drome as well as type 2 diabetes mellitus was more prevalent among women as compared with men. Because their results were not gender-specific, it is not known whether 72Met variant was strongly associated with the above phenotypes in women.

Kuzuya et al. [13] reported a significantly higher frequency of the 72Met allele in the higher BMI (25 and more) group of middle-aged men than in the normal-weight group, suggesting that not only the gender but also the extent of obesity (obese or non-obese) should be considered.

Recently, Zavarella et al. [24] reported on the protective effect of ghrelin in insulin resistance by the 72Met allele in a Caucasian (Italian) population consisting of obese and normal weight subjects; the Met/Met minor homozygotes had lower values of triglycerides, fasting insulin, HOMA-IR, and a higher total ghrelin level. We could not observe such effects in Leu72Met. They also reported that the T/T minor homozygotes of −604C>T had decreasing values of fasting insulin and HOMA-IR. We have not yet analysed −604T>C (rs27647): however, if LD is maintained between −604T>C and −994C>T, our results in the −994 minor homozygotes are not in agreement with theirs. Preproghrelin gene polymorphism may affect insulin metabolism differently according to the race and gender of the subjects or extent of obesity (obese or non-obese).

We are particularly interested in the relationship existing among the SNPs, plasma ghrelin concentration, and susceptibility to diabetes or obesity. Unfortunately, the plasma ghrelin was not measured in the present study. Ando and his colleague [25] reported on the association of higher acylated ghrelin (active form) concentration and body weight, BMI, fat area, waist circumference, skinfold thickness, and a lower HDL cholesterol in Japanese women with the +3056C allele. They stated that the 72Met carriers also had a higher acylated ghrelin but to a lesser extent than the +3056C carriers. Some groups reported a higher ghrelin concentration in the 72Met carriers [24]. These results predict that a higher ghrelin predisposes to obesity in the subjects with the −1500G - −1062C - −994T haplotype as well. Above mentioned Choi et al. [11] used biochemical experiments and found that a transcription factor, myoD, preferentially binds to the region with −1062C, although they described that the region with −1062G had a 1.7-fold higher promoter activity of preproghrelin than that with −1062C.

About the reason why preproghrelin gene SNP may relate to diabetes in obese men, we suppose that higher ghrelin affects serum glucose homeostasis via androgens. Dezaki et al. described that besides effects on appetites and energy homeostasis, ghrelin is also involved in regulating insulin release and glucose metabolism: ghrelin inhibits insulin release [26]. As shown in Table 4, male +3056C/C homozygotes indeed had lower insulin and C-peptide levels on an average, though these were not significant.

Haploview enables the study of LD among SNPs and the estimation of frequencies of the analysed haplotype. Our D' values of the LD are mostly in agreement with those of Ando et al. [12] or of Choi et al. [11]. We observed 2 haplotype blocks in this region: block 1, −1500C>G - −1062G>C - −994C>T and block 2: Leu72Met - +3056T>C. These SNPs shows a strong LD in each haploblocks. This is probably because not only the coding-rich region but also the promoter region also has functional importance.

In conclusion, the +3056C/C minor homozygotes were associated with predisposition to diabetes mellitus in obese men. In obese women, the −1500G - −1062C - −994T haplotype was correlated with visceral adiposity, and the 72Met - +3056C haplotype was associated with susceptibility to obesity via aberrant fat metabolism (reduction of serum cholesterol). Thus, polymorphisms of the preproghrelin gene are suggested to be closely related to diabetes and obesity in obese Japanese. Further studies are required to evaluate these findings and to elucidate the underlying biological mechanisms.

Acknowledgements

This work was supported by grant-in-aid for Scientific Research from the Ministry of Health, Labor and Welfare, Japan. We are thankful to Dr. Masahiro Morioka (Saku Central Hospital) for the kind cooperation.

References

[1] Watanabe S, Morita A, Aiba N, Miyachi M, Sasaki S, Morioka M, et al. Study design of the Saku control obesity program (SCOP). Anti-Aging Med 2007;4(2):70–3.

[2] Morita A, Ohmori Y, Suzuki N, Ide N, Morioka M, Aiba N, et al. Anthropometric and clinical findings in obese people in Saku control obesity program (SCOP). Anti-Aging Med 2008;5(1):13–6.

[3] Yamada K, Takezawa J, Morita A, Matsumura Y, Watanabe S. DNA polymorphism of obese people in Saku Control Obesity Program (SCOP). Anti-Aging Med 2008;4(2):63–9.

[4] Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, et al. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science 2005;310(5750):996–9.

[5] Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature 2000;407(6806):908–13.
[6] Ukkola O, Ravussin E, Jacobson P, Snyder EE, Changnon M, Sjostrom L, et al. Mutations in the preproghrelin/ghrelin gene associated with obesity in humans. J Clin Endocrinol Metab 2001;86(8):3996–9.
[7] Korbonits M, Gueorguiev M, O'Grady E, Lecoeur C, Swan DC, Mein CA, et al. A variation in the ghrelin gene increases weight and decreases insulin secretion in tall, obese children. J Clin Endocrinol Metab 2002;87(8):4005–8.
[8] Hinney A, Hoch A, Geller F, Schafer H, Siegfried W, Goldschmidt H, et al. Ghrelin gene: identification of missense variants and a frameshift mutation in extremely obese children and adolescents and healthy normal weight students. J Clin Endocrinol Metab 2002;87(6):2716.
[9] A haplotype map of the human genome. Nature 2005;437(7063):1299–320.
[10] Qin ZS, Niu T, Liu JS. Partition-ligation-expectation-maximization algorithm for haplotype inference with single-nucleotide polymorphisms. Am J Hum Genet 2002;71(5):1242–7.
[11] Choi HJ, Cho YM, Moon MK, Choi HH, Shin HD, Jang HC, et al. Polymorphisms in the ghrelin gene are associated with serum high-density lipoprotein cholesterol level and not with type 2 diabetes mellitus in Koreans. J Clin Endocrinol Metab 2006;91(11):4657–63.
[12] Ando T, Komaki G, Naruo T, Okabe K, Takii M, Kawai K, et al. Possible role of preproghrelin gene polymorphisms in susceptibility to bulimia nervosa. Am J Med Genet B Neuropsychiatr Genet 2006;141B(8):929–34.
[13] Kuzuya M, Ando F, Iguchi A, Shimokata H. Preproghrelin Leu72Met variant contributes to overweight in middle-aged men of a Japanese large cohort. Int J Obes (Lond) 2006;30(11):1609–14.
[14] Tang NP, Wang LS, Yang L, Gu HJ, Zhu HJ, Zhou B, et al. Preproghrelin Leu72Met polymorphism in Chinese subjects with coronary artery disease and controls. Clin Chim Acta 2008;387(1–2):42–7.
[15] Zou CC, Huang K, Liang L, Zhao ZY. Polymorphisms of the ghrelin/obestatin gene and ghrelin levels in Chinese children with short stature. Clin Endocrinol (Oxf) 2008;69(1):99–104.
[16] Kim SY, Jo DS, Hwang PH, Park JH, Park SK, Yi HK, et al. Preproghrelin Leu72Met polymorphism is not associated with type 2 diabetes mellitus. Metabolism 2006;55(3):366–70.
[17] Puder JJ, Monaco SE, Sen Gupta S, Wang J, Ferin M, Warren MP. Estrogen and exercise may be related to body fat distribution and leptin in young women. Fertil Steril 2006;86(3):694–9.
[18] Morita Y, Iwamoto I, Mizuma N, Kuwahata T, Matso T, Yoshinaga M, et al. Precedence of the shift of body-fat distribution over the change in body composition after menopause. J Obstet Gynaecol Res 2006;32(5):513–6.
[19] Pagotto U, Gambineri A, Pelusi C, Genghini S, Cacciari M, Otto B, et al. Testosterone replacement therapy restores normal ghrelin in hypogonadal men. J Clin Endocrinol Metab 2003;88(9):4139–43.
[20] Greenman Y, Rouach V, Limor R, Gilad S, Stern N. Testosterone is a strong correlate of ghrelin levels in men and postmenopausal women. Neuroendocrinology 2009;89(1):79–85.
[21] Christo K, Cord J, Mendes N, Miller KK, Goldsteint MA, Klibanski A, et al. Acylated ghrelin and leptin in adolescent athletes with amenorrhea, eumenorrheic athletes and controls: a cross-sectional study. Clin Endocrinol (Oxf) 2008;69(4):628–33.
[22] Misra M. Bone density in the adolescent athlete. Rev Endocr Metab Disord 2008;9(2):139–44.
[23] Steinle NI, Pollin TI, O'Connell JR, Mitchell BD, Shuldiner AR. Variants in the ghrelin gene are associated with metabolic syndrome in the Old Order Amish. J Clin Endocrinol Metab 2005;90(12):6672–7.
[24] Zavarella S, Petrone A, Zampetti S, Gueorguiev M, Spoletini M, Mein CA, et al. A new variation in the promoter region, the −604 C>T, and the Leu72Met polymorphism of the ghrelin gene are associated with protection to insulin resistance. Int J Obes (Lond) 2008;32(4):663–8.
[25] Ando T, Ichimaru Y, Konjiki F, Shoji M, Komaki G. Variations in the preproghrelin gene correlate with higher body mass index, fat mass, and body dissatisfaction in young Japanese women. Am J Clin Nutr 2007;86(1):25–32.
[26] Dezaki K, Sone H, Yada T. Ghrelin is a physiological regulator of insulin release in pancreatic islets and glucose homeostasis. Pharmacol Ther 2008;118(2):239–49.

糖尿病性腎症を防ぐ食事療法

渡邊 昌

(社)生命科学振興会理事長

糖尿病患者の10～15%が糖尿病性腎症に移行し、慢性腎不全(CKD)の大きな要因となっている。透析学会の試算では毎年、腎透析に入る糖尿病患者は1.5万人から2万人と予測されている。2008年から2012年までの累積患者数は21万人を超え、1兆円以上の医療費を使うことになる。糖尿病患者はエネルギーコントロールを強いられることが多く、たんぱく尿が現れると0.8 g/kg体重当たりのたんぱく食を勧められるようになる。一方で低たんぱく食を成功させるには必要十分なエネルギー摂取が必要となるので、おそらく患者も栄養士も戸惑うことが多いと思われる。糖尿病腎症の進行阻止に必要な栄養療法について考えてみたい。

変わるたんぱく質摂取推奨量

日本人の肥満や生活習慣病の増加に、戦後の食生活の変化、特に動物性脂肪の摂取増が原因とされることが多いが、私は動物性たんぱく質摂取の増加も寄与していると思うようになった[1]。日本ではたんぱく質の所要量は時代を追うごとに低くなっている[2]。国連傘下のFAOとWHOは栄養問題に関して、1971年と1981年にエネルギーとたんぱく質についてガイドラインを出し、1985年にはWHOテクニカルレポートとして広く世間に周知させた。

たんぱく質は1g 4 kcalと計算されるが、エネルギーが十分摂れていれば、たんぱく質が脱アミノ反応によってTCA回路で燃焼されることはない。そこで、たんぱく質の摂取量については科学的見地から見直され、2002年にたんぱく質アミノ酸の摂取量について専門家によるワーキンググループが組まれ、2007年にWHOテクニカルレポートとして報告された[3]。

食事摂取基準2010ではWHOのたんぱく質平衡維持量を検討し、平均すると0.65 g/kg体重/日となるので、この値をもって窒素平衡維持量としている。WHOではこの量をもとに標準偏差の2倍を足して体重当たり0.83 g/kgをたんぱく質摂取の安全量としている。日本の摂取基準では、これに基づき成人の1日たんぱく質摂取量を男性60 g、女性50 gとしてある。

これに基づいて人体の食餌必要量が算出された[2]。1日の標準食餌という概念を提唱したのはミュンヘン大学の生理学者フォイトで、彼は労働者の消費する食品の燃焼熱や成分を測定して得た平均値を基に1日に熱量は2,976kcal、たんぱく質118 g、脂肪56 g、炭水化物500 gを摂ることが必要と提案した。当時は摂取量を測定し、それを必要量と考えたのである。この値は明治時代にドイツ医学を導入した際に日本へもたらされた。

それに対し、エール大学のチッテンデンは1902年11月から1日にたんぱく質を30 gないし35 gの食事を7ヶ月間続け、これだけ摂れば自分自身の窒素平衡を保てることを証明した。チッテンデンの食事は体格から計算するとエ

表1　1985年基準と今回のアミノ酸摂取基準比較

アミノ酸	2007WHO		1985FAO/WHO/UN	
	mg/kg per day	mg/kg protein	mg/kg per day	mg/g protein
Histidine	10	15	8～12	15
Isoleucine	20	30	10	15
Leucine	39	59	14	21
Lysine	30	45	12	16
Methionine + cystaine	15	22	13	20
Methionine	10	16	–	–
Cystaine	4	6	–	–
Phenylalanine + tyrosine	25	38	14	21
Threonine	15	23	7	11
Tryptophan	4	6	3.5	5
Valine	26	39	10	15
必須アミノ酸総量	194	277	99.5	141

1体重1kg当たり0.66gの摂取たんぱく質量としての血漿量105mgから換算

表2　アミノ酸必要量と食品中のアミノ酸量（mg）

	必要量 mg/gN	体重 60kgの人	牛乳	納豆	さんま	黒まぐろ	和牛肩	豚肩	鶏卵
量/g	1	6.3	0.51	2.6	2.9	4.2	3.2	3.1	2
Ileu	30	1188	175	751	836	na	969	977	701
Leu	59	2336	300	na	na	na	na	na	na
Lys	45	1782	263	na	na	na	na	na	926
Met	16	634	87	260	592	700	551	567	453
Cys	6	238	29	258	204	261	242	226	330
Phe・Tyr	38	1504	279	1589	1361	1815	1519	1460	1422
Thr	23	911	134	606	656	na	969	914	886
Trp	6	238	42	242	210	295	229	229	194
Val	39	1544	212	839	976	na	na	na	800
His	15	225	93	496	na	na	840	na	330
Arg			100	934	na	na	na	na	825
Ala			106	693	na	na	na	na	743
Asp酸			256	na	na	na	na	na	na
Glu酸			620	na	na	na	na	na	na
Gly			62	693	868		872	889	453
Pro			315	895	651	844	807	819	486
Ser			155	722	710	971	840	756	857

na:データなし

ネルギーが約300 kcal不足になり、そのため窒素平衡がやや高めで負になっている。この結果の詳細は1904年に公刊された「栄養の生理学的経済」の中に詳述されている[1]。

たんぱく質摂取量の古典的論争

20世紀の初頭に、直接、間接に食品の熱量を測定して、これに基づいて人体の理論上の食餌必要量が算出された[2]。1日の標準食餌という概念を提唱したのはミュンヘン大学の生理学者フォイトで、彼は労働者の消費する食品の燃焼熱や成分を測定して得た平均値を基に1日に熱量は2,976 kcal、たんぱく質118 g、脂肪56 g、炭水化物500 gを摂ることが必要と提案した。当時は摂取量を測定し、それを必要量と考えたのである。この値は明治時代にドイツ医学を導入した際に日本へもたらされた。

それに対し、エール大学のチッテンデンは1902年11月から1日にたんぱく質を30 gないし35 gの食事を7ヵ月間続け、これだけ摂れば自分自身の窒素平衡を保てることを証明した。チッテンデンの食事は体格から計算するとエネルギーが約300 kcal不足になり、そのため窒素平衡がやや高めで負になっている。この結果の詳細は1904年に公刊された「栄養の生理学的経済」の中に記述されている[1]。

日本人の高たんぱく食信仰

日本人はもともとたんぱく質摂取量が少なかったので、戦前の富国強兵政策の下ではたんぱく質摂取量を高めに設定した。戦後も三食運動の普及により、たんぱく質は筋肉を作るという誤った考えが広まり、特に動物性たんぱく質摂取は戦後1日10 gから50 gへと約5倍になった。

しかし、たんぱく質が分解されて生じるアンモニアや尿素は生体にとって毒性を発揮する。食事摂取基準2010にまとめられているが、40歳以下の健康な成人に1日体重あたり1.9～2.2 gのたんぱく質を一定期間摂取させると、インスリンの感受性低下、酸・シュウ酸塩・カルシウムの尿排泄増加、糸球体濾過量増加、骨吸収の増加、血漿グルタミン濃度の低下など、好ましくない代謝変化が生じる[5]。

また、65歳以上の男性に1日体重あたり2 g以上のた

んぱく質を摂取させると、血中尿素窒素が10.7 mmol/L以上に上昇し、高窒素血症が発症することが報告されている。これらの報告から、成人においては年齢にかかわらず、たんぱく質摂取は2.0 g/kg体重/日未満に留めるのが適当である。

個人の推定エネルギー必要量

個人の推定エネルギー必要量とは「当該年齢、性別、身長、体重、及び健康な状態を損なわない身体活動量を有する人において、エネルギー出納（成人の場合、エネルギー摂取量－エネルギー消費量）が0（ゼロ）となる確率が最も高くなると推定される、習慣的なエネルギー摂取量の1日当たりの平均値」と定義されている。わかりやすい指標は体重が一定なら良いということになる。

食事摂取基準2010では標準体重にあると思われる男女の基礎代謝量を年代ごとに出し、それに活動量をかけて年齢ごとの必要量を示している。この基準は健康人を対象にしているが、病人にも応用されていることが多い。

私たちは栄養指導にもっと個人単位のエネルギー必要量をわかりやすく示せるようにテイラーメイド・ヌトリション(tailor-made nutrition)を提唱してきた。これは新しいエネルギー単位として氷1 kgの融解熱（80 kcal）を1単位として定義した。そうすると、男女共、成人以降なら高齢に至るまで「体重x 0.4」単位でよいことを発見した[6]。これは糖尿病の食品交換表で使われている単位でもあり、すでに普及している単位と重なる。

食品の製造業者、供給者が一体となって食品にこの単位表示を付けてくれれば、エネルギー摂取をコントロールしなければならない人は容易に自分で必要なエネルギー摂取をコントロールできる。この表示に炭水化物、たんぱく質、脂肪、野菜・果物のマーカーとして抗酸化価を色で示す案も提唱しているが、4色のバランスをとって食べれば、必要な栄養素がおおむね充足して摂れる。

たんぱく質のエネルギー

たんぱく質1 gを4 kcalと計算するのは問題がないのであろうか？　たんぱく質をボンベカロリメーターで燃やすと、1 g当たりカゼインは7.10 kcal、卵白は5.74 kcal、赤身肉は5.78 kcalである。たんぱく質は食事性発熱量が30%程度あるので0.7をかけるとそれぞれ4.97、4.18、4.46となる。アトウオーターは吸収率を0.9として4.0 kcalに丸めたのである。さらに排泄される尿素は、2.54 kcalの熱量を持つので、たんぱく質の燃焼に役立つエネルギーは実際には2.8 kcal/g程度しかない可能性もある。この程度なら50 g摂ったとしても75 kcal程度であり、2,000 kcal摂る人なら7％にしかならないので無視しても許容範囲となる。

そうすると、必要なエネルギーは炭水化物と脂肪から摂らねばならないことになるが、その比率をどれくらいにすればよいのかは今後の検討課題である。6：4あるいは、ケトン食を考えれば1：2くらいの範囲に収まると思われるが、高エネルギー低たんぱく質の新しい食品を開発する必要があるかもしれない。

糖尿病患者への食事指導

尿たんぱくが陽性となりCKDの可能性を持つ糖尿病患者については上記の点を再考慮せねばならない。糖尿病患者は肥満のことが多く、エネルギー摂取の制限を受けていることが多いからである。腎不全への進行を阻止するためにはできるだけ低たんぱく食にする必要がある。

少なくても0.5 g/kg程度を目標にする。必要なエネルギー摂取を確保できればたんぱく質摂取量は必要最小限でよいはずである[7,8]。窒素平衡から見ると0.3 g/kg程度でも可能と思われる。実際、私のところに来る適正たんぱく食普及会の人たちはそれ以下の人もいて、GFR 30 ml以下で何年もCrを維持し差し支えなく日常生活をしている。Kopple[8]はGFRが25 ml/min以下の人に0.28 g/kg/日、たんぱく質量で20 g/日を勧め、不足分はBCAAかαケト酸で補給することを提案している[8]。

最近、話題となっているのが、尿素の再利用である。たんぱく質摂取が70 g/日以上摂取されている場合、75％は尿に、25％は結腸からリサイクルされる[3]。腸内細菌が80％をアミノ酸にするからである。たんぱく質摂取量が35 g/日以下の場合はリサイクルに回る尿素が増える。これが計算値以下の低たんぱく食に耐えられる理由であろう。

糖尿病の患者はIgA腎症などに由来する腎不全に比べコントロールが難しい。血糖値の高いことは、糸球体がhyper-filtration（排泄過剰）の状態にあり、水分等の再吸収が間に合わず、グルコースの血中濃度が高い状態が続く。空腹時血糖、HbA1c、インスリン抵抗性、hyper-filtration（排泄過剰）などの改善は重要である[9,10]。血圧のコントロールが一番大きな課題である。

アミノ酸摂取量とたんぱく質摂取量

食事摂取基準2010のアミノ酸摂取量はWHOのガイドラインを踏襲している。これを食品に換算するとどれくらいの量になるのであろうか。窒素1g(たんぱく質6.25g)当たり多いアミノ酸で数10mg含まれていればよい。代表的な食品を表に示すと、牛乳たんぱく質は窒素1g当たり8～10倍の必要アミノ酸量がある。魚や肉に関しても10倍以上の必須アミノ酸が含まれるので、体重60kgの人で考えれば肉・魚を150gも摂れば必要な必須アミノ酸を含み、アミノ酸所要量は達成できることになる。たんぱく質量は15～20gあれば必須アミノ酸はまかなえることになり、少ないのはリシンくらいなのでリシンの多い鶏卵をたまに摂ればよい。非必須アミノ酸については食品成分表に掲載のないものが多いが、多くの肉や魚には必須アミノ酸の倍近い量が含まれているので、生体内での重要性を考えればこれらも含まれた形で食品から摂取するのが望ましい。とことん低たんぱく食を追及するなら、できるだけアミノ酸構成の良いたんぱく質を選び、不足しそうな制限アミノ酸はサプリメントから摂るという方法も可能であろう[11]。

おわりに

たんぱく質、アミノ酸の栄養学は、腎不全の進行を予防するための低たんぱく食や特殊なアミノ酸機能を個々に判断して摂取しようという新しい研究領域を広げている。いずれにしても薬剤のみでは病状をコントロールするのは難しく、生活習慣として食事療法を行わなければ腎機能を保てない。糖尿病薬の多くは腎不全状態には禁忌となるので、食事療法は非常に重要である。この分野の研究の蓄積が必要である。なお、フォイトとチッテンデンの論争を知ることは重要と思われるが、成書2を参考にしていただければ幸いである。

文　献

1 渡邊昌．食の随想 温故知食「長寿村、短寿村から学ぶこと」．医と食 2009; 1(2): 38-9.
2 渡邊昌．栄養学原論．南江堂，東京，2009.
3 WHO/FAO Expert Committee. Protein and Amino Acid Requirements in Human Nutrition. World Health Organization. Geneve, 2007.
4 Chittenden RH. Physiological Economy in Nutrition with Special Reference to the Minimal Proteid Requirement of the Healthy Man: An Experimental Study. Frederick A. Stokes. NY, 1904.
5 食事摂取基準 2010. http://www.nih.go.jp/
6 Kopple JD. McCollum Award Lecture, 1996: protein-energy malnutrition in maintenance dialysis patients. Am J Clin Nutr 1997; 65: 1544-57.
7 Mandayam S, Mitch WE. Dietary protein restriction benefits patients with chronic kidney disease. Nephrology 2006; 11: 53-7.
8 Kopple JD, Massry SG: Nutritional Management of Renal Disease. Williams & Wilkins, Baltimore, MD, 1996.
9 Jones SL, Kontessis P, Wiseman M, et al. Protein intake and blood glucose as modulators of GFR in hyperfiltering diabetic patients. Kid Int 1992; 41: 1620-8.
10 渡邊昌、たんぱく質・アミノ酸の適正摂取量、食品学会誌 2009(印刷中).

Current concept of protein and amino acid requirements

Shaw Watanabe, Life Science Promotion Association

The question of how much intake of protein being considered adequate was debated by Voit and Chittenden in the early 20th century. Since World War II, periodic reports by the FAO and WHO have suggested values for energy and protein requirements; here, we consider the findings of the recent WHO technical report no.935 in relation to the Japanese Dietary Reference Intake 2010. Patients with renal failure need to take low protein diet to save thein renal function. The report also gives required intake of essential amino acids. Various amino acids have special roles in specific metabolic pathways, such as sulfur metabolism in the methionin cycle, arginin in the intestinal-renal axis, etc. Non-essential amino acids are also known to play important roles, such as arginin in the urea cycle, glutamic acid for immune function, serin for neuronal growth, etc. Peptide supplements have recently become commercially available; their rapid absorption may make them suitable for treatment of protein-energy malnutrition (PEM) in elderly patients. The use of such newly appeared dietary agents could be integrated into "functional nutriology," in which the antioxidant action of proteins should also be considered. *Clinical & Functional Nutriology 2009; 1(3): 23-26*

Androgen Receptor-Dependent Activation of Endothelial Nitric Oxide Synthase in Vascular Endothelial Cells: Role of Phosphatidylinositol 3-Kinase/Akt Pathway

Jing Yu, Masahiro Akishita, Masato Eto, Sumito Ogawa, Bo-Kyung Son, Shigeaki Kato, Yasuyoshi Ouchi, and Tetsuro Okabe

Departments of Integrated Traditional Medicine (J.Y., T.O.) and Geriatric Medicine (M.A., M.E., S.O., B.-K.S., Y.O.), Graduate School of Medicine, and the Institute of Molecular and Cellular Bioscience (S.K.), The University of Tokyo, Tokyo 113-8655, Japan

The mechanisms of testosterone-induced vasodilatation are not fully understood. This study investigated the effect of testosterone on nitric oxide (NO) synthesis and its molecular mechanism using human aortic endothelial cells (HAEC). Testosterone at physiological concentrations (1–100 nM) induced a rapid (15–30 min) increase in NO production, which was associated with phosphorylation and activation of endothelial NO synthase (eNOS). Then, the involvement of the androgen receptor (AR), which is abundantly expressed in HAEC, was examined. The effect of testosterone on eNOS activation and NO production were abolished by pretreatment with an AR antagonist nilutamide and by transfection with AR small interference RNA. In contrast, testosterone-induced eNOS phosphorylation was unchanged by pretreatment with an aromatase inhibitor or by transfection with ERα small interference RNA. 5α-Dihydrotestosterone, a nonaromatizable androgen, also stimulated eNOS phosphorylation. Next, the signaling cascade that leads to eNOS phosphorylation was explored. Testosterone stimulated rapid phosphorylation of Akt in a time- and dose-dependent manner, with maximal response at 15–60 min. The rapid phosphorylation of eNOS or NO production induced by testosterone was inhibited by Akt inhibitor SH-5 or by phosphatidylinositol (PI) 3-kinase inhibitor wortmannin. Co-immunoprecipitation assays revealed a testosterone-dependent interaction between AR and the p85α subunit of PI3-kinase. In conclusion, testosterone rapidly induces NO production via AR-dependent activation of eNOS in HAEC. Activation of PI3-kinase/Akt signaling and the direct interaction of AR with p85α are involved, at least in part, in eNOS phosphorylation. (***Endocrinology*** **151: 1822–1828, 2010)**

Steroid hormones play various roles in vascular functions through the specific receptor (1). Although the effects of androgens on the cardiovascular system have been controversial (2), recent epidemiological studies have shown that low testosterone level is associated with incident cardiovascular disease (3, 4) and impaired endothelial vasomotor function (5) in men. Several studies have also shown that short-term administration of testosterone to men with coronary artery disease reduces myocardial ischemia (6–8) and improves endothelial vasomotor function (9, 10). These findings suggest beneficial effects of testosterone on the cardiovascular systems and endothelium in men. Testosterone causes acute vasorelaxation *in vitro* and *in vivo* in animals and in humans (11–14), but the precise mechanisms of testosterone-induced vasorelaxation are still unknown. Some suggest the role of nitric oxide (NO) (12, 15), but others have denied the involvement of NO (16, 17).

Androgen receptor (AR) is a member of the nuclear receptor superfamily, which exerts its effects by modifying

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2009-1048 Received September 2, 2009. Accepted January 15, 2010.
First Published Online March 1, 2010

Abbreviations: AR, Androgen receptor; DAF-2DA, 4,5-diaminofluorescin diacetate; DHT, 5α-dihydrotestosterone; eNOS, endothelial NO synthase; ERα, estrogen receptor-α; HAEC, human aortic endothelial cells; L-NAME, nitro-L-arginine methyl ester; NOx, nitrite/nitrate; NT, nontargeting; PI, phosphatidylinositol; siRNA, small interference RNA.

gene expression (18). The signal transduction pathways activated by AR has not been defined well in the cardiovascular system. We have previously reported that AR is involved in endothelial NO synthase (eNOS) activation induced by ginsenoside-Rb1, a steroid hormone-like herb, through phosphatidylinositol (PI) 3-kinase/Akt signaling (19). In the present study, we investigated the effect of testosterone on eNOS activation and NO production and further explored the role of AR and its signaling pathway in human aortic endothelial cells (HAEC).

Materials and Methods

Cell culture

HAEC (derived from a 50-yr-old man, tissue acquisition no. 14506, lot no. 6F3753; Cambrex BioScience, Inc., Walkersville, MD) were maintained in EBM-2 (Clonetics, Walkersville, MD) medium supplemented with 10% fetal bovine serum, a growth factor cocktail [0.1% human epidermal growth factor, 0.04% hydrocortisone, 0.4% human fibroblast growth factor-B, 0.1% vascular endothelial growth factor, 0.1% R^3-IGF-1 [an 83-amino acid analog of IGF-1 comprising the complete human IGF-1 sequence with the substitution of an Arg (R) for the Glu (E) at position three, hence R^3 and a 13-amino acid extension peptide at the N terminus], 0.1% ascorbic acid, 0.1% (Gentamicin, Amphotericin B)-1000, 0.1% heparin], and penicillin (100 U/ml)/streptomycin (100 μg/ml). For the experiments, HAEC at the fifth to seventh passage were seeded in collagen-coated two-chamber slides [for 4,5-diaminofluorescin diacetate (DAF-2DA) experiment], six-well plates [for nitrite/nitrate (NOx) determination], or 6-cm dishes (for immunoblotting and other experiments), respectively, at a concentration of 10^4 cells/cm^2 and grown to subconfluence. Then the cells were incubated in phenol red- and growth factor cocktail-free EBM-2 medium supplemented with 1% dextran-coated charcoal-stripped fetal bovine serum (condition medium) for 6 h to diminish steroids and to obtain growth arrest. In the inhibition experiments, the cells were pretreated with inhibitors for 60 min before the stimuli. Dimethylsulfoxide (0.01–0.02%) was used as a vehicle and a solvent for reagents used in this study.

Detection of NO

NO production was examined using NO-sensitive fluorescent dye DAF-2DA (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). Briefly, cells were seeded in collagen-coated two-chamber slides in culture medium (2 ml) until subconfluence. Growth-

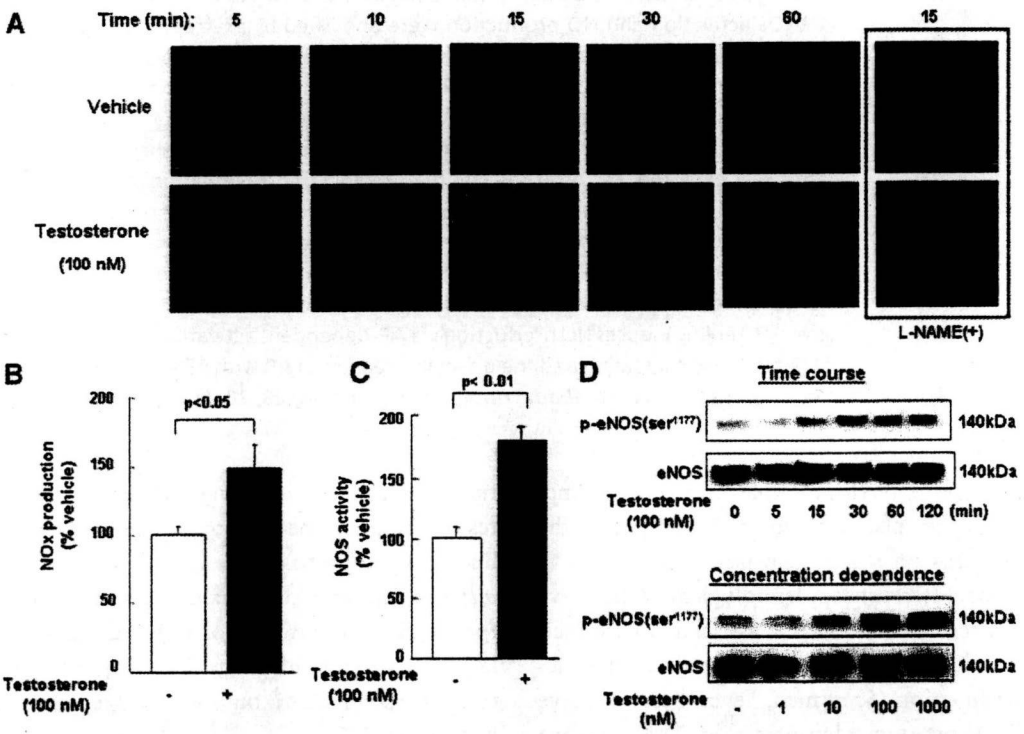

FIG. 1. Testosterone rapidly stimulates NO production and NOS activation in HAEC. A, Growth-arrested HAEC were loaded with DAF-2DA before treatment with or without testosterone for the indicated times. After testosterone treatment, cells were fixed in 2% paraformaldehyde and viewed using a fluorescence microscope. Emission of green light is indicative of NO production. In some groups of cells, L-NAME (0.5 mM) was added 30 min before loading cells with DAF-2DA. B, NOx concentration in the supernatants was measured as stable metabolites of NO at 2 h after stimulation with testosterone. Data were converted to percentage of vehicle and expressed as mean ± SEM of three independent experiments using different cell preparations (n = 4). C, After testosterone or vehicle was added to the medium for 30 min, cells were homogenized, and the activity of NOS was measured by the ability of NOS to convert [^{3}H]L-arginine to [^{3}H]L-citrulline as described in *Materials and Methods*. Data were converted to percentage of vehicle and expressed as mean ± SEM of three independent experiments using different cell preparations (n = 3). D, Growth-arrested HAEC were incubated with testosterone or vehicle for the indicated times or with the indicated concentrations of testosterone for 30 min. Phosphorylation of eNOS at Ser1177 (p-eNOS), and the total eNOS levels in cell lysates were analyzed by immunoblotting. Representative blots are shown, and the results were confirmed by at least three independent experiments. A representative result of at least three independent experiments are shown in A and D.

arrested cells were loaded with DAF-2DA (5 μM for 30 min at 37 C) and then rinsed three times with Hanks' balanced salt solution (Hanks' buffer; Sigma-Aldrich, St. Louis, MO), kept in the dark, and maintained at 37 C in condition medium (2 ml). After 90 min, cells were treated with testosterone or other stimuli. For inhibition experiments, the inhibitors were added 60 min before stimuli. Green fluorescence intensity was visualized with a laser-scanning confocal microscopy system [Bio-Rad (Hercules, CA) Laser Sharp2000] connected to a CCD camera and a computer system. Emission of green light (510 nm) from cells excited by light at 488 nm is indicative of NO production as a result of the reaction of DAF-2DA with NO. Accumulation of NO from HAEC in the culture medium was measured as the levels of NOx, oxidized products of NO, using a fluorometric 2,3-diaminonaphthalene kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan). Briefly, cells were seeded in collagen-coated six-well plates in cultural medium (2 ml) until subconfluence. Growth-arrested cells were stimulated with testosterone for 2 h in the condition medium (1 ml/well). Cell- and debris-free supernatants were applied for the measurement to total NOx concentrations. NOx concentrations were calculated according to the manufacturer's protocol.

NOS activity assay

NOS activity was quantified by measurement of the conversion of L-arginine to L-citrulline using an NOS assay kit (Calbiochem, EDM Biosciences, Inc., La Jolla, CA). Briefly, cells were seeded in collagen-coated 6-cm dishes and grown until subconfluent and growth arrested. At 30 min after stimulation with testosterone, cells were harvested and lysed. The concentration of protein in cell lysate was adjusted to 10 μg/μl. According to the manufacturer's protocol, total cell lysate and reaction mixture were incubated with 1 μCi/μl L-[2,3,4,5-^{3}H]arginine (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) for 60 min at 30 C, and then, converted citrulline was separated from the mixture by passing the mixture through a column of equilibrated cups. The eluate was sampled in scintillation liquid. Extracts incubated with an NOS inhibitor, nitro-L-arginine methyl ester (L-NAME, 1 mM), served as the blank. Converted NOS activity was calculated by subtracting the value of the blank from that of the sample.

Small interference RNA (siRNA) transfection

siRNA duplexes (Santa Cruz Biotechnology, Santa Cruz, CA) against AR (accession no. sc-29204) and estrogen receptor-α (ERα) (accession no. sc-29305) were used for directed knockdown of protein expression. Nontargeting (NT) scrambled siRNA (Santa Cruz; accession no. sc-37007) was used as a control siRNA. HAEC were seeded in collagen-coated 6-cm dishes and grown in culture medium without antibiotics. At 50–60% confluence, cells were transfected with 5 nM AR siRNA, 10 nM

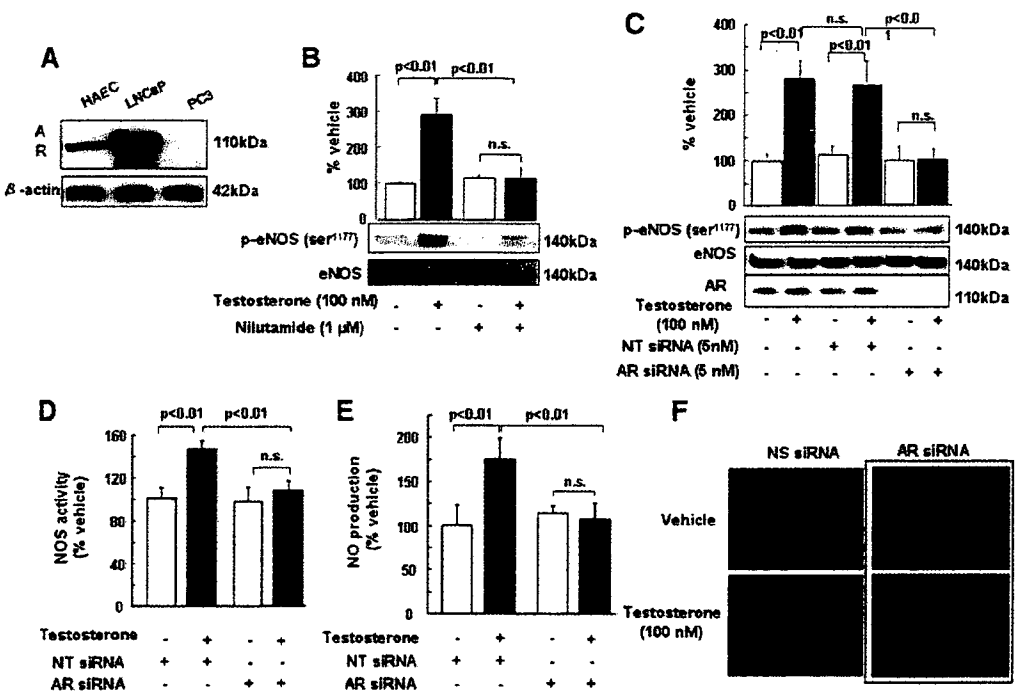

FIG. 2. AR is involved in testosterone-induced eNOS phosphorylation. A, AR expression was examined in HAEC, AR-positive LNCaP cells, and AR-negative PC3 cells using immunoblotting (*upper panel*). The membrane was stripped and re-immunoblotted with anti-β-actin antibody for monitoring equal amounts of protein from each sample (*lower panel*). B, Cells were treated with testosterone or vehicle for 30 min in the presence or absence of nilutamide. Phosphorylation of eNOS at Ser1177 (p-eNOS), and protein levels of eNOS were analyzed using immunoblotting. C–F, Growth-arrested HAEC were transfected with 5 nM AR siRNA or 5 nM nontargeting scrambled (NT) siRNA for 24 h as described in *Materials and Methods*. Cells were treated with testosterone or vehicle for 30 min. C, Phosphorylated eNOS at Ser1177 (p-eNOS) and total eNOS and AR were analyzed using immunoblotting. D, NOS activity was measured by the ability of NOS to convert [^{3}H]L-arginine to [^{3}H]L-citrulline as described. E, NOx concentration in the supernatants was measured as described in *Materials and Methods*. F, After transfection with siRNA, growth-arrested cells were loaded with DAF-2DA and treated with or without testosterone for 15 min. NO production was examined as described in *Materials and Methods*. A representative result from three independent experiments is shown in A–C and F. Data represent mean ± SEM of the p-eNOS/eNOS ratio of quantified densities from three independent experiments; representative blots are shown in B and C. Data were converted to fold over vehicle and expressed as mean ± SEM of three independent experiments using different cell preparations (n = 3) in D and E. n.s., Not significant.

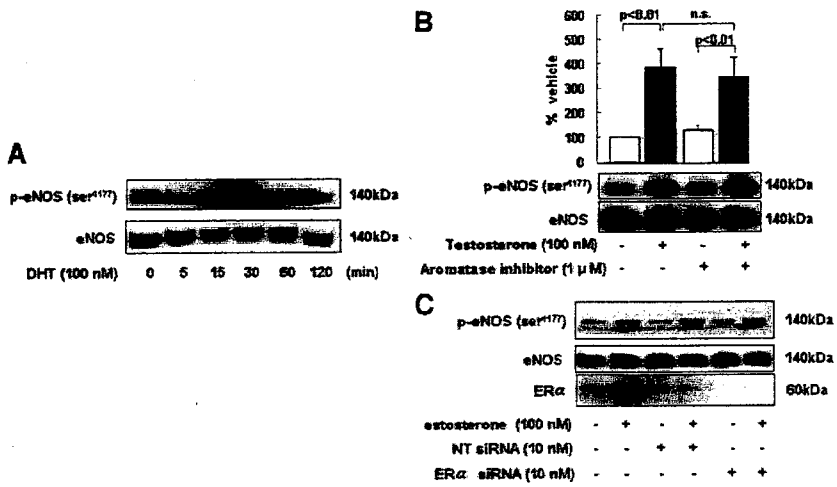

FIG. 3. Estradiol/ER pathway is not involved in testosterone-induced eNOS phosphorylation. A, Growth-arrested HAEC were treated with DHT or vehicle for the indicated times. B, Growth-arrested cells were treated with testosterone or vehicle for 30 min in the presence or absence of aromatase inhibitor. Data represent mean ± SEM of the phosphorylated eNOS (p-eNOS)/eNOS ratio of quantified densities from three independent experiments. A representative blot is shown. n.s., Not significant. C, Cells were treated with testosterone or vehicle for 30 min after transfection of siRNA against ERα or nontargeting scrambled (NT) siRNA (10 nM). A–C, Phosphorylation of eNOS at Ser1177 (p-eNOS), total eNOS, and ERα in cell lysates were analyzed using immunoblotting. A representative result from three independent experiments is shown.

ERα siRNA, or NT siRNA using HiPerFect transfection reagent (QIAGEN, Valencia, CA) in 1 ml transfection medium (Santa Cruz). Two hours later, 3 ml culture medium was added, and incubation was performed for another 22 h. Cells were washed with Hanks' buffer and used for the experiments.

Immunoprecipitation and immunoblotting

Immunoprecipitation assays were performed according to the standard protocol. Briefly, treated cells were washed twice in ice-cold Hanks' buffer and lysed with RIPA lysis buffer (Tris-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.004% sodium azide, protease inhibitor cocktail, and sodium orthovanadate). The volume of all samples was brought up to 1 ml by the addition of lysis buffer. Equal amounts of total cell lysates (800 μg protein per sample) were precleared with protein A/G agarose beads (Santa Cruz) at 4 C for 30 min and then incubated with anti-p85α antibody (B-9, 1 μg/mg cellular protein; Santa Cruz) at 4 C overnight with end-over-end rotation. A cell lysate with antimouse IgG antibody served as a negative control. After this period, protein A/G agarose beads were added and incubated for another 2–4 h at 4 C. After removal of the lysates by centrifugation, the immunoprecipitates were washed four times with PBS containing 1% Nonidet P-40 and resuspended in 2% electrophoresis sample buffer (Santa Cruz), separated on precast SDS-PAGE, and transferred to polyvinyl difluoride membranes.

Antibodies against AR (N-20; Santa Cruz), ERα (Ab-10; Thermo Fisher Scientific, Fremont, CA), phospho-eNOS (Ser1177), and eNOS/NOS type III (BD Transduction Laboratories, Lexington, KY), phospho-Akt and Akt (Cell Signaling Technology, Beverly, MA) were used for immunoblotting as described previously (19).

Data analysis

Values are expressed as mean ± SEM in the text and figures. Statistical comparisons were performed using ANOVA with *post hoc* Fisher's protected least significant difference test. Differences with a value of $P < 0.05$ were considered statistically significant.

Results

Testosterone stimulates rapid NO production and eNOS activation in HAEC

After the addition of testosterone (Sigma-Aldrich), a significant increase in green fluorescence was observed at 5 min. Maximal stimulation of NO production was observed at 15–30 min, but this response was abolished by pretreatment of cells with an NOS inhibitor, L-NAME (Fig. 1A). The concentration of NOx, stable metabolites of NO, in the culture medium was also increased up to approximately 1.5-fold by testosterone (100 nM) treatment for 2 h (Fig. 1B). Testosterone increased NOS activity, as measured by determination of L-citrulline converted from L-arginine, after 30 min of treatment (Fig. 1C). Also, testosterone induced eNOS phosphorylation (Ser1177) in a time- and dose-dependent manner, with no influence on eNOS protein levels (Fig. 1D).

AR mediates testosterone-induced eNOS activation

We investigated the role of AR in the effect of testosterone on eNOS activation. First, we confirmed the endogenous expression of AR in HAEC by immunoblotting in comparison with an AR-positive prostate cancer cell line, LNCaP, and an AR-negative cell line, PC3 (Fig. 2A). Pretreatment with nilutamide, an AR antagonist, abolished the testosterone-induced rapid phosphorylation of eNOS (Fig. 2B). Next, we applied siRNA for loss-of-function analysis of AR. Associated with the efficient knockdown of AR expression, testosterone-induced eNOS phosphorylation (Fig. 2C), NOS activation (Fig. 2D), and NO production (Fig. 2, E and 2F) were abolished by transfection with AR siRNA but were not by control NT siRNA. We then examined the effect of nonaromatizable 5α-dihydrotestosterone (DHT, 100 nM; Sigma-Aldrich) on eNOS phosphorylation. DHT also induced eNOS phosphorylation (Fig. 3A). In contrast, conversion to estradiol or ERα does not seem to play a role, because neither the aromatase inhibitor (1 μM) nor transfection of ERα siRNA affected testosterone-induced eNOS phosphorylation (Fig. 3, B and C).

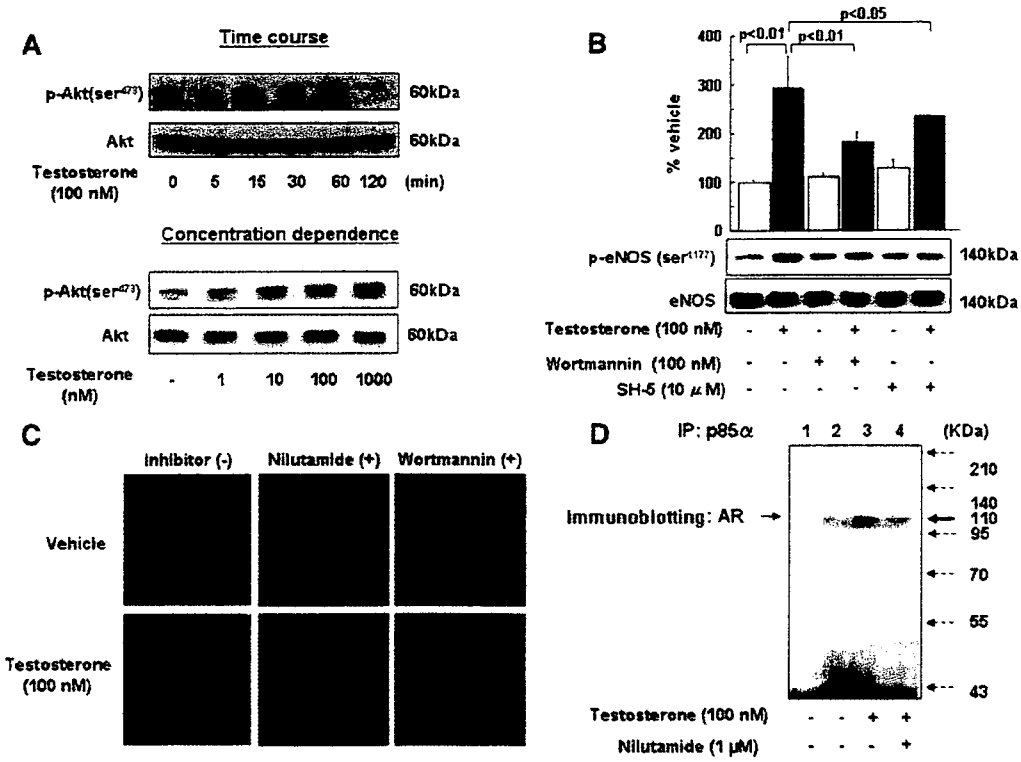

FIG. 4. PI3-kinase/Akt pathway is involved in testosterone-induced eNOS phosphorylation and NO production. A, Growth-arrested HAEC were treated with vehicle or testosterone for the indicated times or with the indicated concentrations of testosterone for 30 min. Phosphorylation of Akt at Ser473 (p-Akt) and total Akt level in cell lysates were analyzed by immunoblotting. B, Wortmannin or SH-5 was added 60 min before the cells were treated with testosterone for 30 min. Phosphorylation of eNOS at Ser-1177 (p-eNOS), and total eNOS levels in cell lysates were analyzed. Data represent mean ± SEM of the p-eNOS/eNOS ratio of quantified densities from three independent experiments. A representative blot is shown. C, Growth-arrested cells were loaded with DAF-2DA and treated with or without testosterone for 15 min in the presence or absence of wortmannin or nilutamide. D, Growth-arrested HAEC were treated with testosterone or vehicle for 30 min in the presence or absence of nilutamide. Cell extracts were immunoprecipitated with anti-p85α antibody (lanes 2–4) or IgG (lane 1) and separated by SDS-PAGE, and AR was detected with anti-AR antibody. A representative result from three independent experiments is shown in A, C, and D.

PI3-kinase/Akt pathway is involved in eNOS phosphorylation and NO production induced by testosterone

Akt, a serine/threonine kinase, is a key effector of PI3-kinase signaling, directing eNOS phosphorylation (20, 21). Therefore, we examined the role of PI3-kinase/Akt in testosterone-induced eNOS phosphorylation. Testosterone rapidly phosphorylated Akt in a time- and dose-dependent manner, with no influence on Akt protein levels (Fig. 4A). Pretreatment with a PI3-kinase inhibitor wortmannin or an Akt inhibitor SH-5 significantly attenuated testosterone-induced eNOS phosphorylation (Fig. 4B). NO production was also inhibited by wortmannin (Fig. 4C).

PI3-kinase consists of the regulatory subunit p85α and the catalytic subunit p110 (22, 23), and the direct interaction between ERα and p85α initiates eNOS activation (24). Thus, we examined whether AR could interact with p85α, using co-immunoprecipitation assays. As shown in Fig. 4D, AR was associated with p85α in a ligand-dependent and AR antagonist-sensitive manner. These results indicate that testosterone stimulates AR binding to the p85α subunit of PI3-kinase, leading to Akt and eNOS activation.

Discussion

In the present study, we demonstrated that testosterone rapidly induces NO production, associated with the phosphorylation/activation of eNOS. Endothelium-derived NO has been shown to modulate a variety of vascular functions, including vasodilation, inhibition of endothelial cell death, inhibition of platelet aggregation, and attenuation of leukocyte infiltration (25). Thus, NO production by testosterone may account, at least in part, for the vasodilatory (11–14) and antiischemic (6–8) action of testosterone as well as the association of endogenous testosterone with endothelial vasomotor function (5) and protection against cardiovascular disease (3, 4) in men.

In endothelial cells, eNOS is a key enzyme for NO production upon the conversion of the substrate L-arginine to L-citrulline (26), and the phosphorylation of eNOS at

serine-1177 is known to increase enzyme activity (27–29). The increase of NO production by testosterone was abolished by pretreatment of cells with an NOS inhibitor, L-NAME (Fig. 1A), indicating the involvement of eNOS in testosterone-stimulated NO production.

Although most of the biological actions of testosterone may be mediated by AR, some of them may be mediated by ER, after conversion to estradiol (30, 31). Indeed, estrogens have been shown to rapidly activate eNOS and stimulate NO production in an ERα-dependent manner (24, 32). In our study, however, the role of both aromatase and ERα in testosterone-induced eNOS phosphorylation was negligible. In addition, nonaromatizable DHT also elicited a significant eNOS phosphorylation. Moreover, eNOS phosphorylation, NOS activation, and NO production induced by testosterone were reversed by pretreatment with an AR antagonist nilutamide or by transfection of AR siRNA. Taken together, it can be concluded that the effects of testosterone on eNOS phosphorylation and NO production are AR dependent. In our preliminary experiments, the expression of AR protein was not altered up to 4 h after testosterone or DHT treatment, although AR expression appeared to increase 24 h later (data not shown). Accordingly, the level of AR expression may not have influenced the rapid response of eNOS and NO to testosterone.

Multiple signal transduction pathways, including phosphoinositide-3 kinase /Akt kinase converge to regulate eNOS activity by phosphorylation (20, 33). In the present study, we showed that testosterone-induced eNOS phosphorylation is activated by the PI3-kinase/Akt pathway. We also demonstrated that AR interacts with the regulatory subunit p85α of PI3-kinase in endothelial cells. The binding was increased by testosterone and inhibited by an AR antagonist. Although we have not identified the sites of interaction between AR and p85α, Sun *et al.* (34) have demonstrated that the N terminus of AR binds to the C-terminal Src-homology 2 domain (SH2 domain) of p85α in LNCaP cells. Accordingly, AR might bind to p85α in a similar fashion in endothelial cells.

Others have also investigated the mechanisms underlying the vasodilatory action of testosterone (15–18). Their results have been controversial, although many studies suggest the involvement of NO. For instance, Chou *et al.* (12) and Costarella *et al.* (15) reported that in canine coronary arteries and rat thoracic aorta, relaxation in response to testosterone was attenuated by a NOS inhibitor, L-NAME (10, 11), suggesting a role for NO. In contrast, Honda *et al.* (16) and Tep-areenan *et al.* (17) have shown that vasorelaxation to testosterone is endothelium dependent but is not L-NAME sensitive. Yue *et al.* (11) and others (1, 35, 36) demonstrated that vasodilatation induced by pharmacological concentrations of testosterone is independent of the vascular endothelium and AR. The different mechanistic findings underlying the vasodilatory action of testosterone between our study and others (11, 16, 17, 35, 36) are unclear but may be attributable to the experimental conditions such as type of cells, tissue preparations, dose of testosterone, and inhibitors used. In our study, we used primary cultured HAEC; thus, we did not examine how produced NO acts on the arterial wall and vascular smooth muscle cells. Additional studies are required for better understanding of actions of testosterone on the cardiovascular system and the role of AR and its signaling pathways.

In summary, we found that testosterone *per se* stimulates rapid production of NO through AR-dependent activation of eNOS in HAEC. Activation of PI3-kinase/Akt signaling and the direct interaction of AR with p85α are involved, at least in part, in the phosphorylation of eNOS.

Acknowledgments

We thank Ms. Yuki Ito for technical assistance and Prof. Satoshi Inoue, Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, for providing LNCaP and PC3 cells.

Address all correspondence and requests for reprints to: Masahiro Akishita, M.D., Ph.D., 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.

This study was supported in part by Health and Labor Sciences Research Grants (H17-Choju-046) from the Ministry of Health, Labor, and Welfare of Japan and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture, and Sports of Japan (21390220 and 20249041).

Disclosure Summary: The authors have nothing to disclose.

References

1. Christ M, Wehling M 1998 Cardiovascular steroid actions: swift swallows or sluggish snails? Cardiovasc Res 40:34–44
2. Vitale C, Mendelsohn ME, Rosano GM 2009 Gender differences in the cardiovascular effect of sex hormones. Nat Rev Cardiol 6:532–542
3. Khaw KT, Dowsett M, Folkerd E, Bingham S, Wareham N, Luben R, Welch A, Day N 2007 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 116:2694–2701
4. Laughlin GA, Barrett-Connor E, Bergstrom J 2008 Low serum testosterone and mortality in older men. J Clin Endocrinol Metab 93: 68–75
5. Akishita M, Hashimoto M, Ohike Y, Ogawa S, Iijima K, Eto M, Ouchi Y 2007 Low testosterone level is an independent determinant of endothelial dysfunction in men. Hypertens Res 30:1029–1034
6. Webb CM, Adamson DL, de Zeigler D, Collins P 1999 Effect of

acute testosterone on myocardial ischemia in men with coronary artery disease. Am J Cardiol 83:437–439, A9
7. Rosano GM, Leonardo F, Pagnotta P, Pelliccia F, Panina G, Cerquetani E, della Monica PL, Bonfigli B, Volpe M, Chierchia SL 1999 Acute anti-ischemic effect of testosterone in men with coronary artery disease. Circulation 99:1666–1670
8. English KM, Steeds RP, Jones TH, Diver MJ, Channer KS 2000 Low-dose transdermal testosterone therapy improves angina threshold in men with chronic stable angina: a randomized, double-blind, placebo-controlled study. Circulation 102:1906–1911
9. Ong PJ, Patrizi G, Chong WC, Webb CM, Hayward CS, Collins P 2000 Testosterone enhances flow-mediated brachial artery reactivity in men with coronary artery disease. Am J Cardiol 85:269–272
10. Kang SM, Jang Y, Kim JY, Chung N, Cho SY, Chae JS, Lee JH 2002 Effect of oral administration of testosterone on brachial arterial vasoreactivity in men with coronary artery disease. Am J Cardiol 89:862–864
11. Yue P, Chatterjee K, Beale C, Poole-Wilson PA, Collins P 1995 Testosterone relaxes rabbit coronary arteries and aorta. Circulation 91:1154–1160
12. Chou TM, Sudhir K, Hutchison SJ, Ko E, Amidon TM, Collins P, Chatterjee K 1996 Testosterone induces dilation of canine coronary conductance and resistance arteries in vivo. Circulation 94:2614–2619
13. Webb CM, McNeill JG, Hayward CS, de Zeigler D, Collins P 1999 Effects of testosterone on coronary vasomotor regulation in men with coronary heart disease. Circulation 100:1690–1696
14. English KM, Jones RD, Jones TH, Morice AH, Channer KS 2002 Testosterone acts as a coronary vasodilator by a calcium antagonistic action. J Endocrinol Invest 25:455–458
15. Costarella CE, Stallone JN, Rutecki GW, Whittier FC 1996 Testosterone causes direct relaxation of rat thoracic aorta. J Pharmacol Exp Ther 277:34–39
16. Honda H, Unemoto T, Kogo H 1999 Different mechanisms for testosterone-induced relaxation of aorta between normotensive and spontaneously hypertensive rats. Hypertension 34:1232–1236
17. Tep-areenan P, Kendall DA, Randall MD 2002 Testosterone-induced vasorelaxation in the rat mesenteric arterial bed is mediated predominantly via potassium channels. Br J Pharmacol 135:735–740
18. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM 1995 The nuclear receptor superfamily: the second decade. Cell 83:835–839
19. Yu J, Eto M, Akishita M, Kaneko A, Ouchi Y, Okabe T 2007 Signaling pathway of nitric oxide production induced by ginsenoside Rb1 in human aortic endothelial cells: a possible involvement of androgen receptor. Biochem Biophys Res Commun 353:764–769
20. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM 1999 Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 399:601–605
21. Hisamoto K, Ohmichi M, Kurachi H, Hayakawa J, Kanda Y, Nishio Y, Adachi K, Tasaka K, Miyoshi E, Fujiwara N, Taniguchi N, Murata Y 2001 Estrogen induces the Akt-dependent activation of endothelial nitric-oxide synthase in vascular endothelial cells. J Biol Chem 276:3459–3467
22. Fry MJ 1994 Structure, regulation and function of phosphoinositide 3-kinases. Biochim Biophys Acta 1226:237–268
23. Vanhaesebroeck B, Stein RC, Waterfield MD 1996 The study of phosphoinositide 3-kinase function. Cancer Surv 27:249–270
24. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK 2000 Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 407:538–541
25. Arnal JF, Dinh-Xuan AT, Pueyo M, Darblade B, Rami J 1999 Endothelium-derived nitric oxide and vascular physiology and pathology. Cell Mol Life Sci 55:1078–1087
26. Moncada S, Higgs A 1993 The L-arginine-nitric oxide pathway. N Engl J Med 329:2002–2012
27. Gallis B, Corthals GL, Goodlett DR, Ueba H, Kim F, Presnell SR, Figeys D, Harrison DG, Berk BC, Aebersold R, Corson MA 1999 Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor LY294002. J Biol Chem 274:30101–30108
28. Boo YC, Kim HJ, Song H, Fulton D, Sessa W, Jo H 2006 Coordinated regulation of endothelial nitric oxide synthase activity by phosphorylation and subcellular localization. Free Radic Biol Med 41:144–153
29. Mount PF, Kemp BE, Power DA 2007 Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. J Mol Cell Cardiol 42:271–279
30. Nathan L, Shi W, Dinh H, Mukherjee TK, Wang X, Lusis AJ, Chaudhuri G 2001 Testosterone inhibits early atherogenesis by conversion to estradiol: critical role of aromatase. Proc Natl Acad Sci 98:3589–3593
31. Sasano H, Murakami H, Shizawa S, Satomi S, Nagura H, Harada N 1999 Aromatase and sex steroid receptors in human vena cava. Endocr J 46:233–242
32. Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn ME, Shaul PW 1999 Estrogen receptor α mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. J Clin Invest 103:401–406
33. Michell BJ, Griffiths JE, Mitchelhill KI, Rodriguez-Crespo I, Tiganis T, Bozinovski S, de Montellano PR, Kemp BE, Pearson RB 1999 The Akt kinase signals directly to endothelial nitric oxide synthase. Curr Biol 9:845–848
34. Sun M, Yang L, Feldman RI, Sun XM, Bhalla KN, Jove R, Nicosia SV, Cheng JQ 2003 Activation of phosphatidylinositol 3-kinase/Akt pathway by androgen through interaction of p85α, androgen receptor, and Src. J Biol Chem 278:42992–43000
35. Perusquía M, Hernández R, Morales MA, Campos MG, Villalón CM 1996 Role of endothelium in the vasodilating effect of progestins and androgens on the rat thoracic aorta. Gen Pharmacol 27:181–185
36. Sigler LH, Tulgan J 1943 Treatment of angina pectoris by testosterone propionate. NY State J Med 43:1424–1428

Geriatrics Gerontology 1st Impact Factor in June 2010 Medline Indexed

Geriatr Gerontol Int 2010; **10:** *32–39*

ORIGINAL ARTICLE: EPIDEMIOLOGY, CLINICAL PRACTICE AND HEALTH

Age-related changes in plasma androgen levels and their association with cardiovascular risk factors in male Japanese office workers

Shiho Fukai,[1] Masahiro Akishita,[1] Mariko Miyao,[1] Kiyoshi Ishida,[2] Kenji Toba[3] and Yasuyoshi Ouchi[1]

[1]Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, [2]Clinic, Asahi Mutual Life Insurance Company, and [3]Department of Geriatric Medicine, Kyorin University School of Medicine, Tokyo, Japan

Aim: To assess the age-related change in plasma androgen levels in healthy middle-aged men and whether any clinical parameters are associated with the hormonal change.

Methods: The study was comprised of male Japanese office-workers aged 40–64 years, who had undergone an annual health check-up in 2002 and 2007 (96 and 76 men, respectively). Body mass index and blood pressure were measured, and serum concentration of lipids, glucose and uric acid in addition to plasma total testosterone, free testosterone and dehydroepiandrosterone sulfate (DHEA-S) levels were determined in the morning after an overnight fast. The 5-year hormonal changes and their associations with clinical parameters were analyzed in 33 men who repeated the examination at both check-ups. The cross-sectional associations of hormonal levels with clinical parameters were also investigated.

Results: Age was negatively associated with free testosterone ($r = -0.399$, $P < 0.001$ in 2002; $r = -0.458$, $P < 0.001$ in 2007) and DHEA-S ($r = -0.233$, $P = 0.02$ in 2002; $r = -0.336$, $P < 0.01$ in 2007) but not with total testosterone, while the 5-year changes of free testosterone and DHEA-S levels were not significant and showed no associations with major cardiovascular risk factors. Cross-sectionally, after adjustment for age, linear regression analysis showed a positive association between free testosterone and blood hemoglobin and a negative association between total testosterone and serum uric acid.

Conclusion: In Japanese middle-aged men, 5-year androgen decline is too subtle to detect, and endogenous androgen levels seem to have relatively weak association with cardiovascular risk profiles. **Geriatr Gerontol Int 2010; 10: 32–39.**

Keywords: aging male, dehydroepiandrosterone sulfate, hypogonadism, testosterone.

Introduction

The concept of age-related androgen deficiency in men, namely late-onset hypogonadism (LOH),[1] has opened public awareness of the significance of men's health. LOH is not a rare condition in aging men, although often left undiagnosed and untreated. Recently, not only

Accepted for publication 1 July 2009.

Correspondence: 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

 | doi: 10.1111/j.1447-0594.2009.00552.x

endocrinologists but also geriatricians and general physicians are required to be more vigilant in diagnosing and treating symptomatic hypogonadism as the front line of health-care delivery. Based on current guidelines,[1] assessing plasma testosterone levels are recommended when an adult man exhibits signs of hypogonadism such as feelings of low energy, decreased libido, decreased muscle strength, and also as part of normal medical screening in men starting at age 40–50 years to establish a baseline. The application of androgen replacement therapy (ART) should be discussed for symptomatic patients with low testosterone level. However, reliable standard values of androgen levels in healthy men are essential to determine the application of ART.

Age-associated decline in plasma androgen concentration has been confirmed by a large series of cross-sectional studies. Reference range of serum total testosterone (TT) and free testosterone (FT) in Japanese male adults has also been established.[2] Several longitudinal studies have also found that TT and FT concentrations fall by 0.8% and 2% per year, respectively, in middle-aged men, contributing to an increasing prevalence of LOH with advancing age.[3–5] These surveys, however, have been performed based on samples of Caucasian men in the Western countries; therefore, additional data are needed to elucidate the longitudinal change in Japanese middle-aged men, especially whose testosterone levels are declining gradually.

In addition to the age-associated decline in plasma androgen levels, large inter- subject variations are seen at all ages. Although the mechanisms of this variability have not been completely elucidated, several physiological factors or chronic diseases such as obesity,[6–8] diabetes mellitus,[9–13] hypertension[14–17] and dyslipidemia[12,14,18] appear to play a role. In the present study, we aimed first to assess the age-related change in plasma androgen levels in healthy Japanese middle-aged men, and second to elucidate whether any clinical parameters measured in health check-ups are associated with the hormonal change both in cross-sectional and longitudinal surveys.

Methods

Study design and participants

This survey was conducted as a part of annual health check-ups of office workers at a company located in Tokyo, Japan, in 2002 and 2007. A total of 139 men aged 40–64 years who had undergone the health check-up (96 men in 2002 [mean ± standard deviation age = 52.7 ± 5.9] and 76 men in 2007 [51.5 ± 6.8 years]) were enrolled. Among the 96 men who attended in 2007, 33 men repeated the check-up in 2007 (Fig. 1). Those under testosterone or dehydroepiandrosterone (DHEA) replacement, those being treated with androgen deprivation therapy and those with malignancies including prostate cancer were excluded. The study protocol was approved by the ethics committee of the Graduate School of Medicine, University of Tokyo. Each subject gave a written informed consent for the enrollment in this study.

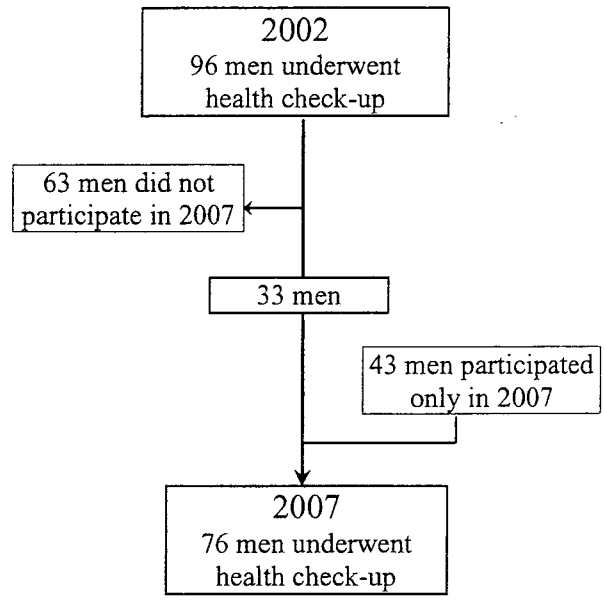

Figure 1 Participant flow diagram.

Annual health check-ups

A history was taken, physical examination including brachial blood pressure in addition to laboratory tests were performed in all subjects. Height and weight were measured with participants wearing light clothing without shoes, and body mass index (BMI) was calculated as weight in kg/m^2. Waist circumference was measured only in 2007. Blood sampling was performed in the morning (10.00–12.00 hours) of the health check-up after an overnight fast, to measure plasma hormones in addition to blood cell counts and blood chemical parameters.

Laboratory assays

Blood cell counts and blood chemical parameters were assayed by a commercial laboratory (Byotaiseiri Laboratory, Tokyo, Japan). Plasma TT and DHEA sulfate (DHEA-S) concentrations were determined using sensitive radioimmunoassay (RIA) in 2002 and chemiluminescence immunoassays (CLIA) method in 2007 by commercial laboratories (SBS [Tokyo, Japan] and SRL [Tokyo, Japan], respectively). In order to compare the measurements in both years, the following conversion formulas provided by SRL were applied: y (CLIA) =

1.03 × (RIA) + 14, the coefficient of determination (R^2) was 0.96 for TT and y (CLIA) = 0.10 × (RIA) + 2.23, R^2 was 0.97 for DHEA-S. Plasma FT concentrations were measured using the RIA method in both years, with the same measurement reagent (Mitsubishi Kagaku Iatron, Tokyo, Japan), although in different laboratories; SBS in 2002 and SRL in 2007. Hence, the following conversion formula was applied to adjust the slight difference between the two laboratories: y(SRL) = 0.95 (SBS) + 0.94, with an R^2 of 0.98. The intra-assay coefficients of variation for these measurements were less than 5%.

Statistical analysis

Data were analyzed using SPSS statistical software (ver. 11.0). The values are expressed as the means ± standard deviation in the text. Pearson's correlation coefficients were used to assess the relationship between plasma androgen levels and age. A paired Student's *t*-test was used to assess the 5-year change in hormone levels and clinical and biochemical parameters. Differences in the characteristics of the subjects of two health check-up year groups were compared with an unpaired Student's *t*-test. Standardized regression coefficients from multivariate linear regression analysis of potential cardiovascular disease risk profiles in relation to age and plasma androgen levels were determined. $P < 0.05$ was considered statistically significant.

Results

The subject profiles of 139 men who underwent annual health check-ups in 2002 and in 2007 are summarized in Table 1. Of 2002 and 2007 subjects, 27% and 29% were obese (BMI > 25) and 34% and 38% had hypertension, respectively. None of the subjects had a TT level below 8 nmol/L (the range may benefit from testosterone treatment[1]), and only 4.2% and 5.3% of subjects in each year (aged 47–58 years in 2002 and 44–59 years in 2007) had TT levels between 8 and 12 nmol/L (the range might require hormone therapy after ruling out other causes[1]).

In cross-sectional analysis, using the linear regression model, age was negatively associated with plasma concentration of FT and DHEA-S but not with TT (Fig. 2). The estimated 5-year decline of plasma hormone levels, calculated with the linear regression equations from the samples in 2002 and 2007, were −1.27 and −1.29 pg/mL (−4.41 and −4.50 pmol/L) for FT, and −16.1 and −23.4 μg/dL (−0.44 and −0.64 μmol/L) for DHEA-S, respectively. However, the 5-year changes of FT and DHEA-S levels were not significant in the longitudinal survey of 33 men, which was started when the average age of the subjects was 49 years (Table 2). Moreover, regression analysis showed no significant associations between each hormonal change and neither major cardiovascular risk factors at baseline nor their 5-year changes (data not shown).

Cross-sectionally, after adjustment for age, linear regression analysis demonstrated a positive association between FT and hemoglobin and a negative association between TT and uric acid, while androgen levels did not show statistical interactions with other health parameters (Table 3). Multiple regression analysis revealed that the negative association between TT and uric acid level was independent of age, BMI, estimated glomerular filtration rate and blood hemoglobin ($\beta = -0.257$, $P < 0.01$ in 2002; $\beta = -0.226$, $P < 0.05$ in 2007). The

Table 1 Characteristics of subjects in 2002 and 2007

Characteristics	Subjects in 2002	Subjects in 2007	*P*-value
n	96	76	
Age, years	52.7 ± 5.9	51.5 ± 6.8	0.234
Systolic blood pressure, mmHg	127 ± 16	124 ± 13	0.189
Diastolic blood pressure, mmHg	79 ± 10	80 ± 12	0.430
Body mass index, kg/m^2	23.6 ± 2.7	24.0 ± 3.0	0.301
Total cholesterol, nmol/L	5.34 ± 0.75	5.67 ± 0.73	0.005
HDL cholesterol, nmol/L	1.42 ± 0.31	1.58 ± 0.39	0.003
Triglyceride, nmol/L	1.56 ± 1.03	1.54 ± 1.08	0.924
Fasting glucose, nmol/L	5.77 ± 1.33	5.44 ± 0.89	0.060
Plasma hormone levels			
Total testosterone, nmol/L	22.0 ± 5.1	20.8 ± 5.6	0.167
Free testosterone, pmol/L	46.8 ± 12.8	55.9 ± 13.5	<0.001
DHEA-S, μmol/L	5.5 ± 2.2	6.2 ± 2.6	0.052

Data are shown as mean ± standard deviation. *P*-values represent differences between measurements of 2002 and 2007 with unpaired Student's *t*-test. DHEA-S, dehydroepiandrosterone sulfate; HDL, high-density lipoprotein.

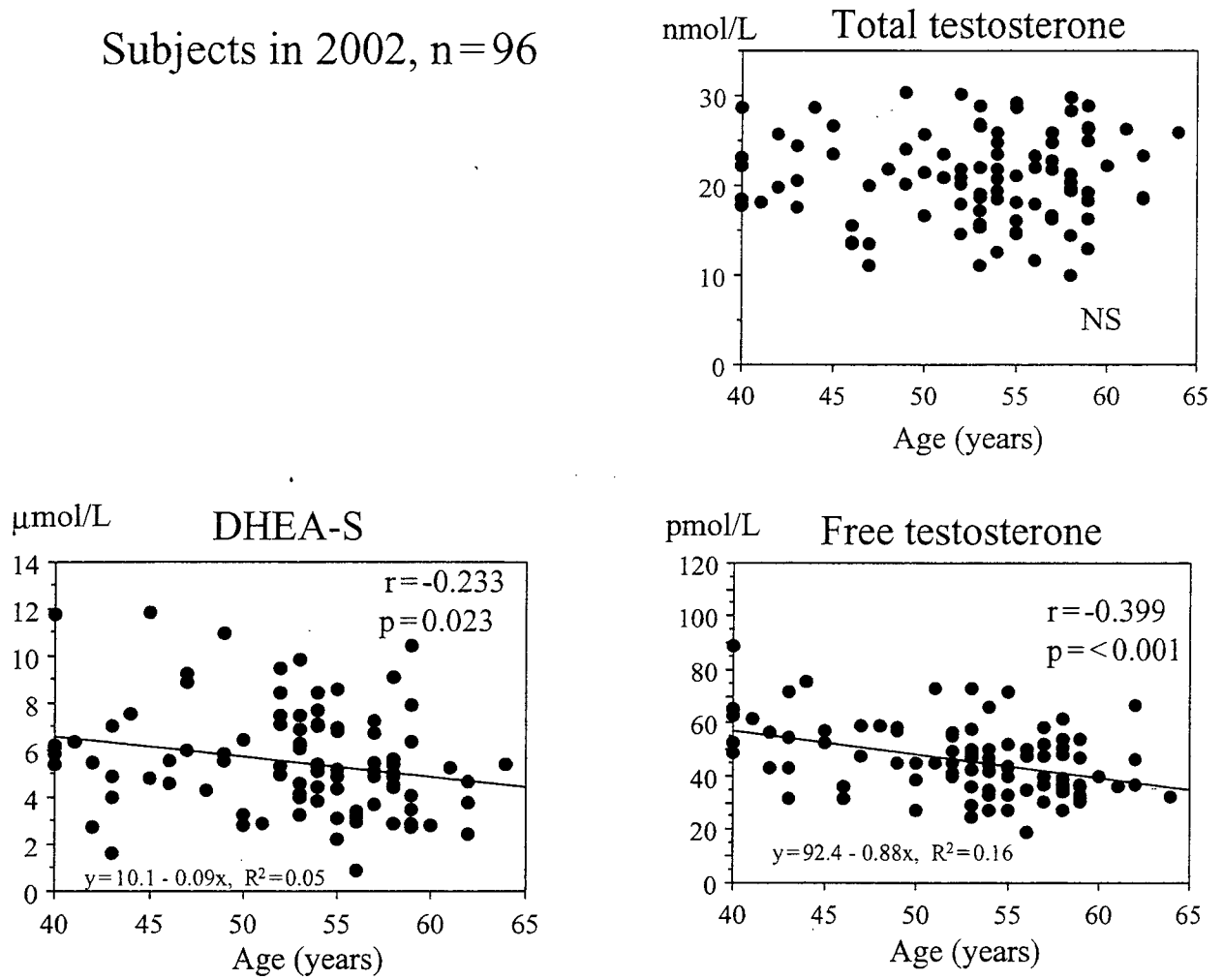

Figure 2 Scatter plots illustrate the correlations between age and plasma androgen concentrations. DHEA-S, dehydroepiandrosterone sulfate; NS, not significant.

statistical result was similar after waist circumference was entered as a covariate into the multiple regression model instead of BMI in 2007 ($\beta = -0.234$, $P < 0.05$). With respect to the association between FT and hemoglobin, there was a significant correlation after adjusting for age and BMI in the subjects in 2007 ($\beta = 0.288$, $P < 0.01$); however, statistical significance disappeared in the subjects in 2002 ($\beta = 0.140$, $P = 0.176$).

Discussion

The present study demonstrated that 5-year androgen decline from age 40–50 years among Japanese middle-aged men is very small with large individual variations. In addition, in these healthy men, endogenous androgen levels were not significantly associated with cardiovascular risk profiles neither cross-sectionally nor longitudinally, except that TT was inversely correlated with serum uric acid in both 2002 and 2007 subjects.

Despite the results from previous longitudinal studies that TT, FT and DHEA-S concentrations decline with aging in men;[3–5,19,20] the 5-year changes in these hormonal levels were not significant in the present study. The estimated annual decline of FT level, calculated with the linear regression equations from our cross-sectional samples (Fig. 2), was approximately –2.5% per year. However, the number of samples needed in the longitudinal study was calculated to be approximately 91 in power analysis, and the sample size was not enough to enable statistical judgment due to large standard deviation. Further, although not significantly, the mean FT level increased after 5 years compared to baseline; this could be attributed to the relatively short follow-up period or to too small number of subjects to detect the subtle hormonal decline with considerably large between-subject variations.

In the current study, as shown in Table 3, no clear associations were observed between plasma androgen

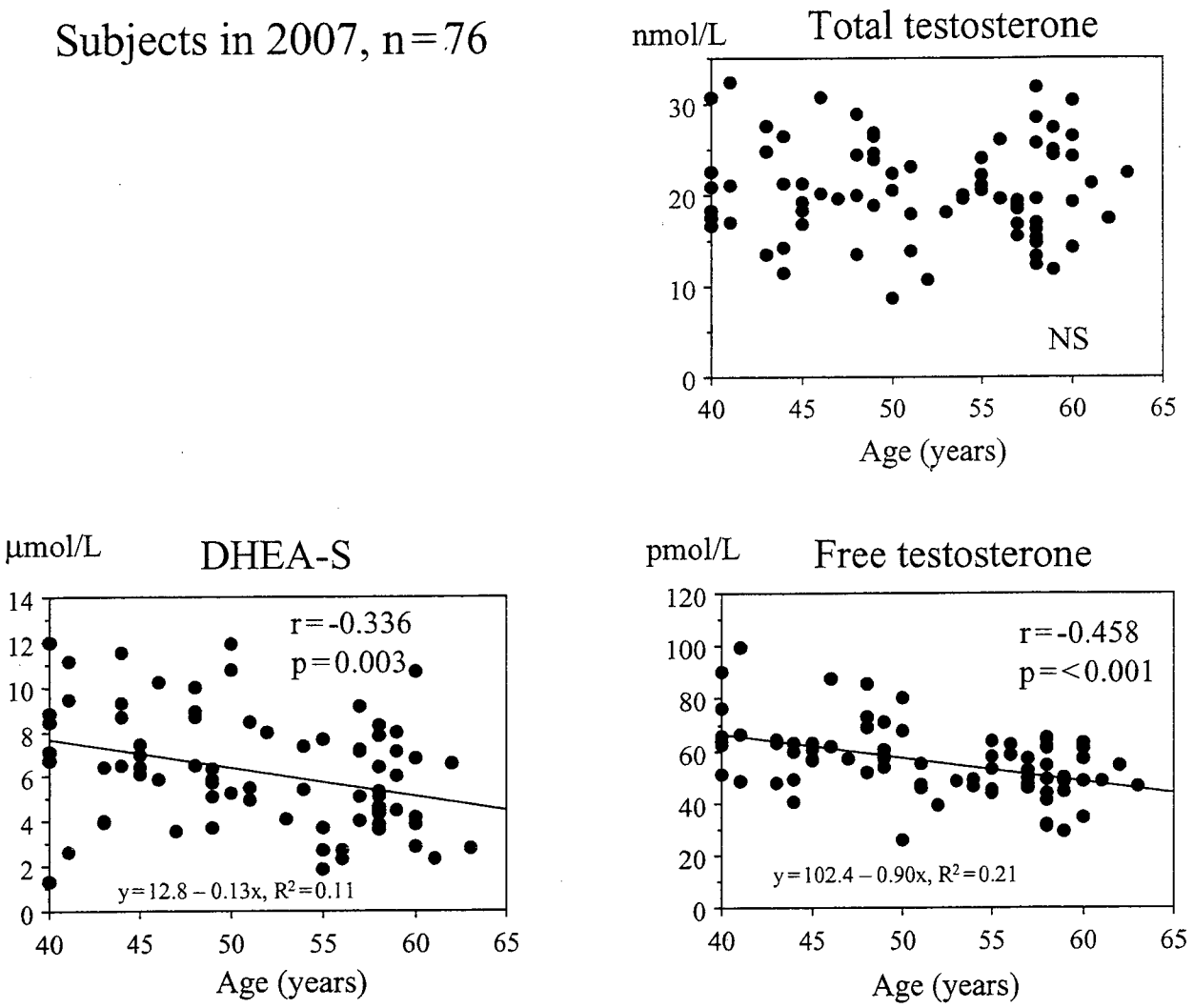

Figure 2 Continued.

levels and the cardiovascular risk profiles. There were some inconsistent results in 2002 and 2007, such as the association between DHEA-S and blood pressure. These associations are relatively weak and lack reproducibility, although statistically significant; thus, we think that the association between androgen levels and cardiovascular risk profiles are not of clinical significance. Low testosterone level in men has been inversely related to obesity or waist circumference in the previous studies;[6–8,21] however, others studies showed no correlation.[22,23] Besides obesity, low testosterone level has been associated with components of the metabolic syndrome, and a bidirectional interaction has been found between decline of testosterone and the metabolic syndrome.[24,25] In the present study, only 27% and 29% of the subjects (in 2002 and in 2007, respectively) were obese, and the substantial proportion of the subjects were normotensive, not diabetic, without dyslipidemia, and only 10% of the subjects in 2007 (when waist circumference measurements were obtained) were diagnosed with metabolic syndrome. More than 95% of our subjects showed plasma TT and FT levels within the reference range;[2,26] thus, the associations of cardiovascular risks with androgen levels might be blunted in non-obese, relatively healthy middle-aged men.

Although weak, a positive association between FT and blood hemoglobin was found, and it could be attributed to testosterone's erythropoietic activity. The stronger association of hemoglobin with active form of testosterone (bioavailable T) rather than with TT level has also been reported.[27] Testosterone had been postulated to act directly on bone marrow at the level of polychromatophilic erythroblasts and enhance the synthesis of ribosomal RNA or its precursors, and

Table 2 Clinical and biochemical characteristics and sex hormone levels at baseline and after 4 years in 33 middle-aged men

Characteristics	Baseline	After 5 years	*P*-value
Age, years	49.3 ± 5.4 (40–58)	54.3 ± 5.4 (45–63)	
Body mass index, kg/m^2	23.5 ± 2.8 (18.3–30.4)	23.3 ± 2.8 (18.3–30.7)	0.391
Systolic blood pressure, mmHg	121 ± 17 (90–152)	124 ± 15 (95–164)	0.142
Diastolic blood pressure, mmHg	78 ± 10 (60–100)	78 ± 12 (54–110)	0.842
Total cholesterol, mmol/L	5.12 ± 0.78 (4.27–7.40)	5.49 ± 0.57 (5.49–7.40)	0.006
HDL cholesterol, mmol/L	1.45 ± 0.24 (1.06–1.97)	1.48 ± 0.28 (1.12–2.15)	0.133
Triglyceride, mmol/L	1.28 ± 0.54 (0.53–2.46)	1.34 ± 0.99 (0.54–5.18)	0.734
Fasting glucose, mmol/L	5.66 ± 1.44 (4.44–11.98)	5.55 ± 1.11 (3.61–9.32)	0.565
Uric acid, mmol/L	0.35 ± 0.07 (0.16–0.48)	0.36 ± 0.07 (0.21–0.49)	0.129
Plasma hormone levels			
Total testosterone, nmol/L	21.8 ± 4.7 (11.9–30.5)	20.7 ± 5.6 (8.7–31.9)	0.227
Free testosterone, pmol/L	49.6 ± 13.2 (27.0–75.3)	53.6 ± 11.8 (26.0–85.4)	0.110
DHEA-S, μmol/L	5.5 ± 2.2 (2.1–11.4)	5.4 ± 2.1 (2.3–11.4)	0.508

Data are shown as mean ± standard deviation (range). *P*-values represent differences between measurements of 2002 and 2007 with paired Student's *t*-test. DHEA-S, dehydroepiandrosterone sulfate; HDL, high-density lipoproteins.

Table 3 Age-adjusted association between plasma androgen levels and potential cardiovascular disease risk profile of middle-aged men from cross-sectional samples in both years

	Total testosterone		Free testosterone		DHEA-S	
	2002	2007	2002	2007	2002	2007
Body mass index	−0.188	−0.087	0.030	0.103	0.046	0.082
Waist circumference	NE	−0.081	NE	0.109	NE	−0.021
Systolic blood pressure	−0.056	−0.177	0.169	0.108	0.077	0.237*
Diastolic blood pressure	−0.078	−0.088	0.124	0.147	0.109	0.295*
Hemoglobin	0.116	0.053	0.263*	0.367**	0.090	0.349**
Fasting glucose	−0.045	−0.040	−0.068	−0.121	−0.182	−0.076
Total cholesterol	0.028	−0.149	0.178	0.037	−0.084	0.122
HDL cholesterol	−0.063	0.083	−0.050	−0.149	−0.037	−0.107
Triglyceride	−0.030	−0.193	0.274*	0.201	0.128	0.258*
Uric acid	−0.259*	−0.245*	−0.030	−0.109	0.303**	0.113

*$P < 0.05$, **$P < 0.01$. Data are presented as standardized regression coefficients. DHEA-S, dehydroepiandrosterone sulfate; HDL, high-density lipoproteins; NE, not examined.

stimulate nuclear ribonuclease,[28] by stimulating erythropoietin[29,30] or in other erythropoietin-independent mechanisms.[27,31,32]

Cross-sectionally, after adjustment for age, linear regression analysis showed a negative association between TT and serum uric acid. To date, most observational studies have found a positive association, while a few have reported a negative association,[33] between uric acid and testosterone. Also, androgen deprivation therapy showed reduced uric acid level.[34] Moreover, one prospective study has demonstrated that uric acid levels had fallen significantly in male- to-female transsexual persons after 1 year of hormone therapy.[35] These results have been attributed to purine production induced by testosterone in addition to estrogen's uricosuric (enhancement in urinary urate excretion) effect, and also thought to be the reason for the sex difference in serum uric acid levels between men and premenopausal women.[36,37] On the contrary, our results have shown a weak negative association between TT and serum uric acid in both 2002 and 2007. Several studies suggested that over-inflow of free fatty acid to the liver from visceral fat may be linked to de novo purine synthesis, which may accelerate the uric acid production,[38,39] and that hyperinsulinemia may reduce urinary urate excretion in parallel with a decrease in urinary sodium excretion.[40] In fact, simple regression analysis revealed that uric acid was correlated with BMI ($r = 0.431$, $P < 0.001$ in 2002; $r = 0.245$, $P < 0.05$ in 2007) and with waist circumference ($r = 0.221$, $P < 0.05$

in 2007). However, multiple regression analysis revealed that the negative association between TT and uric acid level was independent of BMI and waist circumference. Insulin level was not measured in the present study; however, a future study should further investigate whether insulin resistance would be an intervening factor between TT and uric acid, and longer longitudinal studies and ART could resolve this question.

A limitation of the present study should be noted. The number of subjects and the length of follow-up period might have been insufficient. In order to detect the subtle hormonal decline with considerably large between-subject variations, more subjects and a longer period are required.

In summary, in a group of Japanese middle-aged men with normal range of androgen levels, 5-year androgen decline was not significant. Also, endogenous androgen levels were not associated with cardiovascular risk factors in these relatively healthy subjects. However, we still do not have enough information about the critical level for starting ART, optimal dose, target testosterone level to be reached or long-term safety; thus, we suggest further investigation into the longitudinal change in hormonal levels in Japanese healthy men with aging.

Acknowledgments

We would like to thank Ms Sonoko Omi for her technical assistance. This study was supported by grants from Health and Labor Sciences Research Grants (H17-Choju-046, H18-Choju-031) from the Ministry of Health, Labor and Welfare of Japan; a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan (20249041); grants from NOVARTIS Foundation for Gerontological Research and Yamaguchi Endocrine Research Association and Mitsui Sumitomo Insurance Welfare Foundation.

References

1 Wang C, Nieschlag E, Swerdloff RS *et al.* ISSAM ISA, EAU, EAA and ASA recommendations, investigation, treatment and monitoring of late-onset hypogonadism in males. *Eur J Endocrinol* 2008; **159**: 507–514.
2 Iwamoto T, Yanase T, Horie H, Namiki M, Okuyama A. Late-onset hypogonadism (LOH) and androgens: Validity of the measurement of free testosterone levels in the diagnostic criteria in Japan. *Int J Urol* 2009; **16**: 168–174.
3 Feldman HA, Longcope C, Derby CA *et al.* Age trends in the level of serum testosterone and other hormones in middle-aged men: longitudinal results from the Massachusetts Male Aging Study. *J Clin Endocrinol Metab* 2002; **87**: 589–598.
4 Harman SM, Metter EJ, Tobin JD *et al.* Longitudinal effects of aging on serum total and free testosterone levels in healthy men: Baltimore Longitudinal Study of Aging. *J Clin Endocrinol Metab* 2001; **86**: 724–731.
5 Muller M, den Tonkellar I, Thijssen JHH, Grobbee DE, van der Schouw YT. Endogenous sex hormones in men aged 40–80 years. *European Journal of Endocrinology* 2003; **149**: 583–589.
6 Wajchenberg BL. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocr Rev* 2000; **21**: 697–738.
7 Couillard C, Gagnon J, Bergeron J *et al.* Contribution of body fatness and adipose tissue distribution to the age variation in plasma steroid hormone concentrations in men: the HERITAGE family study. *J Clin Endocrinol Metab* 2000; **85**: 1026–1031.
8 Khaw KT, Barrett-Connor E. Lower endogenous androgens predict central adiposity in men. *Ann Epidemiol* 1992; **2**: 675–682.
9 Barrett-Connor E, Khaw KT, Yen SS. Endogenous sex hormone levels in older adult men with diabetes mellitus. *Am J Epidemiol* 1990; **132**: 895–901.
10 Andersson B, Vermeulen A, Marin P, Bjorntorp P, Lissner L. Testosterone concentrations in women and men with NIDDM. *Diabetes Care* 1994; **17**: 405–411.
11 Chang TC, Tung CC, Hsiao YL. Hormonal changes in elderly men with non-insulin-dependent diabetes-mellitus and the hormonal relationships to abdominal adiposity. *Gerontology* 1994; **40**: 260–267.
12 Simon D, Charles MA, Nahoul K *et al.* Association between plasma total testosterone and cardiovascular risk factors in healthy adult men: the Telecom Study. *J Clin Endocrinol Metab* 1997; **82**: 682–685.
13 Stellato RK, Feldman HA, Hamdy O *et al.* Testosterone, sex hormone? binding globulin, and the development of type 2 diabetes in middle-aged men: prospective results from the Massachusetts Male Aging Study. *Diabetes Care* 2000; **23**: 490–494.
14 Barrett-Connor E, Khaw KT. Endogenous sex hormones and cardiovascular disease in men. A prospective population-based study. *Circulation* 1988; **78**: 539–545.
15 Fogari R, Preti P, Zoppi A *et al.* Serum testosterone levels and arterial blood pressure in the elderly. *Hypertens Res* 2005; **28**: 625–630.
16 Khaw KT, Barrett-Connor E. Blood pressure and endogenous testosterone in men: an inverse relationship. *J Hypertens* 1988; **6**: 329–332.
17 Hughes GS, Mathur RS, Margolius HS. Sex steroid hormones are altered in essential hypertension. *J Hypertens* 1989; **7**: 181–187.
18 Hak AE, Witteman JC, de Jong FH *et al.* Low levels of endogenous androgens increase the risk of atherosclerosis in elderly men: the Rotterdam study. *J Clin Endocrinol Metab* 2002; **87**: 3632–3639.
19 Bjornerem A, Straume B, Midtby M *et al.* Endogenous sex hormones in relation to age, sex, lifestyle factors, and chronic diseases in a general population: the Tromso Study. *J Clin Endocrinol Metab* 2004; **89**: 6039–6047.
20 Tannenbaum C, Barrett-Connor E, Laughlin GA *et al.* A longitudinal study of dehydroepiandrosterone sulphate (DHEAS) change in older men and women: the Rancho Bernardo Study. *Eur J Endocrinol* 2004; **151**: 717–725.
21 Svartberg J, von Mühlen D, Sundsfjord J, Jorde R. Waist circumference and testosterone levels in community dwelling men. The Tromsø study. *Eur J Epidemiol* 2004; **19**: 657–663.
22 Pasquali R, Casimirri F, Cantobelli S *et al.* Effect of obesity and body fat distribution on sex hormones and insulin in men. *Metabolism* 1991; **40**: 101–104.
23 Leenen R, van der Koy K, Seidell JC, Deurenberg P, Kopperschaar HPF. Visceral fat accumulation in relation