Table 1
Demographics and baseline characteristics

	Placebo (n = 35)	Rosuvastatin			Bezafibrate 200 mg bid (n = 27)
		5 mg (n = 32)	10 mg (n = 34)	20 mg (n = 26)	
Age (years), mean (S.D.)	51.6 (10.2)	50.4 (11.2)	51.6 (11.5)	55.8 (11.4)	50.5 (12.2)
Male/female, n (%)	21 (60.0)/14 (40.0)	22 (68.8)/10 (31.3)	26 (76.5)/8 (23.5)	20 (76.9)/6 (23.1)	21 (77.8)/6 (22.2)
Body mass index (kg/m²), mean (S.D.)	26.0 (3.0)	25.0 (2.8)	26.1 (3.1)	25.8 (3.1)	26.0 (3.0)
Fredrickson type, n (%)					
IIb	15 (42.9)	13 (40.6)	12 (35.3)	8 (30.8)	10 (37.0)
IV	20 (57.1)	19 (59.4)	22 (64.7)	18 (69.2)	17 (63.0)
Concomitant disease, n (%)					
Hypertension	11 (31.4)	10 (31.3)	14 (41.2)	12 (46.2)	10 (37.0)
Diabetes mellitus	2 (5.7)	4 (12.5)	2 (5.9)	4 (15.4)	1 (3.7)
Coronary artery disease	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

placebo group. Five of these patients (three in the rosuvastatin 5 mg group, one in the rosuvastatin 10 mg group and one in the bezafibrate group) discontinued treatment because of adverse events. A total of 15 patients were withdrawn according to study-specific discontinuation criteria (5 due to increased TG levels to ≥800 mg/dL, 9 due to decreased LDL-C levels to ≤50 mg/dL, and 1 due to both), 1 patient did not fulfill eligibility criteria, and 2 patients were not willing to continue in the study.

3.1. Efficacy

Mean baseline TG levels ranged from 334 to 398 mg/dL. Compared with placebo, TG was reduced by 30.1% with rosuvastatin 5 mg, 30.1% with rosuvastatin 10 mg and 32.3% with rosuvastatin 20 mg (all $p \leq 0.0001$), with no evidence of a dose–response (Table 2; Fig. 1). Reductions in TG were evident by week 2 and were sustained through week 8 without marked change. In terms of raw means, rosuvastatin across

its dose range reduced TG by 29.1–31.1% from baseline, compared with 45.4% for bezafibrate. LDL-TG and VLDL-TG were reduced in all rosuvastatin groups with no apparent dose–response, and no change in HDL-TG was observed (Table 2).

Non-HDL-C, LDL-C, and TC were all significantly reduced with rosuvastatin, compared with placebo, with evidence of a dose effect for each (Table 3); HDL-C was significantly increased at each rosuvastatin dose with no evidence of dose-related effects. Apo B was reduced in all rosuvastatin groups with evidence of a dose effect, while apo A-1 was increased in all rosuvastatin groups with no evidence of a dose effect; however, statistical comparisons with placebo were not performed for these parameters (Table 4). Lipid ratios were all markedly reduced in an apparent dose-related manner with rosuvastatin treatment. The inflammatory marker hs-CRP was reduced by medians of 22.9–38.5% with rosuvastatin treatment, compared with an increase of 3.5% in the placebo group.

Table 2
Changes in TG levels from baseline at 8 weeks

	Placebo (n = 35)	Rosuvastatin			Bezafibrate[a] 200 mg bid (n = 27)
		5 mg (n = 32)	10 mg (n = 34)	20 mg (n = 26)	
TG					
Baseline (mg/dL), mean (S.D.)	334 (118)	336 (125)	338 (151)	398 (120)	355 (126)
% Change, mean (S.D.)	+1 (30)	−29 (22)	−29 (23)	−31 (39)	−45 (21)
Difference with placebo, estimate [95% CI][b]	–	−30 [−47, −13] $P = 0.0001$	−30 [−47, −13] $P < 0.0001$	−32 [−50, −14] $P = 0.0001$	–
VLDL-TG					
Baseline (mg/dL), mean (S.D.)	197.7 (85.6)	201.3 (92.1)	207.5 (145.3)	243.7 (94.5)	225.0 (117.5)
% Change, mean (S.D.)	+11.7 (45.8)	−22.6 (33.4)	−19.9 (41.7)	−24.7 (54.6)	−48.0 (24.5)
LDL-TG					
Baseline (mg/dL), mean (S.D.)	51.5 (16.9)	54.1 (20.2)	56.6 (24.3)	57.3 (22.7)	54.5 (18.3)
% Change, mean (S.D.)	+12.3 (49.9)	−25.6 (22.4)	−34.0 (24.7)	−31.7 (16.7)	−15.3 (23.1)
HDL-TG					
Baseline (mg/dL), mean (S.D.)	22.5 (7.2)	24.3 (9.6)	22.9 (9.9)	26.4 (9.9)	24.8 (11.0)
% Change, mean (S.D.)	+3.2 (29.8)	−1.9 (29.0)	−0.4 (29.2)	+1.7 (40.5)	−22.0 (30.2)

[a] Results for bezafibrate provided as a benchmark comparator; only raw means available in the table.

[b] Results of ANOVA model including treatment (placebo and rosuvastatin 5, 10 and 20 mg) as fixed effect. Changes are shown as differences between rosuvastatin and placebo. p-Values were calculated only for TG.
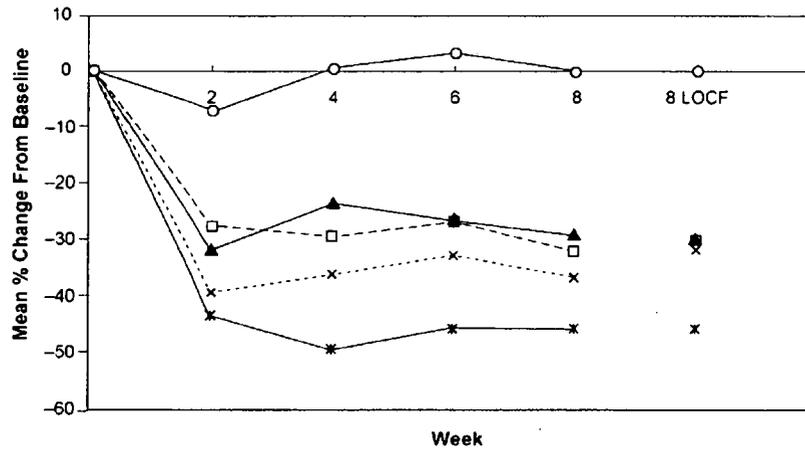
Fig. 1. Mean % change in TG from baseline at 2, 4, 6 and 8 weeks and at 8 weeks with last observation carried forward (LOCF) in patients receiving placebo or rosuvastatin 5, 10 or 20 mg once daily, or bezafibrate 200 mg twice daily (○, placebo; □, rosuvastatin 5 mg qd; ▲, rosuvastatin 10 mg qd; ·×·, rosuvastatin 20 mg qd; ✕, bezafibrate 200 mg bid).

When compared with bezafibrate (raw means), rosuvastatin produced superior results with respect to changes in non-HDL-C and LDL-C, while generating smaller improvements in HDL-C (12.4–16.7% for rosuvastatin across its dose range versus 19.6% for bezafibrate) (Tables 3 and 4). Hs-CRP also exhibited dose-related reduction with rosuvastatin treatment (22.9–38.5%).

Table 3
Changes in lipid/lipoprotein profiles and hs-CRP from baseline at 8 weeks

	Placebo (n = 35)	Rosuvastatin			Bezafibrate[a] 200 mg bid (n = 27)
		5 mg (n = 32)	10 mg (n = 34)	20 mg (n = 26)	
Non-HDL-C					
Baseline (mg/dL), mean (S.D.)	202.7 (52.1)	190.1 (34.5)	192.3 (49.6)	191.9 (33.9)	192.7 (35.6)
% Change, mean (S.D.)	+1.4 (9.3)	−36.8 (13.7)	−41.0 (16.0)	−44.3 (17.6)	−2.0 (12.1)
vs. placebo, estimate [95% CI][b]	–	−38.1 [−46.5, −29.8]	−42.3 [−50.5, −34.1]	−45.6 [−54.5, −36.8]	–
LDL-C					
Baseline (mg/dL), mean (S.D.)	138.1 (49.7)	124.4 (35.8)	125.9 (43.1)	115.9 (29.6)	122.8 (34.9)
% Change, mean (S.D.)	+3.6 (15.5)	−31.9 (17.8)	−38.1 (18.6)	−41.0 (18.3)	+29.3 (30.2)
vs. placebo, estimate [95% CI][b]	–	−35.5 [−45.7, −25.2]	−41.7 [−51.8, −31.6]	−44.6 [−55.5, −33.7]	–
TC					
Baseline (mg/dL), mean (S.D.)	246.0 (54.9)	231.9 (35.2)	232.2 (52.6)	234.2 (36.9)	234.3 (34.3)
% Change, mean (S.D.)	+1.3 (8.2)	−27.1 (11.3)	−31.4 (13.1)	−33.3 (12.8)	+2.1 (9.9)
vs. placebo, estimate [95% CI][b]	–	−28.4 [−35.1, −21.7]	−32.7 [−39.3, −26.1]	−34.7 [−41.8, −27.6]	–
HDL-C					
Baseline (mg/dL), mean (S.D.)	43.2 (7.4)	41.8 (7.4)	39.8 (7.2)	42.3 (8.5)	41.6 (9.0)
% Change, mean (S.D.)	+1.8 (10.7)	+14.5 (16.9)	+12.4 (16.3)	+16.7 (15.7)	+19.6 (13.6)
vs. placebo, estimate [95% CI][b]	–	+12.7 [+3.9, +21.5]	+10.7 [+2.0, +19.3]	+15.0 [+5.7, +24.4]	–
RLP-C					
Baseline (mg/dL), mean (S.D.)	14 (6)	15 (11)	17 (16)	17 (10)	15 (9)
% Change, mean (S.D.)	+8 (58)	−48 (25)	−49 (24)	−50 (46)	−43 (27)
SD-LDL					
Baseline (mg/dL), mean (S.D.)	13 (5)	11 (6)	11 (8)	13 (4)	12 (6)
% Change, median	−7	−16	−22	−34	−33
hs-CRP					
Baseline (mg/dL), mean (S.D.)	0.115 (0.139)	0.120 (0.127)	0.138 (0.136)	0.166 (0.161)	0.096 (0.105)
% Change, median	+3.5	−22.9	−37.8	−38.5	−1.7

[a] Results for bezafibrate provided as a benchmark comparator; only raw means available in the table.
[b] Results of ANOVA model including treatment (placebo and rosuvastatin 5, 10 and 20 mg) as fixed effect. Changes are shown as differences between rosuvastatin and placebo. p-Values were calculated only for TG.

Table 4

Changes in apolipoprotein profiles and lipid ratios from baseline at 8 weeks

	Placebo (n = 35)	Rosuvastatin			Bezafibrate[a] 200 mg bid (n = 27)
		5 mg (n = 32)	10 mg (n = 34)	20 mg (n = 26)	
Apo B					
Baseline (mg/dL), mean (S.D.)	134.2 (33.4)	125.5 (23.0)	128.5 (37.6)	125.2 (19.6)	127.5 (21.7)
% Change, mean (S.D.)	+2.8 (10.1)	−29.1 (12.7)	−34.3 (14.0)	−35.3 (15.3)	+2.4 (12.7)
Apo A-I					
Baseline (mg/dL), mean (S.D.)	142.1 (22.4)	140.0 (19.2)	134.6 (16.8)	145.3 (26.3)	144.3 (36.8)
% Change, mean (S.D.)	+1.3 (7.3)	+7.8 (9.7)	+7.9 (15.9)	+9.8 (11.3)	+7.5 (9.1)
Apo C-III					
Baseline (mg/dL), mean (S.D.)	18.5 (6.2)	18.0 (4.8)	17.6 (5.8)	20.5 (5.2)	19.9 (7.8)
% Change, mean (S.D.)	+3.9 (19.2)	−18.7 (19.4)	−17.5 (26.7)	−16.6 (25.7)	−32.6 (13.6)
Non-HDL-C:HDL-C					
Baseline, mean (S.D.)	4.74 (1.15)	4.67 (1.15)	4.90 (1.25)	4.66 (1.04)	4.88 (1.51)
% Change, mean (S.D.)	+0.5 (13.1)	−43.7 (14.5)	−46.2 (17.6)	−50.2 (21.1)	−17.0 (13.8)
LDL-C:HDL-C					
Baseline, mean (S.D.)	3.21 (1.08)	3.03 (0.92)	3.17 (0.95)	2.79 (0.72)	3.12 (1.16)
% Change, mean (S.D.)	+1.9 (12.0)	−39.6 (17.4)	−43.9 (18.6)	−47.7 (20.7)	+8.7 (24.8)
TC:HDL-C					
Baseline, mean (S.D.)	5.74 (1.15)	5.67 (1.15)	5.90 (1.25)	5.66 (1.04)	5.88 (1.51)
% Change, mean (S.D.)	+0.2 (10.6)	−35.5 (11.5)	−38.0 (14.2)	−41.2 (17.1)	−13.9 (10.9)
Apo B:apo A-I					
Baseline, mean (S.D.)	0.96 (0.26)	0.92 (0.21)	0.96 (0.26)	0.88 (0.18)	0.94 (0.28)
% Change, mean (S.D.)	+1.6 (9.0)	−33.9 (12.9)	−37.9 (15.8)	−40.0 (18.4)	−3.6 (17.4)

[a] Results for bezafibrate provided as a benchmark comparator; only raw means available in the table.

3.2. Safety

Treatment-emergent adverse events occurred in 57.1% of placebo recipients, 51.1% of rosuvastatin recipients and 59.3% of bezafibrate recipients (Table 5). Adverse events considered related to study treatment occurred in 8.6% of placebo recipients, 10.9% of rosuvastatin recipients and 14.8% of bezafibrate recipients. Serious adverse events occurred in five patients receiving rosuvastatin. One patient receiving rosuvastatin 5 mg had abnormal liver function, which was later confirmed by biopsy to be due to autoimmune hepatitis. In the rosuvastatin 10 mg group, one patient had acute cholecystitis, one had small cell lung cancer, and

one had a rib fracture. One rosuvastatin 20 mg patient had a benign salivary gland neoplasm. Four serious adverse events other than abnormal liver function were considered unrelated to study treatment.

No serious adverse events were seen in the bezafibrate patients. The four rosuvastatin patients discontinuing treatment because of adverse events included the patient with acute cholecystitis and the patient with abnormal liver function noted above. One patient receiving rosuvastatin 5 mg discontinued treatment because of worsening constipation, which was judged related to study treatment. Another patient receiving rosuvastatin 5 mg discontinued treatment because of numbness in the hand, which was considered unrelated

Table 5

Treatment-emergent adverse events (AEs)

	Placebo (n = 35)	No. (%) of patients rosuvastatin				Bezafibrate 200 mg bid (n = 27)
		5 mg (n = 32)	10 mg (n = 34)	20 mg (n = 26)	All (n = 92)	
Any AE	20 (57.1)	14 (43.8)	17 (50.0)	16 (61.5)	47 (51.1)	16 (59.3)
Serious AE leading to death	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Serious AE	0 (0.0)	1 (3.1)	3 (8.8)	1 (3.8)	5 (5.4)	0 (0.0)
Discontinuation of study treatment due to AE	0 (0.0)	3 (9.4)	1 (2.9)	0 (0.0)	4 (4.3)	1 (3.7)
Treatment-related AE	3 (8.6)	3 (9.4)	2 (5.9)	5 (19.2)	10 (10.9)	4 (14.8)
Most frequent AEs, reported for >4% of patients in any treatment group						
Nasopharyngitis	7 (20.0)	3 (9.4)	3 (8.8)	3 (11.5)	9 (9.8)	4 (14.8)
Arthralgia	0 (0.0)	0 (0.0)	2 (5.9)	2 (7.7)	4 (4.3)	0 (0.0)
Constipation	1 (2.9)	2 (6.3)	1 (2.9)	1 (3.8)	4 (4.3)	1 (3.7)
Myalgia	1 (2.9)	0 (0.0)	2 (5.9)	2 (7.7)	4 (4.3)	0 (0.0)

Table 6
Changes in mean creatinine values and number of subjects with an increase in creatinine ≥30% from baseline at 8 weeks

	Placebo (n = 35)	Rosuvastatin			Bezafibrate 200 mg bid (n = 27)
		5 mg (n = 32)	10 mg (n = 34)	20 mg (n = 26)	
Creatinine					
Baseline (mg/dL), mean (S.D.)	0.759 (0.182)	0.794 (0.186)	0.804 (0.158)	0.813 (0.150)	0.804 (0.175)
Change (mg/dL), mean (S.D.)	−0.012 (0.072)	0.008 (0.058)	−0.006 (0.075)	−0.004 (0.067)	0.083 (0.094)
≥30% increase (n, %)	0	0	0	0	0

to study treatment. The most common adverse events (>4% frequency) in rosuvastatin patients irrespective of causality assessment were nasopharyngitis, arthralgia, constipation and myalgia. The most frequent adverse event (>4% frequency) in bezafibrate patients was nasopharyngitis.

Clinically significant increases in ALT (defined as increases >3 times ULN on two separate occasions at least 2 days apart) were observed in two rosuvastatin patients, including the patient described above who was subsequently diagnosed with autoimmune hepatitis, and in one patient receiving rosuvastatin 20 mg (increases at weeks 2 and 6). No patients had serum creatine kinase elevations >10 times ULN, and no cases of myopathy or rhabdomyolysis were observed. No patients had increases in creatinine of ≥30% from baseline, and there were no notable differences among treatment groups with regard to changes in mean creatinine levels (Table 6). No proteinuria (defined as shift from "none/trace" at baseline to "≥++") was observed in study patients at weeks 4, 8, or at the time of withdrawal. Transient hematuria was observed in three patients receiving rosuvastatin 5 mg, one receiving 10 mg and one receiving 20 mg.

4. Discussion

In this study, rosuvastatin 5–20 mg reduced TG levels in hypertriglyceridemic Japanese patients by 30–32% over 8 weeks, compared with placebo, with no evidence of a dose-related effect. Reductions were observed after 2 weeks of treatment and were maintained throughout the 8 weeks. Rosuvastatin treatment also produced significant beneficial changes in other lipid parameters, compared with placebo, including non-HDL-C reductions of 38–46%, LDL-C reductions of 35–45%, TC reductions of 28–35% and HDL-C increases of 11–15%, as well as marked reductions in VLDL- and LDL-TG fractions and lipid ratios from baseline. Median levels of the inflammatory marker hs-CRP were reduced by 22.9–38.5% with rosuvastatin treatment. A bezafibrate arm was included in the current study to provide guidance in the design of a phase III trial of rosuvastatin in a hypertriglyceridemic population. Treatment with bezafibrate 400 mg/day produced greater improvement in TG and HDL-C than rosuvastatin but, as expected, had substantially less beneficial effects on other atherogenic markers, including a 2.0% decrease in non-HDL-C from baseline, and a 29% increase in LDL-C.

A trial of Western hypertriglyceridemic patients [5] showed that 10 mg of rosuvastatin provided greater TG reductions than did 5 mg (37% versus 18%), although there was little difference in treatment effect at doses above 10 mg (37% reduction with 20 mg, 40% with 40 mg and 40% with 80 mg). However, median decreases in TG for the 5-, 10-, 20-, 40- and 80-mg doses were 21, 37, 37, 43 and 46%, respectively, suggesting a modest dose–response relationship. Data from the current study in Japanese patients do not suggest a dose effect in TG reduction, with very similar magnitudes of reduction being observed at 5-, 10- and 20-mg doses.

This study showed that rosuvastatin 5 mg produced beneficial reductions in TG levels, although higher doses did not provide greater TG reductions. However, dose-related improvements in other atherogenic lipids – including non-HDL-C, LDL-C, apo B, and atherogenic:anti-atherogenic lipoprotein ratios – were observed in these hypertriglyceridemic patients, which is consistent with results from previous studies of rosuvastatin in hypercholesterolemic Japanese patients [7,8].

Although bezafibrate did not provide a marked beneficial effect on lipid parameters such as LDL-C and non-HDL-C, it produced substantial reductions in TG and increase in HDL-C. These findings are consistent with other major studies of fibrates, which demonstrated that fibrates do not appreciably change the concentration of LDL-C [10–12], but rather change particle size distribution, reducing small LDL subfractions and increasing peak particle size [13–15].

The differences between rosuvastatin and bezafibrate in reducing non-HDL-C levels are of particular importance for patients with elevated TG. Non-HDL-C provides a measure of all atherogenic (apo B-containing) lipoproteins, including VLDL remnant lipoproteins, and is more widely available than apo B measurement; there are some data to suggest that non-HDL-C may improve prediction of cardiovascular disease risk over LDL-C [16,17]. The US National Cholesterol Education Program Adult Treatment Panel III guidelines [3] recommend the reduction in non-HDL-C as a secondary target of lipid-lowering therapy after LDL-C goals have been met. Others have suggested that apo B is also an important indicator of cardiovascular risk and in fact may be superior to either LDL-C or non-HDL-C in this regard [18–20].

The coefficients of variation for percentage change of HDL-C, HDL-TG and apo A-I are relatively high. However, the data tends to suggest qualitative differences in the way the two drugs affect HDL. HDL-TG was reduced by about

20% with bezafibrate but rosuvastatin had little or no effect on this parameter. Moreover, the percentage increases in apo A-I with rosuvastatin seem to be greater than with bezafibrate relative to the changes in HDL-C. These compositional changes may reflect differences in the mechanisms by which statins and fibrates elevate HDL.

Complementing previous studies of rosuvastatin in a Japanese population [7,8], the present investigation further supports the comparable efficacy of rosuvastatin in lowering lipid levels in this ethnic group compared with others. These observations are particularly noteworthy in light of the well-documented population differences in rosuvastatin plasma exposure between Western and Asian subjects (1.6–2.3-fold greater rosuvastatin exposure in Asians compared with Western subjects) [21].

The ethnic differences in rosuvastatin disposition also appeared to have no effect on susceptibility to known adverse events. Rosuvastatin was well tolerated in the study, with no indication of dose-related increases in adverse events or laboratory abnormalities. Liver function abnormalities attributable to rosuvastatin treatment were rare, there were no cases of proteinuria and no evidence of renal impairment, and no cases of myopathy or rhabdomyolysis were observed.

In summary, rosuvastatin 5–20 mg reduced TG by 30–32% in Japanese hypertriglyceridemic patients, with no evidence of a dose-related effect on TG reduction. Rosuvastatin treatment also provided large dose-related decreases in LDL-C, robust increases in HDL-C and large improvements in other atherogenic lipid parameters. Many hypertriglyceridemic patients require improvements in other lipid parameters, and these parameters should guide selection of rosuvastatin dose. Rosuvastatin was markedly superior with respect to changes in non-HDL-C and LDL-C improvements, while bezafibrate demonstrated greater improvemnets in TG and HDL-C levels. Rosuvastatin treatment was well tolerated and raised no specific safety concerns in hypertriglyceridemic patients.

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Original Article

Polymorphisms of Apolipoprotein E and Methylenetetrahydrofolate Reductase in the Japanese Population

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Aim: The aim of this study is to analyze the effect of apolipoprotein E (apo E) and methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms on serum lipid and homocysteine levels in the general Japanese population.
Methods: We analyzed the polymorphisms in individuals randomly selected from among participants of Serum Lipid Survey 2000.
Results: The frequency of the $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$ alleles of *APOE* was 4.2, 85.3, and 10.5%, respectively. Individuals with the genotype $\varepsilon 4/\varepsilon 4$ had the highest total and low-density lipoprotein (LDL) cholesterol levels, while those with $\varepsilon 2/\varepsilon 2$ had the lowest. Individuals with the $\varepsilon 2/\varepsilon 2$ and $\varepsilon 2/\varepsilon 4$ genotypes had higher remnant-like particles (RLP)-cholesterol levels than those with $\varepsilon 2\varepsilon 3$, $\varepsilon 3\varepsilon 3$, and $\varepsilon 3\varepsilon 4$. There was a trend for individuals with the $\varepsilon 2/\varepsilon 4$ and $\varepsilon 2/\varepsilon 2$ genotypes to have higher triglyceride levels, although the difference was not significant. The presence of the T allele in a *MTHFR* polymorphism (C667T) was associated with higher homocysteine levels, which is more prominent in men than in women.
Conclusion: Thus in our large-scale analysis we have shown that RLP-cholesterol is better associated with *APOE* genotype than triglyceride and the effect of the T allele on *MTHFR* polymorphism (C667T) homocysteine levels is more prominent in men than in women among Japanese.

Introduction

Apolipoprotein E (apo E) is an important structural constituent of serum chylomicrons, very low-density lipoproteins, and high-density lipoproteins (HDL) and plays a critical role in lipoprotein metablism, where it can facilitate the clearance of remnant lipoprotein and cellular efflux of cholesterol[1]. Apo E has three polymorphisms, ε2, ε3, and ε4, which affect lipoprotein metabolism and atherosclerosis[2]. The ε4 allele is associated with higher low-density lipoprotein (LDL) cholesterol levels than the other alleles and with a higher incidence of coronary heart disease[3]. Apo E4 is also shown to be involved in the development of Alzheimer's disease[4], while homozygosity for apo E2 is associated with the development of type III hyperlipidemia[5].

We also studied the *MTHFR* gene because its polymorphisms affect serum homocysteine levels and homocysteine is also associated with cardiovascular disease and Alzheimer's disease[6-9]. An elevated homocysteine level is associated with coronary heart disease and the C677T polymorphism in the *MTHFR* gene results in reduced *MTHFR* enzyme activity and reduced methylation of homocysteine to methioneine resulting in mild hyperhomocysteinemia[10]. Although several studies have examined the incidence of *APOE* and *MTHFR* polymorphisms[8, 11], these has been no large-scale study to determine the incidence of *APOE* and *MTHFR* polymorphisms and their association with lipoprotein profiles and homocysteine levels in the general Japanese population. In 2000, we conducted a lipid survey in the Japanese population, 12,839 people all over the country. In this survey, we examined *APOE* and *MTHFR* gene polymorphisms to determine the incidence of each and its relationship with lipid profiles and homocysteine levels in the Japanese.

Methods

Design and Data Collection

This work is part of Serum Lipid Level Survey 2000 from various parts of Japan. The Ethics committee, Graduate School and Faculty of Medicine, Kyoto University approved the study protocol and all subjects provided written informed consent for participation in the gene analysis. The handling of DNA samples followed the guidelines from the Ministry of Health, Labor, and Welfare. In Serum Lipid Survey 2000, a total of 12,839 subjects were recruited at 36 hospitals across the country. The subjects in the present study were participants in the survey at 9 hospitals from whom informed content for genotyping was sought. Of the 12,839 subjects, 2,267 (17.7%) with no lipid-altering medication were randomly selected for the present study. Among the 2,267 participants, we examined serum homocysteine levels and *MTHFR* gene polymorphisms in 505 participants.

Laboratory Methods

All serum and blood samples were obtained in the fasting state. All lipid and other analyses were conducted on venous blood samples within one week of collection at BML (Saitama, Japan). Serum cholesterol and TG levels were measured by enzymatic assay. HDL-cholesterol and LDL-cholesterol levels were measured enzymatically with a kit from Daiichi Kagaku Co. Ltd. (Tokyo, Japan). The results of lipid analyses in the four surveys were indirectly standardized according to the criteria of the CDC Lipid Standardization Program[12]. The serum homocysteine level was assayed by high performance liquid chromatography with fluorescent detection as described by Ubbink *et al.*[13]. DNA was extracted with a QIAamp DNA blood kit (Qiagen, Hilden, Germany).

Detection of gene Mutations by Invader® Assay

We used the Invader® assay to screen for mutations of the *APOE* and *MTHFR* genes, as previously described. In brief, the probe/Invader®/MgCl2 mixture was prepared by combining 3 μL of primary probe/Invader® mix and 5 μL of 22.5 mM MgCl2 per reaction. The primary probes/Invader® mixture contained 3.5 μmol/L wild primary probe, 3.5 μmol/L mutant primary probe, 0.35 μmol/L Invader® oligonucleotide, and 10 mmol/L MOPS. Eight microliters of primary probe/Invader®/MgCl2 mixture was added per well of a 96-well plate. Seven microliters of 5 fmol/L synthetic target oligonucleotides, 10 μg/mL yeast tRNA (no target blank), and genomic DNA (15 ng/μL) were added, and denatured by incubation at 95℃ for 10 min. After 15 μL of mineral oil (Sigma, St. Louis, MO) was overlayed into all reaction wells, the plate was incubated isothermally at 63℃ for 4 h in a DNA thermalcycler (PTC-200; MJ Research, Watertown, MA) and then kept at 4℃ until fluorescence were measured. The intensity of the fluorescence was measured with a fluorescence microtiter plate reader (Cytoflour 4000; Applied Biosystems) with excitation at 485 nm/20 nm (Wavelength/Bandwidth) and emission at 530 nm/25 nm for FAM; and excitation at 560 nm/20 nm and emission at 620 nm/40 nm for RED. The genotyping was analyzed by calculating the ratio of net counts with wild primary probe to net counts with mutant primary probe. The probes used in this study were designed and synthesized by Third Wave Technologies, Inc (Madison, WI).

Data Analyses

Differences in means were evaluated with an analysis of variance. The analysis was performed with the statistical Package for Social Sciences (SPSS Japan Inc. ver. 11.5, Tokyo, Japan).

Results

We investigated the frequency and phenotypic association of *APOE* gene polymorphisms of 2,267 subjects. We found that the SNPs were in Hardy-Weinberg equilibrium. As previously described, the mean age, total cholesterol, TG, HDL-cholesterol, and LDL-cholesterol levels in this population were similar to the levels for all 12,839 patients in Serum Lipid Survey 2000[14]. We also found that the medians of total, LDL-, and HDL-cholesterol levels did not differ appreciably from the means, thereby excluding gross right-hand tailing of the distribution (data not shown). These data indicate that the participants in the gene analysis are representative of the general Japanese population.

The genotype and allelic frequency of *APOE* polymorphisms are presented in **Table 1**. The frequency of the ε2, ε3, and ε4 alleles was 4.2, 85.3, and 10.5%, respectively. As in other studies, the genotypes ε2ε2,

ε2ε4, and ε4ε4 were quite rare. High frequencies of the ε3 allele are also found in Chinese, but the frequency is lower in Caucasians[15].

We next examined the association of the *APOE* genotype and lipid profiles in these participants. As shown in **Table 2**, all the lipid parameters and blood glucose differed significantly among these genotypes by ANOVA. Total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, and RLP-cholesterol levels were different among the groups. The *p* values are shown in the right column. According to the post-hoc analysis, the total cholesterol level was significantly lower for genotype ε2ε2 than ε4ε4 and genotype ε2ε3 than ε3ε3, ε2ε4, or ε4ε4. The HDL-cholesterol level was significantly higher for ε2ε3 than ε2ε4. The LDL-cholesterol level was significantly lower for genotypes ε2ε2 and ε2ε3 than for ε3ε3, ε3ε4, and ε4ε4. The RLP-cholesterol level was significantly higher for ε2ε2 than ε2ε3, ε3ε3, ε3ε4, or ε4ε4 and for genotype ε2ε4 than ε2ε3, ε3ε3, or ε3ε4, although there was no significant difference in triglyceride levels according to the post-hoc analysis. Blood glucose or age did not differ significantly among the groups.

We next examined the association of the *MTHFR* C667T polymorphism with serum homocysteine levels in 505 samples randomly selected from 2,267 samples. As shown in **Table 3**, the incidence of the CC, CT, and TT genotypes was 33.9, 46.1, and 20.0%, respectively. The TT genotype was significantly associated with higher homocysteine levels in men and women, and statistical significance was found between CC and TT and between CT and TT by a post-hoc analysis. However, the difference was more prominent in men.

Table 1. Genotype and allele frequency of *APOE* gene in Japanese.

genotype	n	%	alleles	n	%
ε2/ε2	9	0.4	ε2	192	4.2
ε2/ε3	155	6.8	ε3	3,868	85.3
ε2/ε4	19	0.8	ε4	474	10.5
ε3/ε3	1,653	72.9			
ε3/ε4	407	18.0			
ε4/ε4	24	1.1			

Discussion

There, we have shown in a large-scale study, the

Table 2. Mean of serum lipid levels and blood glucose in each genotype of *APOE* in Japanese.

	ε2/ε2	ε2/ε3	ε2/ε4	ε3/ε3	ε3/ε4	ε4/ε4	total	*p* value
	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	
T-cho	165.0 ± 23.8	189.7 ± 3.00	202.9 ± 12.6	201.8 ± 0.92	206.8 ± 1.95	223.3 ± 9.18	202.1 ± 0.81	<0.0001
TG	171.4 ± 52.8	118.8 ± 8.55	189.0 ± 53.3	117.0 ± 2.41	128.0 ± 5.16	127.9 ± 18.9	119.8 ± 2.13	0.023
HDL-c	51.2 ± 9.51	63.6 ± 1.92	53.0 ± 3.07	59.8 ± 0.41	58.0 ± 0.82	61.9 ± 3.48	59.7 ± 0.36	0.007
LDL-c	70.5 ± 5.63	101.9 ± 2.75	117.2 ± 8.07	118.5 ± 0.91	120.5 ± 1.93	131.5 ± 7.97	117.7 ± 0.79	<0.0001
RLP-c	22.9 ± 1.15	4.4 ± 0.37	12.5 ± 7.59	4.7 ± 0.17	5.2 ± 0.33	4.1 ± 0.58	4.8 ± 0.15	<0.0001
FBS	121.3 ± 19.5	104.7 ± 3.27	110.6 ± 9.37	103.9 ± 0.94	103.3 ± 2.17	88.6 ± 2.54	103.9 ± 0.83	0.461
age	52.8 ± 10.1	49.5 ± 2.11	50.8 ± 53.2	46.7 ± 0.69	47.4 ± 1.30	43.2 ± 4.61	47.1 ± 0.58	0.659

T-cho: total cholesterol (mg/dL), TG: triglyceride (mg/dL), HDL-c: HDL-cholesterol (mg/dL), LDL-c: LDL-cholesterol (mg/dL), RLP-c: remnant-like particles cholesterol (mg/dL), FBS: fasting blood sugar (mg/dL), SEM: standard error of the mean

Table 3. Genotype frequency of the *MTHFR* gene and its association with serum homocysteine levels in Japanese.

total

genotype	n	%	mean	SEM	
CC	171	33.9	10.9	0.3	$p < 0.001$
CT	233	46.1	11.6	0.24	
TT	101	20.0	15.7	1.23	
total	505	100	12.2	0.29	

male

genotype	n	%	mean	SEM	
CC	92	33.6	10.7	0.36	$p < 0.001$
CT	132	48.2	12.9	0.35	
TT	50	18.2	19.8	2.41	
total	274	100	13.4	0.52	

female

genotype	n	%	mean	SEM	
CC	79	34.2	10.2	0.43	$p = 0.005$
CT	101	43.7	10.1	0.27	
TT	51	22.1	11.9	0.57	
total	231	100	10.5	0.23	

SEM: standard error of the mean

frequency of the *APOE* genotype in the Japanese and its association with serum lipid levels. Frequencies of *APOE* genotypes are highly heterogenous among various populations. Epidemiological data indicate that the frequency of the $\varepsilon 3$ allele is higher in Japanese and Chinese than in Caucasians, while the frequency of the $\varepsilon 4$ allele is lower in Asians than Caucasians[3, 16]. Our data indicate that the frequency of the $\varepsilon 3$ allele is quite consistent with previous reports in Japanese[8, 11, 16, 17], and is slightly higher than that of Icelandic and Hungarian populations and much higher than that in the Finnish population[15].

Our study confirmed that the $\varepsilon 4$ allele is associated with higher, and the $\varepsilon 2$ allele is associated with lower, LDL cholesterol levels. Although there was a trend for individuals with the genotypes $\varepsilon 2/\varepsilon 4$ and $\varepsilon 2/\varepsilon 2$ to have higher triglyceride levels, it was not statistically significant by a post-hoc analysis, probably because triglyceride levels are highly variable. However, individuals with $\varepsilon 2/\varepsilon 4$ and $\varepsilon 2/\varepsilon 2$ had significantly higher RLP-cholesterol levels than did those with the other genotypes, indicating that RLP-cholesterol might be better correlated with *APOE* genotype. Although in this study we could not compare the body

mass index of $\varepsilon 2/\varepsilon 2$ homozygotes, it would be intriguing to know whether individuals with the $\varepsilon 2/\varepsilon 4$ and $\varepsilon 2/\varepsilon 2$ genotypes have metabolic abnormalities, such as abdominal obesity and insulin resistance, because they have higher triglyceride, RLP-cholesterol, and blood glucose levels.

Elevated levels of homocysteine have been considered a risk for cardiovascular disease. Our study is consistent with other studies that show higher homocysteine levels in people with the TT genotype. However, the relationship between the C677T *MTHFR* polymorphism and cardiovascular disease is still controversial. Because our study population is made up of healthy volunteers, a prospective study is necessary to determine which genotype is associated with cardiovascular risk.

In summary, we have provided the largest database of gene polymorphisms related to lipid metabolism and homocysteine in the general Japanese population. A prospective study is necessary to determine the contribution of these gene polymorphisms to cardiovascular risk in Japanese.

Acknowledgements

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β2-Adrenergic receptor regulates Toll-like receptor-4-induced nuclear factor-κB activation through β-arrestin 2

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Summary

Toll-like receptors (TLRs) play an important role in innate immunity while, β2-adrenergic receptors (β2AR) provide the key linkages for the sympathetic nervous system to regulate the immune system. However, their role in macrophages remains uncertain. Here, we demonstrate the cross-talk between β2AR and TLR signalling pathways. Expression of β2AR was down-regulated by TLR4 ligand lipopolysaccharide (LPS) stimulation. To investigate the physiological consequence of this down-regulation RAW264 cells, a macrophage cell line, were transfected with a β2AR expression vector (RAWar). Both LPS-stimulated inducible nitric oxide synthase (NOS II) expression and NO production were markedly suppressed in the RAWar cells. The activation of nuclear factor-κB (NF-κB) and degradation of the inhibitor of NF-κB (IκBα) in response to LPS were markedly decreased in these cells. The level of β-arrestin 2, which regulates β2AR signalling, was also reduced in RAW264 cells after stimulation with LPS, but not in RAWar cells. Overexpression of β-arrestin 2 (RAWarr2) also inhibited NO production and NOS II expression. Furthermore, we demonstrated that β-arrestin 2 interacted with cytosolic IκBα and that the level of IκBα coimmunoprecipitated by anti-β-arrestin 2 antibodies was decreased in the RAW264 cells but not in RAWar or RAWarr2 cells. These findings suggest that LPS-stimulated signals suppress β2AR expression, leading to down-regulation of β-arrestin 2 expression, which stabilizes cytosolic IκBα and inhibits the NF-κB activation essential for NOS II expression, probably to ensure rapid and sufficient production of NO in response to microbial attack.

Keywords: β2-adrenergic receptor; monocytes/macrophages; nitric oxide; nuclear factor-κB; toll-like receptor

Introduction

The ability of the innate immune system to recognize and respond to microbial components has been chiefly attributed to a family of type I transmembrane receptors termed Toll-like receptors (TLRs) that are expressed abundantly on antigen-presenting cells such as macrophages and dendritic cells and can discriminate among the distinct molecular patterns associated with microbial components.[1,2] The TLR-initiated activation of nuclear factor-κB (NF-κB) is essential for the regulation of induc-ible nitric oxide synthase (NOS II) and several proinflammatory cytokines, which are produced in response to invading pathogens. The NO produced by NOS II has a number of important biological functions, including roles in host defence against intracellular pathogens and tumour-cell killing. Although this basic definition is still accepted, over the past decade NO has been shown to play a much more diverse role not only in the immune system but also in other organ systems, including both beneficial and detrimental effects.[3,4] For example, the systemic inflammatory response syndrome, which includes

1

severe septic shock and multiple organ system failure, remains a leading cause of death in critically ill patients. Therefore, it is necessary to clarify the molecular mechanisms of TLR-initiated signalling that lead to NO production in response to microbial components.

Nuclear factor-κB is found predominantly in the cytoplasm complexed with members of the inhibitor of NF-κB (IκB) family. The release of NF-κB from IκB proteins is an essential step in the generation of transcriptionally competent NF-κB. The consensus is that IκB proteins mask the nuclear localization signals of NF-κB proteins, thereby regulating NF-κB activity, primarily by limiting their nuclear translocation. Recent studies, however, have indicated that IκBα is detected in both the nucleus and cytoplasm and that although the NF-κB complexes shuttle between the nucleus and cytoplasm under all conditions, they are unable to bind DNA because of their association with proteins of the IκB family.[5–7] Nuclear IκBα is not sensitive to signal-induced degradation. Therefore, following stimulation, NF-κB activities are dependent on the level of cytoplasmic NF-κB/IκBα complexes.

Recently, we demonstrated that the level of β$_2$-adrenergic receptor (β$_2$AR) expression influences TLR4 signalling.[8] β$_2$AR is a member of a family of G protein-coupled receptors (GPCRs) and is the key link involved in immune system regulation via the sympathetic nervous system.[9,10] Primary and secondary lymphoid organs, such as the thymus, spleen and lymph nodes, receive extensive sympathetic/noradrenergic innervation, and lymphocytes, macrophages and many other immune cells bear functional β$_2$AR. Therefore, β$_2$AR stimulation regulates pro-inflammatory cytokine production, lymphocyte traffic and proliferation, and antibody secretion through cyclic adenosine monophosphate (cAMP) generation and protein kinase A (PKA) activation.[10,11] However, the role of β$_2$AR in the TLR signalling pathway in macrophages remains vague. On the other hand, arrestins are cytosolic proteins that play a critical role in the regulation of GPCR signalling.[12,13] Recent studies have shown that they also interact with their partner molecules in a variety of signalling pathways, including NF-κB signalling.[14–16] In the present study, we investigated the physiological consequence of the down-regulation of β$_2$AR expression in macrophages and analysed the cross-talk between the signalling of β$_2$AR and TLRs.

Materials and methods

Cell culture

The murine macrophage cell line RAW264 (RCB0535) was purchased from RIKEN Cell Bank (Ibaraki, Japan) and cultured as described in our previous study.[17] The cells were stimulated with 1 μg/ml lipopolysaccharide (LPS) from *Escherichia coli* 055 (Sigma-Aldrich, St Louis,

MO). Cell viability was assessed using the trypan blue dye exclusion test and cell size was measured by flow cytometric analysis of forward light scatter characteristics using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described elsewhere.[18] The NF-κB oligonucleotide probe (5'-AGT TGA GGG GAC TTT CCC AGG-3') was purchased from Promega (Madison, WI) and labelled with biotin at its 3' end. The nuclear protein (2 μg) and excess amounts of labelled oligonucleotide probes were incubated in 20 μl EMSA buffer [20 mM HEPES, pH 7·6, 10 mM (NH$_4$)$_2$SO$_4$, 1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid (EDTA), 0·2% Tween, 30 mM KCl, 1 μg poly (dI-dC), 1 μg poly L-lysine] at room temperature for 15 min, electrophoresed in 7% polyacrylamide gels, transferred onto the Biodyne Plus Membane (Pall BioSupport Division, Port Washington, NY), and cross-linked in ultraviolet light. To detect signals, the blots were incubated with streptavidin–horseradish peroxidase conjugate in a blocking reagent for 15 min and with a chemiluminescent reagent for 5 min. The blots were then exposed to Kodak X Omat AR film (GE Healthcare Bio-Science, Piscataway, NJ).

Western blotting analysis

Cell membrane proteins were prepared using the Plasma Membrane Protein Extraction Kit (Bio Vision, Mountain View, CA). Cytoplasmic protein extracts were prepared as described previously (30). The protein concentration was determined using the Bradford reagent (BioRad, Hercules, CA), and equal amounts of membrane proteins or cytoplasmic proteins were loaded. The samples were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred on to polyvinylidene difluoride membranes (Applied Biosystems, Foster City, CA). The membranes were blocked with 10% nonfat dried milk in Tris-buffered saline and incubated with goat polyclonal antibodies against β$_2$AR, goat polyclonal antibodies against β-arrestin 2, or rabbit polyclonal antibodies against IκBα and NOS II (Santa Cruz Biotechnology, Santa Cruz, CA); this was followed by incubation with appropriate secondary antibodies (horseradish peroxidase-conjugated rabbit anti-goat or goat anti-rabbit immunoglobulin G; Dako, Kyoto, Japan). To ensure equal protein loading, the membranes were incubated with rabbit anti-actin or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology) for the detection of cytoplasmic or cell surface GAPDH[19] after stripping. Immunoreactivity was visualized using an enhanced chemiluminescence reagent (ECL; GE Healthcare Bio-Science).

Immunoprecipitation

The cells were lysed with lysis buffer (20 mM Tris–HCl, pH 7·6, 150 mM NaCl, 2 mM EDTA, 0·5% Nonidet P-40 and protease inhibitors). The samples were clarified by centrifugation at 21 000 g at 4° for 30 min. The protein concentration was determined using the Bradford reagent (Bio-Rad). β-Arrestin 2 was immunoprecipitated with anti-β-arrestin 2 monoclonal antibodies (Santa Cruz Biotechnology) from equal samples, followed by treatment with 10 μl protein G–Sepharose beads (GE Healthcare Bio-Science). After extensive washing, the complexes were analysed by SDS–PAGE and Western blotting by using rabbit polyclonal antibodies against IκBα.

Determination of nitrite concentration

Nitrite in the cell culture supernatants was measured using the assay system of Ding et al.[20] The nitrite concentration was calculated by comparison with sodium nitrite, which was used as a standard. In some experiments, 200 μM pyrrolidine dithiocarbamate (PDTC, Sigma) was added to the cultures.

Determination of intracellular cAMP concentration

Cells were cultured with or without LPS for 6 hr and were stimulated with Salbutamol (1×10^{-6} M) for the final 30 min. Cell supernatants were then removed and cells were lysed. Intracellular cAMP was determined with a commercially available enzyme immunoassay (GE Healthcare Bio-Science).

Real-time polymerase chain reaction (PCR)

Total cellular RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and aliquots of 2 μg were reverse-transcribed with ReverScript I (Wako Pure Chemical Industries, Osaka, Japan) and an oligo-dT(15-mer) (Roche Diagnostics, Indianapolis, IN) at 42° for 50 min. The complementary DNAs (cDNAs) were amplified by PCR under the following conditions using the oligonucleotide primers and cycles listed in Table 1: 94° for 30 seconds, 55° for 30 seconds, and 72°

for 30 seconds for NOS II and 18S ribosomal RNA (rRNA), and 94° for 30 seconds, 60° for 30 seconds, and 72° for 30 seconds for total and transfected β₂AR and β-arrestin 2. The quantity of the cDNA template included in these reactions and the number of amplification cycles were optimized to ensure that the reactions were stopped during the linear phase of product amplification, thus permitting semiquantitative comparisons of messenger RNA (mRNA) abundance between different RNA preparations.

β₂AR and β-arrestin 2 plasmid constructs and stable transfection

Full-length murine β₂AR (β_2ar) and β-arrestin 2 ($\beta arrestin2$) cDNAs were obtained by PCR using the primers 5′-GCTGAATGAAGCTTCCAGGA-3′ (sense) and 5′-GCCTGTATTACAGTGGCGAG-3′ (antisense) for β₂AR and 5′-GGCGGGCGGAGGGCGGCGAG-3′ (sense) and 5′-CGTCCTAGCAGAACTGGTCA-3′ (antisense) for β-arrestin 2. The amplified β₂AR and β-arrestin 2 fragments were subcloned into the pGEM-T Easy vector (Promega) and then into NotI-digested pcDNA4 (Invitrogen, Carlsbad, CA). The amplified PCR products were sequenced using an automatic DNA sequencer (Applied Biosystems). The plasmid DNA used for transfection was prepared using the EndoFree Plasmid Kit (Qiagen). RAW264 cells were transfected with the pcDNA4 vector, pcDNA4-β₂ar, or pcDNA4-βarrestin2 using LipofectAMINE Reagent (Invitrogen). Selection was initiated in a medium containing 500 μg/ml Zeocine (Invitrogen).

Luciferase assays

The full-length murine NOS II promoter fragment was cloned into the pGL3-enhancer luciferase reporter gene vector (Promega) (pGL3-NOS II) as described previously.[21] RAW264 cells were transfected using the LipofectAMINE Reagent with constructs containing the luciferase reporter gene, and luciferase activity was determined using the Dual Luciferase Assay System Kit (Promega) as described elsewhere.[21] Activity was normalized relative to an internal cotransfected constitutive control (*Renilla* luciferase expression vector, pRL-TK; Promega). In some

Table 1. Oligonucleotide sequences used for polymerase chain reaction

	Forward	Reverse	Cycle
β₂AR	GGAGCAGGATGGGCGGACGG	GCCTTCCATGCCTGGGGGAT	34
Transfected β₂AR	GGAGCAGGATGGGCGGACGG	TGGTGATGGTGATGATGACC	34
β-arrestin 2	GCAGCCAGGACCAGAGGACA	CCACGCTTCTCTCGGTTGTC	35
NOS II	CTTCCGAAGTTTCTGGCAGCAGCG	GAGCCTCGTGGCTTTGGGCTCCTC	26
18S	GAGAAACGGCTACCACATCC	CCCAAGATCCAACTACGAGC	26

β₂AR, β₂-adrenergic receptor; NOS II, nitric oxide synthase II.

experiments, RAW264 cells were transiently cotransfected with the NF-κB-responsive promoter reporter–luciferase construct pNF-κB-Luc (Clontech, Palo Alto, CA) or pGL3-NOS II and pcDNA4-β2ar or IκBα dominant-negative vector pCMV-IκBαM (Clontech).

Statistical analysis

Student's *t*-test for unpaired samples was used to compare two means. For more than two groups, statistical significance of the data was assessed by analysis of variance. Where significant differences were found, individual comparisons were made between groups using the *t*-statistic and adjusting the critical value according to the Bonferroni method. Differences were considered significant at $P < 0.05$. Data in the text and figures are expressed as means ± SEM.

Results

Preventing the down-regulation of β2AR inhibits LPS-stimulated NOS II expression

Levels of both β2AR protein and β2AR mRNA were markedly decreased in RAW264 cells following LPS stim-

ulation (Fig. 1a). To investigate the role of β2AR down-regulation in response to LPS, a stable β2AR transfectant (RAWar) and a vector control (RAWvec) were established. Although the levels of both β2AR protein and mRNA expression were notably decreased in RAWvec cells following LPS stimulation, the down-regulation of β2AR expression was prevented in the RAWar cells (Fig. 1b). The transfected β2AR protein did not have a tag sequence capable of modifying β2AR function so the protein levels of only transfected β2AR could not be analysed. The mRNA levels of transfected β2AR were low in unstimulated RAWar cells but markedly increased in the cells following LPS stimulation (Fig. 1c). In our previous study, we showed that the levels of both protein and mRNA of transfected cDNA cloned into the pcDNA4 vector were low in unstimulated RAW264 cells but were markedly increased in the cells following LPS stimulation.[17] Therefore, it appears that total β2AR expression in unstimulated RAWar cells was not much higher than in RAWvec cells and that the decrease in intrinsic β2AR expression in the LPS-stimulated RAWar cells was masked by the increased expression of transfected β2AR as the result of the LPS stimulation. Although, the intracellular cAMP concentration in RAWar cells stimulated with salbutamol was similar to that in RAWvec cells, LPS
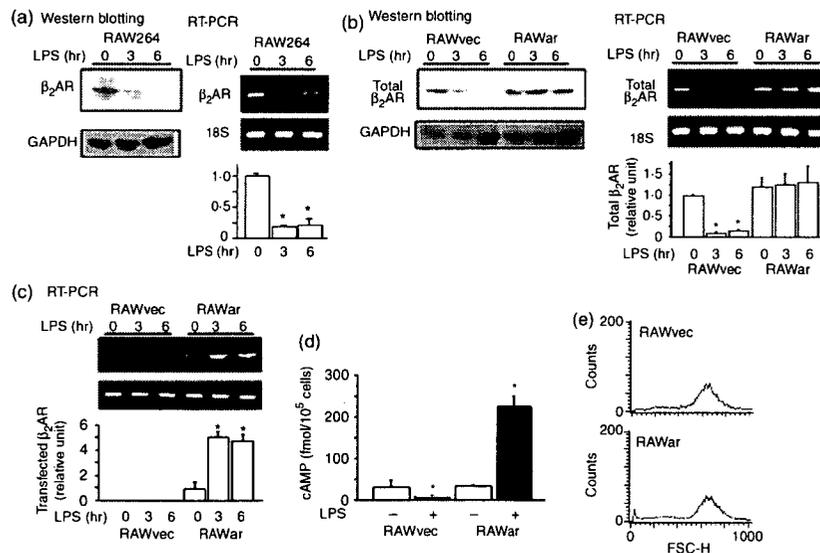
Figure 1. Lipopolysaccharide (LPS) stimulation down-regulates β2-adrenergic receptor (β2AR) expression. (a) RAW264 cells were stimulated with LPS. The protein levels of β2AR and GAPDH (loading control) in the plasma membrane were analysed by Western blotting (left panel). The β2AR messenger RNA (mRNA) and 18S ribosomal RNA (rRNA; loading control) were analysed by reverse transcription–polymerase chain reaction (RT-PCR; right upper panel). Bar graphs show the relative intensity of the PCR bands from three separate experiments (mean ± SEM) (right lower panel). *$P < 0.01$ versus 0 hr. (b) RAW264 cells were transfected with the β2ar construct or vector alone. The protein levels of β2AR and GAPDH (left panel) and mRNA expressions of β2AR and 18S rRNA (right upper panel) were analysed as in (a). Bar graphs show the relative intensities of the PCR bands from three separate experiments (mean ± SEM) (right lower panel). *$P < 0.01$ versus 0 hr. (c) mRNA expressions of β2AR and 18S rRNA (upper panel) were analysed as in (a). Bar graphs show the relative intensities of the PCR bands from three separate experiments (mean ± SEM) (lower panel). *$P < 0.01$ versus 0 hr. (d) Cells were cultured with or without LPS for 6 hr and were stimulated with salbutamol (1×10^{-6} M) for the final 30 min. Then, intracellular cyclic AMP concentrations were analysed. *$P < 0.05$ versus without LPS. (e) Cell size was measured by flow cytometric analysis of forward light scatter characteristics (FSC).
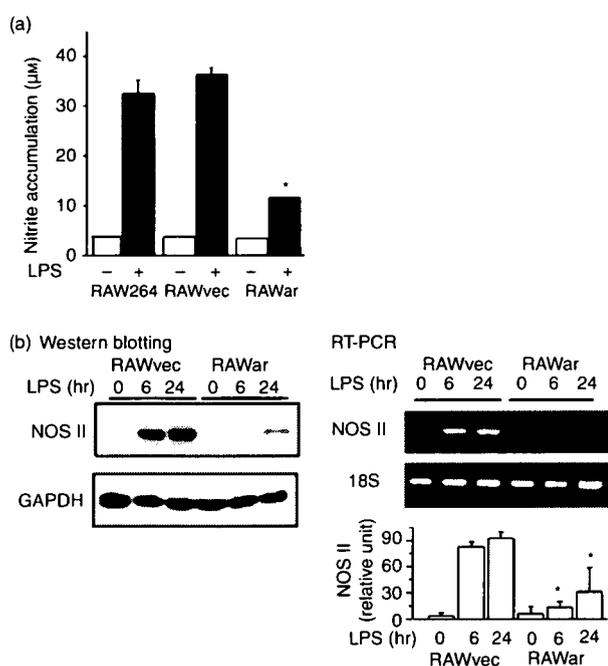
stimulation decreased the accumulation of intracellular cAMP in RAWvec cells but increased it in RAWar cells (Fig. 1d), suggesting that the transfected β₂AR was functionally active. Similar histograms of the distribution of forward light scatter characteristics were observed in RAWvec and RAWar cells, suggesting that the β₂AR transfection did not alter the cell size (Fig. 1e). In addition, cell viabilities were more than 98% in both cells.

The effects of forced β₂AR expression on NO production were examined. The nitrite concentration in the culture supernatants of the LPS-stimulated RAWar cells was considerably lower than in the culture supernatants of the RAWvec cells (Fig. 2a). After stimulation with LPS for 6 hr, a distinct 130 000 molecular weight NOS II protein band was observed in the RAWvec cells but not in the RAWar cells (Fig. 2b). Although a protein band corresponding to NOS II was observed in the RAWar cells after stimulation with LPS for 24 hr, the expression level was apparently lower than in the RAWvec cells. Similar

(a)

(b) Western blotting

RT-PCR

Figure 2. Forced β₂-adenergic receptor (β₂AR) expression suppresses nitric oxide (NO) production and nitric oxide synthase II (NOS II) expression. (a) Cells were stimulated with lipopolysaccharide (LPS) for 24 hr, and nitrite accumulation in the supernatants was measured using the Griess reagent. The results are expressed as means ± SEM from three-well cultures. *P < 0·001 versus LPS-stimulated RAW264 or RAWvec cells. (b) The protein levels of NOS II and GAPDH (left panel) and messenger RNA expressions of NOS II and 18S ribosomal RNA were analysed as in A (right upper panel). Bar graphs show the relative intensity of the polymerase chain reaction bands from four separate experiments (mean ± SEM) (right lower panel). *P < 0·01 versus corresponding RAWvec cells. Data shown are representative of three or four separate experiments.

results were obtained on reverse transcription PCR analysis of NOS II mRNA expression (Fig. 2b).

Preventing the down-regulation of β₂AR inhibits LPS-stimulated NF-κB activation.

Next, the effects of forced β₂AR expression on NF-κB activation in response to LPS were analysed. As illustrated in Fig. 3(a), marked NF-κB activation was observed in the RAWvec cells stimulated with LPS for 3 and 6 hr but not in the RAWar cells. The level of cytoplasmic IκBα was decreased in the RAWvec cells after LPS stimulation for 6 hr but this level was not decreased in the RAWar cells (Fig. 3b). To further confirm the role of β₂AR in LPS-stimulated NF-κB activation, the effects of forced β₂AR expression on NF-κB-dependent gene transcription were analysed. NF-κB-mediated-luciferase reporter activity (Fig. 3c) and NOS II promoter activity (Fig. 3d) after stimulation with LPS were inhibited in cells that were cotransfected with the pcDNA4-β₂ar construct (AR) as well as in cells cotransfected with pCMV-IκBαM (DN-κB). These findings suggested that β₂AR functions as a negative regulator of NF-κB activation by inhibiting IκBα degradation in LPS-stimulated macrophages. Previously, it has been shown that PDTC blocks NF-κB activation by inhibiting IκBα degradation and subsequently the translocation of NF-κB subunits to the nucleus.[22] To elucidate the effects of NF-κB activation on the expression of the responsive gene, Nos2, PDTC was added to the RAW264 cell cultures at several time-points after the addition of LPS, and accumulation of NO in the supernatants was analysed after LPS stimulation for 24 hr. As illustrated in Fig. 3(e), when PDTC was added to cultures at 0–9 hr after the addition of LPS, the NO concentrations in these cultures were markedly lower than those in cultures stimulated with LPS for 24 hr without PDTC (right column), indicating that continuous NF-κB activation is essential for adequate NOS II induction.
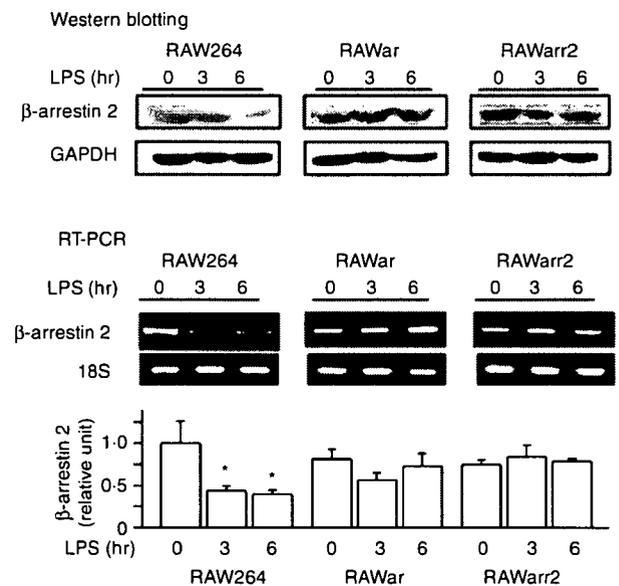
β₂AR regulates NF-κB activation through β-arrestins

As β-arrestin 2 has been reported to interact with IκBα,[15,16] we examined whether β-arrestin 2 participates in the β₂AR-mediated regulation of IκBα degradation and NF-κB activation in response to LPS. The expression of β-arrestin 2 was also down-regulated in the LPS-stimulated RAW264 cells (Fig. 4, left panels). Forced β₂AR expression abolished the down-regulation of β-arrestin 2 expression (middle panels), suggesting that β-arrestin 2 expression was regulated by β₂AR. Deletion of β₂AR by small interfering RNA (siRNA) decreased β-arrestin 2 expression (data not shown), supporting the theory that β-arrestin 2 expression is regulated by β₂AR. To investigate the role of β-arrestin 2 down-regulation in response to LPS, a stable β-arrestin 2 transfectant (RAWarr2) was
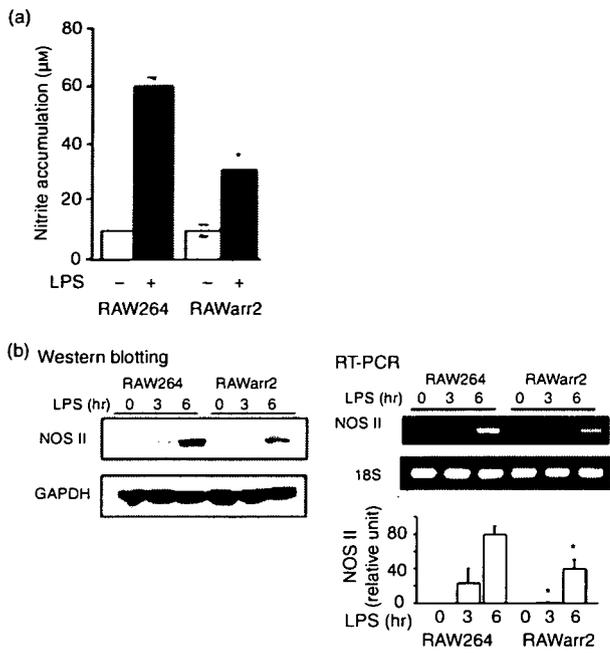
(a)

(b)

(c)

(d)

(e)

Western blotting

RT-PCR

Figure 4. Lipopolysaccharide (LPS) stimulation down-regulates β-arrestin 2 expression. RAW264, RAWar, and RAWarr2 cells were stimulated with LPS, and the protein levels of β-arrestin 2 and GAPDH (upper panel) and messenger RNA expressions of β-arrestin 2 and 18S ribosomal RNA (middle panel) were analysed as in Fig. 1(a). Bar graphs show the relative intensity of the band from three separate experiments (mean ± SEM) (lower panel). *P < 0·01 versus 0 hr.

established (Fig. 4, right panels). Since transfection with the vector did not influence NO production (Fig. 1c), cells transfected with β-arrestin 2 were compared with RAW264 cells. As shown in the RAWar cells (Fig. 2), NO production (Fig. 5a) and NOS II protein and mRNA expressions (Fig. 5b) were definitely decreased in the RAWarr2 cells.

Anti-β-arrestin 2 antibodies coimmunoprecipitated IκBα in RAW264 cells before, but not after, LPS stimulation for 6 hr (Fig. 6). On the other hand, the amount of IκBα coprecipitated by anti-β-arrestin 2 antibodies was not reduced but rather was increased in the RAWar and RAWarr2 cells after LPS stimulation, indicating that the LPS-stimulated down-regulation of β₂AR and β-arrestin 2 is essential for IκBα degradation.

Discussion

In this study, we investigated the role played by β₂AR in the antimicrobial responses of macrophages. First, we demonstrated that β₂AR expression is decreased by LPS

Figure 5. Forced β-arrestin 2 expression suppresses nitric oxide (NO) production and nitric oxide synthase II (NOS II) expression. (a) Cells were stimulated with lipopolysaccharide (LPS) for 24 hr, and nitrite accumulation in the supernatants was measured using the Griess reagent. The results are expressed as means ± SEM from three-well cultures. *P < 0·001 versus LPS-stimulated RAW264 cells. (b) The protein levels of NOS II and GAPDH (left panel) and messenger RNA expressions of NOS II and 18S ribosomal RNA (light upper panel) were analysed as in Fig. 1(a). Bar graphs show the relative intensity of the polymerase chain reaction bands from three separate experiments (mean ± SEM) (right lower panel). *P < 0·01 versus corresponding RAW264 cells. Data shown are representative of three to four separate experiments.
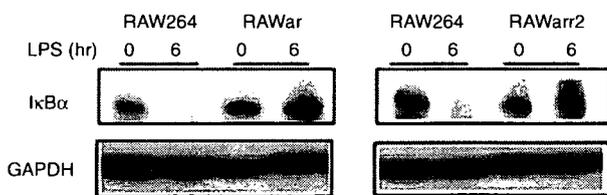
Figure 6. β-arrestin 2 interacts with cytosolic inhibitor of NF-κB (IκBα). Before and after stimulation with lipopolysaccharide (LPS) for 6 hr, cells were lysed and immunoprecipitated with anti-β-arrestin 2 antibodies. Western blotting analysis was performed using anti-IκBα antibodies (upper panel). The protein levels of GAPDH in equal amounts of lysates were used for control (lower panel).

stimulation. To investigate the role of β_2AR down-regulation in response to LPS directly, we established a macrophage cell line, RAWar. Prevention of the down-regulation of β_2AR expression in RAWar cells resulted in reduced NO production, suggesting that the LPS-associated down-regulation of β_2AR expression plays an important role in NO production in macrophages.

Decreases in NOS II mRNA expression were observed in the RAWar cells, indicating that NOS II expression was transcriptionally down-regulated by forced β_2AR expression. Prevention of the down-regulation of β_2AR expression in the RAWar cells resulted in a marked decrease in NF-κB activation and inhibited cytosolic IκBα degradation, indicating that the forced β_2AR expression inhibited LPS-induced NF-κB activation by IκBα stabilization.

On the other hand, β-arrestins, which are universally expressed members of the arrestin family, are the major regulators of GPCR signalling and bind to activated GPCRs, causing receptor desensitization and internalization.[14] Recently, β-arrestins have been shown to play functional roles in the regulation of a variety of signalling pathways and in the mediation of cross-talk between signalling pathways. Moreover, there is accumulating evidence that β-arrestin 2, which is expressed abundantly in the spleen, is functionally involved in some important immune responses.[23–26] We have demonstrated that β-arrestin 2 is down-regulated in LPS-stimulated RAW264 cells. Down-regulation of β-arrestin 2 was abolished in RAWar cells, suggesting that β-arrestin 2 expression is regulated by β_2AR. These findings suggest that β_2AR participates in signal transduction pathways from TLR4 by regulating the level of β-arrestin 2 expression. Meanwhile, the amount of IκBα coimmunoprecipitated by anti-β-arrestin 2 antibodies was decreased in the RAW264 cells after their stimulation with LPS but not in the RAWar or RAWarr2 cells, suggesting that β_2AR inhibited LPS-induced NF-κB activation by stabilizing IκBα through β-arrestin 2. The release of NF-κB following the degradation of IκBα proteins is an essential step in the generation of transcriptionally competent NF-κB. In addition, NF-κB activity following stimulation is dependent on the level of cytoplasmic NF-κB/IκBα complexes free from stabilizing factors. Therefore, the following appear likely: (1) LPS-stimulated signals suppress β_2AR expression, (2) the reduction of β_2AR results in the down-regulation of β-arrestin 2 expression, (3) β-arrestin 2 stabilizes cytoplasmic IκBα and inhibits NF-κB activation (so reduction in the level of β-arrestin 2 accelerates IκBα degradation and NF-κB activation in LPS-stimulated cells) and (4) nuclear translocation of NF-κB enhances NOS II expression.

The cross-talk between β_2AR and the TLR signalling pathways is schematically summarized in Fig. 7.

Catecholamines increase cAMP via β_2AR activation, and PKA activation inhibits NF-κB-induced transcription by phosphorylating cAMP responsive element binding protein (CREB), which competes with p65 for the limited amounts of CREB-binding protein (CBP) (Fig. 7a(ii)).[27] However, β_2AR agonists did not suppress NO production (unpublished observation). In the present study, we demonstrated that LPS stimulation suppressed the cAMP accumulation in RAWvec cells stimulated with a β_2AR
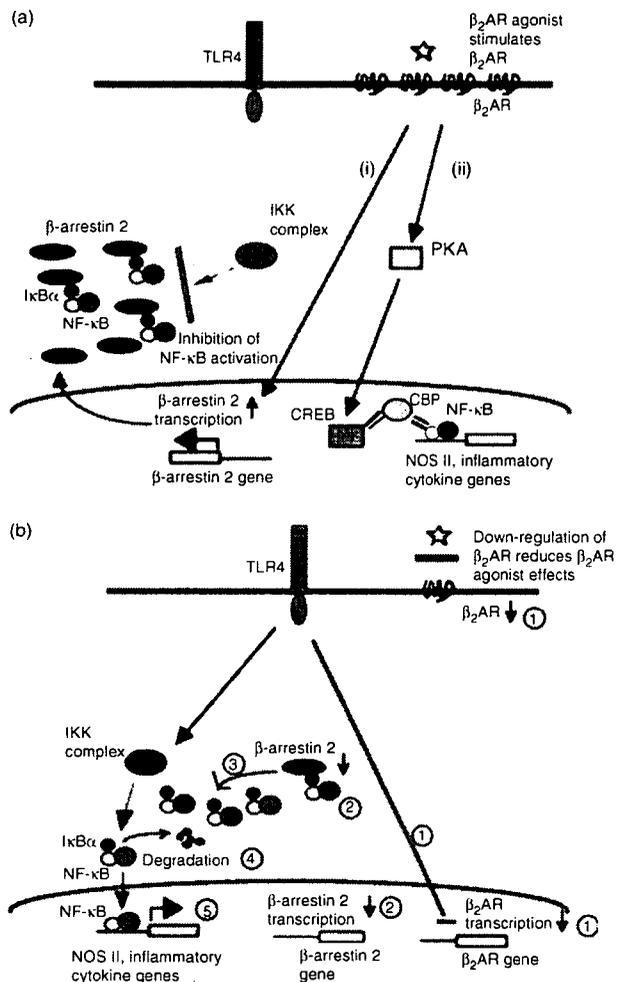
Figure 7. Cross-talk between β2-adrenergic receptor (β2AR) and Toll-like receptor (TLR) signalling pathways. (a) β2AR agonists suppress nuclear factor-κB (NF-κB) activation by increasing cytoplasmic β-arrestin 2, which stabilizes the NF-κB/inhibitor of NF-κB (IκBα) complexes in cytoplasm (i) or by activating cAMP response element binding protein (CREB), which then produces competition between CREB-binding protein (CBP) and NF-κB in the nucleus (ii). (b) TLR4-dependent signals lead to the following steps both in the presence or absence of β2AR agonists: ① TLR4-dependent down-regulation of β2AR expression, ② down-regulation of β-arrestin 2, ③ release of NF-κB/IκBα complexes in the cytoplasm, ④ degradation of IκBα, and ⑤ translocation of NF-κB to the nucleus and transcription of its target genes.

agonist. In addition, we showed that prevention of the down-regulation of β2AR inhibits the degradation of IκBα through β-arrestin 2, which stabilizes IκBα in the steady state (Fig. 7a(ii)). Therefore, the down-regulation of expression of both β2AR and β-arrestin 2 by the TLR4-dependent pathway might provide a mechanism for 'escaping' anti-proinflammatory signals, such as the β2AR–cAMP–PKA pathway[27] or the β2AR–β-arrestin 2–IκBα pathway. As the levels of β2AR ligands vary under

different conditions, understanding the cross-talk between TLRs and β2AR pathways may have both physiological and pathophysiological importance. Taken together, the observations of the present study regarding the regulation of TLR4 signalling through β2AR appear to provide another therapeutic target for the regulation of inflammatory disease conditions.

Acknowledgements

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A novel GIP receptor splice variant influences GIP sensitivity of pancreatic β-cells in obese mice

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Harada N, Yamada Y, Tsukiyama K, Yamada C, Nakamura Y, Mukai E, Hamasaki A, Liu X, Toyoda K, Seino Y, Inagaki N. A novel GIP receptor splice variant influences GIP sensitivity of pancreatic β-cells in obese mice. Am J Physiol Endocrinol Metab 294: E61–E68, 2008. First published October 30, 2007; doi:10.1152/ajpendo.00358.2007.— Gastric inhibitory polypeptide (GIP) is an incretin that potentiates insulin secretion from pancreatic β-cells by binding to GIP receptor (GIPR) and subsequently increasing the level of intracellular adenosine 3',5'-cyclic monophosphate (cAMP). We have identified a novel GIPR splice variant in mouse β-cells that retains intron 8, resulting in a COOH-terminal truncated form (truncated GIPR). This isoform was coexpressed with full-length GIPR (wild-type GIPR) in normal GIPR-expressing tissues. In an experiment using cells transfected with both GIPRs, truncated GIPR did not lead to cAMP production induced by GIP but inhibited GIP-induced cAMP production through wild-type GIPR (n = 3–4. P < 0.05). Wild-type GIPR was normally located on the cell surface, but its expression was decreased in the presence of truncated GIPR, suggesting a dominant negative effect of truncated GIPR against wild-type GIPR. The functional relevance of truncated GIPR in vivo was investigated. In high-fat diet-fed obese mice (HFD mice), blood glucose levels were maintained by compensatory increased insulin secretion (n = 8. P < 0.05), and cAMP production (n = 6. P < 0.01) and insulin secretion (n = 10. P < 0.05) induced by GIP were significantly increased in isolated islets, suggesting hypersensitivity of the GIPR. Total GIPR mRNA expression was not increased in the islets of HFD mice, but the expression ratio of truncated GIPR to total GIPR was reduced by 32% compared with that of control mice (n = 6. P < 0.05). These results indicate that a relative reduction of truncated GIPR expression may be involved in hypersensitivity of GIPR and hyperinsulinemia in diet-induced obese mice.

gastric inhibitory polypeptide; gastric inhibitory polypeptide receptor; alternative splicing; dominant negative effect; obesity

OBESITY LEADS TO INSULIN RESISTANCE, characterized by fasting hyperinsulinemia and excessive insulin secretion after meal ingestion in the attempt to maintain euglycemia (25). Obesity is an important risk factor in progression to type 2 diabetes mellitus (14) and also in cardiovascular disease (16), and reduction of obesity can normalize hyperinsulinemia and impede the progression of diabetes and arteriosclerosis.

Incretins are a group of peptide hormones released from the gastrointestinal tract into the circulation in response to meal ingestion that potentiate glucose-stimulated insulin secretion and include gastric inhibitory polypeptide (GIP), also called glucose-dependent insulinotropic polypeptide (24). GIP is secreted from the K cells of the duodenum and proximal jejunum upon meal ingestion and binds to the GIP receptor (GIPR) on the surface of pancreatic β-cells, adipose tissues, and osteoblasts to stimulate insulin secretion (21), fat accumulation (20), and bone formation (30) by increasing the level of intracellular adenosine 3',5'-cyclic monophosphate (cAMP).

Previously, we found that GIPR-deficient mice exhibit insufficient compensatory insulin secretion upon high-fat loading (21), indicating that GIP plays a critical role in maintaining the blood glucose level by inducing hypersecretion of insulin in diet-induced obesity. Increased GIP signaling in obesity might be due to hypersecretion of GIP from K cells or hypersensitivity of GIPR to GIP at the β-cells. An increased blood GIP level in obesity has been reported in some studies (3, 6) but is controversial (27, 28), and altered GIPR sensitivity in obesity has not been investigated.

GIPR is the G protein-coupled receptor (GPCR) that belongs to the secretin-vasoactive intestinal peptide receptor family (31, 33). The gene encoding the human GIPR contains 14 exons (33); the rat and mouse GIPR-encoding genes contain 15 exons (2, 21). Alternative splicing is a frequent occurrence in the transcriptome in higher eukaryotic cells and can alter the structure of the encoded protein and dramatically increase the efficiency of the proteome in regulating cell function. In the present study, we report a novel splice variant GIPR expressed in mouse pancreatic β-cells and the investigation of its functional significance in hypersensitivity of GIPR in high-fat diet-induced obese mice.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice (7 wk old) were obtained from Shimizu (Kyoto, Japan). The animals were fed control fat chow (CFD: 10% fat, 20% protein, and 70% carbohydrate by energy) or high-fat chow (HFD: 45% fat, 20% protein, and 35% carbohydrate by energy) for 10 wk. The energy density of both diets was 3.57 kcal/g. After a 16-h fast, oral glucose tolerance tests (OGTTs) (2 g/kg body wt) were performed in CFD and HFD mice. Blood glucose and plasma insulin levels were measured in samples taken at the indicated times. Blood glucose levels were determined by the glucose oxidase method. Plasma insulin levels were determined using enzyme immunoassay (Shibayagi, Gunma, Japan). Animal care and procedures were approved by the Animal Care Committee of Kyoto University.