use. Exclusion of the patients with cancer-related hemorrhage did not fundamentally influence the analytical results (data not shown).

This small case–control study showed no association of admission as a result of gastrointestinal hemorrhage with the use of antithrombotic drugs or aspirin among older patients. As most of the patients were managed by geriatricians in our department, the finding might be limited to the particular facility or cohort, but might not be extended to the general population. It is suggested, however, that geriatricians can make an appropriate decision on the indication and management of antithrombotic drugs for older patients. Although no studies have shown comparable findings in terms of gastrointestinal bleeding, geriatric evaluation and management has been reported to be effective to reduce serious adverse drug events.[4] A recent review on the management of antiplatelet agents[5] also recommended comprehensive strategies to reduce the risk of hemorrhagic complications. Prospective studies with a large sample size are required to confirm this issue. Nevertheless, it is certain that the use of antithrombotic medications should be carefully determined by considering the risk/benefit balance of each patient.

References

1 Garcia Roriguez LA, Jick H. Risk of upper gastrointestinal bleeding and perforation associated with individual non-steroidal anti-inflammatory drugs. *Lancet* 1994; **343**: 769–772.
2 Boyd CM, Darer J, Boult C, Fried LP, Boult L, Wu AW. Clinical practice guidelines and quality of care for older patients with multiple comorbid diseases: implications for pay for performance. *JAMA* 2005; **294**: 716–724.
3 Man-Son-Hing M, Laupacis A. Anticoagulant-related bleeding in older persons with atrial fibrillation: physicians' fears often unfounded. *Arch Intern Med* 2003; **163**: 1580–1586.
4 Schmader KE, Hanlon JT, Pieper CF *et al.* Effects of geriatric evaluation and management on adverse drug reactions and suboptimal prescribing in the frail elderly. *Am J Med* 2004; **116**: 394–401.
5 Kalyanasundaram A, Lincoff AM. Managing adverse effects and drug-drug interactions of antiplatelet agents. *Nat Rev Cardiol* 2011; 8: 592–600.

Hypertension Research (2012), 1–7

www.nature.com/hr

REVIEW SERIES

Hormonal effects on blood vessels

Masahiro Akishita[1] and Jing Yu[2]

The incidence of cardiovascular disease (CVD) is lower in younger women than in men of the same age, but it increases after menopause, implicating the atheroprotective action of endogenous estrogen. Although observational studies have suggested the efficacy of estrogen therapy in postmenopausal women, placebo-controlled, randomized trials, such as the Women's Health Initiative, have not confirmed effects of estrogen therapy on CVD. Conversely, basic, experimental research has progressed and provided mechanistic insight into estrogen's action on blood vessels. By contrast, the vascular effects of androgens remain poorly understood and have been controversial for a long time. In recent years, an increasing body of evidence has suggested that androgens may exert protective effects against the development of atherosclerosis, at least in elderly men. Epidemiological studies have shown that the incidence of and mortality due to CVD were increased in elderly men with low testosterone levels, although the efficacy of androgen therapy remains unknown. Furthermore, recent experimental studies have demonstrated the direct action of androgens on the vasculature. In this review, we illustrate the effects of sex steroids on the cardiovascular system, focusing on the action of testosterone on the blood vessels.
Hypertension Research advance online publication, 2 February 2012; doi:10.1038/hr.2012.4

Keywords: cardiovascular disease; endothelium; estrogen; testosterone; vascular smooth muscle

INTRODUCTION

Since the 1940s, it has been recognized that sex steroids have important roles in the cardiovascular system.[1,2] A number of epidemiological studies have shown that sex differences are apparent in the incidence of atherosclerotic disease. The incidence of cardiovascular diseases (CVDs), such as hypertension and coronary artery disease, is lower in younger women than in men of the same age.[3–5] However, it rises after menopause and, with age, catches up to that among men. These phenomena have been explained by the atheroprotective action of endogenous estrogen and its deprivation in postmenopausal women. In the past 20–30 years, many studies have suggested the efficacy of hormone replacement therapy (HRT) in postmenopausal women for the prevention of CVD and the putative vasoprotective effects of estrogen. However, reports from the Heart and Estrogen-Progestin Replacement Study (HERS)[6] and the Women's Health Initiative (WHI)[7] denied the efficacy of estrogen therapy in CVD.

By contrast, the actions of androgens on the cardiovascular system remain unclear. In the process of atherosclerosis, androgens may exert complex effects on vessel walls. Both beneficial and detrimental effects have been reported. For many years, it was widely believed that androgens have unfavorable roles in the development of atherosclerosis. Recently, however, the link between androgen deficiency and atherosclerosis has been demonstrated in a number of studies.[8–10] Various epidemiological and experimental studies have also demonstrated that androgens exert beneficial influences on CVD via the direct and indirect action of androgens on the blood vessels.

As the effects of estrogen on the cardiovascular system have been extensively studied and reviewed,[11–14] we allocated a small portion of our research to estrogen, highlighting recent developments. A larger part of this review focuses on androgens, particularly testosterone, to discuss the biological role of testosterone in vascular physiology and pathology in aging men.

ACTION OF ESTROGEN ON THE CARDIOVASCULAR SYSTEM

Effects of estrogen on cardiovascular risk factors

A number of studies have reported that estrogen therapy in postmenopausal women decreases the serum levels of both total and low-density lipoprotein cholesterol while raising high-density cholesterol and triglycerides, primarily by influencing the expression of hepatic apoprotein genes.[11,15] Also, estrogen inhibits the lipid peroxidation of low-density lipoprotein *in vitro* and *in vivo*.[16,17] Furthermore, estrogen can modulate glucose metabolism and prevent other risk factors for CVD, such as obesity (Table 1).[18,19]

Direct vascular action of estrogen

Two estrogen receptor (ER) subtypes, ERα and ERβ, have been identified and are expressed in the vasculature, and experimental studies have demonstrated the vasodilator effects of estrogen/ER through their action on the endothelium, smooth muscle and extracellular matrix. Estrogen enhances endothelium-dependent vasorelaxation via increased release of nitric oxide (NO),[20–22] endothelium-derived hyperpolarizing factor[23] and PGI_2[24,25] and decreased production of endothelin-1 (Table 1).[26] Several studies have demon-

[1]Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan and [2]Department of Integrated Traditional Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
Correspondence: Dr M Akishita, Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: akishita-tky@umin.ac.jp
Received 5 September 2011; revised 17 November 2011; accepted 12 December 2011

Table 1 Anti-atherosclerotic effects of estrogen

Risk factors	*Vascular action*
Lipid metabolism	Endothelium-dependent vasorelaxation
HDL cholesterol↑	Nitric oxide↑
LDL cholesterol↓	Endothelin-1↓
Lp (a)↓	EDHF↑
Anti-oxidant	PGI_2↑
Glucose metabolism	Inhibition of EC apoptosis
Anti-obese	Endothelium-independent vasorelaxation
	Calcium antagonistic
	Inhibition of VSMC migration/proliferation

Abbreviations: EC, endothelial cell; EDHF, endothelium-derived hyperpolarizing factor; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VSMC, vascular smooth muscle cell.

strated that estrogen inhibits calcium influx[27,28] and stimulates calcium efflux[29] in vascular smooth muscle cells (VSMCs), leading to endothelium-independent vasodilation. Moreover, estrogen inhibits neointima formation in response to balloon injury[30,31] and perivascular cuff placement.[32] Endothelial regeneration,[33] inhibition of endothelial apoptosis[34] and inhibition of VSMC migration and proliferation[32] may account for the inhibitory effects of estrogen on neointima formation. Analyses of knockout mice for ERα and ERβ have provided more information regarding the molecular mechanism of estrogen's action on the blood vessels.[5] Recent progress in nuclear receptor research has also clarified the non-genomic action of estrogen on the vasculature,[14] such as the direct interaction of ERα with the regulatory subunit of phosphatidylinositol-3-OH kinase.[35]

Role of the novel ER G protein coupled receptor 30 (GPR30) in the cardiovascular system

In addition to the two classical ER subtypes, ERα and ERβ, a third membrane-bound and G-protein-coupled ER, GPR30, has been identified in human vascular endothelial cells (ECs) and smooth muscle cells.[36–38] Haas *et al.*[37] reported that G-1, a selective stimulator of GPR30, acutely blocked vasoconstrictor-induced changes in intracellular calcium concentrations and vascular tone, resulting in lowering of blood pressure in normotensive rats. Similar vasodilator effects of GPR30 have been confirmed in other studies.[39–41] It has also been reported that stimulation of GPR30 blocks VSMC proliferation.[37,42]

The vasodilator action of G-1 may be mediated by NO-independent[40] and NO-dependent[37,39,40] pathways; the latter involves GPR30-induced endothelial NO synthase (eNOS) phosphorylation.[43] Also, G-1 decreases nicotinamide adenine dinucleotide phosphate-stimulated superoxide production by the carotid and intracranial arteries, indicating the scavenging effects of GPR30 on superoxide anions.[39] In the heart, G-1 reduces ischemia/reperfusion injury and preserves cardiac function through the phosphatidylinositol 3-kinase/Akt and extracellular signal-regulated kinase pathways and by eNOS phosphorylation.[44,45] Treatment with G-1 for 2 weeks reduced the expression of angiotensin II type 1 receptor and angiotensin-converting enzyme.[40] The non-selective ER antagonist ICI 182780 and selective ER modulators, such as tamoxifen and raloxifene, have been shown to act as GPR30 ligands.[46] Moreover, both GPR30 and ER are required for estrogen action in some situations, whereas GPR30 can act alone in the absence of ER,[46,47] suggesting a complex network between GPR30 and ER.

HRT and CVD

Observational studies have suggested that HRT decreases the risk of CVD in postmenopausal women.[48,49] However, large-scale, placebo-controlled, randomized trials, such as the HERS[6] and the WHI,[7] did not confirm the findings of the observational studies. In the WHI, HRT with conjugated equine estrogen plus medroxyprogesterone acetate increased the incidence of CVD instead, particularly in women older than 60 years of age, although women who started HRT soon after menopause tended to have a decreased risk for coronary heart disease.[50]

Additional data from other studies have supported the concept that the vasoprotective effects of estrogen are evident only when hormone therapy is initiated soon after the onset of menopause and before the development of atherosclerosis. In a meta-analysis of hormone therapy, CVD mortality was lower in younger women on hormone therapy (mean age of 55 years old) than in age-matched controls.[51] Women aged 50–59 years who were enrolled in the conjugated equine estrogen trial of the WHI had significantly lower scores for coronary artery calcification 8.7 years after randomization than with placebo.[52]

Two ongoing clinical trials, the Kronos Early Estrogen Prevention Study[53] and the Early Versus Late Intervention Trial with Estradiol Study (available at http://clinicaltrials.gov/ct2/show/NCT00114517; accessed 16 November 2011), were designed to examine the timing, dosage, route and limited duration of administration on patients' cardiovascular outcomes and to prove the benefits of HRT in atherosclerosis when HRT is initiated soon after menopause. In the near future, these trials will provide additional insight into HRT and cardiovascular health in younger postmenopausal women.

ASSOCIATION OF LOW TESTOSTERONE LEVELS WITH CVD

Plasma testosterone levels decrease with aging, and >20% of healthy men older than 60 years of age have testosterone levels below the standard range in young men aged 20–30 years.[54,55] Lower testosterone levels are associated with cognitive dysfunction, muscle weakness, anemia, osteoporosis, mood disturbances and impaired general and sexual health in aging men.[56,57] Recently, many studies have demonstrated the relationship of testosterone with CVD, indicating a consistent inverse relationship between endogenous testosterone and adverse cardiovascular events.

A case–control study among 117 Indian men aged 30–60 years with old myocardial infarction showed that testosterone concentrations were significantly lower in the patients with myocardial infarction than in the control subjects.[58] Similar results were reported in men with acute myocardial infarction.[59] Cross-sectional results from the Massachusetts Male Aging Study (1709 men aged 40–70 years) showed that serum total and free testosterone levels bear an inverse relationship with CVD, independent of cardiovascular risk factors.[60] Recently, epidemiological studies have found that low testosterone levels are a predictor of all-cause and cardiovascular mortality in elderly men.[61,62] These findings were followed by studies investigating the incidence of CVD and testosterone levels.[63,64] According to these observations, endogenous testosterone appears to exert beneficial effects on the cardiovascular system.

ASSOCIATION OF LOW TESTOSTERONE WITH SURROGATE MARKERS OF ATHEROSCLEROSIS

The mechanisms underlying the epidemiological associations of low testosterone with CVD are complex and poorly understood. However, it is assumed that endogenous testosterone has physiological effects on the blood vessels and exerts atheroprotective effects. Actually, an increasing body of evidence has shown that low levels of endogenous

androgens are associated with atherosclerosis progression in elderly men. Carotid artery intima-media thickness, a common marker of clinical and subclinical atherosclerosis, has been shown to be correlated inversely with testosterone levels.[65–67] Demirbag *et al.*[68] reported a similar finding by examining the intima thickness of the thoracic aorta in older men. Similarly, in the Rotterdam Study population, Hak *et al.*[69] demonstrated that both bioavailable and total testosterone levels were negatively associated with calcified deposits in the abdominal aorta in men older than 55 years of age.

Arterial stiffness, measured as pulse wave velocity or augmentation index, is a predictor of cardiovascular events.[70] Yaron *et al.*[71] reported that age- and blood pressure-adjusted pulse wave velocity was significantly higher in hypogonadal men. Similarly, low testosterone levels in male hemodialysis patients were associated with increases in pulse wave velocity and CVD mortality.[72] Clinical and preclinical evidence exists linking endothelial dysfunction to androgen deficiency. In 187 Japanese men aged 47 ± 15 (s.d.) years, flow-mediated dilatation of the brachial artery, a reliable marker of endothelial function, was positively correlated with plasma testosterone levels, independent of other atherosclerosis risk factors.[73] Comparable results were reported from Europe[74] and specifically from Turkey.[75]

CLINICAL EFFECTS OF ANDROGEN REPLACEMENT THERAPY

As early as the 1940s, Lesser[2] demonstrated that testosterone administration alleviates symptoms and ECG abnormalities in men with angina. Subsequent studies have shown that short-term testosterone administration in men with coronary artery disease results in coronary artery dilation and resistance to ischemia. Indeed, testosterone infusion into the coronary arteries induces vasodilation,[76] and intravenous administration of testosterone reduces the exercise-induced ischemic response in men with stable angina.[77,78] Furthermore, acute administration of testosterone in men with chronic heart failure reduces peripheral vascular resistance and cardiac afterload, resulting in an increased cardiac index.[79] Chronic administration of testosterone also improves functional capacity and symptoms in heart failure patients.[80]

Several reports have shown that testosterone administration improves arterial stiffness and endothelial vasomotor function in men. Testosterone replacement in hypogonadal men results in acute (48 h) and chronic (3 months) decreases in pulse wave velocity.[71] It was also reported that testosterone replacement in men with coronary heart disease and low plasma testosterone decreased radial and aortic augmentation indices.[81] Acute intravenous infusion[82] and 8-week oral administration of testosterone[83] improved flow-mediated vasodilation of the brachial artery.

Testosterone therapy in hypogonadal men with type 2 diabetes mellitus suppressed the production of inflammatory cytokines by circulating monocytes.[84] A randomized, placebo-controlled, double-blind trial of 184 men with hypogonadism and metabolic syndrome showed that intramuscular administration of testosterone undecanoate decreased plasma levels of interleukin-1β, tumor necrosis factor-α and C-reactive protein in association with reductions in body mass index and waist circumference, while interleukin-6 and interleukin-10 did not change significantly.[85]

Taken together, testosterone administration, at least in hypogonadal men, may have a favorable vascular effect, including endothelium-dependent or -independent vasodilation and reduction of arterial stiffness and inflammatory markers. In contrast, the effects of testosterone replacement on the progression of carotid intima-media thickness or other atherosclerotic lesions, as well as on CVD risk,[86] are unknown.

DIRECT EFFECTS OF TESTOSTERONE ON VASCULAR WALLS

Risk factors, such as metabolic syndrome, may partly explain the association of low testosterone with CVD. As the relationship between testosterone and metabolic syndrome has been extensively reviewed,[87,88] this section focuses on the direct effects of testosterone on the vascular wall and the underlying molecular mechanism.

As mentioned above, testosterone therapy can improve vascular function and several markers of atherosclerosis in men. Therefore, vascular ECs, VSMCs and macrophages may be targets of androgen's actions. Indeed, androgen receptor (AR) has been shown to be expressed in these cells.[89–91]

Effects of testosterone on animal models of atherosclerosis and neointima formation

It has been demonstrated that the administration of testosterone in castrated male rabbits that were fed a high-cholesterol diet reduced aortic atherosclerosis, largely independent of plasma lipids.[92,93] In addition, neointima formation after coronary balloon injury in swine was increased by castration and was reversed by testosterone replacement.[94] Regarding the role of AR, conflicting findings have been reported. Nathan *et al.*[95] demonstrated the inhibitory effects of testosterone on fatty streak formation in castrated low-density lipoprotein receptor-deficient male mice, but the effects of testosterone were abrogated by treatment with an aromatase inhibitor, suggesting that estradiol converted from testosterone had a major role. Conversely, Qiu *et al.*[91] showed that nonaromatizable dihydrotestosterone suppressed atherosclerosis formation in castrated male rabbits, indicating a role for AR. Exaggerated vascular remodeling in AR-deficient mice, in response to angiotensin II infusion, also suggests an important role for AR.[96] A recent study by Bourghardt *et al.*[97] may provide a hint in addressing this issue. They administered testosterone in AR-deficient mice with apolipoprotein E-deficient backgrounds and showed that testosterone reduced atherosclerotic lesions, both in AR-deficient and castrated wild-type male mice, but testosterone was less effective in AR-deficient mice, suggesting AR-dependent and -independent mechanisms.

Effects of testosterone on ECs

Several reports have implicated the effects of testosterone on endothelial regeneration. Cai *et al.*[98] demonstrated that testosterone induced time- and dose-dependent proliferation of human aortic ECs via an AR-dependent pathway. In young hypogonadal men, low testosterone levels were associated with a small number of endothelial progenitor cells,[99] and testosterone replacement was able to increase the number of progenitor cells.[100] The synthesis and release of vasoactive substances by EC may have a role in these effects. Of the substances synthesized by EC, NO is a critical molecule that regulates vascular tone and atherosclerosis, and it is a major target of testosterone. It has been reported that testosterone-induced endothelium-dependent vasodilation is mediated in part by NO.[101] We recently demonstrated that testosterone rapidly induces NO production via AR-mediated activation of eNOS in human aortic ECs.[89] Furthermore, we showed that AR directly interacts with the p85 subunit of phosphatidylinositol 3-kinase, resulting in phosphorylation/activation of Akt/eNOS signaling. Taking together with our preliminary observation about the involvement of extracellular signal-regulated kinase 1/2 signaling and [Ca2+]i in AR-dependent eNOS activation, quite similar signaling pathways to those for estrogen can be proposed for testosterone (Figure 1), although some of these pathways should be verified in further studies. The genomic action of testosterone in ECs has not been studied extensively.

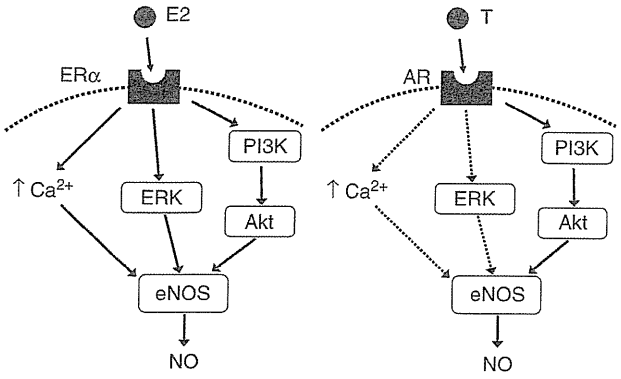

Figure 1 Signal transduction pathways of eNOS activation by estradiol and testosterone in vascular endothelial cells. AR, androgen receptor; E2, estradiol; eNOS, endothelial NO synthase; ERα, estrogen receptor α; ERK, extracellular signal-regulated kinase; NO, nitric oxide; PI3K, phosphatidylinositol 3-kinase; T, testosterone. Dotted curves indicate the plasma membrane. Dotted arrows indicate probable but undetermined pathways.

It has been reported that testosterone increases the number of ECs secreting endothelin-1,[102] although its contribution to the modulation of vascular tone and of CVD is unknown. Testosterone at physiological concentrations seems to have a beneficial influence on the hemostatic system through tissue plasminogen activator expression and inhibition of plasminogen activator inhibitor type 1 secretion by human umbilical vein ECs.[103]

Effects of testosterone on VSMCs

Most of the rapid vasodilator effects of testosterone are endothelium independent and thus are attributable to its action on VSMCs. In particular, vasodilator responses to pharmacological concentrations of testosterone seem to be AR independent. Yue *et al.*[104] reported that the relaxing response of rabbit coronary arteries to testosterone was significantly inhibited by the potassium-channel inhibitor barium chloride but not by the inhibition of NO synthesis or by removal of the endothelium. Several groups have shown that testosterone inhibits the agonist-induced rise of [Ca2+]i in VSMCs, as has been documented for estrogen. Crews and Khalil[28] reported that testosterone at supra-physiological doses (10–100 pmol l^{-1}) significantly suppresses the vasoconstriction of porcine coronary artery strips induced by prostaglandin F2α or by KCl, in parallel with the inhibition of Ca2+ entry. Hall *et al.*[105] demonstrated, using the A7r5 VSMC cell line, that testosterone and dihydrotestosterone selectively suppressed Ca2+ entry via L-type Ca2+ channels. Similar results have been reported in different experimental conditions by other groups.[106–108]

The involvement of potassium channels in testosterone-induced vasodilatation has also been studied by many researchers.[109–111] Cairrao *et al.*[112] reported that an AR antagonist, flutamide, and an adenosine triphosphate-sensitive potassium-channel inhibitor, glibenclamide, had no influence on the testosterone relaxant effect, whereas a voltage-sensitive potassium-channel inhibitor, 4-aminopyridine, decreased this effect of testosterone. Opening of voltage-sensitive potassium channels induces hyperpolarization of the plasma membrane, which in turn may lead to the closing of L-type Ca2+ channels. These pharmacological studies, most of which used chemical inhibitors, may be strengthened by studies employing molecular-targeting strategies.

Accumulation of VSMCs in damaged vascular layers is a critical process in the development of atherosclerosis and is closely related to hypertension and its complications. Many, but not all, of the previous studies indicated that testosterone might inhibit VSMC growth. Hanke *et al.*[113] reported, using an *ex vivo* organ culture system, that testosterone at 10–100 ng ml^{-1} significantly inhibited neointima formation in association with increased expression of AR in endothelium-denuded rabbit aortic rings after 21 days of incubation. Somjen *et al.*[114] demonstrated the dose-dependent inhibitory effects of dihydrotestosterone and membrane-impermeable testosterone on DNA synthesis in cultured VSMCs derived from the human umbilical artery, suggesting a role for membrane AR. The above-mentioned study by Tharp *et al.*[94] showed that the expressions of protein kinase C delta and p27 (kip1) were increased in coronary artery sections of testosterone-treated swine.

Androgen-responsive genes directly regulated by AR in VSMCs have not been determined, except for AR itself. However, we recently found that growth arrest-specific gene 6 was transactivated by testosterone in human VSMCs via binding of AR to the promoter region of the growth arrest-specific gene 6.[90] In this study, testosterone inhibited inorganic phosphate-induced VSMC apoptosis, leading to the suppression of VSMC calcification. To further elucidate the mechanism underlying the effects of testosterone on the cardiovascular system, identification of androgen-responsive genes in VSMCs, as well as in ECs, is required in future studies.

Natoli *et al.*[115] investigated, using human aortic VMSCs, and found that testosterone significantly reduced collagen and fibrillin-1 deposition, while it had no effect on elastin. They also found that testosterone increased the expression of matrix metalloproteinase-3, which has an important role in vascular remodeling.

POSSIBLE HARMFUL EFFECTS OF TESTOSTERONE ON BLOOD VESSELS

Although many studies have shown the beneficial effects of testosterone on the blood vessels, as mentioned above, other studies have suggested that long-term administration of testosterone may elicit harmful effects, especially vasoconstriction via upregulation of thromboxane A2,[116] norepinephrine synthesis,[117] angiotensin II[118] and endothelin-1.[102] It has been also reported that testosterone accelerates vascular remodeling[119] and stimulates renal prohypertensive processes, including the renin–angiotensin–aldosterone system.[120] Recent meta-analyses have revealed that CVD events were not different between testosterone and placebo groups,[86,121] indicating the complexity of testosterone therapy, as was shown for estrogen therapy in women.

TESTOSTERONE DEFICIENCY AND CVD IN WOMEN

An age-related reduction in circulating levels of androgens occurs in women as well.[122] However, it is unclear whether this decline adversely affects vascular health in women. Higher serum testosterone concentrations, within the physiological range, have been associated with lower carotid intima-media thickness,[123] suggesting potential protective effects of endogenous testosterone on cardiovascular health in pre- and postmenopausal women. Conversely, it is well known that women with polycystic ovary syndrome, who exhibit high androgen levels, are at a higher risk for CVD. Some studies have reported that high testosterone is associated with an adverse CVD risk factor profile in postmenopausal women, irrespective of polycystic ovary syndrome.[3,124] Polymorphism of the (CAG)n repeat of the AR gene was associated with CVD and risk factor profiles in postmenopausal women.[125] Thus far, evidence is lacking for an association of testosterone with CVD events in women, and it is uncertain whether testosterone could be used as a postmenopausal hormone therapy.

CONCLUSION

In this review, we illustrated the sex hormones' effects on the cardiovascular system, focusing on the action of testosterone on the blood vessels. Endogenous androgens, as well as estrogen, may display favorable effects on the vasculature, but whether HRT protects aging men and women from CVD is still unknown. Although testosterone administration seems to have diverse or contradictory effects in younger men and women, androgen therapy may provide hope for elderly hypogonadal men. This issue will remain unclear unless clinical trials of testosterone therapy are conducted. Also, progress in basic research on hormonal effects on blood vessels is essential to understanding the role of sex hormones in the development of CVD.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grants received from Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan (21390220, 20249041).

1 Sitigler LH, Tulgan J. Treatment of angina pectoris by testosterone propionate. *NY State J Med* 1943; **43**: 1424–1428.
2 Lesser MA. Testosterone propionate therapy in one hundred cases of angina pectoris. *J Clin Endocrinol Metab* 1946; **6**: 549–557.
3 Barrett-Connor E. Sex differences in coronary heart disease. Why are women so superior? The 1995 Ancel Keys Lecture. *Circulation* 1997; **95**: 252–264.
4 Reckelhoff JF. Gender differences in the regulation of blood pressure. *Hypertension* 2001; **37**: 1199–1208.
5 Mendelsohn ME, Karas RH. Molecular and cellular basis of cardiovascular gender differences. *Science* 2005; **308**: 1583–1587.
6 Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, Vittinghoff E. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *JAMA* 1998; **280**: 605–613.
7 Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *JAMA* 2002; **288**: 321–333.
8 Phillips GB, Pinkerbell BH, Jing TY. The association of hypotestosteronemia with coronary artery disease in men. *Arterioscler Thromb* 1994; **14**: 701–706.
9 English KM, Mandour O, Steeds RP, Diver MJ, Jones TH, Channer KS. Men with coronary artery disease have lower levels of androgens than men with normal coronary angiograms. *Eur Heart J* 2000; **2**: 890–894.
10 Rosano GM, Sheiban I, Massaro R, Pagnotta P, Marazzi G, Vitale C, Mercuro G, Volterrani M, Aversa A, Fini M. Low testosterone levels are associated with coronary artery disease in male patients with angina. *Int J Impot Res* 2007; **19**: 176–182.
11 Mendelsohn ME, Karas RH. The protective effects of estrogen on the cardiovascular system. *N Engl J Med* 1999; **340**: 1801–1811.
12 Klouche M. Estrogens in human vascular diseases. *Ann NY Acad Sci* 2006; **1089**: 431–443.
13 Yang XP, Reckelhoff JF. Estrogen, hormonal replacement therapy and cardiovascular disease. *Curr Opin Nephrol Hypertens* 2011; **20**: 133–138.
14 Mendelsohn ME, Karas RH. Rapid progress for non-nuclear estrogen receptor signaling. *J Clin Invest* 2010; **120**: 2277–2279.
15 Yamakawa-Kobayashi K, Somekawa Y, Fujimura M, Tomura S, Arinami T, Hamaguchi H. Relation of the -514C/T polymorphism in the hepatic lipase gene to serum HDL and LDL cholesterol levels in postmenopausal women under hormone replacement therapy. *Atherosclerosis* 2002; **162**: 17–21.
16 Kuohung W, Shwaery GT, Keaney Jr JF. Tamoxifen, esterified estradiol, and physiologic concentrations of estradiol inhibit oxidation of low-density lipoprotein by endothelial cells. *Am J Obstet Gynecol* 2001; **184**: 1060–1063.
17 Santanam N, Shern-Brewer R, McClatchey R, Castellano PZ, Murphy AA, Voelkel S, Parthasarathy S. Estradiol as an antioxidant: incompatible with its physiological concentrations and function. *J Lipid Res* 1998; **39**: 2111–2118.
18 Liang YQ, Akishita M, Kim S, Ako J, Hashimoto M, Iijima K, Ohike Y, Watanabe T, Sudoh N, Toba K, Yoshizumi M, Ouchi Y. Estrogen receptor beta is involved in the anorectic action of estrogen. *Int J Obes Relat Metab Disord* 2002; **26**: 1103–1109.
19 Lijnen HR. Murine models of obesity and hormonal therapy. *Thromb Res* 2011; **127** (Suppl 3): S17–S20.
20 Kauser K, Rubanyi GM. Potential cellular signaling mechanisms mediating upregulation of endothelial nitric oxide production by estrogen. *J Vasc Res* 1997; **34**: 229–236.
21 Hashimoto M, Akishita M, Eto M, Ishikawa M, Kozaki K, Toba K, Sagara Y, Taketani Y, Orimo H, Ouchi Y. Modulation of endothelium-dependent flow-mediated dilatation of the brachial artery by sex and menstrual cycle. *Circulation* 1995; **92**: 3431–3435.
22 Geary GG, Krause DN, Duckles SP. Estrogen reduces mouse cerebral artery tone through endothelial NOS- and cyclooxygenase-dependent mechanisms. *Am J Physiol Heart Circ Physiol* 2000; **279**: H511–H519.
23 Liu MY, Hattori Y, Fukao M, Sato A, Sakuma I, Kanno M. Alterations in EDHF-mediated hyperpolarization and relaxation in mesenteric arteries of female rats in long-term deficiency of oestrogen and during oestrus cycle. *Br J Pharmacol* 2001; **132**: 1035–1046.
24 Jun SS, Chen Z, Pace MC, Shaul PW. Estrogen upregulates cyclooxygenase-1 gene expression in ovine fetal pulmonary artery endothelium. *J Clin Invest* 1998; **102**: 176–183.
25 Sherman TS, Chambliss KL, Gibson LL, Pace MC, Mendelsohn ME, Pfister SL, Shaul PW. Estrogen acutely activates prostacyclin synthesis in ovine fetal pulmonary artery endothelium. *Am J Respir Cell Mol Biol* 2002; **26**: 610–616.
26 Akishita M, Kozaki K, Eto M, Yoshizumi M, Ishikawa M, Toba K, Orimo H, Ouchi Y. Estrogen attenuates endothelin-1 production by bovine endothelial cells via estrogen receptor. *Biochem Biophys Res Commun* 1998; **251**: 17–21.
27 Han SZ, Karaki H, Ouchi Y, Akishita M, Orimo H. 17 Beta-estradiol inhibits Ca2+ influx and Ca2+ release induced by thromboxane A2 in porcine coronary artery. *Circulation* 1995; **91**: 2619–2626.
28 Crews JK, Khalil RA. Antagonistic effects of 17 beta-estradiol, progesterone, and testosterone on Ca2+ entry mechanisms of coronary vasoconstriction. *Arterioscler Thromb Vasc Biol* 1999; **19**: 1034–1040.
29 Prakash YS, Togaibayeva AA, Kannan MS, Miller VM, Fitzpatrick LA, Sieck GC. Estrogen increases Ca2+ efflux from female porcine coronary arterial smooth muscle. *Am J Physiol* 1999; **276**: H926–H934.
30 Chen SJ, Li H, Durand J, Oparil S, Chen YF. Estrogen reduces myointimal proliferation after balloon injury of rat carotid artery. *Circulation* 1996; **93**: 577–584.
31 Krom YD, Pires NM, Jukema JW, de Vries MR, Frants RR, Havekes LM, van Dijk KW, Quax PH. Inhibition of neointima formation by local delivery of estrogen receptor alpha and beta specific agonists. *Cardiovasc Res* 2007; **73**: 217–226.
32 Akishita M, Ouchi Y, Miyoshi H, Kozaki K, Inoue S, Ishikawa M, Eto M, Toba K, Orimo H. Estrogen inhibits cuff-induced intimal thickening of rat femoral artery: effects on migration and proliferation of vascular smooth muscle cells. *Atherosclerosis* 1997; **130**: 1–10.
33 Krasinski K, Spyridopoulos I, Asahara T, van der Zee R, Isner JM, Losordo DW. Estradiol accelerates functional endothelial recovery after arterial injury. *Circulation* 1997; **95**: 1768–1772.
34 Sudoh N, Toba K, Akishita M, Ako J, Hashimoto M, Iijima K, Kim S, Liang YQ, Ohike Y, Watanabe T, Yamazaki I, Yoshizumi M, Eto M, Ouchi Y. Estrogen prevents oxidative stress-induced endothelial cell apoptosis in rats. *Circulation* 2001; **103**: 724–729.
35 Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 2000; **407**: 538–541.
36 Haas E, Meyer MR, Schurr U, Bhattacharya I, Minotti R, Nguyen HH, Heigl A, Lachat M, Genoni M, Barton M. Differential effects of 17beta-estradiol on function and expression of estrogen receptor alpha, estrogen receptor beta, and GPR30 in arteries and veins of patients with atherosclerosis. *Hypertension* 2007; **49**: 1358–1363.
37 Haas E, Bhattacharya I, Brailoiu E, Damjanović M, Brailoiu GC, Gao X, Mueller-Guerre L, Marjon NA, Gut A, Minotti R, Meyer MR, Amann K, Ammann E, Perez-Dominguez A, Genoni M, Clegg DJ, Dun NJ, Resta TC, Prossnitz ER, Barton M. Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity. *Circ Res* 2009; **104**: 288–291.
38 Meyer MR, Prossnitz ER, Barton M. The G protein-coupled estrogen receptor GPER/GPR30 as a regulator of cardiovascular function. *Vascul Pharmacol* 2011; **55**: 17–25.
39 Broughton BR, Miller AA, Sobey CG. Endothelium-dependent relaxation by G protein-coupled receptor 30 agonists in rat carotid arteries. *Am J Physiol Heart Circ Physiol* 2010; **298**: H1055–H1061.
40 Lindsey SH, Carver KA, Prossnitz ER, Chappell MC. Vasodilation in response to the GPR30 agonist G-1 is not different from estradiol in the mRen2. Lewis female rat. *J Cardiovasc Pharmacol* 2011; **57**: 598–603.
41 Meyer MR, Baretella O, Prossnitz ER, Barton M. Dilation of epicardial coronary arteries by the G protein-coupled estrogen receptor agonists G-1 and ICI 182,780. *Pharmacology* 2010; **86**: 58–64.
42 Takahashi K, Ohmichi M, Yoshida M, Hisamoto K, Mabuchi S, Arimoto-Ishida E, Mori A, Tsutsumi S, Tasaka K, Murata Y, Kurachi H. Both estrogen and raloxifene cause G1 arrest of vascular smooth muscle cells. *J Endocrinol* 2003; **178**: 319–329.
43 Rowlands DJ, Chapple S, Siow RC, Mann GE. Equol-stimulated mitochondrial reactive oxygen species activate endothelial nitric oxide synthase and redox signaling in endothelial cells: roles for F-actin and GPR30. *Hypertension* 2011; **57**: 833–840.
44 Deschamps AM, Murphy E, Sun J. Estrogen receptor activation and cardioprotection in ischemia reperfusion injury. *Trends Cardiovasc Med* 2010; **20**: 73–78.
45 Filice E, Recchia AG, Pellegrino D, Angelone T, Maggiolini M, Cerra MC. A new membrane G protein-coupled receptor (GPR30) is involved in the cardiac effects of 17beta-estradiol in the male rat. *J Physiol Pharmacol* 2009; **60**: 3–10.
46 Prossnitz ER, Maggiolini M. Mechanisms of estrogen signaling and gene expression via GPR30. *Mol Cell Endocrinol* 2009; **308**: 32–38.
47 Prossnitz ER, Barton M. The G-protein-coupled estrogen receptor GPER in health and disease. *Nat Rev Endocrinol* 2011; **7**: 715–726.

48 Grodstein F, Stampfer MJ, Colditz GA, Willett WC, Manson JE, Joffe M, Rosner B, Fuchs C, Hankinson SE, Hunter DJ, Hennekens CH, Speizer FE. Postmenopausal hormone therapy and mortality. *N Engl J Med* 1997; **336**: 1769–1775.
49 Grodstein F, Manson JE, Colditz GA, Willett WC, Speizer FE, Stampfer MJ. A prospective, observational study of postmenopausal hormone therapy and primary prevention of cardiovascular disease. *Ann Intern Med* 2000; **133**: 933–941.
50 Rossouw JE, Prentice RL, Manson JE, Wu L, Barad D, Barnabei VM, Ko M, LaCroix AZ, Margolis KL, Stefanick ML. Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. *JAMA* 2007; **297**: 1465–1477.
51 Salpeter SR, Cheng J, Thabane L, Buckley NS, Salpeter EE. Bayesian meta-analysis of hormone therapy and mortality in younger postmenopausal women. *Am J Med* 2009; **122**: 1016–1022.
52 Manson JE, Allison MA, Rossouw JE, Carr JJ, Langer RD, Hsia J, Kuller LH, Cochrane BB, Hunt JR, Ludlam SE, Pettinger MB, Gass M, Margolis KL, Nathan L, Ockene JK, Prentice RL, Robbins J, Stefanick ML. Estrogen therapy and coronary-artery calcification. *N Engl J Med* 2007; **356**: 2591–2602.
53 Miller VM, Black DM, Brinton EA, Budoff MJ, Cedars MI, Hodis HN, Lobo RA, Manson JE, Merriam GR, Naftolin F, Santoro N, Taylor HS, Harman SM. Using basic science to design a clinical trial: baseline characteristics of women enrolled in the Kronos Early Estrogen Prevention Study (KEEPS). *J Cardiovasc Transl Res* 2009; **2**: 228–239.
54 Harman SM, Metter EJ, Tobin JD, Pearson J, Blackman MR. 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.
55 Feldman HA, Longcope C, Derby CA, Johannes CB, Araujo AB, Coviello AD, Bremner WJ, McKinlay JB. 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.
56 Lamberts SW, van den Beld AW, van der Lely AJ. The endocrinology of aging. *Science* 1997; **278**: 419–424.
57 Kaufman JM, Vermeulen A. The decline of androgen levels in elderly men and its clinical and therapeutic implications. *Endocr Rev* 2005; **26**: 833–876.
58 Sewdarsen M, Vythilingum S, Jialal I, Desai RK, Becker P. Abnormalities in sex hormones are a risk factor for premature manifestation of coronary artery disease in South African Indian men. *Atherosclerosis* 1990; **83**: 111–117.
59 Mohamad MJ, Mohammad MA, Karayyem M, Hairi A, Hader AA. Serum levels of sex hormones in men with acute myocardial infarction. *Neuro Endocrinol Lett* 2007; **28**: 182–186.
60 Feldman HA, Johannes CB, McKinlay JB, Longcope C. Low dehydroepiandrosterone sulfate and heart disease in middle-aged men: cross-sectional results from the Massachusetts Male Aging Study. *Ann Epidemiol* 1998; **8**: 217–228.
61 Khaw KT, Dowsett M, Folkerd E, Bingham S, Wareham N, Luben R, Welch A, Day N. Endogenous testosterone and mortality due to all causes, cardiovascular disease, and cancer in men: European prospective investigation into cancer in Norfolk (EPIC-Norfolk) Prospective Population Study. *Circulation* 2007; **116**: 2694–2701.
62 Laughlin GA, Barrett-Connor E, Bergstrom J. Low serum testosterone and mortality in older men. *J Clin Endocrinol Metab* 2008; **93**: 68–75.
63 Vikan T, Schirmer H, Njølstad I, Svartberg J. Endogenous sex hormones and the prospective association with cardiovascular disease and mortality in men: the Tromsø Study. *Eur J Endocrinol* 2009; **161**: 435–442.
64 Akishita M, Hashimoto M, Ohike Y, Ogawa S, Iijima K, Eto M, Ouchi Y. Low testosterone level as a predictor of cardiovascular events in Japanese men with coronary risk factors. *Atherosclerosis* 2010; **210**: 232–236.
65 Muller M, van de Beld AW, Bots ML, Grobbee DE, Lamberts SWJ, van der Schouw YT. Endogenous sex hormones and progression of carotid atherosclerosis in elderly men. *Circulation* 2004; **109**: 2074–2079.
66 Van de Beld AW, Bots ML, Janssen JAMLL, Pols HAP, Lamberts SWJ, Grobbee DE. Endogenous hormones and carotid atherosclerosis in elderly men. *Am J Epidemiol* 2003; **157**: 25–31.
67 Makinen J, Jarvisalo MJ, Pollanen P, Perheentupa A, Irjala K, Koskenvuo M, Makinen J, Huhtaniemi I, Raitakari OT. Increased carotid atherosclerosis in andropausal middle-aged men. *J Am Coll Cardiol* 2005; **45**: 1603–1608.
68 Demirbag R, Yilmaz R, Ulucay A, Unlu D. The inverse relationship between thoracic aortic intima media thickness and testosterone level. *Endocr Res* 2005; **31**: 335–344.
69 Hak AE, Witteman JC, de Jong FH, Geerlings MI, Hofman A, Pols HA. Low levels of endogenous androgens increase the risk of atherosclerosis in elderly men: the Rotterdam study. *J Clin Endocrinol Metab* 2002; **87**: 3632–3639.
70 Vlachopoulos C, Aznaouridis K, Stefanadis C. Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis. *J Am Coll Cardiol* 2010; **55**: 1318–1327.
71 Yaron M, Greenman Y, Rosenfeld JB, Izkhakov E, Limor R, Osher E, Shenkerman G, Tordjman K, Stern N. Effect of testosterone replacement therapy on arterial stiffness in older hypogonadal men. *Eur J Endocrinol* 2009; **160**: 839–846.
72 Kyriazis J, Tzanakis I, Stylianou K, Katsipi I, Moisiadis D, Papadaki A, Mavroeidi V, Kagia S, Karkavitsas N, Daphnis E. Low serum testosterone, arterial stiffness and mortality in male haemodialysis patients. *Nephrol Dial Transplant* 2011; **26**: 2971–2977.
73 Akishita M, Hashimoto M, Ohike Y, Ogawa S, Iijima K, Eto M, Ouchi Y. Low testosterone level is an independent determinant of endothelial dysfunction in men. *Hypertens Res* 2007; **30**: 1029–1034.
74 Mäkinen JI, Perheentupa A, Irjala K, Pöllänen P, Mäkinen J, Huhtaniemi I, Raitakari OT. Endogenous testosterone and brachial artery endothelial function in middle-aged men with symptoms of late-onset hypogonadism. *Aging Male* 2011; **14**: 237–242.
75 Yilmaz MI, Sonmez A, Qureshi AR, Saglam M, Stenvinkel P, Yaman H, Eyileten T, Caglar K, Oguz Y, Taslipinar A, Vural A, Gok M, Unal HU, Yenicesu M, Carrero JJ. Endogenous testosterone, endothelial dysfunction, and cardiovascular events in men with nondialysis chronic kidney disease. *Clin J Am Soc Nephrol* 2011; **6**: 1617–1625.
76 Webb CM, McNeill JG, Hayward CS, de Zeigler D, Collins P. Effects of testosterone on coronary vasomotor regulation in men with coronary heart disease. *Circulation* 1999; **100**: 1690–1696.
77 Rosano GM, Leonardo F, Pagnotta P, Pelliccia F, Panina G, Cerquetani E, della Monica PL, Bonfigli B, Volpe M, Chierchia SL. Acute anti-ischemic effect of testosterone in men with coronary artery disease. *Circulation* 1999; **99**: 1666–1670.
78 Webb CM, Adamson DL, de Zeigler D, Collins P. Effect of acute testosterone on myocardial ischemia in men with coronary artery disease. *Am J Cardiol* 1999; **83**: 437–439.
79 Pugh PJ, Jones TH, Channer KS. Acute haemodynamic effects of testosterone in men with chronic heart failure. *Eur Heart J* 2003; **24**: 909–915.
80 Malkin CJ, Pugh PJ, West JN, van Beek EJ, Jones TH, Channer KS. Testosterone therapy in men with moderate severity heart failure: a double-blind randomized placebo controlled trial. *Eur Heart J* 2006; **27**: 57–64.
81 Webb CM, Elkington AG, Kraidly MM, Keenan N, Pennell DJ, Collins P. Effects of oral testosterone treatment on myocardial perfusion and vascular function in men with low plasma testosterone and coronary heart disease. *Am J Cardiol* 2008; **101**: 618–624.
82 Ong PJ, Patrizi G, Chong WC, Webb CM, Hayward CS, Collins P. Testosterone enhances flow-mediated brachial artery reactivity in men with coronary artery disease. *Am J Cardiol* 2000; **85**: 269–272.
83 Kang SM, Jang Y, Kim JY, Chung N, Cho SY, Chae JS, Lee JH. Effect of oral administration of testosterone on brachial arterial vasoreactivity in men with coronary artery disease. *Am J Cardiol* 2002; **89**: 862–864.
84 Corrales JJ, Almeida M, Burgo R, Mories MT, Miralles JM, Orfao A. Androgen-replacement therapy depresses the *ex vivo* production of inflammatory cytokines by circulating antigen-presenting cells in aging type-2 diabetic men with partial androgen deficiency. *J Endocrinol* 2006; **189**: 595–604.
85 Kalinchenko SY, Tishova YA, Mskhalaya GJ, Gooren LJ, Giltay EJ, Saad F. Effects of testosterone supplementation on markers of the metabolic syndrome and inflammation in hypogonadal men with the metabolic syndrome: the double-blinded placebo-controlled Moscow study. *Clin Endocrinol (Oxf)* 2010; **73**: 602–612.
86 Haddad RM, Kennedy CC, Caples SM, Tracz MJ, Boloña ER, Sideras K, Uraga MV, Erwin PJ, Montori VM. Testosterone and cardiovascular risk in men: a systematic review and meta-analysis of randomized placebo-controlled trials. *Mayo Clin Proc* 2007; **82**: 29–39.
87 Traish AM, Miner MM, Morgentaler A, Zitzmann M. Testosterone deficiency. *Am J Med* 2011; **124**: 578–587.
88 Corona G, Rastrelli G, Vignozzi L, Mannucci E, Maggi M. Testosterone, cardiovascular disease and the metabolic syndrome. *Best Pract Res Clin Endocrinol Metab* 2011; **25**: 337–353.
89 Yu J, Akishita M, Eto M, Ogawa S, Son BK, Kato S, Ouchi Y, Okabe T. Androgen receptor-dependent activation of endothelial nitric oxide synthase in vascular endothelial cells: role of phosphatidylinositol 3-kinase/AKT pathway. *Endocrinology* 2010; **151**: 1822–1828.
90 Son BK, Akishita M, Iijima K, Ogawa S, Maemura K, Yu J, Takeyama K, Kato S, Eto M, Ouchi Y. Androgen receptor-dependent transactivation of growth arrest-specific gene 6 mediates inhibitory effects of testosterone on vascular calcification. *J Biol Chem* 2010; **285**: 7537–7544.
91 Qiu Y, Yanase T, Hu H, Tanaka T, Nishi Y, Liu M, Sueishi K, Sawamura T, Nawata H. Dihydrotestosterone suppresses foam cell formation and attenuates atherosclerosis development. *Endocrinology* 2010; **151**: 3307–3316.
92 Bruck B, Brehme U, Gugel N, Hanke S, Finking G, Lutz C, Benda N, Schmahl FW, Haasis R, Hanke H. Gender-specific differences in the effects of testosterone and estrogen on the development of atherosclerosis in rabbits. *Arterioscler Thromb Vasc Biol* 1997; **17**: 2192–2199.
93 Alexandersen P, Haarbo J, Byrjalsen I, Lawaetz H, Christiansen C. Natural androgens inhibit male atherosclerosis: a study in castrated, cholesterol-fed rabbits. *Circ Res* 1999; **84**: 813–819.
94 Tharp DL, Masseau I, Ivey J, Ganjam VK, Bowles DK. Endogenous testosterone attenuates neointima formation after moderate coronary balloon injury in male swine. *Cardiovasc Res* 2009; **82**: 152–160.
95 Nathan L, Shi W, Dinh H, Mukherjee TK, Wang X, Lusis AJ, Chaudhuri G. Testosterone inhibits early atherogenesis by conversion to estradiol: critical role of aromatase. *Proc Natl Acad Sci* 2001; **98**: 3589–3593.
96 Ikeda Y, Aihara K, Yoshida S, Sato T, Yagi S, Iwase T, Sumitomo Y, Ise T, Ishikawa K, Azuma H, Akaike M, Kato S, Matsumoto T. Androgen-androgen receptor system protects against angiotensin II-induced vascular remodeling. *Endocrinology* 2009; **150**: 2857–2864.
97 Bourghardt J, Wilhelmson AS, Alexanderson C, De Gendt K, Verhoeven G, Krettek A, Ohlsson C, Tivesten A. Androgen receptor-dependent and independent atheroprotection by testosterone in male mice. *Endocrinology* 2010; **151**: 5428–5437.
98 Cai J, Hong Y, Weng C, Tan C, Imperato-McGinley J, Zhu YS. Androgen stimulates endothelial cell proliferation via an androgen receptor/VEGF/cyclin A-mediated mechanism. *Am J Physiol Heart Circ Physiol* 2011; **300**: H1210–H1221.

99 Foresta C, Caretta N, Lana A, De Toni L, Biagioli A, Ferlin A, Garolla A. Reduced number of circulating endothelial progenitor cells in hypogonadal men. *J Clin Endocrinol Metab* 2006; **91**: 4599–4602.
100 Foresta C, Zuccarello D, De Toni L, Garolla A, Caretta N, Ferlin A. Androgens stimulate endothelial progenitor cells through an androgen-mediated pathway. *Clin Endo* 2008; **68**: 284–289.
101 Costarella CE, Stallone JN, Rutecki GW, Whittier FC. Testosterone causes direct relaxation of rat thoracic aorta. *J Pharmacol Exp Ther* 1996; **277**: 34–39.
102 Pearson LJ, Yandle TG, Nicholls MG, Evans JJ. Regulation of endothelin-1 release from human endothelial cells by sex steroids and angiotensin-II. *Peptides* 2008; **9**: 1057–1061.
103 Jin H, Lin J, Fu L, Mei YF, Peng G, Tan X, Wang DM, Wang W, Li YG. Physiological testosterone stimulates tissue plasminogen activator and tissue factor pathway inhibitor and inhibits plasminogen activator inhibitor type 1 release in endothelial cells. *Biochem Cell Biol* 2007; **85**: 246–251.
104 Yue P, Chatterjee K, Beale C, Poole-Wilson PA, Collins P. Testosterone relaxes rabbit coronary arteries and aorta. *Circulation* 1995; **91**: 1154–1160.
105 Hall J, Jones RD, Jones TH, Channer KS, Peers C. Selective inhibition of L-type Ca2+ channels in A7r5 cells by physiological levels of testosterone. *Endocrinology* 2006; **147**: 2675–2680.
106 Jones RD, English KM, Jones TH, Channer KS. Testosterone-induced coronary vasodilatation occurs via a non-genomic mechanism: evidence of a direct calcium antagonism action. *Clin Sci (Lond)* 2004; **107**: 149–158.
107 Alvarez E, Cairrão E, Morgado M, Morais C, Verde I. Testosterone and cholesterol vasodilation of rat aorta involves L-type calcium channel inhibition. *Adv Pharmacol Sci* 2010; **2010**: 534184.
108 Scragg JL, Jones RD, Channer KS, Jones TH, Peers C. Testosterone is a potent inhibitor of L-type Ca(2+) channels. *Biochem Biophys Res Commun* 2004; **318**: 503–506.
109 Honda H, Unemoto T, Kogo H. Different mechanisms for testosterone-induced relaxation of aorta between normotensive and spontaneously hypertensive rats. *Hypertension* 1999; **34**: 1232–1236.
110 Tep-areenan P, Kendall DA, Randall MD. Testosterone-induced vasorelaxation in the rat mesenteric arterial bed is mediated predominantly via potassium channels. *Br J Pharmacol* 2002; **135**: 735–740.
111 Yildiz O, Seyrek M, Gul H, Un I, Yildirim V, Ozal E, Uzun M, Bolu E. Testosterone relaxes human internal mammary artery *in vitro*. *J Cardiovasc Pharmacol* 2005; **45**: 580–585.
112 Cairrão E, Alvarez E, Santos-Silva AJ, Verde I. Potassium channels are involved in testosterone-induced vasorelaxation of human umbilical artery. *Naunyn Schmiedebergs Arch Pharmacol* 2008; **376**: 375–383.
113 Hanke H, Lenz C, Hess B, Spindler KD, Weidemann W. Effect of testosterone on plaque development and androgen receptor expression in the arterial vessel wall. *Circulation* 2001; **103**: 1382–1385.
114 Somjen D, Kohen F, Gayer B, Kulik T, Knoll E, Stern N. Role of putative membrane receptors in the effect of androgens on human vascular cell growth. *J Endocrinol* 2004; **180**: 97–106.
115 Natoli AK, Medley TL, Ahimastos AA, Drew BG, Thearle DJ, Dilley RJ, Kingwell BA. Sex steroids modulate human aortic smooth muscle cell matrix protein deposition and matrix metalloproteinase expression. *Hypertension* 2005; **46**: 1129–1134.
116 Masuda A, Mathur R, Halushka PV. Testosterone increases thromboxane A2 receptors in cultured rat aortic smooth muscle cells. *Circ Res* 1991; **69**: 638–643.
117 Kumai T, Tanaka M, Watanabe M, Nakura H, Kobayashi S. Influence of androgen on tyrosine hydroxylase mRNA in adrenal medulla of spontaneously hypertensive rats. *Hypertension* 1995; **26**: 208–212.
118 Reckelhoff JF, Zhang H, Granger JP. Testosterone exacerbates hypertension and reduces pressure-natriuresis in male spontaneously hypertensive rats. *Hypertension* 1998; **31**: 435–439.
119 Kienitz T, Quinkler M. Testosterone and blood pressure regulation. *Kidney Blood Press Res* 2008; **31**: 71–79.
120 Yanes LL, Sartori-Valinotti JC, Iliescu R, Romero DG, Racusen LC, Zhang H, Reckelhoff JF. Testosterone-dependent hypertension and upregulation of intrarenal angiotensinogen in Dahl salt-sensitive rats. *Am J Physiol Renal Physiol* 2009; **296**: F771–F779.
121 Fernández-Balsells MM, Murad MH, Lane M, Lampropulos JF, Albuquerque F, Mullan RJ, Agrwal N, Elamin MB, Gallegos-Orozco JF, Wang AT, Erwin PJ, Bhasin S, Montori VM. Clinical review 1: adverse effects of testosterone therapy in adult men: a systematic review and meta-analysis. *J Clin Endocrinol Metab* 2010; **95**: 2560–2575.
122 Longcope C, Franz C, Morello C, Baker R, Johnston Jr CC. Steroid and gonadotropin levels in women during the peri-menopausal years. *Maturitas* 1986; **8**: 189–196.
123 Bernini GP, Sgró M, Moretti A, Argenio GF, Barlascini CO, Cristofani R, Salvetti A. Endogenous androgens and carotid intimal-medial thickness in women. *J Clin Endocrinol Metab* 1999; **84**: 2008–2012.
124 Brand JS, van der Schouw YT. Testosterone, SHBG and cardiovascular health in postmenopausal women. *Int J Impot Res* 2010; **22**: 91–104.
125 Saltiki K, Cimponeriu A, Garofalaki M, Sarika L, Papathoma A, Stamatelopoulos K, Alevizaki M. Severity of coronary artery disease in postmenopausal women: association with the androgen receptor gene (CAG)n repeat polymorphism. *Menopause* 2011; **18**: 1225–1231.

Geriatrics Gerontology 1st Impact Factor in June 2010 Medline Indexed

Geriatr Gerontol Int 2011

ORIGINAL ARTICLE

Polypharmacy as a risk for fall occurrence in geriatric outpatients

Taro Kojima,[1] Masahiro Akishita,[1] Tetsuro Nakamura,[2] Kazushi Nomura,[1] Sumito Ogawa,[1] Katsuya Iijima,[1] Masato Eto[1] and Yasuyoshi Ouchi[1]

[1]Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, and [2]Research Institute of Aging Science, Tokyo, Japan

Objective: To investigate the predictors of falls, such as comorbidity and medication, in geriatric outpatients in a longitudinal observational study.

Methods: A total of 172 outpatients (45 men and 126 women, mean age 76.9 ± 7.0 years) were evaluated. Physical examination, clinical history and medication profile were obtained from each patient at baseline. These patients were followed for up to 2 years and falls were self-reported to their physicians. The factors associated with falls were analyzed statistically.

Results: A total of 32 patients experienced falls within 2 years. On univariate analysis, older age, osteoporosis, number of comorbid conditions and number of drugs were significantly associated with falls within 2 years. On multiple logistic regression analysis, the number of drugs was associated with falls, independent of age, sex, number of comorbid conditions and other factors that were significantly associated in univariate analysis. A receiver–operator curve evaluating the optimal cut-off value for the number of drugs showed that taking five or more drugs was a significant risk.

Conclusion: In geriatric outpatients, polypharmacy is associated with falls. Intervention studies are needed to clarify the causal relationship between polypharmacy, comorbidity and falls. **Geriatr Gerontol Int 2011; ••: ••–••.**

Keywords: bone/musculo-skeletal, elderly, falls, geriatric medicine, internal medicine, polypharmacy.

Introduction

Previous studies have assessed the risk factors for falls in community-dwelling elderly,[1–3] but not in geriatric outpatients, and history of falls, physical ability and living environment were found to be predictors of falls. Outpatients have different characteristics from community-dwelling elderly, and previous studies have not assessed whether medical comorbidity and therapeutic drugs might be risk factors for falls. Falls in patients on medication are complicated, because some drugs, such as aspirin, can cause serious bleeding when they have injurious falls, and others, such as antihypertensive[4] and hypoglycemic[5,6] agents, can cause falls.

Previously, we reported that polypharmacy was associated with the tendency for falls using four indices of fall tendency in a cross-sectional setting in geriatric outpatients,[7] though that study did not evaluate fall occurrences, and also not in a longitudinal manner. Therefore, we aimed at investigating whether polypharmacy was predictive of fall occurrences in a prospective fashion. For this purpose, we followed geriatric outpatients for up to 2 years, and assessed whether polypharmacy is a risk for fall occurrence, together with other risks.

Accepted for publication 19 October 2011.

Correspondence: Dr Masahiro Akishita MD PhD, Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Email: akishita-tky@umin.ac.jp

The validity of two novel indices of fall tendency, the 22 items fall risk index[8] and the 13 points simple screening test,[3] which were used in our previous study, have been confirmed in community-dwelling elderly, but not in geriatric outpatients. Therefore, in the present investigation, the association of these two indices with falls was also evaluated to confirm their validity in geriatric outpatients in a longitudinal study.

Methods

Patients

From 2006 to 2007, a total of 190 consecutive patients aged 65 years or older who were receiving treatment for chronic diseases, such as hypertension, dyslipidemia, diabetes and osteoporosis, who were seen every 2–4 weeks at the outpatient clinic of the Research Institute of Aging Science, Tokyo, were enrolled. All the patients were able to walk independently and their condition was stable. Patients who had acute illness or overt dementia were excluded. Anthropometric and medical information including past history of stroke, myocardial infarction, malignancy and prescribed drugs was obtained from each patient at baseline from the medical chart recorded by the physician in charge. However, 18 patients were excluded, because they were lost to follow up soon after enrolment and the medical information was not fully obtained. All prescribed drugs had not been changed in the included patients for at least 2 months before enrolment. The patients were followed up for 2 years.

Occurrence of falls

During the follow-up period, the patients and their family members responded to the annual questionnaire asking about the occurrence of falls within the past year. The questionnaire was repeated for 2 years.

Indices of fall tendency

After enrolment, the patients were examined for two indices to investigate the fall tendency. These were (i) a questionnaire of the 22 items portable fall risk index;[8] and (ii) the 13 points simple screening test to assess the fall tendency.[3]

Ethical consideration

The present study was approved by the Institutional Review Board of the Research Institute of Aging Science. We obtained written consent from all participants and/or their guardians.

Data analysis and statistical methods

Values are expressed as mean ± standard deviation. In order to analyze the relationship between falls and comorbidity or drugs, variables were compared using Student's *t*-test or χ^2-test as appropriate. Significant factors found in univariate analysis were included in multivariate logistic regression analysis to determine the association of falls with other variables. Receiver–operating curve (ROC) analysis was carried out to identify the optimal cut-off value of the number of drugs for predicting falls within 2 years. The value with the highest sum of sensitivity and specificity was used as the optimal cut-off value. Logistic regression analysis was carried out to assess the validity of the two indices of fall tendency, adjusted by age and sex. *P*-values <0.05 were considered statistically significant. Data were analyzed using JMP version 8.0.1 (SAS Institute, Cary, North Carolina, USA).

Results

Baseline medical information and two indices of fall tendency were evaluated in 172 patients (Table 1). Drugs prescribed in less than 5% of the patients are not shown. Because only patients who were in a stable condition and were able to walk independently were included, patients with Parkinson's disease, severe paresis or painful arthralgia were not included. Calcium channel blockers prescribed in the present study were all long-acting agents, and the prescribed aspirin dosage was 100 mg in all cases. Only a few patients were receiving insulin therapy, sulfonylureas, angiotensin converting enzyme inhibitors, β-blockers, α-blockers, non-steroidal anti-inflammatory drugs or anticoagulants. No patients were taking neuroleptics or antiparkinsonian drugs.

After 1 year, all patients, except for one who died of congestive heart failure, were followed up ($n = 171$, follow-up rate 99.4%). Falls occurred in 22 patients. Only a higher age was associated with falls within 1 year on univariate analysis (non-fallers: 76.4 ± 6.8 years, fallers: 81.0 ± 6.9 years, $P = 0.004$).

After another year (2 years after enrolment), one patient had died of lung cancer, and five patients were lost to follow up. A total of 165 patients were evaluated (follow-up rate 95.9%), and 10 patients had fallen during the second year; thus a total of 32 patients had fallen within 2 years. As shown in Table 2, higher age, osteoporosis, number of comorbid conditions and number of drugs were significant factors associated with falls. To determine the association of falls with these significant factors, multivariate logistic regression analysis was carried out, and as shown in Table 2, the number of drugs was the only factor that was significantly associated with falls within 2 years.

As polypharmacy was assumed to be a risk for falls within 2 years, the cut-off of the number of the drugs was analyzed. Figure 1 shows the ROC curves to define the optimal cut-off point in relation to falls within

Table 1 Characteristics and univariate analysis of association with fallers and non-fallers within 2 years and risk factors

Total		Non-fallers ($n = 133$)	Fallers ($n = 32$)	P-value (Fallers vs. Non-fallers)
Age (years)	77.0 ± 7.0	76.3 ± 6.9	80.0 ± 6.9	0.007
Body mass index (kg/cm^2)	22.7 ± 3.2	22.7 ± 3.3	22.7 ± 3.1	0.98
No. comorbid conditions	1.9 ± 1.1	1.8 ± 1.1	2.3 ± 0.9	0.009
No. drugs	3.2 ± 2.8	2.8 ± 2.7	4.9 ± 2.5	<0.0001
Female ($n = 122$)	–	72.9%	78.1%	0.66
Hypertension ($n = 106$)	–	62.4%	71.8%	0.41
Dyslipidemia ($n = 76$)	–	47.3%	40.6%	0.56
Diabetes ($n = 23$)	–	12.8%	18.8%	0.40
Osteoporosis ($n = 59$)	–	30.8%	56.3%	0.01
History of stroke ($n = 6$)	–	2.3%	9.4%	0.09
History of myocardial infarction ($n = 3$)	–	0.8%	6.3%	0.10
History of cancer ($n = 8$)	–	5.3%	3.1%	0.99
Calcium channel blocker ($n = 59$)	–	33.3%	46.9%	0.16
Angiotensin II receptor blocker ($n = 56$)	–	33.3%	37.5%	0.68
Statin ($n = 40$)	–	23.5%	28.1%	0.65
Aspirin ($n = 31$)	–	19.0%	24.1%	0.61
Bisphosphonate ($n = 9$)	–	4.6%	9.4%	0.38
H2-blocker ($n = 9$)	–	3.8%	12.1%	0.80
Proton pump inhibitor ($n = 11$)	–	5.3%	12.1%	0.23
Hypnotic ($n = 31$)	–	16.7%	28.1%	0.14

Values are expressed as mean ± SD ($n = 165$).

Table 2 Logistic regression analysis of association of falls within 2 years with age, sex, other significant factors found in univariate analysis, and polypharmacy

	Unadjusted odds ratio (95% CI)	Adjusted odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Age (/1 year)	1.08 (1.03–1.13)†	1.06 (0.99–1.13)	1.06 (0.99–1.13)
Sex (male = 0, female = 1)	1.39 (0.56–3.48)	0.98 (0.29–3.23)	0.75 (0.23–2.38)
Osteoporosis ($n = 0$, Y = 1)	3.12 (1.43–6.84)†	2.76 (0.92–7.38)	3.02 (0.96–6.15)
No. comorbid conditions (/disease)	1.63 (1.14–2.32)*	0.90 (0.55–1.47)	0.99 (0.62–1.56)
No. drugs (/drug)	1.29 (1.12–1.48)‡	1.30 (1.08–1.57)*	–
Five or more drugs ($n = 0$, Y = 1)	5.04 (2.25–11.3)‡	–	4.50 (1.66–12.2)†

*$P < 0.05$, †$P < 0.005$, ‡$P < 0.0005$. CI, confidence interval.

2 years: the area under the ROC was 0.731, and the optimal cut-off value of the number of drugs was five (sensitivity 0.576, specificity 0.788). Logistic regression analysis showed that taking five or more drugs was significantly associated with an increased risk of falls (odds ratio 4.5, 95% CI 1.7–12.2) after adjustment for age, sex, osteoporosis and number of comorbid conditions (Table 2).

Also, the association between falls and two indices of fall tendency was evaluated to confirm the validity of each index in geriatric outpatients. As both indices included the questionnaire asking whether patients were "taking five or more drugs," the number of drugs was excluded from this analysis because of duplication in the statistical model. As shown in Table 3, the 22 items fall risk index showed a tendency towards an association with falls within 2 years, odds ratio 1.12 (95% CI 1.00–1.26; $P = 0.05$), whereas the 13 points screening test was significantly associated with falls after adjustment for age, sex and other factors significantly associated in the univariate analysis. Therefore, these indices are considered to be good predictors of falls in geriatric outpatients, as has been shown in community-dwelling elderly subjects.

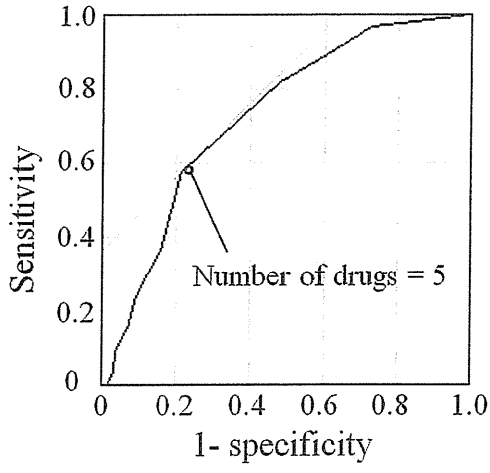

Figure 1 Receiver–operating curves to define optimal cut-off value of number of drugs at baseline in relation to falls within 2 years. Area under the curve was 0.731, optimal cut-off value of the number of drugs was five (sensitivity = 57.6%, specificity = 78.8%).

Discussion

The risk of falls has been assessed in community-dwelling elderly, and history of falls, physical ability and living environment were found to be predictors of falls. Also, in nursing home residents, cognitive function, gait disturbance and urinary incontinence are reported to be risk factors for falls,[9,10] and length of stay, disease condition, surgical procedures and some specific drugs are reported to be risk factors in hospital inpatients.[11,12]

Nevertheless, the risks in geriatric outpatients have not been sufficiently assessed, although assessment of fall risk in geriatric outpatients is important; their medical conditions or drugs might cause falls, and drugs, such as antiplatelet agents or anticoagulants, might cause critical bleeding after a fall. Also, physicians could prevent falls in their patients by giving advice during regular consultations, if risk factors are identified.

In our previous cross-sectional study assessing geriatric outpatients, polypharmacy was significantly correlated with indices of fall tendency, and the present follow-up study of geriatric outpatients showed the impact of polypharmacy on falls within 2 years. Statistical analyses showed that polypharmacy was a risk factor for falls, independent of age, sex and comorbidity.

Besides polypharmacy, several medications and comorbid conditions have been reported as risks for falls.[13–22] Among these, diabetes,[5,6] insomnia,[13] hypnotics,[13–15] antiarrhythmics[22] and antihypertensive agents[14] were not significantly associated with fall risk in the present study. Just 11 patients (45.9% of diabetic patients) were prescribed hypoglycemic agents, such as a sulfonylurea ($n = 8$) or insulin ($n = 3$), and the relatively low rate of prescription of hypoglycemic agents might have affected our result. Neither hypnotics nor antihypertensives were associated with falls. This result might be a result of the small sample size. Anti-arrhythmics were taken by just three patients (digoxin: $n = 2$, class IA anti-arrhythmic drug: $n = 1$). Other drugs, such as major tranquillizers,[14] antidepressants[17,18] and antiparkinsonian agents,[19,22] might increase fall risk; however, no patient used these drugs in the present study. In the present study, most of the patients were in a stable condition throughout the 2 years, though their drugs were changed gradually according to their medical conditions during the observation period. We only used the number of drugs at baseline for statistical analysis; however, the number of drugs increased from 3.2 ± 2.8 to 3.9 ± 3.0 during the 2 years. There were 17 patients whose number of drugs had been decreased, 70 patients not changed and 78 patients increased. The number of drugs after 2 years was also associated with falls ($P < 0.0005$). The optimal cut-off point for the number of drugs was again five (area under ROC curve 0.780, sensitivity 0.576, specificity 0.788). Furthermore, the changes in number of drugs were also associated with falls ($P < 0.05$), and the optimal cut-off point for the change in number of drugs was +1 (area under ROC curve 0.649, sensitivity 0.727, specificity 0.409).

Table 3 Logistic regression analysis of association between 2-year fall occurrences with two indices of fall tendency; 22 items fall risk index and 13 points simple screening test

	Unadjusted odds ratio (95% CI)	Adjusted odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Age (/year)	1.08 (1.03–1.15)**	1.06 (0.99–1.13)	1.06 (1.00–1.13)
Sex (male = 0, female = 1)	1.39 (0.56–3.48)	0.75 (0.23–2.43)	0.79 (0.24–2.56)
Osteoporosis ($n = 0$, Y = 1)	3.12 (1.43–6.84)**	2.56 (0.96–6.82)	2.61 (0.98–6.95)
No. comorbid conditions (/disease)	1.63 (1.14–2.32)*	1.24 (0.83–1.86)	1.32 (0.88–1.97)
Fall risk index (/item)	1.23 (1.11–1.37)***	1.12 (1.00–1.26)	–
Simple screening test (/point)	1.19 (1.06–1.33)**	–	1.14 (1.01–1.29)*

*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$. CI, confidence interval.

Consequently, polypharmacy, especially taking five or more drugs, should be considered a risk for falls.

There were several limitations of the present study. First, the falls were self-reported by the patients. Although all the patients had no overt dementia, they might have forgotten the incident of falling. We attempted to count the total fall occurrences in each patient; however, we could not differentiate the repeated falls in the second year from the fall occurrence in the first year. In fact, we asked 22 patients who reported falls in the first year about fall occurrence during the second year, but they did not accurately recall whether they experienced falls in the first or second year. Second, five patients were lost to follow up at 2 years for unknown reasons. The follow-up ratio was acceptable, although some of the patients might have fallen, have been no longer able to come to the clinic and moved to nursing homes. This might have slightly influenced the result. Also, the cause of falls in polypharmacy patients is not explained. Potentially inappropriate medications, which could cause adverse drug reactions, are usually seen in patients with polypharmacy, and falls might be the consequence of adverse drug reactions, such as dizziness, instability and light-headedness. Pathophysiological assessments and drug-reducing interventions are expected to elucidate the causal relationship.

Additionally, we showed that the 22-item fall risk index and its simple screening test were useful to predict falls in geriatric outpatients. Although both indices have been validated in community-dwelling elderly people, the present finding also showed their association with fall risk among geriatric outpatients. The difference of statistical significance between fall risk index and simple screening test might be a result of small sample size or the difference in the contribution of each item to total scores between the two indices. "Taking five or more drugs" accounts for only one item out of the 22-item fall risk index; in contrast, the same questionnaire accounts two points in the 13-point simple screening test. Because polypharmacy was a strong risk factor of falls in elderly outpatients in the present study, the proportion of polypharmacy in the scores might have caused the discrepancy. Taken together, it is likely that 13-point screening test was more suitable to our subjects who were taking several medicines.

In summary, the present study showed that geriatric outpatients with polypharmacy were at a high risk of falls, especially those receiving five or more drugs. Our finding might add new information for pharmacotherapy and geriatric research in elderly patients with chronic diseases. Intervention studies examining the effect of drug reduction for the prevention of falls are required in the future.

Acknowledgment

We thank Ms Fumie Tanaka for her excellent technical assistance. This study was financially supported by grants from the Ministry of Health, Labour and Welfare of Japan (H21-Chouju-Ippan-005, H22-Chouju-Shitei-009).

Disclosure statement

The authors declare no conflict of interest.

References

1 Stel VS, Pluijm SM, Deeg DJ, Smit JH, Bouter LM, Lips P. A classification tree for predicting recurrent falling in community- dwelling older persons. *J Am Geriatr Soc* 2003; **51**: 1356–1364.
2 Kojima S, Furuna T, Ikeda N, Nakamura M, Sawada Y. Falls among community-dwelling elderly people of Hokkaido, Japan. *Geriatr Gerontol Int* 2008; **8**: 272–277.
3 Okochi J, Toba T, Takahashi T *et al.* Simple screening test for risk of falls in the elderly. *Geriatr Gerontol Int* 2006; **6**: 223–227.
4 Leipzig RM, Cumming RG, Tinetti ME. Drugs and falls in older people: a systematic review and meta-analysis: II. Cardiac and analgesic drugs. *J Am Geriatr Soc* 1999; **47**: 40–50.
5 Berlie HD, Garwood CL. Diabetes medications related to an increased risk of falls and fall-related morbidity in the elderly. *Ann Pharmacother* 2010; **44**: 712–717.
6 Araki A, Ito H. Diabetes mellitus and geriatric syndromes. *Geriatr Gerontol Int* 2009; **9**: 105–114.
7 Kojima T, Akishita M, Nakamura T *et al.* Association of polypharmacy with fall risk among geriatric outpatients. *Geriatr Gerontol Int* 2011; **11**: 438–444.
8 Toba K, Okochi J, Takahashi T *et al.* Development of a portable fall risk index for elderly people living in the community. *Nippon Ronen Igakkai Zasshi* 2005; **42**: 346–352. (In Japanese.)
9 Kron M, Loy S, Sturm E *et al.* Risk indicators for falls in institutionalized frail elderly. *Am J Epidemiol* 2003; **158**: 645–653.
10 van Doorn C, Gruber-Baldini AL, Zimmerman S *et al.* Dementia as a risk factor for falls and fall injuries among nursing home residents. *J Am Geriatr Soc* 2003; **51**: 1213–1218.
11 Halfon P, Eggli Y, Van Melle G, Vagnair A. Risk of falls for hospitalized patients: a predictive model based on routinely available data. *J Clin Epidemiol* 2001; **54**: 1258–1266.
12 Tanaka M, Suemaru K, Ikegawa Y *et al.* Relationship between the risk of falling and drugs in an academic hospital. *Yakugaku Zasshi* 2008; **128**: 1355–1361.
13 Ensrud KE, Blackwell TL, Redline S *et al.* Sleep disturbances and frailty status in older community-dwelling men. *J Am Geriatr Soc* 2009; **57**: 2085–2093.
14 Leipzig RM, Cumming RG, Tinetti ME. Drugs and falls in older people: a systematic review and meta-analysis: I. Psychotropic drugs. *J Am Geriatr Soc* 1999; **47**: 30–39.
15 Woolcott JC, Richardson KJ, Wiens MO *et al.* Meta-analysis of the impact of 9 medication classes on falls in elderly persons. *Arch Intern Med* 2009; **169**: 1952–1960.

16 Tinetti ME, Speechley M, Ginter SF. Risk factors for falls among elderly persons living in the community. *N Engl J Med* 1988; **319**: 1701–1707.
17 Kelly KD, Pickett W, Yiannakoulias N *et al.* Medication use and falls in community-dwelling older persons. *Age Ageing* 2003; **32**: 503–509.
18 Thapa PB, Gideon P, Cost TW, Milam AB, Ray WA. Antidepressants and the risk of falls among nursing home residents. *N Engl J Med* 1998; **339**: 875–882.
19 Bloem BR, Steijns JA, Smits-Engelsman BC. An update on falls. *Curr Opin Neurol* 2003; **16**: 15–26.
20 Arai H, Akishita M, Teramoto S *et al.* Incidence of adverse drug reactions in geriatric units of university hospitals. *Geriatr Gerontol Int* 2005; **5**: 293–297.
21 Iwata M, Kuzuya M, Kitagawa Y, Suzuki Y, Iguchi A. Underappreciated predictors for postdischarge mortality in acute hospitalized oldest-old patients. *Gerontology* 2006; **52**: 92–98.
22 Baranzini F, Diurni M, Ceccon F *et al.* Fall-related injuries in a nursing home setting: is polypharmacy a risk factor? *BMC Health Serv Res* 2009; **9**: 228.

OPEN ACCESS Freely available online

PLoS one

Testosterone Deficiency Accelerates Neuronal and Vascular Aging of SAMP8 Mice: Protective Role of eNOS and SIRT1

Hidetaka Ota[1], Masahiro Akishita[1]*, Takuyu Akiyoshi[1], Tomoaki Kahyo[2], Mitsutoshi Setou[2], Sumito Ogawa[1], Katsuya Iijima[1], Masato Eto[1], Yasuyoshi Ouchi[1]

1 Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo, Japan, **2** Hamamatsu University School of Medicine, Department of Molecular Anatomy, Hamamatsu, Shizuoka, Japan

Abstract

Oxidative stress and atherosclerosis-related vascular disorders are risk factors for cognitive decline with aging. In a small clinical study in men, testosterone improved cognitive function; however, it is unknown how testosterone ameliorates the pathogenesis of cognitive decline with aging. Here, we investigated whether the cognitive decline in senescence-accelerated mouse prone 8 (SAMP8), which exhibits cognitive impairment and hypogonadism, could be reversed by testosterone, and the mechanism by which testosterone inhibits cognitive decline. We found that treatment with testosterone ameliorated cognitive function and inhibited senescence of hippocampal vascular endothelial cells of SAMP8. Notably, SAMP8 showed enhancement of oxidative stress in the hippocampus. We observed that an NAD^+-dependent deacetylase, SIRT1, played an important role in the protective effect of testosterone against oxidative stress-induced endothelial senescence. Testosterone increased eNOS activity and subsequently induced SIRT1 expression. SIRT1 inhibited endothelial senescence via up-regulation of eNOS. Finally, we showed, using co-culture system, that senescent endothelial cells promoted neuronal senescence through humoral factors. Our results suggest a critical role of testosterone and SIRT1 in the prevention of vascular and neuronal aging.

Citation: Ota H, Akishita M, Akiyoshi T, Kahyo T, Setou M, et al. (2012) Testosterone Deficiency Accelerates Neuronal and Vascular Aging of SAMP8 Mice: Protective Role of eNOS and SIRT1. PLoS ONE 7(1): e29598. doi:10.1371/journal.pone.0029598

Editor: Gian Paolo Fadini, University of Padova, Medical School, Italy

Received August 10, 2011; **Accepted** December 1, 2011; **Published** January 4, 2012

Funding: This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan (20249041, 21390220, 21790621). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: akishita-tky@umin.ac.jp

Introduction

Advancing age is the most significant risk factor for the development of cognitive impairment [1,2]; however, what age-related changes underlie this effect remains uncertain. With advancing age, men experience a significant decrease in the circulating level of testosterone. Although studies have shown alterations in mood, libido, and cognition resulting from testosterone deficiency [3], the full range of consequences of age-related testosterone loss remains incompletely defined. In a small clinical study of men recently diagnosed with cognitive impairment, testosterone treatment improved performance on cognitive tests [4]. In a prospective longitudinal study using subjects from the Baltimore Longitudinal Study on Aging, men who developed Alzheimer disease (AD) were observed to exhibit low testosterone levels 5–10 years prior to the clinical diagnosis of AD [5]. With a relationship between age-related testosterone decline in men and increased risk for cognitive impairment reasonably well established, a critical issue is how testosterone contributes to the pathogenesis of cognitive decline with aging. The most likely hypothesis is through the regulation of accumulation of amyloid ß (Aß) peptides, which are widely believed to be the critical initiating step in the pathogenesis of AD. However, it is becoming increasingly clear that not all aspects of cognitive decline can be explained by Aß [6,7]. Findings from such diverse lines of investigations as neuroimaging and clinical trials suggest that non-Aß factors also contribute to memory deficit in aged men.

In *S. cerevisiae*, the *Sir2* (silent information regulator-2) family of genes governs budding exhaustion and replicative life span [8,9]. *Sir2* has been identified as an NAD^+-dependent histone deacetylase and is responsible for maintenance of chromatin silencing and genome stability. Mammalian sirtuin 1 (*Sirt1*), the closest homolog of *Sir2*, regulates the cell cycle, senescence, apoptosis and metabolism, by interacting with a number of molecules such as p53. As recently reported, overexpression of SIRT1 in the brain improved the memory deficit in a mouse model of AD via activation of the transcription of α-secretase [10].

An increasing body of evidence suggests the presence of a link between cognitive decline and vascular dysfunction, especially atherosclerosis [11]. Senescence of endothelial cells is involved in endothelial dysfunction and atherogenesis, and SIRT1 has been recognized as a key regulator of vascular endothelial homeostasis, controlling angiogenesis, endothelial senescence, and dysfunction [12–14].

In the present study, we demonstrated that cognitive impairment in senescence-accelerated mouse prone 8 (SAMP8), a model of cognitive decline with aging, is associated with endothelial senescence in the hippocampus and is ameliorated by testosterone

replacement. SIRT1 plays an important role in prevention of endothelial senescence induced by oxidative stress [13]. We suggest that the protection against endothelial senescence in the hippocampus through up-regulation of testosterone and SIRT1 could contribute to a novel therapeutic strategy against cognitive decline with aging.

Results

Treatment with dihydrotestosterone ameliorated cognitive function of SAMP8

In order to assess the effects of testosterone on cognitive function, we used an in vivo model of aging, SAMP8, and a control counterpart strain, SAMR1. SAMP8 was originally derived from AKR/J strain, litters of which show the characteristic of cognitive decline with aging. These mice exhibit age-related deficits in learning and memory at an early age, and are considered a suitable animal model to study aging and memory deficit. Body weight, appearance, and plasma testosterone level of SAMR1 and SAMP8 at 12 weeks of age were determined. Body weight and appearance did not differ between SAMR1 and SAMP8, but plasma testosterone level in SAMP8 was lower than that in SAMR1 (Figure 1A). By determining the time required to find the platform (escape latency) as a function of days of training in the Morris water maze, we observed a marked decline in performance in SAMP8 compared with SAMR1 (Figure 1B). Because testosterone acts in part through aromatase-dependent conversion to estradiol, non-aromatizable dihydrotestosterone (DHT) was used to examine a direct role of androgens through androgen receptor (AR). SAMP8 treated with DHT showed significantly reduced escape latency time compared with untreated SAMP8. There was no difference in swim speed between the groups; however, % time in the quadrant was increased in DHT-treated SAMP8 (Figure 1B). These results indicate that DHT treatment ameliorated cognitive dysfunction in SAMP8. The water-maze is appropriate for hippocampal-dependent paradigms. However, DHT administration may affect behavior and how animals respond to different stimuli. Therefore, we performed an open field test to examine locomotion, exploratory behavior, and anxiety. No significant effect of DHT on locomotor performance was observed in SAMR1 and SAMP8, whereas SAMR1 moved significantly more compared with SAMP8 (Figure 1C). The ratio of the distance travelled in the central area to that in the total area in the open- field, an indirect measure of exploratory behavior and anxiety [15], was also observed. In SAMP8, DHT increased this ratio (Figure 1C), suggesting that DHT promoted exploratory behavior and diminished anxiety.

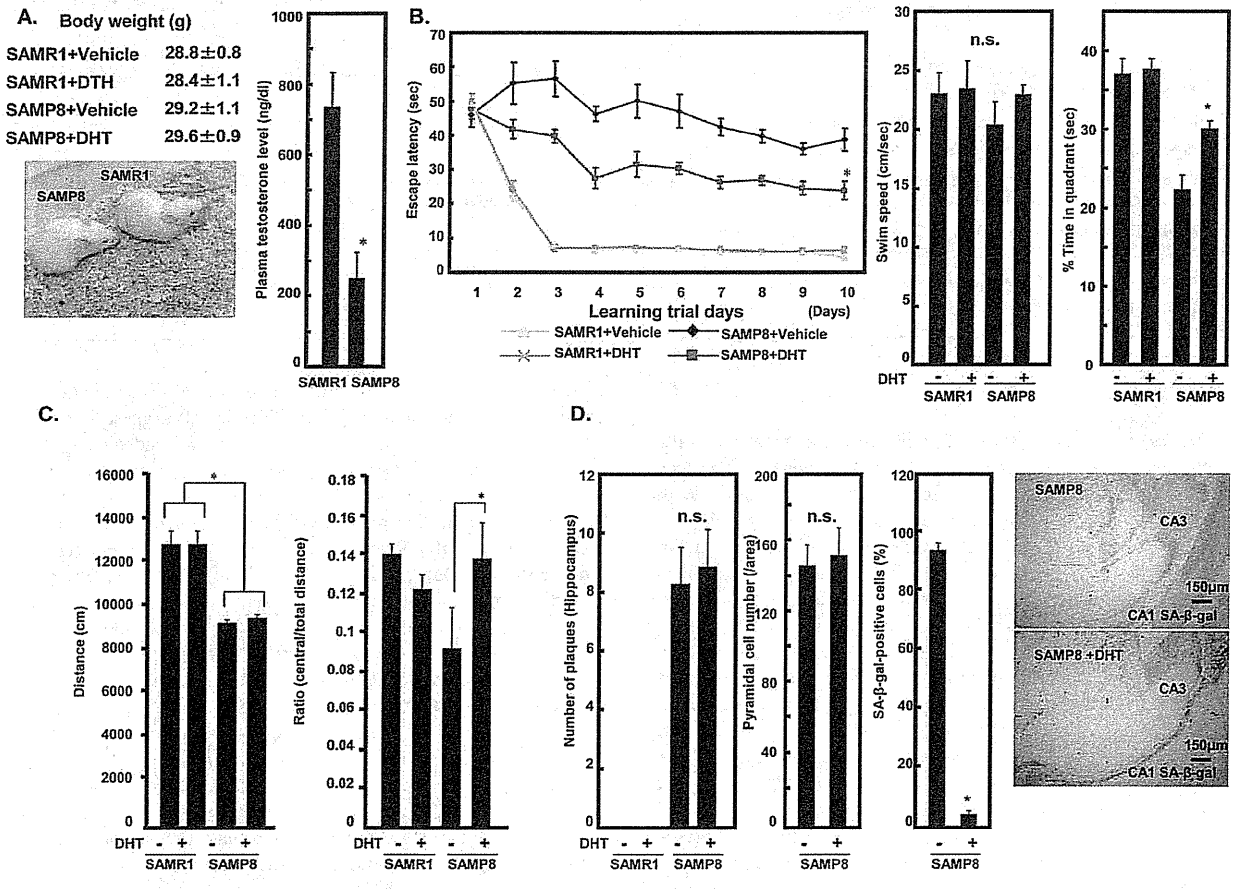

Figure 1. Testosterone deficiency causes senescence of hippocampus and cognitive impairment in SAMP8 mice. A. Body weight, appearance, and plasma testosterone level of male SAMR1 and SAMP8 mice at 12 weeks of age. **B.** Escape latency of SAMR1 (N = 10) and SAMP8 mice (N = 10). Male mice were treated daily for 2 weeks with DHT (500 μg s.c) before trials. Swim speed during quadrant test on day 10. **C.** Total distance and the ratio of central/total distance were measured in open field tests. **D.** Number of amyloid ß plaques, pyramidal cells, and SA-ßgal-positive cells in CA1 and CA3 areas of hippocampus in SAMR1 and SAMP8. (*$p<0.05$, n.s: not significant).
doi:10.1371/journal.pone.0029598.g001

Next, we assessed the number of amyloid ß plaques, pyramidal cells, and SA-ßgal-positive cells in CA1 and CA3 areas of the hippocampus in these mice (Figure 1D). The number of plaques was increased in SAMP8 compared with SAMR1, but was unaltered by treatment with DHT. The number of SA-βgal-stained cells was significantly increased in SAMP8 compared with SAMR1, but treatment with DHT prevented this in SAMP8 despite no difference in pyramidal cell number (Figure 1D).

DHT treatment increased protein and mRNA expression of SIRT1 in SAMP8

Furthermore, to estimate the role of testosterone deficiency in SAMP8, we examined the effect of testosterone supplementation on cognitive function in much older SAMR1 and SAMP8. Similarly to young mice, we observed a marked decline in performance in SAMP8 compared with SAMR1 at 18 months of age. SAMP8 implanted with testosterone pellets showed significantly reduced escape latency time compared with placebo-treated SAMP8 (Figure 2A). Plasma testosterone level in SAMP8 at 18 months of age was lower than that in SAMR1, but implanted mice showed recovery to the level in young mice (Figure 2A). These results indicated that similar to DHT, testosterone also showed the improvement of cognitive function in SAMP8. Next, we examined the cause of low plasma testosterone in SAMP8. SAMP8 showed no testicular atrophy (Figure S1A), but more senescent phenotypes in Leydig cells, which produce testosterone in testes, than SAMR1 (Figure 2B). Moreover, we tried to allotransplant testes from SAMR1 to SAMP8 (Figure S1B). Although performance gradually responded to treatment up to 8–10 weeks, castrated SAMR1 showed a marked decline in performance whereas recipient SAMP8 showed cognitive improvement (Figure 2C).

As recently reported, overexpression or activation of SIRT1 inhibits cellular senescence and protects cellular function in various cell lines [13,16]. Therefore, we examined SIRT1 expression in the hippocampus of SAMP8 with or without DHT treatment, at 12 weeks of age. DHT treatment increased the protein and mRNA expression of SIRT1 in SAMP8 (Figure 2D). To investigate further the involvement of AR, we examined the expression of AR in SAMR1 and SAMP8 brains. The expression of AR was more abundant in the hippocampus than in other brain regions of SAMR1 and SAMP8 (Figure 2E).

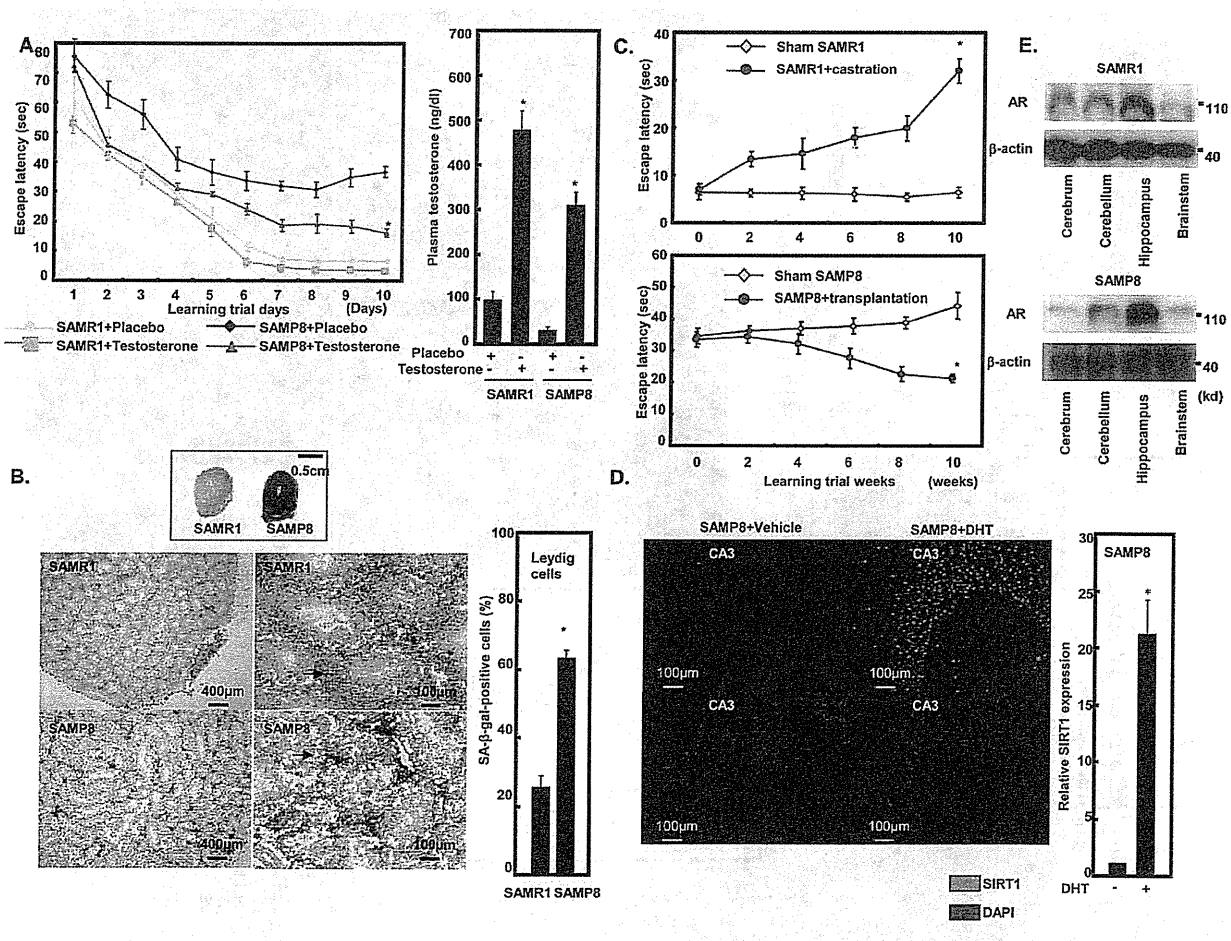

Figure 2. Supplementation of testosterone improves cognitive function in SAMP8 mice. A. Escape latency and plasma testosterone level of male SAMR1 (N = 10) and SAMP8 mice (N = 10) at 18 months of age. These mice were implanted subcutaneously with a placebo or a 21-day-release 2.5 mg testosterone pellet in the dorsal neck. **B.** Number of SA-βgal-stained Leydig cells in testes in SAMR1 and SAMP8. Arrows indicate Leydig cells. Representative SA-βgal-stained testes from SAMR1 and SAMP8. **C.** Escape latency of castrated SAMR1 (upper, N = 5) and recipient SAMP8 (lower, N = 5). Observation (0–10 weeks) was started from 3 weeks after operation. **D.** SIRT1 expression in hippocampus of SAMP8 with or without DHT treatment. Immunofluorescent staining for SIRT1 (green) and DAPI (blue). **E.** Expression of AR in SAMR1 and SAMP8 brains. (*p<0.05).
doi:10.1371/journal.pone.0029598.g002

Oxidative stress was increased in hippocampal cells of SAMP8

Oxidative stress may be closely related to senescence and age-related diseases. Also, an increase in oxidative stress has been suggested to be one of the earliest pathological changes in the brain in conditions with cognitive impairment such as AD [17]. Then, we examined the level of oxidative stress, using the SAMR1 and SAMP8 hippocampus at 12 weeks of age. SAMP8 hippocampus showed an increase in the level of oxidative stress compared with SAMR1 as judged by detection of carbonylated proteins. DHT treatment decreased carbonylated proteins in the SAMP8 hippocampus (Figure 3A). In parallel, the concentration of the neurotransmitter acetylcholine in hippocampal lysates was decreased in SAMP8 compared with that in SAMR1, and DHT treatment prevented this (Figure 3B).

Testosterone and DHT acts on vascular endothelial cells and stimulates the PI3K/Akt pathway, leading to eNOS activation through direct interaction of AR [18,19]. The eNOS/SIRT1 axis is recognized as one of the fundamental determinants of endothelial senescence, and SIRT1 acts as a driver of cellular stress resistance [20]. To examine the influence of DHT treatment on endothelial cells, we determined the degree of senescence and the expression of SIRT1 in endothelial cells around the CA3 area of the hippocampus. DHT-treated SAMP8 showed a reduction of SA-βgal-stained endothelial cells and increased SIRT1 expression compared to untreated SAMP8 (Figure 3C and D). To confirm that these cells were endothelial cells, not neuronal cells, cerebral microvessels were isolated from SAMR1 and SAMP8. In parallel with immunohistological staining, SAMP8 showed a reduction of SIRT1 expression compared to SAMR1, and DTH treatment increased SIRT1 expression compared to that in untreated SAMP8 (Figure 3E). These results suggest that vascular endothelial senescence in the hippocampus may be related to the memory deficit in SAMP8. Since testosterone and DHT activates eNOS, a NOS inhibitor, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), and N^5-(1-Imino-3-butenyl)-L-ornithine (L-VNIO), a

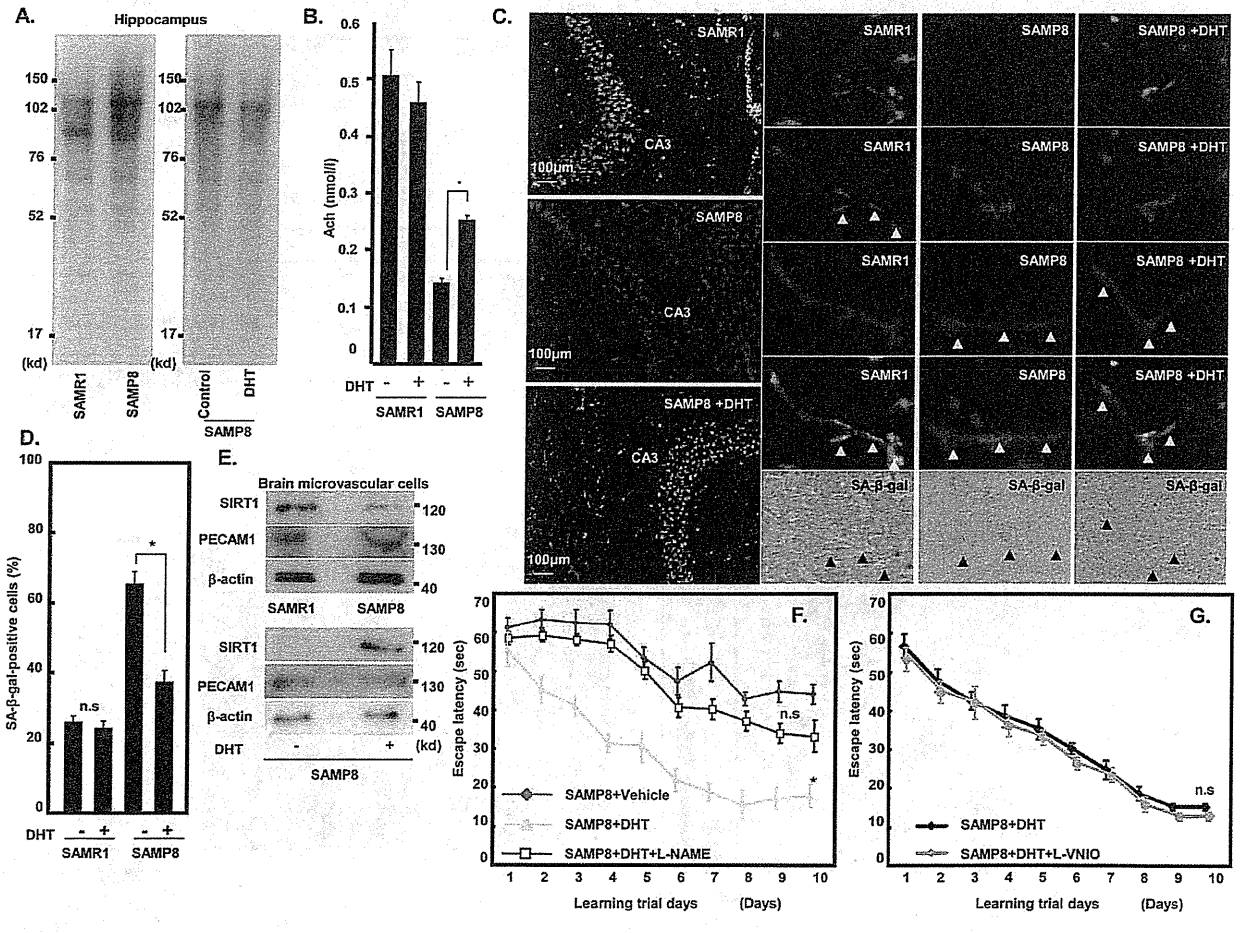

Figure 3. Senescent endothelial cells of hippocampus are decreased by treatment with DHT. A. Oxidative stress level was measured by detection of carbonyl groups introduced into proteins. **B.** Acetyl-choline concentration was measured by a colorimetric method. **C.** SA-βgal-stained endothelial cells and SIRT1 expression in CA3 area of hippocampus in SAMR1 and SAMP8 with or without DHT treatment. Immunofluorescent staining for SIRT1 (green), PECAM-1 (red), and DAPI (blue). **D.** Number of SA-βgal-stained endothelial cells in CA3 area of hippocampus in SAMR1 and SAMP8 with or without DHT treatment. **E.** Expression of SIRT1, PECAM-1, and β-actin was analyzed using cerebral micro vascular cells. **F.** Escape latency of SAMR1 (N = 10) and SAMP8 mice (N = 10). Male mice were treated daily for 2 weeks with DHT (500 μg s.c) and L-NAME (20 mg/kg gavage) before trials. **G.** Escape latency of SAMR1 (N = 5) and SAMP8 mice (N = 5). Male mice were treated daily for 2 weeks with DHT (500 μg s.c) and L-VNIO (5 mg/kg IP) before trials. (*$p<0.05$, n.s: not significant).
doi:10.1371/journal.pone.0029598.g003

selective neuronal NOS (nNOS) inhibitor, were applied to examine the involvement of NOS in this process. L-NAME abrogated the effects of DHT on cognitive function (Figure 3F). In contrast, L-VNIO did not change the effect of DHT (Figure 3G). These results suggest that eNOS/SIRT1 in endothelial cells may play an important role in the protective effect of testosterone against senescence of the hippocampus.

SIRT1 plays an important role in the protective effect of testosterone against endothelial senescence

Following the animal experiments, we examined whether testosterone inhibited endothelial senescence *in vitro* using cultured cells. We induced premature endothelial senescence by addition of H_2O_2 100 μmol/L for 1 hour. DHT or testosterone treatment inhibited SA-βgal activity and the morphological appearance of senescence (Figure 4A). We observed that oxidative stress decreased eNOS and SIRT1 and increased PAI-1 expression, and DHT or testosterone treatment prevented these changes and increased the phosphorylation of eNOS at Ser1177 (Figure 4B). Overexpression of SIRT1 significantly inhibited oxidative stress-induced senescence, and DHT accelerated the effect of SIRT1 through phosphorylation of eNOS at Ser1177 (Figure 4C). To determine the role of endogenous SIRT1, DHT-treated endothelial cells were transfected with SIRT1 siRNA or treated with sirtinol, a chemical inhibitor of SIRT1. SIRT1 siRNA or sirtinol abrogated the effect of DHT on SA-βgal activity (Figure 4D). We previously reported that testosterone activated eNOS [18], and eNOS activation promoted SIRT1 expression [21]. Accordingly, we examined the role of eNOS in the protective effect of testosterone. We observed that DHT or testosterone treatment increased NOS activity that was reduced by oxidative stress (Figure 4E). Treatment with eNOS siRNA or L-NAME decreased the inhibitory effect of DHT on a senescent phenotype in parallel with SIRT1 expression (Figure 4F and G). These results indicate that eNOS/SIRT1 play an important role in the protective effect of testosterone and DHT against a senescent phenotype.

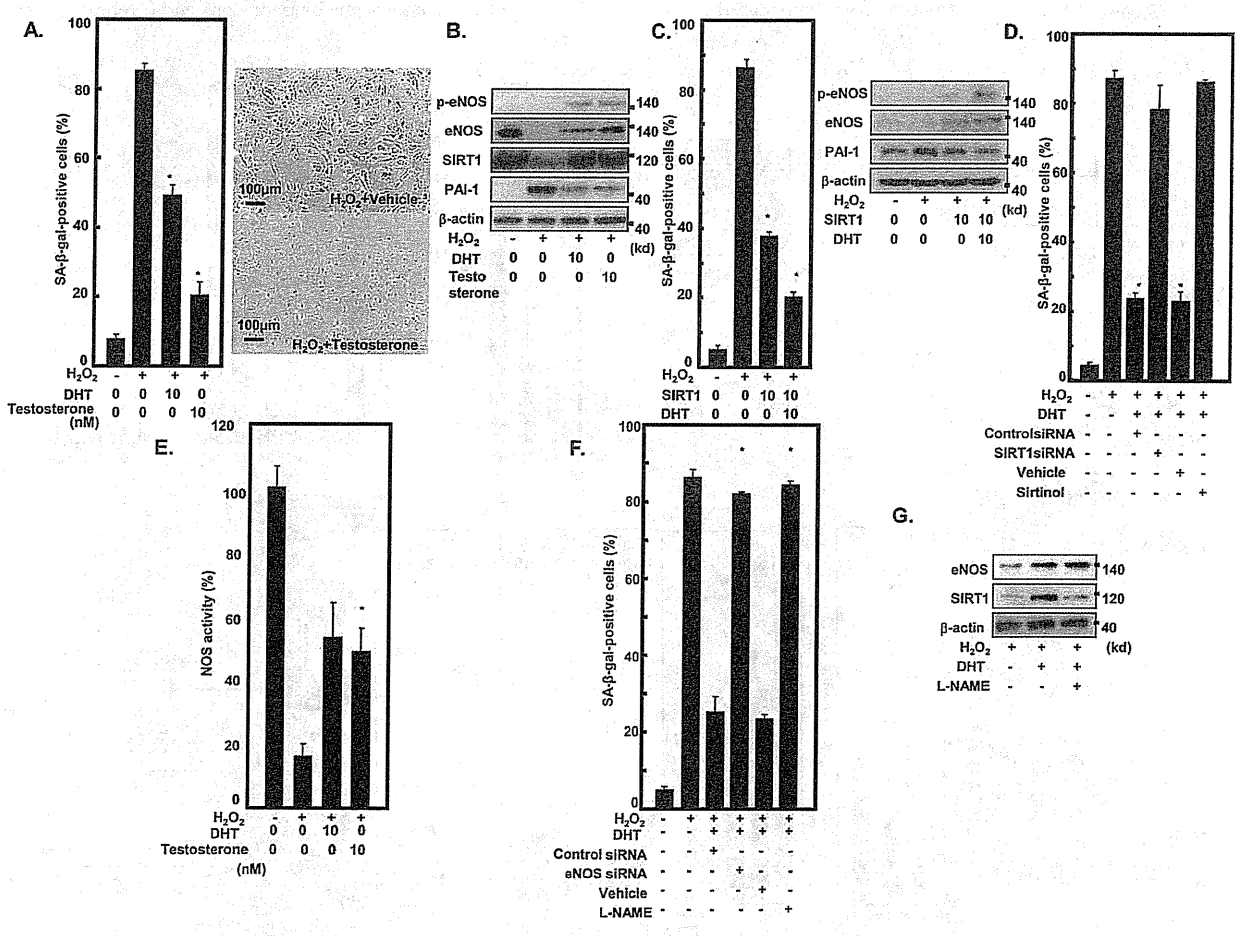

Figure 4. Testosterone inhibits oxidative stress-induced endothelial senescence through eNOS/SIRT1. A. Testosterone inhibited SA-βgal activity and senescent morphological appearance induced by hydrogen peroxide (100 μmol/L). **B.** Expression of eNOS, SIRT1, and PAI-1 in hydrogen peroxide (100 μmol/L)-treated HUVEC under treatment with DHT or testosterone. **C.** Overexpression of SIRT1 and DHT reduced SA-βgal activity. eNOS expression was increased by overexpression of SIRT1, and DHT increased phosphorylation of eNOS (Ser1177). **D.** SIRT1 inhibition by siRNA or sirtinol (100 μmol/L) abrogated the effect of testosterone on SA-βgal activity. **E.** Treatment with testosterone or DHT increased eNOS activity. **F.** eNOS inhibition by siRNA or L-NAME (10 mM) abrogated the effect of testosterone on SA-βgal activity. **G.** Treatment with L-NAME decreased SIRT1 expression in DHT-treated HUVEC. (*$p<0.05$, $N=3$).
doi:10.1371/journal.pone.0029598.g004

Senescent endothelial cells induced by oxidative stress promoted neuronal senescence

Finally, we hypothesized that endothelial senescence promotes senescence of adjacent neuronal cells. To test this hypothesis, we used a co-culture system of endothelial cells (HUVEC) with neuronal cells (mouse hippocampal neuronal cells; MHC) (Figure 5A). Both cells were co-cultured, but were separated by a microporous polycarbonate membrane, for 10 days after endothelial cells were treated with hydrogen peroxide, and the senescent phenotype of MHC was analyzed. We found that the number of SA-βgal-positive cells and the senescent appearance of MHC were increased, and the concentration of acetylcholine in cells was decreased by co-culture with senescent endothelial cells (Figure 5B). In parallel with this, MHC showed increased PAI-1 and p53, and decreased SIRT1 expression (Figure 5C). We also found that senescent endothelial cells showed increased expression of inflammatory cytokines such as IL-6, IL-8, MCP-1, and TNF-α (Figure 5D). Both MHC and HUVEC, or HUVEC alone were treated with testosterone at 3 days before HUVEC were treated with hydrogen peroxide, and both cells were co-cultured for 10 days, and the senescent phenotype of MHC was analyzed. We found that the number of SA-βgal-positive MHC was decreased by treatment of HUVEC with testosterone irrespective of the treatment of MHC with testosterone (Figure 5E). In addition, we found that a SIRT1 activator, resveratrol treatment rescued the senescent phenotype of MHC (Figure 5F). These results suggest that senescent endothelial cells exhibit a senescence-associated secretory phenotype [22], induce neuronal senescence, and testosterone rescues it through up-regulation of SIRT1 (Figure 5G).

Discussion

Testosterone level and cognitive function show a decline with age in men. A series of evidence suggests that this association is not just age related [23]. Results from cell culture and animal studies provide evidence that testosterone could have protective effects on brain function, especially in the hippocampus [24]. Here, we demonstrated that administration of testosterone restored cognitive function in male SAMP8 in association with improvement of the senescent phenotype in the hippocampus and cerebral vessels.

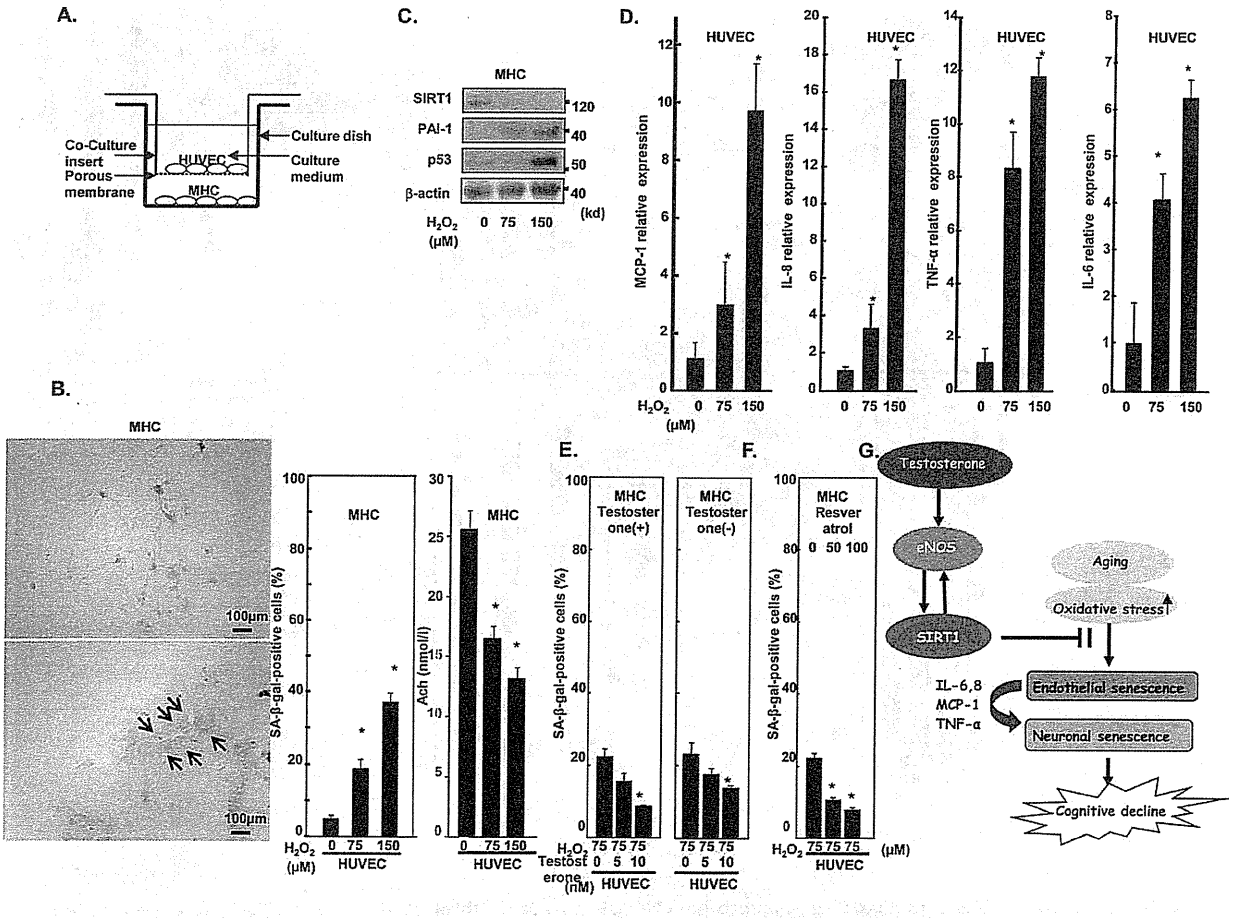

Figure 5. Oxidative stressed-induced endothelial cell senescence promotes adjacent neuronal cell senescence. A. Co-culture cell culture dish. **B.** Number of SA-βgal-stained MHC and senescent appearance of MHC were increased, and acetyl-choline concentration was decreased by co-culture with senescent endothelial cells. Senescent MHC are indicated by arrows. **C.** Expression of SIRT1, PAI-1, p53, and β-actin in MHC co-cultured with senescent endothelial cells. **D.** Expression of IL-6, IL-8, MCP-1, and TNF-α in endothelial cells were analyzed by RT-PCR. **E.** The number of SA-βgal-stained MHC was decreased by treatment with testosterone in both MHC and HUVEC (MHC, testosterone (+)), or HUVEC (MHC, testosterone (−)) alone. **F.** Resveratrol decreased the number of SA-βgal-stained MHC co-cultured with senescent endothelial cells. (*$p<0.05$, N=3). **G.** Hypothetical signal transduction pathways of testosterone in endothelial cells.
doi:10.1371/journal.pone.0029598.g005