth HU, Brownsey R, Parkhouse W, Finegood DT, McIntosh CH, Pederson RA. Long-term treatment with the dipeptidyl peptidase IV inhibitor P32/98 causes sustained improvements in glucose tolerance, insulin sensitivity, hyperinsulinemia, and β-cell glucose responsiveness in VDF (fa/fa) zucker rats. Diabetes 51 (4), 943–950, 2002.

Reimer MK, Holst JJ, Ahrén B. Long-term inhibition of dipeptidyl peptidase IV improves glucose tolerance and preserves islet function in mice. European Journal of Endocrinology/European Federation of Endocrine Societies 146 (5), 717–727, 2002.

Szayna M, Doyle ME, Betkey JA, Holloway HW, Spencer RG, Greig NH, Egan JM. Exendin-4 decelerates food intake, weight gain, and fat deposition in Zucker rats. Endocrinology 141 (6), 1936–1941, 2000.

Thorens B, Dériaz N, Bosco D, DeVos A, Pipeleers D, Schuit F, Meda P, Porret A. Protein kinase A-dependent phosphorylation of GLUT2 in pancreatic beta cells. The Journal of Biological Chemistry 271 (14), 8075–8081, 1996.

Toyoda K, Okitsu T, Yamane S, Uonaga T, Liu X, Harada N, Uemoto S, Seino Y, Inagaki N. GLP-1 receptor signaling protects pancreatic beta cells in intraportal islet transplant by inhibiting apoptosis. Biochemical and Biophysical Research Communications 367 (4), 793–798, 2008.

Tsukiyama K, Yamada Y, Yamada C, Harada N, Kawasaki Y, Ogura M, Bessho K, Li M, Amizuka N, Sato M, Udagawa N, Takahashi N, Tanaka K, Oiso Y, Seino Y. Gastric

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inhibitory polypeptide as an endogenous factor promoting new bone formation after food ingestion. Molecular Endocrinology 20 (7), 1644–1651, 2006.
Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JP, Smith DM, Ghatei MA, Herbert J, Bloom SR. A role for glucagon-like peptide-1 in the central regulation of feeding. Nature 379 (6560), 69–72, 1996.
Wang X, Zhou J, Doyle ME, Egan JM. Glucagon-like peptide-1 causes pancreatic duodenal homeobox-1 protein translocation from the cytoplasm to the nucleus of pancreatic beta-cells by a cyclic adenosine monophosphate/protein kinase A-dependent mechanism. Endocrinology 142 (5), 1820–1827, 2001.
Widmann C, Dolci W, Thorens B. Desensitization and phosphorylation of the glucagon-like peptide-I (GLP-1) receptor by GLP-1 and 4-Phorbol 12-Myristate 13-Acetate. Molecular Endocrinology 10 (1), 62–75, 1996.
Willms B, Werner J, Holst JJ, Orskov C, Creutzfeldt W, Nauck MA. Gastric emptying, glucose responses, and insulin secretion after a liquid test meal: Effects of exogenous glucagon-like peptide-1 (GLP-1)-(7–36) amide in type 2 (noninsulin-dependent) diabetic patients. The Journal of Clinical Endocrinology and Metabolism 81 (1), 327–332, 1996.
Winzell MS, Ahrén B. Durable islet effects on insulin secretion and protein kinase A expression following exendin-4 treatment of high-fat diet-fed mice. Journal of Molecular Endocrinology 40 (2), 93–100, 2008.
Xu G, Kaneto H, Laybutt DR, Duvivier-Kali VF, Trivedi N, Suzuma K, King GL, Weir GC, Bonner-Weir S. Downregulation of GLP-1 and GIP receptor expression by hyperglycemia: Possible contribution to impaired incretin effects in diabetes. Diabetes 56 (6), 1551–1558, 2007.
Yasuda N, Nagakura T, Yamazaki K, Inoue T, Tanaka I. Improvement of high fat-diet-induced insulin resistance in dipeptidyl peptidase IV-deficient Fischer rats. Life Sciences 71 (2), 227–238, 2002.