the ER Ca^{2+}-ATPase inhibitors (21) thapsigargin and cyclopiazonic acid, the ryanodine receptor activator 4-chloro-*m*-cresol, and the protein *N*-glycosylation inhibitor tunicamycin all induced WFS1 protein as shown in Fig. 3. Only brefeldin A had no effect. Ionomycin only weakly induced WFS1 protein. The differing effects of these chemicals, which have different mechanisms of action, may provide insights into the functions of Wfs1. The lack of WFS1 induction with brefeldin A, a Golgi apparatus disruptor, may be related to its instability in solution (22). Although we did not perform Northern blot analysis for each of these reagents, A23187 induced *WFS1* mRNA in fibroblasts (data not shown).

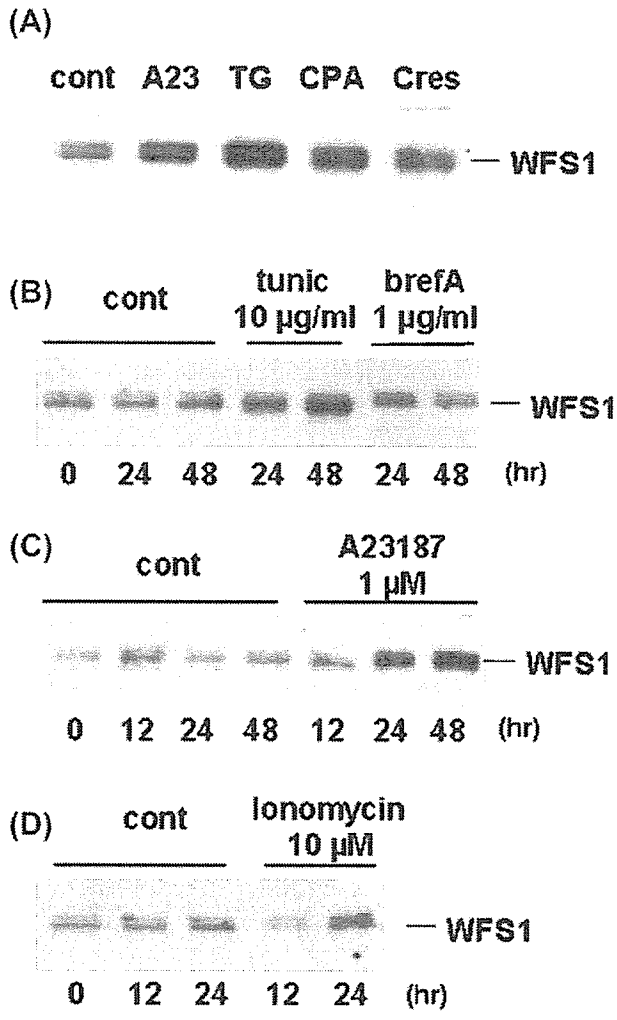

Figure 3 ER stress induces WFS1 protein in fibroblasts. Primary skin fibroblasts were cultured in the presence of (A) A23187 (A23, 1 μmol/l), thapsigargin (TG, 1 μmol/l), cyclopiazonic acid (CPA, 10 μmol/l) and 4-chloro-*m*-cresol (cres, 50 μmol/l) for 48 h. (B–D) Cells were treated for the indicated time periods with (B) tunicamycin (tunic), brefeldin A (brefA), (C) A23187, and (D) ionomycin. cont indicates control. Control samples included a vehicle (DMSO), also administered with all of the drugs. Cells were harvested after the incubation periods, and total cell lysates containing 20 μg protein were subjected to Western blot analysis using anti-WFS1c antibody.

Effects of thapsigargin and tunicamycin on Wfs1 *expression in MIN6 cells*

We next examined the effects of thapsigargin and tunicamycin on the expression of *Wfs1* mRNA in MIN6 cells. Thapsigargin and tunicamycin treatments are known to induce ER stress, and Chop/GADD153 is a transcription factor that plays a role in ER stress-induced apoptotic cell death (23, 24). Phosphorylation of the α-subunit of translation initiation factor-2 (eIF2-α) attenuates protein translation upon ER stress. Although the ER chaperone Bip/GRP78 expression did not change in MIN6 cells (Fig. 4B) probably due to its strong basal expression, thapsigargin and tunicamycin clearly generated ER stress as demonstrated by Chop induction and eIF2-α phosphorylation (Fig. 4A, B). Under these conditions, ER stress-induced caspase-3 activation, an event at the initiation of apoptosis (25), was evidenced by the cleavage of PARP (Fig. 4C). PARP is one of the substrates cleaved by caspase-3. Upon thapsigargin or tunicamycin treatment, the 113 kDa band decreased, and instead, the proteolytic PARP fragment (89 kDa) appeared (Fig. 4C). In association with ER stress induction and caspase-3 activation, *Wfs1* mRNA expression increased (Fig. 4A,D). With thapsigargin, *Wfs1* mRNA started to increase after 6 h and was maximal after 12 h. With tunicamycin, *Wfs1* mRNA induction peaked at 6 h, and then declined. Wfs1 protein was also increased by thapsigargin treatment (Fig. 4B). In contrast, tunicamycin, despite the mRNA induction, did not increase the Wfs1 protein, but decreased it after 24 h (Fig. 4B). This is probably due to the instability of unglycosylated Wfs1 protein (6, 16).

Thapsigargin and tunicamycin enhance human WFS1 *promoter activity in MIN6 cells*

To determine the mechanism of *WFS1* expression, we examined the effects of thapsigargin and tunicamycin on human *WFS1* gene promoter activity by employing transient transfection assays in MIN6 cells. We used a *WFS1* promoter-luciferase construct that contained a 3 kb DNA sequence upstream from the human *WFS1* gene transcription initiation site. The human *WFS1* gene promoter was active in MIN6 cells. Introduction of the *WFS1* promoter-reporter plasmid produced a 20-fold increase in luciferase activity as compared with the promoterless pGL3-Basic vector. Treatment of the cells with thapsigargin or tunicamycin resulted in further 1.3- and 1.5-fold increases in luciferase activity respectively (Fig. 5). We conducted these experiments again using a 1 kb (−1000 to +20) *WFS1* promoter-luciferase reporter gene. The results were essentially the same but the promoter activity was weaker than with the 3 kb construct (data not shown).

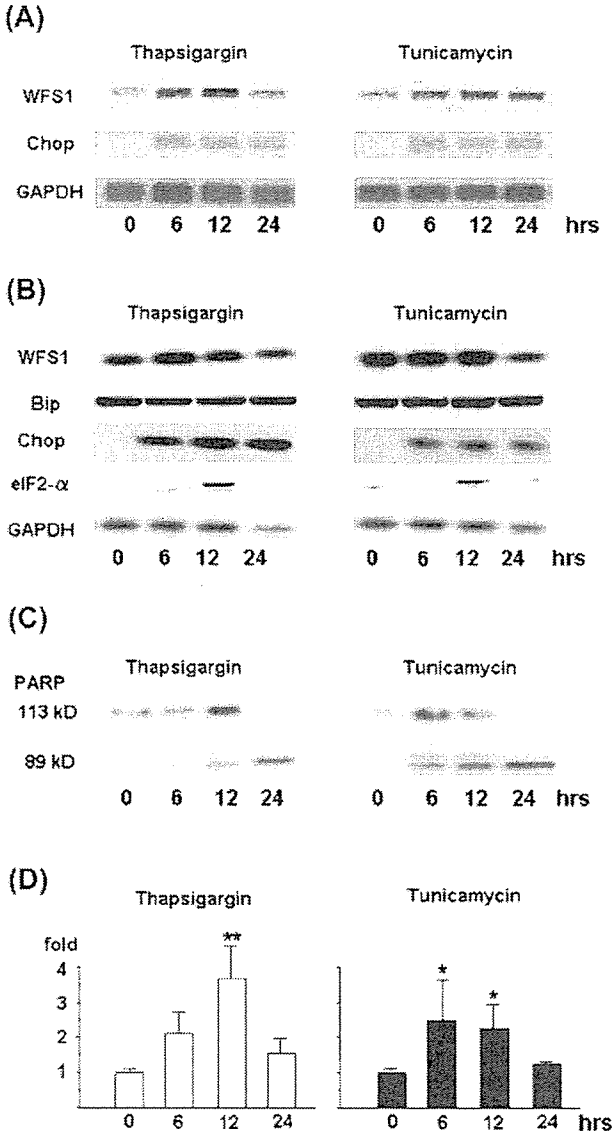

Figure 4 Thapsigargin and tunicamycin increase *Wfs1* mRNA expression in MIN6 cells in association with ER stress and apoptosis induction. MIN6 cells were placed in culture dishes and serum-starved in serum-free DMEM for 12 h, and then treated with thapsigargin (1 μmol/l) or tunicamycin (10 μg/ml) for 6, 12 or 24 h. Dimethyl sulfoxide (DMSO) was used to dissolve thapsigargin and tunicamycin, and the same concentration of DMSO (final, 0.05%) was employed in all experiments, including controls. After incubation, cells were washed once with ice-cold phosphate-buffered saline, and harvested. (A) Ten micrograms RNA were subjected to Northern blot analysis. (B) Total cell lysates containing equal amounts of protein (50 μg) were separated on 10% SDS-PAGE and analyzed by immunoblotting using anti-Wfs1n, anti-Bip (GRP74), anti-Chop or anti-phosphorylated eIF2-α, (C) Total cell lysates containing equal amounts of protein (50 μg) were separated on 10% SDS-PAGE and analyzed by immunoblotting using the anti-PARP antibody. Activated caspase-3 cleaves the 113 kDa PARP, resulting in the appearance of the 89 kDa fragment. (D) Quantification of the *Wfs1* mRNA from the results obtained in (A), shown as means±S.E. ($n = 4$). Statistical analysis, conducted using analysis of variance, indicated that the thapsigargin and tunicamycin treatments significantly increased *Wfs1* mRNA expression at 12 h and at 6 h and 12 h respectively (*$P < 0.05$, **$P < 0.001$).

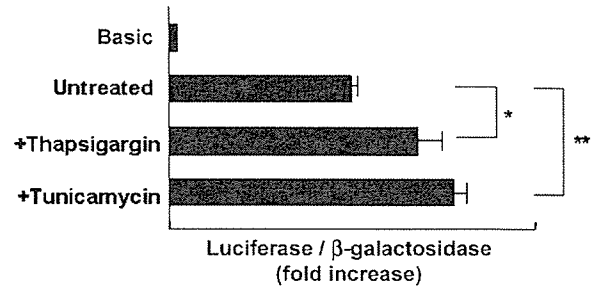

Figure 5 ER stress enhances *WFS1* promoter activity in MIN6 cells. MIN6 cells were transfected with a luciferase reporter plasmid containing a 3.0 kb human *WFS1* gene 5' flanking promoter region (from −3000 to +20) and were exposed to thapsigargin (1 μmol/l) or tunicamycin (10 μg/ml) for 6 h. Beta-galactosidase activity from the co-transfected expression vector pCMVβ was used to calibrate for transfection efficiency. Basic represents luciferase activity from pGL3-Basic (promoterless) vector-transfected cells. Results are expressed as the fold increase as compared with basic (means±S.E. of four independent experiments, each performed in triplicate). *P* values for comparison of results with versus without drug treatments are 0.034 (thapsigargin, *) and 0.005 (tunicamycin, **) (analysis of variance).

Wfs1 *expression is transcriptionally upregulated in β-cells with intrinsic ER stress*

In the Akita mouse, the C96Y mutation of the *ins2* gene disturbs intramolecular disulfide bond formation, resulting in progressive β-cell loss (12). ER stress and subsequent apoptosis are at least partially responsible for this progressive β-cell loss (14). To further examine the association between increased *Wfs1* expression and ER stress, we used mouse insulinoma cells derived from an Akita mouse homozygous for the *ins2* gene C96Y mutation (*Ins2* $^{96Y/Y}$ cell) as a model. *Ins2* $^{WT/WT}$ cells derived from normal littermates served as controls. Doubling of the ER chaperone Bip/GRP78 in *Ins2* $^{96Y/Y}$ cells indicated persistent ER stress in these cells (Fig. 6A). In Ins2$^{96Y/Y}$ cells, Wfs1 protein increased sixfold as compared with that in *Ins2* $^{WT/WT}$ cells (Fig. 6B). *Wfs1* mRNA expression was also increased twofold (data not shown). We next examined *WFS1* promoter activity in these cells. Introduction of the *WFS1* promoter-reporter plasmid into *Ins2* $^{96Y/Y}$ cells approximately doubled luciferase activity as compared with that in wild type *Ins2* $^{WT/WT}$ cells (Fig. 7). Luciferase activity after transfection of the SV40 promoter-reporter plasmid did not differ between *Ins2* $^{96Y/Y}$ and *Ins2* $^{WT/WT}$ cells.

Discussion

Herein, we have documented the localization of Wfs1 expression in the mouse pancreatic islet. Insulin-producing β-cells are the major site of Wfs1 expression, as shown in Ishihara *et al.* (15). Wfs1 expression is also evident in somatostatin-producing δ-cells, but is absent from glucagon producing α-cells and PP-cells. No *Wfs1* expression is observed in pancreatic exocrine acinar

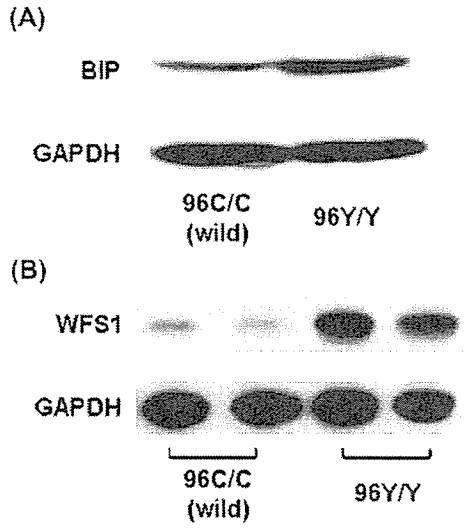

Figure 6 *WFS1* expression is increased in Akita mouse-derived *Ins2*$^{96Y/Y}$ cells. Cell extracts of *Ins2*$^{96Y/Y}$ cells containing equal amounts of protein (50 μg) were separated on 10% SDS-PAGE and analyzed by immunoblotting using (A) anti-Bip, and (B) anti-WFS1n and anti-GAPDH antibodies. Insulinoma cells derived from wild type littermates (wild) were used as the control. In (B), cell extracts were prepared on two separate occasions from cells derived from the same mutant mouse.

cells. A histopathological study of pancreatic islets from Wolfram syndrome patients showed selective loss of insulin-producing β-cells and an apparent preservation of glucagon-producing α-, somatostatin-producing δ-, and PP-cells (26, 27). The histochemical evidence of Wfs1 protein localization in insulin-producing β-cells might provide a histological background explaining the insulin deficiency caused by *WFS1* mutations in Wolfram syndrome patients and suggests that WFS1 protein is necessary for β-cell (28, 29), but not δ-cell survival.

We have also presented evidence herein that ER stress induces *Wfs1* gene expression. Treatment of fibroblasts with A23187, ionomycin, thapsigargin, cyclopiazonic acid, 4-chloro-*m*-cresol or tunicamycin increased *Wfs1* protein levels. Chemical insults by these reagents are known to induce ER stress via disruption of Ca^{2+} homeostasis or inhibition of N-linked glycosylation. Thapsigargin and tunicamycin treatments also induced *Wfs1* mRNA expression in a mouse β-cell line, MIN6 cells. In accordance with the mRNA change, thapsigargin increased Wfs1 protein expression. However, the Wfs1 protein level in MIN6 cells did not change with tunicamycin. This is probably due to Wfs1 being an N-glycosylated protein, and inhibition of glycosylation by tunicamycin decreases its stability (6, 16). Increased *Wfs1* expression in association with ER stress was further demonstrated in another β-cell model with ER stress: *Ins2*$^{96Y/Y}$ cells derived from the Akita mouse. The Akita mouse spontaneously develops early-onset non-obese diabetes with a reduced β-cell mass, which is caused by a conformation-altering missense mutation (Cys96Tyr) in the insulin-2 gene (12, 13). Intramolecular disulfide-bond formation is disrupted in the mutant insulin molecule. It was reported that this misfolded mutant insulin expression constitutively induced ER stress in Akita mouse β-cells (14). We have indeed confirmed increased Bip protein expression in *Ins2*$^{96Y/Y}$ cells as compared with wild type *Ins*$^{WT/WT}$ cells derived from normal littermates. In *Ins2*$^{96Y/Y}$ cells, *Wfs1* mRNA (data not shown) and protein levels (Fig. 6) were both increased. The increased *Wfs1* mRNA (twofold, data not shown) was consistent with the increased *Wfs1* promoter activity (Fig. 7). Our results provide further evidence, i.e. a detailed analysis, that *Wfs1* expression increases in association with ER stress, especially in the pancreatic β-cells selectively lost in patients with Wolfram syndrome. It is noteworthy that the increase in Wfs1 protein was marked (sixfold) as compared with the modest increase in Bip expression (twofold) in *Ins2*$^{96Y/Y}$ cells. Mechanisms other than ER stress might have further increased Wfs1 protein expression in this cell line.

The increase in *Wfs1* expression is attributable, at least in part, to enhanced *Wfs1* transcription, because both ER stress-inducing chemical insults (MIN6 cells) and intrinsic ER stress (*Ins2*$^{96Y/Y}$cells) stimulated *WFS1* promoter activity as demonstrated by a transient transfection assay using a human *WFS1* promoter-luciferase reporter construct. A cis-acting ER stress responsive element (ERSE) has been identified in the proximal promoter regions of chaperone-encoding genes. This element consists of a consensus sequence of

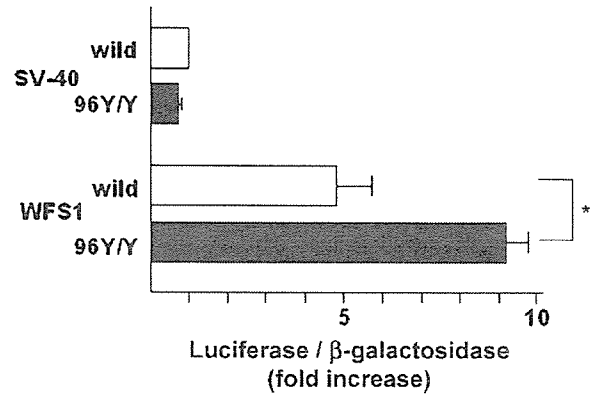

Figure 7 *WFS1* promoter activity is enhanced in *Ins2*$^{96Y/Y}$ cells. *Ins2*$^{96Y/Y}$ cells or wild type (wild) control cells were transfected with a luciferase reporter plasmid containing the 3.0 kb human *WFS1* gene 5′ flanking promoter region (from −3000 to +20), or a control plasmid containing the SV40 promoter-luciferase reporter. The expression vector pCMVβ was co-transfected, and β-galactosidase activity was used to calibrate for transfection efficiency. Results are expressed as fold-increases relative to luciferase/β-galactosidase activities in *Ins2*$^{96Y/Y}$cells as compared with control *Ins2*$^{WT/WT}$ cells in four independent experiments (means±S.E.), each performed in triplicate. *WFS1* promoter activity was significantly increased in *Ins2*$^{96Y/Y}$ cells as compared with control *Ins2*$^{WT/WT}$ cells (*$P = 0.014$, Student's *t*-tests).

CCAAT-N9-CCACG (30). The general transcriptional factor, NF-Y/CBF, binds to the CCAAT motif of the ERSE (31). Once ER stress ensues, p50ATF6 (active form of transcriptional factor ATF6) binds to the CCACG motif of the ERSE (31, 32) resulting in transcriptional induction of ER chaperones. Another ERSE (ERSE-II) with a consensus sequence of ATTGG-N-CCACG has also been identified (32). Although there are six CCAAT motifs in the −2800 to −2300 region of the putative human *WFS1* promoter, we found no ERSE consensus sequences within 3 kb upstream from the transcription initiation site. Further studies will be required to elucidate the mechanism of transcriptional regulation of the *Wfs1* gene via ER stress.

The observations made in this study suggest that Wfs1 protein may be involved in the ER stress response pathway, i.e. the unfolded protein response, in which cells respond by inducing chaperones, attenuating protein translation, and inducing apoptosis. Pancreatic β-cells suffer under chronic ER stress, striving to meet the increasing demands of insulin biosynthesis and secretion. In patients with Wolfram syndrome (26) and in *Wfs1* knock-out mice (15), β-cells were selectively lost from pancreatic islets. Moreover, islets from $Wfs^{-/-}$ mice were highly susceptible to ER stress (thapsigargin and tunicamycin)-induced apoptosis (15). It is tempting to speculate that Wfs1 protein is upregulated in response to ER stress and that it plays a physiological role in protecting cells from ER stress-induced apoptosis. Loss of function mutations of the *Wfs1* gene may cause β-cell loss due to disruption of this protective function. It was recently reported that Wfs1 protein expressed in oocytes exhibited a cation-selective ion channel activity (7). Expression of Wfs1 protein in oocytes increased cytosolic Ca^{2+} levels (7), and islets from $Wfs1^{-/-}$ mice exhibited attenuated glucose-stimulated intracellular Ca^{2+} responses (15). Wfs1 protein may be involved in the maintenance of ER and intracellular Ca^{2+} homeostasis, and its expression is induced under conditions of perturbed homeostasis, including ER stress.

The current findings that Wfs1 protein, which is predominantly expressed in pancreatic islet β-cells, is transcriptionally upregulated by ER stress indicate a link between Wfs1 protein function and ER stress responses. Further investigations utilizing $Wfs1^{-/-}$ mice and $Wfs1^{-/-}$ β-cells will provide insights into Wfs1 protein function and the pathophysiology of Wolfram syndrome.

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References

1 Barrett TG & Bundey SE. Wolfram (DIDMOAD) syndrome. *Journal of Medical Genetics* 1997 **34** 838–841.
2 Barrett TG, Bundey SE & Macleod AF. Neurodegeneration and diabetes: UK nationwide study of Wolfram (DIDMOAD) syndrome. *Lancet* 1995 **346** 1458–1463.
3 Inoue H, Tanizawa Y, Wasson J, Behn P, Kalidas K, Bernal-Mizrachi E, Mueckler M, Marshall H, Donis-Keller H, Crock P, Rogers D, Mihuni M, Kumashiro H, Higashi K, Sobue G, Oka Y & Permutt MA. A gene encoding a transmembrane protein is mutated in patients with diabetes mellitus and optic atrophy (Wolfram syndrome). *Nature Genetics* 1998 **20** 143–148.
4 Strom TM, Hortnagel K, Hofmann S, Gekeler F, Scharfe C, Rabl W, Gerbitz KD & Meitinger T. Diabetes insipidus, diabetes mellitus, optic atrophy and deafness (DIDMOAD) caused by mutations in a novel gene (wolframin) coding for a predicted transmembrane protein. *Human Molecular Genetics* 1998 **7** 2021–2028.
5 Takeda K, Inoue H, Tanizawa Y, Matsuzaki Y, Oba J, Watanabe Y, Shinoda K & Oka Y. WFS1 (Wolfram syndrome 1) gene product: predominant subcellular localization to endoplasmic reticulum in cultured cells and neuronal expression in rat brain. *Human Molecular Genetics* 2001 **10** 477–484.
6 Hofmann S, Philbrook C, Gerbitz K-D & Bauer MF. Wolfram syndrome: structural and functional analyses of mutant and wild-type wolframin, the WFS1 gene product. *Human Molecular Genetics* 2003 **12** 2003–2012.
7 Osman AA, Saito M, Makepeace C, Permutt MA, Schlesinger P & Mueckler M. Wolframin expression induces novel ion channel activity in endoplasmic reticulum membranes and increases intracellular calcium. *Journal of Biological Chemistry* 2003 **26** 52755–52762.
8 Chevet E, Cameron PH, Pelletier MF, Thomas DY & Bergeron JJ. The endoplasmic reticulum: integration of protein folding, quality control, signaling and degradation. *Current Opinion in Structural Biology* 2001 **11** 120–124.
9 Rutkowski DT & Kaufman RJ. A trip to the ER: coping with stress. *Trends in Cell Biology* 2004 **14** 20–28.
10 Oyadomari S, Araki E & Mori M. Endoplasmic reticulum stress-mediated apoptosis in pancreatic β-cells. *Apoptosis* 2002 **7** 335–345.
11 Ron D. Proteotoxicity in the endoplasmic reticulum: lessons from the Akita diabetic mouse. *Journal of Clinical Investigation* 2002 **109** 443–445.
12 Yoshioka M, Kayo T, Ikeda T & Koizumi A. A novel locus, Mody 4, distal to D7Mit189 on chromosome 7 determines early-onset NIDDM in nonobese C57BL/6 (Akita) mutant mice. *Diabetes* 1997 **46** 887–894.
13 Wang J, Takeuchi T, Tanaka S, Kubo SK, Kayo T, Lu D, Takata K, Koizumi A & Izumi T. A mutation in the insulin 2 gene induces diabetes with severe pancreatic β-cell dysfunction in the Mody mouse. *Journal of Clinical Investigation* 1999 **103** 27–37.
14 Oyadomari S, Koizumi A, Takeda K, Gotoh T, Akira S, Araki E & Mori M. Targeted disruption of the Chop gene delays endoplasmic reticulum stress–mediated diabetes. *Journal of Clinical Investigation* 2002 **109** 525–532.
15 Ishihara H, Takeda S, Tamura A, Takahashi R, Yamaguchi S, Takei D, Yamada T, Inoue H, Soga H, Katagiri H, Tanizawa Y & Oka Y. Disruption of the WFS1 gene in mice causes progressive β-cell loss and impaired stimulus-secretion coupling in insulin secretion. *Human Molecular Genetics* 2004 **13** 1159–1170.
16 Yamaguchi S, Ishihara H, Tamura A, Yamada T, Takahashi R, Takei D, Katagiri H & Oka Y. Endoplasmic reticulum stress and

N-glycosylation modulate expression of WFS1 protein. *Biochemical and Biophysical Research Communication* 2004 **3** 250–256.
17 Nozaki J, Kubota H, Yoshida H, Naitoh M, Goji J, Yoshinaga T, Mori K, Koizumi A & Nagata K. The endoplasmic reticulum stress response is stimulated through the continuous activation of transcription factors ATF6 and XBP1 in Ins2 + /Akita pancreatic beta cells. *Genes to Cells* 2004 **9** 261–270.
18 Sheng Z, Kawano J, Yanai A, Fujinaga R, Tanaka M, Watanabe Y & Shinoda K. Expression of estrogen receptors (α,β) and androgen receptor in serotonin neurons of the rat and mouse dorsal raphe nuclei; sex and species differences. *Neuroscience Research* 2004 **49** 185–196.
19 Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y & Yamamura K. Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* 1990 **127** 126–132.
20 Oka Y, Asano T, Shibasaki Y, Kasuga M, Kanazawa Y & Takaku F. Studies with antipeptide antibody suggest the presence of at least two types of glucose transporter in rat brain and adipocyte. *Journal of Biological Chemistry* 1988 **263** 13432–13439.
21 Li WW, Alexandre S, Cao X & Lee AS. Transactivation of the grp78 promoter by Ca^{2+} depletion. A comparative analysis with A23187 and the endoplasmic reticulum $Ca^{(2+)}$-ATPase inhibitor thapsigargin. *Journal of Biological Chemistry* 1993 **268** 12003–12009.
22 Fujiwara T, Oda K, Yokota S, Takatsuki A & Ikehara Y. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *Journal of Biological Chemistry* 1988 **263** 18545–18552.
23 Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL & Ron D. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes and Development* 1998 **12** 982–995.
24 McCullough KD, Martindale JL, Klotz LO, Aw TY & Holbrook NJ. Gadd153 sensitizes cells to endoplasmic reticulum stress by downregulating Bcl2 and perturbing the cellular redox state. *Molecular and Cellular Biology* 2001 **21** 1249–1259.
25 Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M & Lazebnik YA. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 1995 **376** 37–43.
26 Karasik A, O'Hara C, Srikanta S, Swift M, Soeldner JS, Kahn CR & Herskowitz RD. Genetically programmed selective islet beta-cell loss in diabetic subjects with Wolfram's syndrome. *Diabetes Care* 1989 **12** 135–138.
27 Kinsley BT, Swift M, Dumont RH & Swift RG. Morbidity and mortality in the Wolfram syndrome. *Diabetes Care* 1995 **18** 1566–1570.
28 Gerbitz K-D. Reflexions on a newly discovered diabetogenic gene, wolframin (WFS1). *Diabetologia* 1999 **42** 627–630.
29 Minton JA, Rainbow LA, Ricketts C & Barrett TG. Wolfram syndrome. *Reviews in Endocrine and Metabolic Disorders* 2003 **4** 53–59.
30 Yoshida H, Haze K, Yanagi H, Yura T & Mori K. Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. *Journal of Biological Chemistry* 1998 **273** 33741–33749.
31 Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M & Mori K. ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. *Molecular and Cellular Biology* 2000 **20** 6755–6767.
32 Kokame K, Kato H & Miyata T. Identification of ERSE-II, a new cis-acting element responsible for the ATF6-dependent mammalian unfolded protein response. *Journal of Biological Chemistry* 2001 **276** 9199–9205.

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β_2- and β_3-Adrenergic Receptor Polymorphisms Are Related to the Onset of Weight Gain and Blood Pressure Elevation Over 5 Years

Kazuko Masuo, MD, PhD; Tomohiro Katsuya, MD, PhD; Yuxiao Fu, MD; Hiromi Rakugi, MD, PhD; Toshio Ogihara, MD, PhD; Michael L. Tuck, MD

Background—The genes responsible for obesity are candidate genes for obesity-related diseases, such as hypertension. Functional polymorphisms in the β_2- and β_3-adrenergic receptors have been reported to be associated with hypertension and obesity.

Methods and Results—To longitudinally clarify the relevance to alterations in β-adrenergic receptor polymorphisms related to weight gain, blood pressure (BP) elevation, and sympathetic nerve activity as measured by plasma norepinephrine level, we studied 160 young, nonobese, normotensive men. Changes in body weight, BP, plasma norepinephrine levels, and β_2-adrenergic (Arg16Gly, Gln27Glu) and β_3-adrenergic (Trp64Arg) receptor polymorphisms were measured periodically over a 5-year period. Weight gain and BP elevation were defined as ≥10% increases from entry levels over 5 years in body mass index or mean BP. The presence of the Gly16 allele of Arg16Gly was associated with a higher frequency of weight gain and BP elevation over the 5-year period. The subjects carrying the Glu27 allele of Gln27Glu and the Trp64 allele of Trp64Arg had a higher frequency of BP elevation. Significantly higher levels of plasma norepinephrine at entry and at year 5 were observed in the subjects with the Gly16 allele of Arg16Gly and the Glu27 allele of Gln27Glu compared with those without the Gly16 or the Glu27 alleles.

Conclusions—These results demonstrate that the Gly16 allele is related to greater weight gain and BP elevation. Additionally, Glu27 and Trp64 alleles are linked to BP elevation. The subjects carrying the β_2-polymorphisms linked to weight gain and BP elevation also have higher plasma norepinephrine levels that are present at entry before weight gain and BP elevation. These findings suggest that β_2-adrenergic receptor polymorphisms in association with a heightened sympathetic nerve activity could predict the future onset of obesity and hypertension, as shown in the 5-year longitudinal study. (***Circulation.* 2005;111:3429-3434.**)

Key Words: hypertension ■ norepinephrine ■ obesity

Obesity and obesity-related cardiovascular disease are a rapidly growing public health problem,[1] and there is evidence that human obesity and hypertension have strong genetic as well as environmental determinants.[2–4] Reduced energy expenditure and resting metabolic rate are predictive of weight gain, and the sympathetic nervous system participates in regulating energy balance through thermogenesis. The thermogenic effects in obesity have been mainly attributed to the activity of the β_1- and β_2-adrenergic receptors in humans. However, reports of an association of β_2-adrenergic receptor polymorphisms with hypertension and obesity have been discordant.[5–7] Several observations have shown that the Trp64Arg polymorphism of the β_3-adrenergic receptor gene can also be associated with obesity[8–10]; however, this finding has not been confirmed in other studies.[11,12] Few studies have simultaneously taken into account obesity and hypertension as related to polymorphisms of β-adrenoceptor genes in the same study population followed longitudinally for several years. Additionally, plasma norepinephrine levels, as an index of sympathetic nerve activity (SNA), are also included in the present study.

Thus, this study examines the associations of polymorphisms of β-adrenergic receptors with plasma norepinephrine level (index of SNA), weight gain (obesity), and blood pressure (BP) elevation (hypertension) over 5 years in 160 subjects who at entry were young, nonobese, and normotensive.

Methods

Subjects

Subjects were recruited from a cohort of 1121 men who work in a single company in Osaka, Japan, as part of their annual medical

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From the Human Neurotransmitter Laboratory, Baker Heart Research Institute, Melbourne, Victoria, Australia (K.M.); Department of Geriatric Medicine, Osaka University Graduate School of Medicine, Suita City, Osaka, Japan (K.M., T.K., Y.F., H.R., T.O.); and Metabolism and Endocrinology Division, Sepulveda VA Medical Center, and David Geffen UCLA School of Medicine, Los Angeles, Calif (M.L.T.).

Correspondence to Kazuko Masuo, MD, PhD, Baker Heart Research Institute, PO Box 6492, St Kilda Rd Central, Melbourne, Victoria 8008, Australia. E-mail kmasuo@baker.edu.au

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TABLE 1. Polymorphism Genotypic Frequencies in Subjects With Significant Weight Gain (≥10%) and Mean BP Elevation (≥10%) Over 5 Years

	Genotypes			χ^2 Test for 3 Genotypes	χ^2 Test for Alleles
Arg16Gly of β_2-adrenoceptor gene	**Arg16/Arg16**	**Arg16/Gly16**	**Gly16/Gly16**		
Frequency					
With weight gain (n=59)	9 (15.3)	33 (55.9)	17 (28.8)	χ^2=7.98, P=0.019	χ^2=6.31, P=0.012
Without weight gain (n=101)	36 (35.6)	46 (45.5)	19 (18.8)		
With BP elevation (n=41)	4 (9.8)	20 (48.8)	17 (41.4)	χ^2=15.43, P<0.001	χ^2=14.42, P<0.001
Without BP elevation (n=119)	41 (34.5)	59 (49.6)	19 (16.0)		
Gln27Glu of β_2-adrenoceptor gene	**Gln27/Gln27**	**Gln27/Glu27**	**Glu27/Glu27**		
Frequency					
With weight gain (n=59)	50 (84.7)	9 (15.3)	0 (0.0)	...	χ^2=2.89, P=0.089
Without weight gain (n=92)	87 (94.6)	5 (5.4)	0 (0.0)		
With BP elevation (n=41)	32 (78.0)	9 (22.0)	0 (0.0)	...	χ^2=8.36, P=0.004
Without BP elevation (n=110)	105 (95.5)	5 (4.5)	0 (0.0)		
Trp64Arg of β_3-adrenoceptor gene	**Trp64/Trp64**	**Trp64/Arg64**	**Arg64/Arg64**		
Frequency					
With weight gain (n=59)	46 (78.0)	11 (18.6)	2 (3.4)	χ^2=7.41, P=0.025	χ^2=2.39, P=0.122
Without weight gain (n=99)	60 (60.6)	38 (38.4)	1 (1.0)		
With BP elevation (n=41)	35 (85.4)	5 (12.2)	1 (2.4)	χ^2=9.16, P=0.010	χ^2=5.25, P=0.022
Without BP elevation (n=117)	71 (60.7)	44 (37.6)	2 (1.7)		

Values in parentheses are percentage of subjects.

evaluation. Subjects at study entry were excluded who were aged >50 years, had obesity (body mass index [BMI] ≥25 kg/m^2),[13,14] had diabetes mellitus (fasting glucose level >100 mg/dL), and had hypertension (≥140/90 mm Hg). We also excluded subjects who were taking medication for hypertension, hyperlipidemia, hyperuricemia, or other illness. Only subjects who had steady body weight (weight had not changed significantly [<5%] over the past year before the entry period) were enrolled in this study.[15,16] After exclusion, 160 young, nonobese (BMI <25 kg/m^2), normotensive (<140/90 mm Hg) men on no medications were enrolled in the present study. Informed consent was obtained from each subject, as approved by the Ethics Committee of Osaka University Graduate School of Medicine, Osaka, Japan.

Measurements

After an overnight fast of >12 hours, BMI, total body fat mass, ratio of waist circumference to hip circumference (waist-to-hip ratio), BP, heart rate, venous sampling for plasma norepinephrine, and extraction of genomic DNA from leukocytes were taken every year for 5 years. BP and heart rate were measured with the subject in the recumbent position with an automated sphygmomanometer (TM-2713, A&D) with an adjusted cuff size, which had been standardized against a mercury sphygmomanometer. The percent body fat mass was determined with impedance measurements (BF-102, Tanita), and total body fat mass (kg) was calculated according to the following formula: (percent body fat mass/100)×body weight (kg).

Laboratory Determinations

Plasma norepinephrine was measured by high-performance liquid chromatography with a fluorometric method as previously described for this laboratory[17] (intra-assay coefficient of variation=2.1%; interassay coefficient of variation=3.6%; sensitivity=0.06 to 120 nmol/L).

Genotyping

Genotyping was performed by the TaqMan assay as previously described.[18] Two polymorphisms in the β_2-adrenergic receptors (arginine/glycine substitution, Arg16Gly; glutamine/glutamate substitution, Gln27Glu) of the β_2-adrenoceptor gene were studied.[6] One polymorphism (tryptophan/arginine substitution, Trp64Arg) of the β_3-adrenoceptor gene was also studied.[19,20] The probes and primers used in the TaqMan assay were as follows. For single-nucleotide polymorphisms in the β_2-adrenergic receptor gene, the probes and primers were as follows: for Arg16Gly, the probes were CGCATGGCTTCCATTGGGTGC and CGCATGGCTTCTATTGGGTGC, and the primers were GGAACGGCAGCGCCTTCT and CAGGACGATGAGAGACATGACGAT; for Gln27Glu, the probes were CTCGTCCCTTTCCTGCGTGACGT and CTCGTCCCTTTGCTGCGTGACGT (the primers used in this assay were the same as those used for Arg16Gly). For the Trp64Arg single-nucleotide polymorphism in the β_3-adrenergic receptors, the probes were TCTCGGAGTCCAGGCGATGGCCA and CTCGGAGTCCGGGCGATGGCC, and the primers were GGAGGCAACCTGCTGGTCAT and CACGAACACGTTGGTCATGGT.

Statistical Analysis

Genotype frequencies and the Hardy-Weinberg equilibrium were estimated with χ^2 test. Values are shown as mean±SD. All data analyses were performed with SPSS 8.0 for Windows programs. Changes in measured parameters within each group and differences among groups were examined by 2-way ANOVA. When these differences were significant, the Dunnett test was used to determine whether the differences of the mean measured variables at entry and 5 years were significant within the groups and among the groups compared from baseline. Values of P<0.05 were considered significant.

Results

Significant weight gain and BP elevation over 5 years were defined as a ≥10% increase in BMI or mean BP compared with values at entry.[16,21] Fifty-nine subjects had significant weight gain over 5 years, and 41 subjects had significant BP elevation. Table 1 shows the prevalence of weight gain and BP elevation at year 5. No subjects with the Glu27/Glu27 polymorphism of the β_2-adrenoceptor were detected in the present study. The allele frequency of Glu27 of the β_2-

TABLE 2. Characteristics of Subjects at Entry and at Year 5

	Subjects With Weight Gain		Subjects Without Weight Gain		Subjects With BP Elevation		Subjects Without BP Elevation	
	At Entry	At Year 5	At Entry	At Year 5	At Entry	At Year 5	At Entry	At Year 5
Subjects, n	59	59	101	101	41	41	119	119
Smoker/nonsmoker, n	15/44	10/49	22/79	13/88	13/28	8/33	24/95	15/104
Age, y	39±4	44±4‖	40±5	45±5‖	40±4	45±4‖	40±5	45±5‖
BMI, kg/m^2	22.2±1.8	24.6±2.0*§	22.9±1.7	22.4±1.9	22.6±1.6	24.3±2.1†§	22.7±1.7	22.8±1.9
Waist-to-hip ratio	0.92±0.11*	0.97±0.13*§	0.88±0.12	0.91±0.13	0.92±0.09†	0.94±0.11	0.89±0.11	0.92±0.12
Total fat mass, kg	14.1±2.1*	15.6±2.2*§	12.5±1.9	12.9±2.0	13.7±2.0†	15.1±2.0†§	12.8±2.0	13.4±2.2
Systolic BP, mm Hg	127±6	141±8*‖	128±6	131±7	132±7†	146±9‡‖	126±6	131±7
Diastolic BP, mm Hg	77±5*	80±5*	74±5	75±5	74±5	83±6†‖	76±4	75±6
Mean BP, mm Hg	94±5	101±6*§	93±5	94±6	93±6	104±6‡‖	92±6	94±6
Heart rate, bpm	70±5*	72±5	66±5	71±5§	71±4†	73±5	66±5	71±6§
Plasma norepinephrine, pmol/mL	1.18±0.11*	1.41±0.21*§	1.00±0.20	1.26±0.19	1.14±0.12†	1.43±0.24†§	1.01±0.16	1.27±0.18§

Data are mean±SD; n=160.
*$P<0.05$ vs subjects without weight gain; †$P<0.05$, ‡$P<0.01$ vs subjects without BP elevation; §$P<0.05$, ‖$P<0.01$ vs values at entry.

adrenoceptor polymorphism was 4.6%, and that of Arg64 of the β_3-adrenoceptor polymorphism was 17.4%, but all studied loci allele and genotype frequencies were in accordance with the Hardy-Weinberg equilibrium. The frequency distributions for homozygosity for the Arg16 and Gly16 alleles in this study were 28.1% and 22.5%. The frequency distributions for homozygosity for the Gln27 and Glu27 were 90.7% and 0.0%, and the frequency distributions for the Trp64 and Arg64 were 67.1% and 1.9%. The frequency distributions for homozygosity for the Glu27 and the Arg64 in our subjects were similar to those in previous studies in Japanese cohorts but lower than those found in studies in white subjects.[5,6,9,10,22,23] The frequency of Gly16 allele of the β_2-adrenoceptor gene was greater in subjects with weight gain than in those without weight gain. Additionally, the frequency of the Gly16 allele of the β_2-adrenergic receptor gene was significantly greater in subjects who showed a significant BP elevation over 5 years. The frequencies of the Glu27 and Trp64 alleles were higher in subjects with BP elevation than in those without BP elevation (Table 1).

Furthermore, to evaluate the relationships between the β-adrenoceptor alleles and weight gain–related BP elevation, we compared the frequencies of alleles between the groups with and without BP elevation in subjects who significantly gained body weight versus those without weight gain. In subjects who had a significant weight gain, those who also had a significant BP elevation carried a higher frequency of the Gly16 and Glu27 alleles compared with those without a significant BP elevation ($\chi^2=4.73$, $P=0.030$; $\chi^2=6.35$, $P=0.012$, respectively). In subjects who did not gain weight over the 5-year period, the allele frequencies in the 3 genotypes that were studied were similar in subjects with and without a BP elevation over time.

Table 2 shows the demographic characteristics of the 2 groups subdivided by significant weight gain (≥10%) over 5 years or BP elevation at entry and at year 5. At both periods, waist-to-hip ratio, total fat mass, heart rate, and plasma norepinephrine were higher in the group who had significant weight gain and in the group who had a significant rise in mean BP compared with those without weight gain or BP elevation. It is important to note that at entry, BMI and BP were similar between the groups with and without weight gain and BP elevation. Among the 41 subjects with a significant BP elevation, 32 of these individuals also had a significant weight gain.

Subjects were divided into the 2 subgroups in each studied genotype according to the dominant allele. Characteristics between those with and without the dominant allele are shown in Tables 3, 4, and 5. Total body fat mass and waist-to-hip ratio at entry in the subjects carrying the Gly16 allele and Glu allele of the β_2-adrenoceptor gene were greater than in the other genotypes (Tables 3 and 4). BMI and total body fat mass increased significantly in the subjects with the Gly16 allele and Glu27 allele of the β_2-adrenoceptor genes. Subjects who had the Gly16 and Glu27 of the β_2-adrenoceptor gene and the Trp64 of the β_3-adrenoceptor gene had significant increments in mean BP over the 5 years (Tables 3, 4, and 5).

As we have previously reported,[15,16,21] subjects with the most significant weight gain and BP elevation had the highest levels of plasma norepinephrine at entry compared with subjects without weight gain or BP elevation (Table 2). Plasma norepinephrine levels at both entry and year 5 were greater in subjects carrying Gly16 allele and Glu27 allele of the β_2-adrenoceptor genes than in the other genotypes (Tables 3 and 4). Plasma norepinephrine levels increased significantly over the 5-year period in those subjects with the abnormal β-adrenoceptor alleles. The same subjects also had higher plasma norepinephrine levels at entry.

Discussion

The present study shows that the Arg16Gly and the Gln27Glu of the β_2-adrenoceptor and the Trp64Arg of the β_3-adrenoceptor polymorphisms have a substantial influence on future gain in body weight or BP elevation in male subjects who were originally nonobese and normotensive. The subjects carrying the polymorphism for the Gly16, Glu27, and Trp64 alleles show higher frequency in those who had a

TABLE 3. Characteristics of Subjects According to Genotype of Arg16Gly at Entry and at 5 Years

	Without Gly16 Allele (Arg16Arg)		With Gly16 Allele (Arg16Gly+Gly16Gly)	
	At Entry	At Year 5	At Entry	At Year 5
Subjects, n	45	45	115	115
Smoker/nonsmoker, n (%)	10/35 (22.2/77.8)	7/38 (15.6/84.4)	27/88 (23.5/76.5)	16/99 (13.9/86.1)
BMI, kg/m^2	22.6±1.9	22.4±2.8	22.7±1.8	23.5±2.1*‡
Waist-to-hip ratio	0.87±0.10	0.88±0.11	0.91±0.10‡	0.95±0.12*‡
Total fat mass, kg	12.8±2.0	13.2±1.9	13.4±1.9‡	14.1±2.0*‡
Systolic BP, mm Hg	128±5	129±6	127±5	137±7†§
Diastolic BP, mm Hg	73±7	75±5	76±6	79±5‡
Mean BP, mm Hg	92±6	93±5	94±6	98±7*‡
Heart rate, bpm	65±6	69±5	69±6	72±6
Norepinephrine, pmol/mL	0.99±0.16	1.10±0.22	1.09±0.14‡	1.40±0.10†§

Data are mean±SD; n=160.
*P<0.05, †P<0.01 vs value at entry; ‡P<0.05, §P<0.01 vs subjects without Gly16 allele (Arg16Arg genotype).

significant weight gain or BP elevation over the 5-year study. Higher levels of plasma norepinephrine at entry were also seen in the groups with the Gly16 or Glu27 alleles. As we have shown in all studies, a heightened SNA (high mean plasma norepinephrine) predicted subsequent weight gain and BP elevation.[15,16,21] Now we show that the increased SNA is in part determined by the genetic influence of the β_2-adrenergic receptor systems.

Pathophysiological involvement of genetic abnormalities in the β_2-adrenergic receptor system in hypertension and obesity are well described.[5,6,24–26] Among β_2-adrenergic receptor polymorphisms, Arg16Gly and Gln27Glu are considered the most functionally important.[5,6,24–26] Gratze et al[27] found that young normotensive white men homozygous for the Gly16 allele of the β_2-adrenoceptor gene had higher BP and lower peripheral vasodilation after infusion of the β-blocker salbutamol. The Gly16 substitution exaggerates agonist-mediated receptor downregulation.[6,28] Our findings that the Gly16 allele is associated with weight gain and BP elevation associated with higher plasma norepinephrine levels are in accordance with these findings. The subjects who had weight gain–related BP elevation also had higher frequencies of the Gly16 and Glu27 alleles compared with those without BP elevation, suggesting that Gly16/Glu27 is related to obesity-related hypertension. On the other hand, the frequency associations of the Arg16 or Gly16 alleles of the Arg16Gly and the Gln27 or Glu27 alleles of the Gln27Glu with the onset of hypertension and obesity are more controversial.[6] The Glu27 receptor had been shown to be resistant to downregulation compared with Gln27 but when coexpressed with Arg16.[29] We were not able to observe any significant association of the Arg16 and Glu27 alleles with weight gain or BP elevation, probably because of the small sample size of the study.

The β_3-adrenergic receptor system is important in mediating the stimulation of lipolysis by catecholamines in white adipose cells in humans and in the development of obesity.[8–10] It is well documented that weight gain leads to BP elevation,[1,15,16] but there are few investigations about the genetic relations in the β_3-adrenoceptor such as polymor-

TABLE 4. Characteristics of Subjects According to Genotype of Gln27Glu at Entry and at 5 Years

	Without Glu27 Allele (Gln27Gln)		With Glu27 Allele (Gln27Glu)	
	At Entry	At Year 5	At Entry	At Year 5
Subjects, n	137	137	14	14
Smoker/nonsmoker, n (%)	34/103 (24.8/75.2)	22/115 (16.1/83.9)	3/11 (21.4/78.6)	1/13 (7.1/92.9)
BMI, kg/m^2	22.6±1.7	23.0±2.5	23.5±2.1	24.6±3.0*‡
Waist-to-hip ratio	0.89±0.10	0.92±0.11	0.92±0.11	0.99±0.10*‡
Total fat mass, kg	13.0±1.9	13.4±2.0	13.9±1.3‡	14.9±2.3*§
Systolic BP, mm Hg	127±5	135±5*	132±5	138±6*
Diastolic BP, mm Hg	75±5	76±6	77±6	83±5*§
Mean BP, mm Hg	93±5	94±5	95±5	101±6*§
Heart rate, bpm	67±5	71±6	69±5	70±6
Norepinephrine, pmol/mL	1.03±0.20	1.30±0.18*	1.29±0.14‡	1.42±0.19*‡

Data are mean±SD; n=151.
*P<0.05, †P<0.01 vs value at entry; ‡P<0.05, §P<0.01 vs subjects without Glu allele (Gln27Gln genotype).

TABLE 5. Characteristics of Subjects According to Genotype of Trp64Arg at Entry and at 5 Years

	With Trp64 Allele (Trp64Trp+Trp64Arg)		Without Trp64 Allele (Arg64Arg)	
Genotype	At Entry	At Year 5	At Entry	At Year 5
Subjects, n	155	155	3	3
Smoker/nonsmoker, n (%)	36/119 (23.2/76.8)	23/132 (14.6/85.4)	1/2 (33.3/66.7)	0/3 (0.0/100.0)
BMI, kg/m^2	23.1±1.7	23.2±2.7	22.8±0.5	24.0±0.6
Waist-to-hip ratio	0.90±0.06	0.93±0.08	0.90±0.09	0.94±0.10
Total fat mass, kg	13.2±1.8	13.9±2.0	13.1±2.0	13.5±2.0
Systolic BP, mm Hg	127±5	134±6†	126±6	128±7
Diastolic BP, mm Hg	75±5	78±5*	75±5	77±6
Mean BP, mm Hg	93±5	97±5†	92±5	94±6
Heart rate, bpm	68±5	71±6*	67±5	68±6
Norepinephrine, pmol/mL	1.06±0.20	1.31±0.14*	1.03±0.25	1.27±0.23

Data are mean±SD; n=158.
*P<0.05, †P<0.01 vs value at entry.

phisms in Trp64Arg and the association of these polymorphisms with hypertension in obesity.[30] Fujisawa et al[23] have shown in a Japanese population that the allele frequency of Arg64 in hypertensive subjects was similar to that in normotensive subjects. Other investigators have reported in a large Japanese cohort (n=3706) that the subjects with the Arg64/Arg64 genotype had a greater BMI and percent fat mass than those with the in Trp64/Trp64 genotype.[9] Conversely, we did not observe these associations in the genotype of the β_3-adrenoceptor in relation to weight gain–related BP elevation.

In the present study we used plasma norepinephrine levels as an index of SNA. Tuck,[31] Grassi and Esler,[32] and Rahn et al[33] observed that there are different results in SNA values in hypertensive patients depending on the method of SNA measurement, including regional norepinephrine spillover, muscle sympathetic nerve activity (microneurography), and plasma norepinephrine measurements. Spillover methods are considered the "gold standard" for SNA measurements, but in humans these are difficult and invasive measurements. Plasma norepinephrine levels are more practical for large population studies and represent several different processes (secretion, clearance, and reuptake).[3,15,16]

It is known that Asian people (Japanese) have a lower definition of obesity than the World Health Organization BMI cutoff point for obesity (≥30 kg/m^2),[13,14] which is controlled by genotypes. In a Japanese population, a strong association between visceral fat content and the metabolic syndrome has been reported, as seen even in subjects defined as nonobese by BMI but who were obese by CT.[34] In the present study the subjects who had the most significant weight gain and BP elevations also had a greater total body fat mass and waist-to-hip ratio plus higher plasma norepinephrine levels at entry, but BMI was not different between these entry groups. These findings suggest that abdominal obesity might be the link to heightened SNA, which is in part determined genetically by the abnormal β-adrenoceptor polymorphism. Alvarez et al[35,36] have reported that visceral obesity, but not subcutaneous obesity, is best associated with increased SNA. Grassi et al[37] have also found that central obesity is characterized by greater sympathetic activation and impaired baroreceptor sensitivity than peripherally obese or lean subjects.

In summary, these findings are from the first large cohort-based longitudinal study analyzing the effect of genetic variation in the β_2- and β_3-adrenoceptor genes over a fixed time period, showing their strong association with initiation of weight gain and BP elevation. SNA, as seen in plasma norepinephrine accompanying abdominal obesity, may be the major mediator of the β_2-adrenoceptor gene changes.

References

1. Hubert HB, Feinleib M, McNamara PM, Castelli WP. Obesity as an independent risk factor for cardiovascular disease: a 26-year follow-up of participants in the Framingham Heart Study. *Circulation*. 1983;67:969–977.
2. Cui J, Hopper JL, Harrap SB. Genes and family environment explain correlations between blood pressure and body mass index. *Hypertension*. 2002;40:7–12.
3. Masuo K, Mikami H, Ogihara T, Tuck ML. Familial hypertension, insulin, sympathetic activity, and blood pressure elevation. *Hypertension*. 1998;32:96–100.
4. Masuo K, Mikami H, Ogihara T, Tuck ML. Familial obesity, sympathetic activation and blood pressure level. *Blood Pressure*. 2001;10:199–204.
5. Kato N, Sugiyama T, Morita H, Kurihara H, Sato T, Yamori Y, Yazaki Y. Association analysis of β2-adrenergic receptor polymorphisms with hypertension in Japanese. *Hypertension*. 2001;37:286–292.
6. Pereira AC, Floriano MS, Mota GF, Cunha RS, Herkenhoff FL, Mill JG, Krieger JE. β2 Adrenoceptor functional gene variants, obesity, and blood pressure level interactions in the general population. *Hypertension*. 2003;42(pt 2):685–692.
7. van Rossum CT, Hoebee B, Seidell JC, Bouchard C, van Baak MA, de Groot CP, Chagnon M, de Graaf C, Saris WH. Genetic factors as predictors of weight gain in young adult Dutch men and women. *Int J Obes Relat Metab Disord*. 2002;26:517–528.
8. Clement K, Vaisse C, Manning BS, Basdevant A, Guy-Grand B, Ruiz J, Silver KD, Shuldiner AR, Froguel P, Strosberg AD. Genetic variation in the β3-adrenergic receptor and an increased capacity to gain weight in patients with morbid obesity. *N Engl J Med*. 1995;333:352–354.
9. Oizumi T, Daimon M, Saitoh T, Kameda W, Yamaguchi H, Ohnuma H, Igarashi M, Eguchi H, Manaka H, Tominaga M, Kato T. Genotype Arg/Arg, but not Trp/Arg, of the Trp64Arg polymorphism of the β3-adrenergic receptor is associated with type 2 diabetes and obesity in a large Japanese sample. *Diabetes Care*. 2001;24:1579–1583.

10. Strazzullo P, Iacone R, Siani A, Cappuccio FP, Russo O, Barba G, Barbato A, D'Elia L, Trevisan M, Farinaro E. Relationship of the Trp64Arg polymorphism of the beta3-adrenoceptor gene to central adiposity and high blood pressure: interaction with age: cross-sectional and longitudinal findings of the Olivetti Prospective Heart Study. *J Hypertens.* 2001;19:399–406.
11. Buettner R, Schaffler A, Arndt H, Rogler G, Nusser J, Zietz B, Enger I, Hugl S, Cuk A, Scholmerich J, Palitzsch KD. The Try64Arg polymorphism of the β3-adrenergic receptor gene is not associated with obesity or type 2 diabetes mellitus in a large population-based Caucasian cohort. *J Clin Endocrinol Metab.* 1998;83:2892–2897.
12. Gagnon J, Mauriege P, Roy S, Sjostrom D, Chagnon YC, Dionne FT, Oppert JM, Perusse L, Sjostrom L, Bouchard C. The Trp64Arg mutation of the β3 adrenergic receptor gene has no effect on obesity phenotypes in the Quebec Family Study and Swedish Obese Subjects cohorts. *J Clin Invest.* 1996;98:2086–2093.
13. WHO Expert Consultation. Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. *Lancet.* 2004:363:157–163.
14. Kanazawa M, Yoshiike N, Osaka T, Numba Y, Zimmet P, Inoue S. Criteria and classification of obesity in Japan and Asia-Oceania. *Asia Pac J Clin Nutr.* 2002;11(suppl 8):S732–S737.
15. Masuo K, Kawaguchi H, Mikami H, Ogihara T, Tuck ML. Serum uric acid and plasma norepinephrine concentrations predict subsequent weight gain and blood pressure elevation. *Hypertension.* 2003:42:474–480.
16. Masuo K, Mikami H, Ogihara T, Tuck ML. Weight gain–induced blood pressure elevation. *Hypertension.* 2000;35:1135–1140.
17. Willemsen JJ, Ross HA, Jacobs MC, Lenders JW, Thien T, Swinkels LM, Benraad TJ. Highly sensitive and specific HPLC with fluorometric detection for determination of plasma epinephrine and norepinephrine applied to kinetic studies in humans. *Clin Chem.* 1995;41:1455–1460.
18. Ranade K, Chang MS, Ting CT, Pei D, Hsiao CF, Olivier M, Pesich R, Hebert J, Chen YD, Dzau VJ, Curb D, Olshen R, Risch N, Cox DR, Botstein D. High-throughput genotyping with single nucleotide polymorphisms. *Genome Res.* 2001;11:1262–1268.
19. Kadowaki H, Yasuda K, Iwamoto K, Otabe S, Shimokawa K, Silver K, Walston J, Yoshinaga H, Kosaka K, Yamada N, Saito Y, Hagura R, Akanuma Y, Shuldiner A, Yazaki Y, Kadowaki T. A mutation in the β3-adrenergic receptor gene is associated with obesity and hyperinsulinemia in Japanese subjects. *Biochem Biophys Res Commun.* 1995;215:555–560.
20. Walston J, Silver K, Bogardus C, Knowler WC, Celi FS, Austin S, Manning B, Strosberg AD, Stern MP, Raben N, Sorkin JD, Roth J, Shuldiner AR. Time of onset of non–insulin-dependent diabetes mellitus and genetic variation in the β3-adrenergic-receptor gene. *N Engl J Med.* 1995;333:343–347.
21. Masuo K, Mikami H, Ogihara T, Tuck ML. Sympathetic nerve hyperactivity precedes hyperinsulinemia and blood pressure elevation in a young, nonobese Japanese population. *Am J Hypertens.* 1997:10:77–83.
22. Kawamura T, Egusa G, Fujikawa R, Okubo M. Gln27Glu variant of the beta2-adrenergic receptor gene is not associated with obesity and diabetes in Japanese-Americans. *Metabolism.* 2001;50:443–446.
23. Fujisawa T, Ikegami H, Yamato E, Hamada Y, Kamide K, Rakugi H, Higaki J, Murakami H, Shimamoto K, Ogihara T. trp64Arg mutation of beta3-adrenergic receptor in essential hypertension: insulin resistance and the adrenergic system. *Am J Hypertens.* 1997;10:101–105.
24. Large V, Hellstrom L, Reynisdottir S, Lonnqvist F, Eriksson P, Lannfelt L, Arner P. Human beta-2 adrenoceptor gene polymorphisms are highly frequent in obesity and associate with altered adipocyte beta-2 adrenoceptor function. *J Clin Invest.* 1997:100:3005–3013.
25. Castellano M, Rossi F, Giacche M, Perani C, Rivadossi F, Muiesan ML, Salvetti M, Beschi M, Rizzoni D, Agabiti-Rosei E. β2-Adrenergic receptor gene polymorphism, age, and cardiovascular phenotypes. *Hypertension.* 2003;41:361–367.
26. Timmermann B, Mo R, Luft FC, Gerdts E, Busjahn A, Omvik P, Li GH, Schuster H, Wienker TF, Hoehe MR, Lund-Johansen P. Beta2-adrenoceptor genetic variation is associated with genetic predisposition to essential hypertension: the Bergen Blood Pressure Study. *Kidney Int.* 1998;53:1455–1460.
27. Gratze G, Fortin J, Labugger R, Binder A, Kotanko P, Timmermann B, Luft FC, Hoehe MR, Skrabal F. β2-Adrenergic receptor variants affect resting blood pressure and agonist-induced vasodilation in young adult Caucasians. *Hypertension.* 1999;33:1425–1430.
28. Green SA, Turki J, Innis M, Liggett SB. Amino-terminal polymorphisms of the human β2-adrenergic receptor impart distinct agonist-promoted regulatory properties. *Biochemistry.* 1994;33:9414–9419.
29. Martinez FD, Graves PE, Baldini M, Solomon S, Erickson R. Association between genetic polymorphisms of the beta2-adrenoceptor and response to albuterol in children with and without a history of wheezing. *J Clin Invest.* 1997:100:3184–3188.
30. Melis MG, Secchi G, Brizzi P, Severino C, Maioli M, Tonolo G. The Trp64Arg beta3-adrenergic receptor amino acid variant confers increased sensitivity to the pressor effects of noradrenaline in Sardinian subjects. *Clin Sci (Lond).* 2002;103:398–402.
31. Tuck ML. The sympathetic nervous system in essential hypertension. *Am Heart J.* 1986:112:877–886.
32. Grassi G, Esler M. How to assess sympathetic activity in humans. *J Hypertens.* 1999:17:719–734.
33. Rahn KH, Barenbrock M. Hausberg M. The sympathetic nervous system in the pathogenesis of hypertension. *J Hypertens.* 1999;17(suppl 3):S11–S14.
34. Nagaretani H, Nakamura T, Funahashi T, Kotani K, Miyanaga M, Tokunaga K, Takahashi M, Nishizawa H, Kishida K, Kuriyama H, Hotta K, Yamashita S, Matsuzawa Y. Visceral fat is a major contributor for multiple risk factor clustering in Japanese men with impaired glucose tolerance. *Diabetes Care.* 2001;24:2127–2133.
35. Alvarez GE, Ballard TP, Beske SD, Davy KP. Subcutaneous obesity is not associated with sympathetic neural activation. *Am J Physiol.* 2004;287:H414–H418.
36. Alvarez GE, Beske SD, Ballard TP, Davy KP. Sympathetic neural activation in visceral obesity. *Circulation.* 2002;106:2533–2536.
37. Grassi G, Dell'Oro R, Facchini A, Quarti Trevano F, Bolla GB, Mancia G. Effect of central and peripheral body fat mass distribution on sympathetic and baroreflex function in obese normotensives. *J Hypertens.* 2004;22:2363–2369.

β_2-Adrenoceptor Polymorphisms Relate to Insulin Resistance and Sympathetic Overactivity as Early Markers of Metabolic Disease in Nonobese, Normotensive Individuals

Kazuko Masuo, Tomohiro Katsuya, Yuxiao Fu,
Hiromi Rakugi, Toshio Ogihara, and Michael L. Tuck

Background: The genes responsible for insulin resistance are also candidate genes for insulin resistance-related diseases, such as obesity and hypertension. Functional polymorphisms in the β_2- and β_3-adrenergic receptors have been reported to be associated with diabetes, hypertension, and obesity. To clarify the relevance of the β-adrenergic receptor polymorphisms to insulin resistance, we studied their association with polymorphisms of β_2 (Arg16Gly, Gln27Glu) and β_3 (Trp64Arg) adrenoceptor genes.

Methods: We studied 155 young, nonobese Japanese men using the homeostasis model assessment of insulin resistance (HOMA-IR) to divide individuals into insulin-sensitive and insulin-resistant groups. Insulin resistance in the participants was defined as HOMA-IR equal to or greater than the average plus 1 SD of 3.1. There were 69 men who were insulin resistant and 86 men who were insulin sensitive. Body mass index (BMI), blood pressure (BP), plasma glucose, insulin, leptin, norepinephrine (NE) levels, and the polymorphisms of Arg16Gly and Gln27Glu of the β_2- and Trp64Arg of the β_3-adrenoceptor polymorphisms were measured in all participants.

Results: The insulin-resistant group had higher frequency of the Gly16 allele of Arg16Gly compared with the insulin-sensitive group, whereas the frequencies of genotypes or alleles of Gln27Glu and Trp64Arg were similar. The insulin-resistant group had a higher mean HOMA-IR, fasting insulin, NE, and total fat mass compared with levels in the insulin-sensitive group, but the BMI and leptin levels were similar. The subjects carrying the Gly16 allele of the β_2-adrenoceptor gene had a higher mean HOMA-IR, fasting insulin, NE, body fat mass, and BP than those without the Gly16 allele.

Conclusions: The Gly16 mutation of the β_2-adrenoceptor gene is associated with increased insulin resistance, adiposity, and BP accompanied by higher plasma NE levels early in the metabolic disease in developing obesity. These findings show an important role of β_2-adrenoceptor gene polymorphisms in the association of insulin resistance in hypertension and obesity. Am J Hypertens 2005;18:1009–1014 © 2005 American Journal of Hypertension, Ltd.

Key Words: Insulin resistance, sympathetic nerve activity, β_2- and β_3-adrenoceptor polymorphisms, blood pressure, and obesity.

Obesity and hypertension are associated with metabolic disturbances such as insulin resistance, hyperinsulinemia, and dyslipidemia.[1,2] One pathophysiologic significance of early insulin resistance is that insulin has mitogenic properties that can potentiate vascular smooth-muscle growth, promoting structural changes in blood vessels and possibly contributing to atherosclerosis. Thus, insulin resistance may be an important etiologic factor in the cardiovascular risk seen in the development of obesity and hypertension.[1–3]

One major risk is that human obesity and hypertension have well defined genetic determinants such polymorphisms in the β_2- and β_3-adrenergic receptor.[4–12] We have

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From the Human Neurotransmitter Laboratory (KM), Baker Heart Research Institute, Melbourne, Victoria, Australia; Department of Geriatric Medicine (KM, TK, YF, HR, TO), Osaka University Graduate School of Medicine, Suita, Osaka, Japan; and Endocrinology and Metabolism Division (MLT), Sepulveda Veterans Affairs Medical Center and David Geffen, University of California–Los Angeles School of Medicine, Los Angeles, California.

Address correspondence and reprint requests to Dr. Kazuko Masuo, Baker Heart Research Institute, PO Box 6492 St Kilda Road Central, Melbourne, Victoria 8008, Australia; e-mail: kmasuo@baker.edu.au

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reported that insulin resistance and hyperinsulinemia are associated with heightened sympathetic nerve activity[13] and that heightened sympathetic nerve activity, as seen in elevated plasma NE, predicts insulin resistance, subsequent weight gain, and BP elevation.[14–16] In addition, normotensive and normal-weight individuals who have a positive family history of hypertension and obesity also have heightened sympathetic nerve activity.[17,18] These findings imply that sympathetic overactivity defined here as high plasma NE levels are associated with genetic determinants on the β-adrenergic receptor that may contribute to insulin resistance. The present study further examines the relationship between polymorphisms of the β-adrenergic receptors and progression in reduced insulin sensitivity in nonobese, normotensive Japanese men.

Methods

Subjects

A cohort of 1121 men working in Osaka, Japan, as part of their biannual medical evaluation were studied. Subjects were excluded who were >50 years of age, overweight (body mass index [BMI] 25 to 30 kg/m^2) or obese (BMI >30 kg/m^2), had diabetes mellitus (fasting glucose level>100 mg/dL), or hypertension (≥160/95 mm Hg). Additional exclusions were subjects who were taking medications for hypertension, hyperlipidemia, hyperuricemia, or other illness. After exclusion, 155 young men who were nonobese (BMI <25 kg/m^2) and nonhypertensive (BP <160/95 mm Hg) and who were not using any medications were recruited from the cohort. The subjects were subdivided into an insulin-sensitive group and an insulin-resistant group using the homeostasis model assessment of insulin resistance (HOMA-IR) and a cut-off limit of average + 1 SD (2.2 + 0.9) in participants. Because it is well known that recent alterations in plasma insulin, leptin, and NE levels are altered with weight changes, only those subjects who had steady body weight (weight had not changed significantly (<5%) over the past year) were enrolled in the present study.[15,16,19]

The protocol was approved by the Ethics Committee of Osaka University Graduate School of Medicine, Japan, and written informed consent was obtained from all of the subjects.

Measurements

After an overnight fast of 12 h, BMI, total body fat mass, BP, heart rate, and venous sampling for blood glucose, plasma norepinephrine (NE), insulin, leptin, and the extraction of genomic DNA from leukocytes were obtained after a 30-min rest period in the supine position. Lipids profiles (total cholesterol, triglyceride, HDL-cholesterol) and uric acid levels were also evaluated. Both BP and heart rate were measured three times in the supine position by an automated sphygmomanometer (TM-2713, A&D Co. Ltd., Tokyo, Japan), which had been standardized against a mercury sphygmomanometer. The percentage body fat mass was determined with impedance measurements (BF-102, Tanita, Japan), and total body fat mass (kg) was calculated according to the following formula: [percentage body fat mass (%)/100] × body weight (kg). Plasma NE was measured by high-performance liquid chromatography with a fluorometric method (intra-assay coefficient of variation [CV] = 2.1%; inter-assay CV = 3.6%; sensitivity = 0.06 to 120 nmol/L). Plasma immunoreactive insulin was measured by standard radioimmunoassay methods (insulin RIABEAD II, Dinabott; intra-assay CV = 1.9%; inter-assay CV = 2.2%; sensitivity = 0.75 to 300 μU/mL). Plasma leptin was measured by radioimmunoassay (human leptin RIA kit, Linco; intra-assay CV = 5.0%, interassay CV = 4.5%, and sensitivity = 0.03 to 6 nmol/L). The HOMA-IR was defined as the product of fasting plasma insulin (μU/mL) and glucose (mg/dL) divided by 405.[20]

Genotyping

Genotyping was performed by the TaqMan assay, as previously described.[21] Two polymorphisms (arginine/glycine substitution, Arg16Gly, and glutamine/glutamate substitution, Gln27Glu) of the β_2-adrenoceptors[6] and one polymorphism (tryptophan/arginine substitution, Trp64Arg) of the β_3-adrenergic receptor[11,12] were evaluated. For single-nucleotide polymorphisms of the β_2-adrenergic receptor gene, the probes and primers were as follows: for Arg16Gly, the probes were CGCATGGCTTCCATTGGGTGC and CGCATGGCTTCTATTGGGTGC, and the primers were GGAACGGCAGCGCCTTCT and CAGGACGATGAGAGACATGACGAT; for Gln27Glu, the probes were CTCGTCCCTTTCCTGCGTGACGT and CTCGTCCCTTTGCTGCGTGACGT, and the primers used in this assay were the same as those used for Arg16Gly. For the Trp64Arg single-nucleotide polymorphism in the β_3-adrenergic receptors, the probes were TCTCGGAGTCCAGGCGATGGCCA and CTCGGAGTCCGGGCGATGGCC, and the primers were GGAGGCAACCTGCTGGTCAT and CACGAACACGTTGGTCATGGT.

Statistical Analyses

Genotype frequencies and Hardy-Weinberg equilibrium were estimated with χ^2 test. Values are shown as mean ± SD. Differences among groups were examined by the paired or unpaired t test. Multiple regression linear analyses were applied to evaluate the relationship between HOMA-IR as a dependent variable and plasma NE, BMI, total body fat mass, and mean BP (systolic and diastolic BP) as independent variables. Values of $P < .05$ were considered significant.

Results

Prevalence of Insulin Resistance

A total of 69 subjects were insulin resistant and 86 subjects were insulin sensitive as defined by the HOMA-IR. The

insulin-resistant group had a significantly lower frequency of the Arg16/Arg16 genotype (χ^2= 12.38, P = .002) and a higher frequency of the Gly16 allele (χ^2= 5.53, P = .019) in analysis of the β_2-adrenoceptor gene compared with results in the insulin-sensitive group (Fig. 1). Frequencies of each genotype and allele of Gln27Glu and those of Trp64Arg were similar between the insulin-sensitive and insulin-resistant groups.

Profiles of Insulin-Resistant Subjects

The insulin-resistant group had higher HOMA-IR, fasting plasma insulin, NE, total body fat mass, uric acid, total cholesterol, triglyceride, and lower HDL-cholesterol levels, whereas BMI, BP levels, and leptin levels were similar in both groups (Table 1).

Profiles of the Subjects Carrying the Gly16 Allele of the β_2-Adrenoceptor

Insulin resistant subjects had a higher frequency of the Gly16 allele of the β_2-adrenoceptor gene, suggesting the Gly16 allele is related to insulin resistance. Thus, we compared the subjects with and without the Gly16 allele of the β_2-adrenoceptor gene regardless of the status of insulin sensitivity. The HOMA-IR, fasting plasmas insulin, NE, total body fat mass, serum uric acid levels, and systolic, diastolic, and mean BP levels were higher in the subjects with the Gly16 allele (the Arg16/Gly16 + Gly16/Gly16 genotype) compared with values in subjects without the Gly16 allele (the Arg16/Arg16 genotype) of the β_2-adrenoceptor gene (Table 2). When those subjects were subdivided by insulin sensitivity, only the insulin-resistant group with higher fasting plasma insulin ($P < .05$) and NE ($P < .05$) levels were found in the group with the Gly16 allele (Fig. 2).

Multiple Regression Linear Analyses

When HOMA-IR was used as a dependent variable, plasma NE (P = .012), total body fat mass (P = .016), and systolic (P = .034) and mean BP (P = .007) levels were significant determinant variables (R^2 = 0.379, F = 19.96, $P < .001$) in multiple regression linear analysis.

Discussion

To clarify the relationship of β-adrenoceptors polymorphisms, insulin resistance, and plasma NE levels as an index of the sympathetic nervous system activity, we studied profiles of hormones and relations of polymorphisms of β-adrenoceptor genes over time in healthy individuals. We found that the insulin-resistant subjects had higher frequencies of the Gly16 allele of the β_2-adrenoceptors, and that the subjects who carried the Gly16 allele had higher levels of fasting insulin (HOMA-IR), plasma NE, and uric acid. In addition, total body fat mass and BP levels were higher in the subjects with the Gly16 allele in nonobese, nonhypertensive men. These findings suggest 1) that insulin resistance could in part be determined by the genetic variant of the β_2-adrenoceptor gene, and 2) that the β_2-adrenoceptor polymorphism accompanying higher plasma NE levels could increase insulin resistance, adiposity (obesity), and BP elevation.

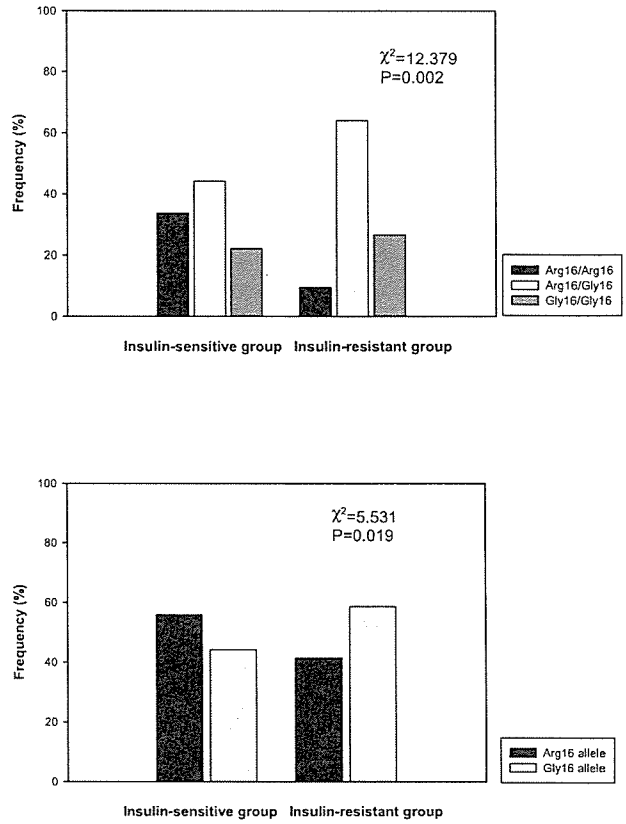

FIG. 1. Frequencies of the genotypes (**upper panel**) and the allele (**lower panel**) at Arg16Gly of the β_2-adrenoceptor gene.

In the present study, we used plasma NE levels as an index of sympathetic nerve activity. Tuck,[22] Grassi and Esler,[23] and Rahn et al[24] reported different results in sympathetic nervous system activity in hypertensive patients according to NE measurement methods, muscle sympathetic nerve activity using microneurography methods, plasma NE measurements, and regional spillover method.[25] Many investigators recommend the regional spillover method as a gold standard for sympathetic nerve activity, but these are difficult and invasive measurements. Plasma NE levels are much more practical for large populations and represent the result of several different processes such as secretion, clearance, and reuptake, especially in large population studies such as cross-sectional design studies[13,17] and in repeated measurements in longitudinal studies.[14–16]

β-Adrenoceptor Polymorphisms Versus Insulin Resistance

Significant evidence has been provided for a strong physiologic relationship between the β_2-adrenoceptor and β_3-adre-

Table 1. Comparisons of values between insulin-sensitive subjects and insulin-resistant subjects

Characteristic	Insulin-Sensitive Subjects	Insulin-Resistant Subjects
Number	86	69
Age (y)	37.0 ± 6.9	36.8 ± 7.8
Body mass index (kg/m^2)	21.6 ± 2.8	22.8 ± 2.8
Total body fat mass (kg)	14.4 ± 4.1	16.1 ± 3.9*
Waist-to-hip circumference ratio	0.90 ± 0.11	0.92 ± 0.13
Systolic blood pressure (mm Hg)	127 ± 12	129 ± 11
Diastolic blood pressure (mm Hg)	78 ± 11	79 ± 12
Mean blood pressure (mm Hg)	94 ± 10	96 ± 12
Heart rates (beats/min)	64 ± 3	65 ± 4
HOMA-IR	1.7 ± 0.9	4.2 ± 0.5†
Plasma insulin (μU/mL)	8.1 ± 2.7	17.1 ± 2.9†
Plasma norepinephrine (pmol/mL)	1.26 ± 0.29	1.74 ± 0.38*
Plasma leptin (ng/mL)	3.9 ± 2.0	4.1 ± 2.1
Blood glucose (mg/dL)	90.7 ± 5.8	93.5 ± 6.0
Total cholesterol (mg/dL)	200 ± 27	216 ± 22*
Triglyceride (mg/dL)	117 ± 35	173 ± 48‡
HDL-cholesterol (mg/dL)	58 ± 13	50 ± 12*
Uric acid (mg/dL)	5.3 ± 1.4	5.8 ± 1.2*

HOMA-IR = homeostasis model of insulin resistance.
* $P < .05$, † $P < .001$, ‡ $P < .01$ versus values in the insulin-sensitive subjects.

noceptor as seen in obesity,[6–9,11,26,27] hypertension,[6,10] and insulin resistance.[11,12] Among β_2- and β_3-adrenoceptor polymorphisms, amino acid substitutions, Arg16Gly and Gln27Glu of the β_2-adrenoceptor polymorphism, and Trp64Arg of the β_3-adrenoceptor polymorphism are also considered functionally important in understanding the genetic relationship among obesity, hypertension, and insulin resistance.

Gratze et al[28] found that young, normotensive, white male subjects homozygous for the Gly16 allele of the β_2-adrenoceptor gene had higher BP and lower peripheral vasodilation in response to the infusion of the β-blocker salbutamol. The β_2-adreoceptor is also expressed in pancreatic β-cells to modulate insulin secretion. Irakashi et al[29] suggested that the Arg16Gly variant of the β_2-adrenoceptor gene has an influence on insulin secretion. In the present study, the subjects with the Gly16 allele of the β_2-adreoceptor gene had higher plasma insulin and NE levels, suggesting that the Gly16 allele of the β_2-adrenoceptor gene is closely linked to insulin-resistant status

Table 2. Comparisons of values between subjects with and without Gly16 allele of the β_2- adrenoceptor gene

Characteristic	Subjects Without Gly16 Allele Arg16/Arg16	Subjects With Gly16 Allele (Arg16/Gly16 + Gly16/Gly16)
Number	45	110
Age (y)	36.2 ± 6.9	37.2 ± 6.5
Body mass index (kg/m^2)	21.6 ± 2.1	22.4 ± 2.5
Total body fat mass (kg)	14.6 ± 3.7	15.5 ± 3.9*
Waist to hip circumference ratio	0.90 ± 0.11	0.91 ± 0.13
Systolic blood pressure (mm Hg)	124 ± 12	129 ± 14*
Diastolic blood pressure (mm Hg)	76 ± 11	80 ± 12*
Mean blood pressure (mm Hg)	92 ± 12	96 ± 8*
Heart rates (beats/min)	63 ± 4	65 ± 3
HOMA-IR	2.5 ± 0.7	3.0 ± 0.5*
Plasma insulin (μU/mL)	10.2 ± 3.7	12.9 ± 2.2*
Plasma norepinephrine (pmol/mL)	1.28 ± 0.29	1.57 ± 0.38*
Plasma leptin (ng/mL)	3.8 ± 2.0	4.1 ± 2.1
Blood glucose (mg/dL)	91.0 ± 5.8	92.3 ± 6.0
Total cholesterol (mg/dL)	201 ± 30	210 ± 27
Triglyceride (mg/dL)	127 ± 43	148 ± 50
HDL-cholesterol (mg/dL)	57 ± 12	53 ± 13
Uric acid (mg/dL)	5.0 ± 1.4	5.7 ± 1.2*

Abbreviation as in Table 1.
* $P < .05$ versus values in the insulin-sensitive subjects.

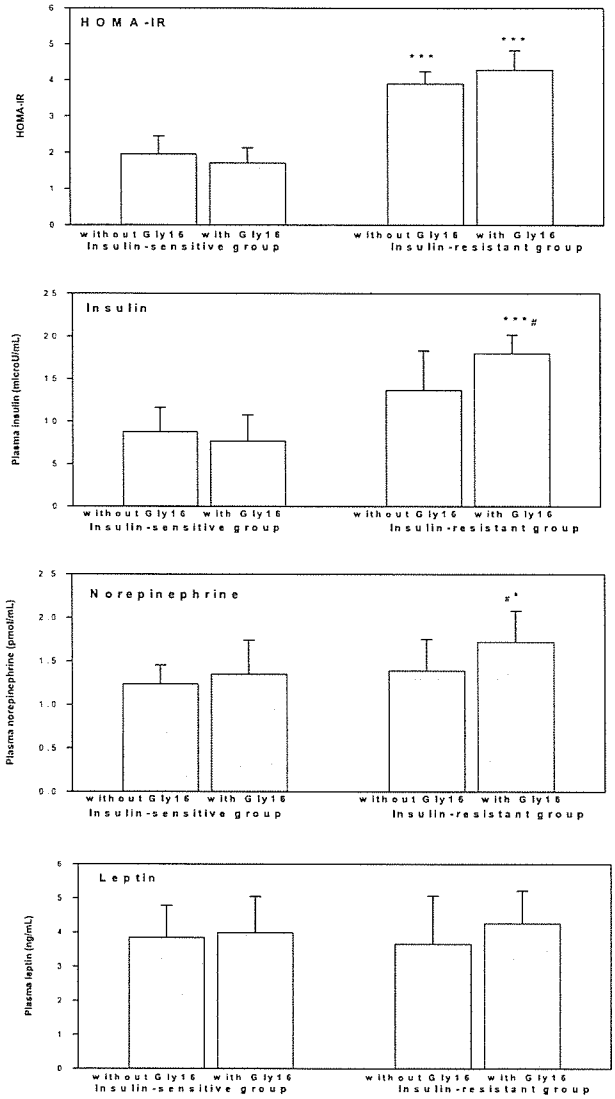

FIG. 2. The homeostasis model assessment of insulin resistance (HOMA-IR) (**top panel**), fasting plasma insulin levels (**second panel from top panel**), supine plasma norepinephrine levels (**second panel from bottom panel**), and plasma leptin levels (**bottom panel**) in the insulin-sensitive group and the insulin-resistant group according to the Gly16 allele of the β_2-adrenoceptor gene. $^{*}P < .05$, $^{***}P < .001$ versus values in the insulin-sensitive subjects. $^{\#}P < .05$ versus values in the subjects without Gly16 allele (carrying Arg16/Arg16 genotype) of the β_2-adrenoceptor gene.

associated with heightened sympathetic nerve activity shown as higher plasma NE levels and BP elevation. Thus, the Gly16 allele could lead to heightened sympathetic nerve activity, insulin resistance, and higher BP and adiposity and could predict these developments in nonobese, nonhypertensive individuals.

The Glu27/Glu27 genotype of the β_2-adrenoceptor gene has a well established association with obesity.[7] Subjects with Glu27 homozygotes have excess body fat and increased fat cell size compared with Gln homozygotes in a white population and also have abdominal obesity and insulin resistance.[26] We did not observe the association of the polymorphism at Gln27Glu of the β_2-adrenoceptor gene with insulin resistance, perhaps because of the very low frequency of the Glu27 allele. In a healthy Japanese population, distribution of the Glu27 allele of the β_2-adrenoceptor gene is different from that in individuals of non-Asian white ethnicity, as previously reported,[30] and the frequency of the Glu27 allele of the β_2-adrenoceptor gene is much lower.

Insulin Resistance Versus Sympathetic Overactivity

The group with the Gly16 allele of the β_2-adrenoceptor gene had a higher total body fat mass and BP levels, and our results in multiple regression analyses showed close relationships between HOMA-IR, plasma NE, total body fat mass, and mean BP. These findings demonstrate that the Gly16 allele that accompanies insulin resistance and heightened sympathetic nerve activity is associated with relatively greater adiposity and BP elevation. In addition, we have previously shown that insulin resistance is strongly related to heightened sympathetic nerve activity, BP elevation, and increased adiposity.[13–15,19] The present study was examined in a cross-sectional design. Hence, we could not discern the relations between genotype, BP elevation, and weight gain; however, we have reported in longitudinal studies that higher levels of plasma NE as a phenotype marker of sympathetic nerve activity predicts subsequent BP elevation and weight gain.[14–16] Taken together, our findings suggest the proposal that the adrenergic receptor defects lead to sympathetic nervous system overactivity that might play a role in the development of insulin resistance, hypertension, and obesity. In conclusion, a polymorphism at Arg16Gly of the β_2-adrenoceptor gene could be linked to insulin resistance and sympathetic nerve overactivity, as in this population of nonobese, nonhypertensive Japanese men.

References

1. Tuck ML: Glucose, insulin, and insulin resistance as biomedical predictors of hypertension. Am J Hypertens 1991;4:638S–641S.
2. Sowers JR: Obesity as a cardiovascular risk factor. Am J Med 2003;115(Suppl 8A):37S–41S.
3. Corry DB, Tuck ML: Glucose and insulin metabolism in hypertension. Am J Nephrol 1996;16:223–236.
4. Cui J, Hopper JL, Harrap SB: Genes and family environment explain correlations between blood pressure and body mass index. Hypertension 2002;40:7–12.
5. Dluhy RG: Screening for genetic causes of hypertension. Curr Hypertens Rep 2002;4:439–444.
6. Pereira A, Floriano MS, Mota GF, Cunha RS, Herkenhoff FL, Mill JG, Krieger JE: β_2 Adrenoceptor functional gene variants, obesity, and blood pressure level interactions in the general population. Hypertension 2003;42:685–692.
7. Large V, Hellstrom L, Reynisdottir S, Lonnqvist F, Eriksson P, Lannfelt L, Arner P: Human beta-2 adrenoceptor gene polymorphisms are highly frequent in obesity and associate with altered adipocyte beta-2 adrenoceptor function. J Clin Invest 1997;100:3005–3012.

8. Clement K, Vaisse C, Manning BS, Basdevant A, Guy-Grand B, Ruiz J, Silver KD, Shuldiner AR, Froguel P, Strosberg AD: Genetic variation in the β_3-adrenergic receptor and an increased capacity to gain weight in patients with morbid obesity. N Engl J Med 1995; 333:352–354.
9. Yoshida T, Sakane N, Umekawa T, Sakai M, Takahashi T, Kondo M: Mutation of β_3-adrenergic receptor gene and responses to treatment of obesity. Lancet 1995;346:1433–1434.
10. Strazzullo P, Iacone R, Siani A, Cappuccio FP, Russo O, Barba G, Barbato A, D'Elia L, Trevisan M, Farinaro E: Relationship of the Trp64Arg polymorphism of the beta3-adrenoceptor gene to central adiposity and high blood pressure interaction with age. Cross-sectional and longitudinal findings in the Olivetti Prospective Heart Study. J Hypertens 2001;19:399–406.
11. Kadowaki H, Yasuda K, Iwamoto S, Otabe S, Shimokawa K, Silver K, Walston J, Yoshinaga H, Kosaka K, Yamada N, Saito Y, Hagura R, Akanuma Y, Shuldiner A, Yazaki Y, Kadowaki T: A mutation in the β_3-adrenergic receptor gene is associated with obesity and hyperinsulinemia in Japanese subjects. Biochem Biophys Res Commun 1995;215:555–560.
12. Walston J, Silver K, Bogardus C, Knowler WC, Celi FS, Austin S, Manning B, Strosberg AD, Stern MP, Raben N, Sorkin JD, Roth J, Shuldiner AR: Time of onset of non-insulin-dependent diabetes mellitus and genetic variation in the β_3-adrenergic-receptor gene. N Engl J Med 1995;333:343–347.
13. Masuo K, Mikami H, Ogihara T, Tuck ML: Prevalence of hyperinsulinemia in young, nonobese, Japanese men. J Hypertens 1997; 15:157–165.
14. Masuo K, Mikami H, Ogihara T, Tuck ML: Sympathetic nerve hyperactivity precedes hyperinsulinemia and blood pressure elevation in a young, nonobese Japanese population. Am J Hypertens 1997;10:77–83.
15. Masuo K, Mikami H, Ogihara T, Tuck ML: Weight gain-induced blood pressure elevation. Hypertension 2000;35:1135–1140.
16. Masuo K, Kawaguchi H, Mikami H, Ogihara T, Tuck ML: Serum uric acid and plasma norepinephrine concentrations predict subsequent weight gain and blood pressure elevation. Hypertension 2003; 42:474–480.
17. Masuo K, Mikami H, Ogihara T, Tuck ML: Familial hypertension, insulin, sympathetic activity, and blood pressure elevation. Hypertension 1998;32:96–100.
18. Masuo K, Mikami H, Ogihara T, Tuck ML: Familial obesity, sympathetic activation and blood pressure level. Blood Press 2001; 10:199–204.
19. Masuo K, Mikami H, Ogihara T, Tuck ML: Weight reduction and pharmacological treatment in obesity hypertensives. Am J Hypertens 2001;14:530–538.
20. Kanauchi M, Yamano S, Kanauchi K, Saito Y: Homeostasis model assessment of insulin resistance, quantitive insulin sensitivity check index, and oral glucose insulin sensitivity index in nonobese, nondiabetic subjects with high-normal blood pressure. J Clin Endocrinol Metab 2003;88:3444–3446.
21. Ranade K, Chang MS, Ting CT, Pei D, Hsiao CF, Olivier M, Pesich R, Hebert J, Chen YD, Dzau VJ, Curb D, Olshen R, Risch N, Cox DR, Botstein D: High-throughput genotyping with single nucleotide polymorphisms. Genome Res 2001;11:1262–1268.
22. Tuck ML: The sympathetic nervous system in essential hypertension. Am Heart J 1988;112:877–886.
23. Grassi G, Esler M: How to assess sympathetic activity in humans. J Hypertens 1999;17:719–734.
24. Rahn KH, Barenbrock M, Hausberg M: The sympathetic nervous system in the pathogenesis of hypertension. J Hypertens 1999; 17(Suppl 3):S11–S14.
25. Kopin IJ, Rundqvist B, Friberg P, Lenders J, Goldstein DS, Eisenhofer G: Different relationships of spillover to release of norepinephrine in human heart, kidney, and forearm. Am J Physiol 1998; 27:R165–R173.
26. Hellstrom L, Large V, Reynisdottir S, Wahrenberg H, Arner P: The different effects of Gln27Glu β_2-adrenoceptor gene polymorphism on obesity in males and in females. J Intern Med 1999;245:253–259.
27. Clement K, Manning BS, Basdevant A, Strosberg AD, Guy-Grand B, Froguel P: Gender effect of the Trp64Arg mutation in the β_3 adrenergic receptor gene on weight gain in morbid obesity. Diabetes Metab 1997;23:424–427.
28. Gratze G, Fortin J, Labugger R, Binder A, Kotanko P, Timmermann B, Luft FC, Hoehe MR, Skarbal F: β_2-adrenergic receptor variants affect resting blood pressure and agonist-induced vasodilation in young adult Caucasians. Hypertension 1999;33:1425–1430.
29. Ikarashi T, Hanyu O, Maruyama S, Souda S, Kobayashi C, Abe E, Ukisu J, Naganuma K, Suzuki A, Toya M, Kaneko S, Suzuki K, Nakagawa O, Aizawa Y: Genotype Gly/Gly of the Arg16Gly polymorphism of the beta2-adrenergic receptor is associated with elevated fasting serum insulin concentrations, but not with acute insulin response to glucose in type 2 diabetic patients. Diabetes Res Clin Pract 2004;63:11–18.
30. Kawamura T, Egusa G, Fujikawa R, Okubo M: Gln27Glu variant of the beta2-adrenergic receptor gene is not associated with obesity and diabetes in Japanese-Americans. Metabolism 2001;50:443–446.

AJH 2005; 18:1508–1516

Rebound Weight Gain as Associated With High Plasma Norepinephrine Levels That Are Mediated Through Polymorphisms in the β2-Adrenoceptor

Kazuko Masuo, Tomohiro Katsuya, Hideki Kawaguchi, Yuxiao Fu, Hiromi Rakugi, Toshio Ogihara, and Michael L. Tuck

Background: A successful weight loss program is essential treatment for obesity-related diseases, but it is well known that the majority of individuals do not succeed in weight loss maintenance. The present study evaluates hormonal mechanisms and the relationship of β2-adrenoceptor polymorphisms involved in individuals who regain weight after initially successful weight loss.

Methods: Overweight Japanese men (n = 154) were enrolled in a 24-month weight loss program. Body mass index (BMI), total body fat mass, plasma norepinephrine (NE) and leptin levels, and β2-adrenoceptor polymorphisms (Arg16Gly, Gln27Glu) were measured every 6 months for the 24-month period. Maintenance of weight loss was defined as significant weight loss (≥10% reduction) from entry weight at 6 months and maintenance of the weight loss for an additional 18 months. Rebound weight gain was defined as significant weight loss at 6 months but subsequent regain of body weight during the next 18 months.

Results: The results showed that 37 subjects maintained weight loss during 24 months, whereas 36 subjects had rebound weight gain. The BMI at entry and calorie intake and physical activity at each period were similar between the two groups. Subjects who maintained weight loss had at entry a significantly lower fat mass and plasma NE levels compared to those with rebound weight gain. Body fat mass, NE, and leptin levels at entry predicted the degree of change in body weight during the 24-month study period. Subjects with rebound weight gain had a significantly higher frequency of the Gly16 allele for the β2-adrenoceptor polymorphism compared to subjects who had a 24-month maintenance of weight loss. Subjects carrying the Gly16 allele also had significantly higher plasma NE, leptin, and body fat mass levels and a greater waist-to-hip ratio both at entry and throughout the study.

Conclusions: A high initial degree of body fat mass and high plasma NE levels as determined by the Gly16 allele for the β2-adrenoceptor polymorphisms predict those individuals who will have rebound weight gain after their initial successful weight loss. Am J Hypertens 2005;18:1508–1516 © 2005 American Journal of Hypertension, Ltd.

Key Words: Rebound weight gain, weight loss resistance, sympathetic nerve activity, leptin, obesity, β2-adrenoceptor polymorphisms.

Weight loss and maintenance of weight loss are the most effective nonpharmacologic treatments for correction of cardiovascular and metabolic risk factors in obese patients.[1–7] However, few obese people succeed in sustained weight loss, and long-term results of weight loss programs are disappointing with a substantial proportion of people regaining most of the weight initially lost.

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There is strong evidence suggesting that human obesity has both genetic and environmental determinants.[8,9] Investigations have reported associations of polymorphisms of the β2- and β3-adrenoceptors in obesity,[10–15] and regulation of thermogenesis is mainly attributed to β2- and β3-adrenergic receptor activity. Increased energy expenditure and increased resting metabolic rate are predictive

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From the Human Neurotransmitter Laboratory, Baker Heart Research Institute (KM), Melbourne, Victoria, Australia; Department of Geriatric Medicine, Osaka University Graduate School of Medicine (KM, TK, HK, YF, HR, TO), Suita City, Osaka, Japan; and Endocrinology and Metabolism Division, Sepulveda VA Medical Center and the David Geffen, UCLA School of Medicine (MLT), Los Angeles, California.

Address correspondence and reprint requests to Dr. Kazuko Masuo, Baker Heart Research Institute, PO Box 6492 St Kilda Road Central, Melbourne, Victoria 8008, Australia; e-mail: kmasuo@baker.edu.au

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of weight loss, and the sympathetic nervous system plays a key role in regulating energy balance through stimulation of thermogenesis. Effects on rates of thermogenesis are also influenced by genetic factors. Few studies have taken into account success in maintenance of weight loss, resistance to weight loss, and rebound weight gain as part of hormonal changes associated with changes in body weight or the polymorphisms of the β-adrenoceptor genes that occur with weight change. We examined weight loss in relation to changes in body fat mass, plasma norepinephrine (NE), leptin, and insulin. In addition, we compared polymorphisms of β2- and β3-adrenoceptor genes in subjects who maintained weight loss during 24 months to those who regain body weight (rebound weight) in a protocol of a defined, constant dietary intake and exercise program.

Methods

Subjects

The weight loss program enrolled 154 overweight (25 kg/m^2 ≤body mass index [BMI] <30 kg/m^2) men, consisting of 89 overweight normotensive men (blood pressure [BP] <140/90 mm Hg) and 65 overweight, untreated mildly hypertensive men (140/90 mm Hg ≤BP <160/95 mm Hg). None of the subjects had diabetes (fasting blood glucose level >100 mg/dL) or other illness including psychological or emotional problems.[16] No subject was taking antihypertensive agents or other medications. Furthermore, no subject had any symptoms of obstructive sleep apnea (ie, breathing pauses every night or almost every night) or extremely loud habitual snoring or sleepiness during the daytime.[17,18] Only subjects whose body weight had not changed for at least the past 2 years (weight change <5%) provided in their biannual medical evaluation records were enrolled in the present study.[4,19] The subjects enrolled in this weight loss program were emotionally stable,[16] and had a similar socioeconomic status. The protocol was approved by the Ethics Committee of Osaka University Graduate School of Medicine, Japan, and written informed consent was obtained from all the subjects.

Study Design

The weight loss program consisted of a low caloric diet (1600 kcal/d, 55% of calories from carbohydrate, 30% from protein, and 15% from fat) and a low sodium diet (7g NaCl per day) and aerobic exercise of more than 1 h daily (eg, walking, jogging, or gym exercise). The subjects attended a 1-h private teaching and counseling session each week for 4 weeks, followed by biweekly 1-h sessions for 23 additional months. All sessions were led by experts in nutrition and exercise counseling. Calorie intake was calculated based on the subjects meal diary, which was assisted by nutritionists. The physical activity was quantified and recorded by the use of step-counters used on a daily basis. Diet and exercise compliance were monitored according to the subjects' own records every 2 weeks and were recorded at private counseling sessions. Compliance to diet and exercise was considered excellent and consistent based on those records.

Methods

After an overnight fast of 12 h and 30 min rest in the supine position, height, body weight, BMI, percentage total body fat mass, BP, heart rate, and venous blood sampling for measurements of blood glucose, plasma NE, leptin, insulin, and the extraction of genomic DNA from leukocytes were obtained. Samples were taken at entry and at 6, 12, and 24 months during the study. The BP and heart rate were measured more than three times in the supine position by an automated sphygmomanometer (TM-2713, A&D, Tokyo, Japan) using an adjusted cuff size based on arm circumference. Recorded BP levels and heart rates were averaged. The percentage body fat mass was determined by impedance measurements (BF-102, Tanita, Tokyo, Japan). Total body fat mass (in kilograms) was calculated according to the following formula: [percentage body fat mass (%)/100] × body weight (kg). Plasma NE was measured after separation by high-performance liquid chromatography using the fluorometric method as previously described in detail,[19] and plasma immunoreactive insulin was measured by a standard radioimmunoassay method as described in detail (insulin RIABEAD II, Dinabott, Tokyo, Japan).[19] Plasma leptin was measured by radioimmunoassay[19] (human leptin RIA kit, Linco, St. Charles, MO, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was defined as the product of fasting plasma insulin (in microunits per milliliter) and glucose (in milligrams per deciliter) divided by 405.[20]

Genotyping

Genotyping was performed by the TaqMan assay, as previously detailed (Applied Biosystems, Foster City, CA, USA).[21] Two polymorphisms (Arg16Gly, Gln27Glu) of the β2-adrenoceptors[13,14] and one polymorphism (Trp64Arg) of the β3-adrenoceptor[22] were evaluated. For single-nucleotide polymorphisms (SNPs) in the β2-adrenoceptor genes, the probes and primers were as follows: for Arg16Gly, the probes were CGCATGGCTTCCATTGGGTGC and CGCATGGCTTCTATTGGGTGC, and the primers were GGAACGGCAGCGCCTTCT and CAGGACGATGAGAGACATGACGAT; and for Gln27Glu, the probes were CTCGTCCCTTTCCTGCGTGACGT and CTCGTCCCTTTGCTGCGTGACGT (the primers used in this assay were the same as those used for Arg16Gly). For the Trp64Arg SNP in the β3- adrenoceptor, the probes were TCTCGGAGTCCAGGCGATGGCCA and CTCGGAGTCCGGGCGATGGCC, and the primers were GGAGGCAACCTGCTGGTCAT and CACGAACACGTTGGTCATGGT.

Table 1. Characteristics in the four study groups according to responses to weight loss

	Weight Loss Maintenance	Rebound	Slow Weight Loss	Weight Loss Resistance
Subjects (*n*)	37	36	60	21
Age at entry (yr)	35 ± 6	37 ± 6	37 ± 6	37 ± 8
Height (m)	1.73 ± 0.05	1.74 ± 0.04	1.74 ± 0.05	1.72 ± 0.05
BMI (kg/m^2)				
Entry	27.1 ± 1.9	27.7 ± 1.6	27.3 ± 1.7	27.4 ± 2.0
6 months	22.8 ± 1.4†**	22.8 ± 0.8†‖**	25.4 ± 2.0‡	26.6 ± 2.2§
12 months	22.6 ± 2.3†**	23.4 ± 2.2*#	24.9 ± 1.9	26.1 ± 2.9‡
18 months	22.4 ± 2.0†‡**	25.1 ± 2.2#	24.1 ± 2.0*#	26.0 ± 2.3‖#
24 months	22.1 ± 1.5†§**	26.4 ± 2.1‖	23.8 ± 1.9*‡**	26.1 ± 2.2‖#
Total body fat mass (kg)				
Entry	21.5 ± 5.4†‡‖	25.6 ± 4.9*	25.9 ± 6.1	27.8 ± 4.2‡
6 months	17.8 ± 4.7†‡‖#	21.0 ± 3.8*#	22.3 ± 5.2	24.3 ± 4.1‡
12 months	16.5 ± 4.1†‡‖#	21.5 ± 4.1#	20.7 ± 4.9#	22.8 ± 3.8#
18 months	15.6 ± 3.8†§**	22.2 ± 4.7‖#	18.7 ± 5.1*‡**	21.9 ± 4.1‖**
24 months	15.2 ± 4.3†§**	22.5 ± 5.0‖#	17.8 ± 4.9*‡**	21.2 ± 4.7‖**
Waist-to-hip ratio				
Entry	1.16 ± 0.10*‖	1.20 ± 0.11	1.23 ± 0.15	1.23 ± 0.15
6 months	0.98 ± 0.09*‖#	1.03 ± 0.10*‖#	1.15 ± 0.14*‡	1.22 ± 0.09‡‖
12 months	0.95 ± 0.10*‡#	1.05 ± 0.09*#	1.05 ± 0.11*#	1.17 ± 0.13‡‖
18 months	0.92 ± 0.09†§**	1.15 ± 0.20‖	1.03 ± 0.13*‡#	1.12 ± 0.11‖#
24 months	0.90 ± 0.12†§**	1.12 ± 0.14‖	0.98 ± 0.12*‡**	1.16 ± 0.13‖#
Systolic BP (mm Hg)				
Entry	136 ± 12	134 ± 10	137 ± 10	133 ± 12
6 months	135 ± 10	134 ± 9	134 ± 9	134 ± 8
12 months	133 ± 9	136 ± 7	134 ± 8	135 ± 8
18 months	128 ± 10*‡	135 ± 8	132 ± 9	134 ± 6
24 months	128 ± 9#	133 ± 8	130 ± 10#	130 ± 11
Diastolic BP (mm Hg)				
Entry	78 ± 10	77 ± 9	79 ± 9	74 ± 11
6 months	77 ± 9	76 ± 7	76 ± 8	73 ± 7
12 months	76 ± 8	77 ± 6	75 ± 8	73 ± 8
18 months	74 ± 9	77 ± 7	74 ± 9	76 ± 7
24 months	73 ± 8#	76 ± 7	72 ± 10#	72 ± 11
Mean BP (mm Hg)				
Entry	98 ± 12	96 ± 10	98 ± 9	94 ± 14
6 months	97 ± 10	96 ± 7	96 ± 9	94 ± 7
12 months	95 ± 9	97 ± 6	95 ± 8	94 ± 8
18 months	92 ± 10	96 ± 8	94 ± 9	95 ± 6
24 months	92 ± 9#	95 ± 8	91 ± 11#	93 ± 13
Heart rate (beats/min)				
Entry	68 ± 7	70 ± 8	68 ± 8	71 ± 7
6 months	66 ± 6	68 ± 7	67 ± 7	71 ± 6
12 months	65 ± 7	69 ± 7	66 ± 8	69 ± 6
18 months	64 ± 5‡	70 ± 8	66 ± 7	68 ± 7
24 months	62 ± 6‡#	68 ± 7	65 ± 7	67 ± 5
Plasma norepinephrine (pmol/mL)				
Entry	1.53 ± 0.32†‡‖	1.87 ± 0.37*‖	2.25 ± 0.31*‡	2.59 ± 0.53‡‖
6 months	1.31 ± 0.30†¶	1.49 ± 0.41†#	2.01 ± 0.33‡	2.22 ± 0.51§
12 months	1.17 ± 0.31†‡¶#	1.63 ± 0.44*	1.78 ± 0.35#	2.07 ± 0.47‡
18 months	1.11 ± 0.32†‖#	1.40 ± 0.51*#	1.54 ± 0.31**	2.02 ± 0.46‡‖#
24 months	1.09 ± 0.28†‡‖**	1.32 ± 0.37*#	1.41 ± 0.37**	1.87 ± 0.48‡‖**
Plasma leptin (ng/mL)				
Entry	7.9 ± 2.8*‖	8.9 ± 2.9*‖	10.1 ± 3.0‡	11.7 ± 2.7‡
6 months	5.8 ± 2.5†#	6.2 ± 2.4†#	7.8 ± 2.9*	10.2 ± 3.0§‖
12 months	4.5 ± 1.9†‖#	5.8 ± 2.3*#	6.8 ± 2.7*#	8.5 ± 2.4‡‖
18 months	4.0 ± 1.7†‡**	6.1 ± 2.6*#	6.0 ± 2.8*#	8.2 ± 3.0‡‖#
24 months	3.7 ± 1.8†‡**	6.7 ± 2.5‖#	5.1 ± 1.7*‡**	7.1 ± 2.9‖#
HOMA-IR				
Entry	2.3 ± 0.5	2.5 ± 0.5	2.5 ± 0.6	2.7 ± 0.6
6 months	2.0 ± 0.3*	2.0 ± 0.4*#	2.3 ± 0.5	2.5 ± 0.5‡
12 months	1.7 ± 0.5‖	2.0 ± 0.5	2.1 ± 0.4	2.2 ± 0.6
18 months	1.7 ± 0.5*#	2.1 ± 0.6	2.0 ± 0.5#	2.2 ± 0.5
24 months	1.4 ± 0.6*‡‖**	2.1 ± 0.5	1.8 ± 0.5**	2.2 ± 0.4#