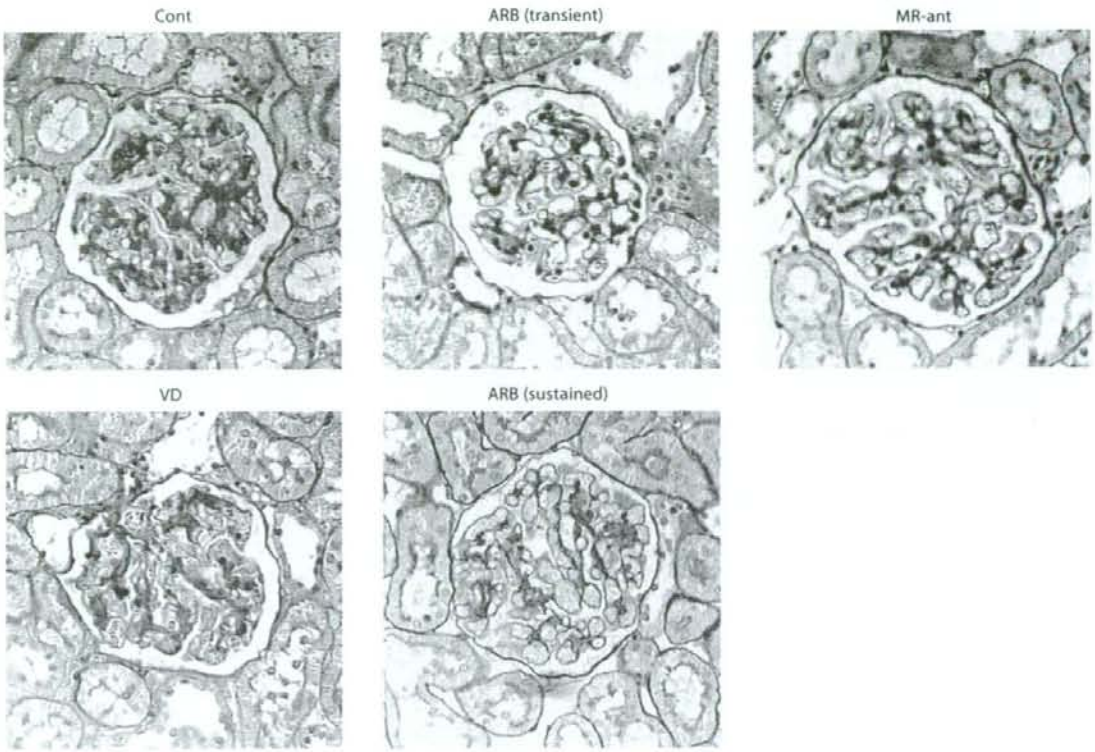

Fig. 3. Light microscopy of renal histology in the different groups. SHR were treated with various antihypertensive agents between 3 and 10 weeks of age and STZ was administered at 16 weeks. Treatments consisted of no treatment (control group = Cont), transient treatment with the angiotensin receptor blocker candesartan cilexetil [ARB (transient)], transient treatment with the mineralocorticoid receptor antagonist potassium canrenoate (MR-ant), transient treatment with the vasodilator hydralazine (VD) or continuous treatment with the angiotensin receptor blocker candesartan cilexetil [ARB (sustained)]. Kidneys were removed at 36 weeks for histological analysis. Representative photomicrographs of paraformaldehyde-fixed sections stained with PAS are shown. Orig. magnif. ×200.

On light microscopy, rats in the control group exhibited glomerular changes of diabetic nephropathy characterized by marked mesangial expansion. These changes were almost completely suppressed in the ARB (sustained) group (fig. 2b, 3). Also, the mesangial expansion score was significantly decreased in both the ARB (transient) and MR-ant groups whereas the histological findings in the vasodilator group were similar to control rats. No significant differences in plasma urea nitrogen or creatinine were found in the different groups (table 1).

Effects of Transient Exposure to Various Antihypertensive Agents on Components of the Renin-Angiotensin System in STZ-Treated SHR

The values of PRA, PAC as well as expression of renal renin, AT1a and AT2 receptor mRNA expression were assessed in order to examine the hypothesis that the observed changes could be explained by changes in these components of the RAAS (fig. 4). A 2.3-fold increase in PRA was found in the ARB (sustained) group but no significant differences were seen in the other groups. The values of renal renin mRNA were also increased in the ARB (sustained) group (tables 2, 3). No significant differ-

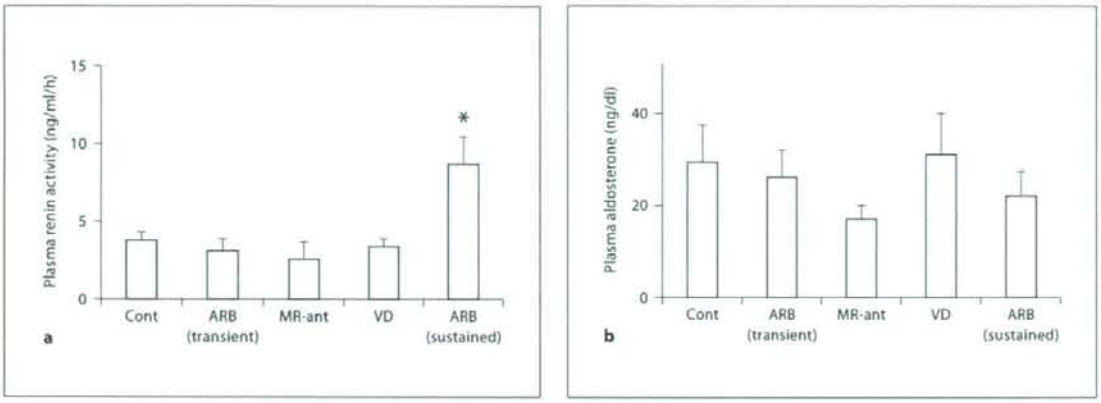

Fig. 4. Effects of transient exposure to various antihypertensive agents on PRA and plasma aldosterone in STZ-treated SHR. SHR were treated with various antihypertensive agents between 3 and 10 weeks of age and STZ was administered at 16 weeks. Treatments consisted of no treatment (control group = Cont), transient treatment with the angiotensin receptor blocker candesartan cilexetil [ARB (transient)], transient treatment with the mineralocorticoid receptor antagonist potassium canrenoate (MR-ant), transient treatment with the vasodilator hydralazine (VD) or continuous treatment with the angiotensin receptor blocker candesartan cilexetil [ARB (sustained)]. Rats were sacrificed at 36 weeks and PRA (**a**) and plasma aldosterone (**b**) measured. An elevated PRA was evident in the ARB (sustained) group but no other significant effects were evident. * $p < 0.05$ vs. Cont.

Table 2. Expression of renal renin, AT1 receptor and AT2 receptor mRNA in the different groups (arbitrary units)

	Group 1	Group 2	Group 3	Group 4	Group 5
Treatment	Cont	ARB (transient)	MR-ant	VD	ARB (sustained)
Renal renin/GAPDH mRNA	1.0 ± 0.3	1.2 ± 0.6	1.8 ± 0.8	1.5 ± 0.5	5.3 ± 0.8*
Renal AT1/GAPDH mRNA	1.0 ± 0.3	1.4 ± 0.1	0.9 ± 0.4	1.8 ± 0.3	1.7 ± 0.2
Renal AT2/GAPDH mRNA	1.0 ± 0.5	1.5 ± 0.8	1.3 ± 0.3	0.8 ± 0.3	1.4 ± 0.7

Results shown are the means ± SEM. Cont = Control treated; ARB (transient) = treated transiently with candesartan cilexetil; MR-ant = treated transiently with potassium canrenoate; VD = treated transiently with hydralazine; ARB (sustained) = treated continuously with candesartan cilexetil.

* $p < 0.05$ vs. Cont.

ences in the renal expression of AT1a or AT2 receptor mRNA were evident between any of the experimental groups.

Discussion

Previous studies have shown that transient treatment of genetically hypertensive rats with a renin-angiotensin system inhibitor results in the suppression of the subsequent development of hypertension. Following the initial studies by Harrap et al. [6], Wu et al. [7] and others, we reported that prepubertal treatment with an ARB can attenuate the development of not only hypertension but also renal injury in both the stroke-prone SHR and Dahl-S rat models [8, 7]. We also showed that prepubertal exposure to the ARB candesartan cilexetil conferred protection against subsequent acquired renal injury induced by the nephrotoxin L-NAME in SHR [10].

Table 3. Summary of changes in SBP, albuminuria and mesangial expansion scores in the different groups

	Group 1	Group 2	Group 3	Group 4	Group 5
Treatment	Cont	ARB (transient)	MR-ant	VD	ARB (sustained)
SBP at age 10 weeks, mm Hg	185 ± 14	140 ± 10**	172 ± 12	136 ± 8**	142 ± 4**
SBP at age 34 weeks, mm Hg	230 ± 7	180 ± 6**	211 ± 11	225 ± 6	138 ± 8**
Urine albumin at age 34 weeks, mg/day	489 ± 117	57 ± 33**	165 ± 51*	393 ± 165	24 ± 15**
Mesangial expansion score at age 34 weeks	30 ± 5	16 ± 2*	15 ± 3*	26 ± 7	4 ± 2**

Results shown are the means ± SEM. Cont = Control treated; ARB (transient) = treated transiently with candesartan cilexetil; MR-ant = treated transiently with potassium canrenoate; VD = treated transiently with hydralazine; ARB (sustained) = treated continuously with candesartan cilexetil.

* $p < 0.05$; ** $p < 0.01$ vs. Cont.

In this study, we examined the effects of administering the ARB candesartan from age 3 to 10 weeks on the subsequent course of hypertension and renal disease in STZ-treated SHR. In the case of the ARB, we found that transient treatment during this 'critical period' caused a significant decrease in BP that was sustained throughout the course of the experiment and which was accompanied by the marked inhibition of the development of albuminuria. Indeed, the urine albumin excretion was similar to that found in the rats treated continuously with ARB.

An important novel finding was that transient treatment with the MR-ant potassium canrenoate (the active metabolite of spironolactone [14]) caused a partial suppression of the development of albuminuria in the absence of any significant effect on BP. Interestingly, the mesangial expansion found in the control rats was significantly greater than that evident in both the groups treated with candesartan and potassium canrenoate. In contrast, no amelioration of mesangial expansion was found in the hydralazine-treated group. These data indicate that transient treatment with MR-ant results in a significant decrease in albuminuria and inhibition of mesangial expansion in the absence of a major effect on BP. This novel finding may have important implications for understanding the mechanisms involved in the development of diabetic nephropathy. In particular, there is considerable controversy in the current literature regarding the relative importance of BP-dependent and -independent mechanisms in the renoprotective actions of RAAS inhibitors. A large number of animal studies, as well as several clinical studies comparing ACEI or ARB with other antihypertensive agents, support a role for BP-independent effects in the renoprotective actions of these agents [5]. However, Casas et al. [4] concluded from a meta-analysis of several trials of ACEI that the renoprotective actions could be explained by the beneficial effect upon BP alone.

In our previous studies using an ARB, we were unable to differentiate between BP-dependent and -independent mechanisms. However, the results of this study demonstrating the suppressive effect of early blockade of the RAAS during the 'critical period' upon subsequent diabetic renal injury strongly suggests that BP-independent factors play a key protective role. It is also true that the BP decrease seen in the ARB (transient) group could provide additional protection. This view is supported by the report by Nagai et al. [15], who found that treatment of type 2 diabetic rats with an ARB from age 4 to 11 weeks did not cause a significant decrease in BP but did attenuate the development of diabetic nephropathy.

The mechanism by which RAAS inhibitors cause BP-independent suppression of glomerular changes remains to be defined. At present, we are conducting extensive gene and protein expression studies to examine the hypothesis that blockade of the RAA system during the 'critical period' in development results in a structural remodeling of the intraglomerular microvasculature, resulting in a diminished susceptibility to the onset of diabetes-induced changes later in life. It is pertinent that a recent study by Baumann et al. [16] reported that spironolactone treatment from age 4 to 8 weeks did not attenuate albuminuria at age 72 weeks in SHR, indicating that the effects of interventions may vary in different disease models and depend upon drug dosage and treatment protocols (the dose of spironolactone in the study was 1 mg/kg which was $^1/_{20}$th of the dose of can-

renoate in this study). On the other hand, the fact that potassium canrenoate did not cause a significant change in BP even during the treatment period is consistent with several studies which have shown that administration of potassium canrenoate or its parent compound spironolactone does not have a major effect on BP in SHR [17–20].

We treated the rats from age 3 to 10 weeks as our previous studies indicated that treatment during this period has a major effect on BP and renal injury later in life. Post-natal renal development (nephrogenesis) is complete during this period from weaning to puberty and yet hypertension and renal arteriolar hypertrophy have not become fully established in the SHR model [6, 21]. Regarding the timing of the interventions, Harrap et al. [6] compared the effects of ACEI from age 2 to 6 weeks, 6 to 10 weeks and 2 to 10 weeks and found that long-term effects were obtained with all of these treatment periods. In this study, we injected STZ and established diabetes at 16 weeks, when the BP changes and renal arteriolar changes are fully established and stable in the SHR model. Further studies are required to examine the effects of inducing diabetes at earlier or later time points.

Clinically, the results of this study may have important implications for the prevention of diabetic renal disease. It is evident that individual diabetic patients differ in their susceptibility to diabetic nephropathy, with some patients being resistant to the development of nephropathy despite having poor glycemic control, whereas other patients develop renal injury early despite relatively good glycemic control [1, 3]. Although the different susceptibility may be genetically defined, the results of this study suggest the possibility that developmental differences in RAAS activity may modulate the susceptibility to renal injury later in life. This could explain why some patients exhibit a marked susceptibility to diabetic nephropathy whereas others are clearly resistant. Furthermore, if this speculation is correct, then clinical intervention at an appropriate time could alter the susceptibility to the development of subsequent nephropathy in individual patients. This would be an important first step in the development of strategies to prevent diabetic nephropathy. One potential approach suggested by this study is to treat hypertensive diabetic patients with an ARB in order to suppress the development of renal injury, whereas treatment with MR-ant may be more appropriate in normotensive diabetic patients. Clinical studies will be required to explore these possibilities.

In summary, we have shown for the first time that treatment of rats during the 'critical period' with ARB suppresses both systemic BP and diabetic nephropathy. In contrast, comparable treatment with MR-ant resulted in significant suppression of diabetic renal changes in the absence of any significant changes in BP in the STZ model of diabetic nephropathy in SHR. No such changes were evident with a vasodilator. These results underscore the importance of BP-independent factors in the pathogenesis of diabetic nephropathy. From a clinical perspective, an important implication of this study is that early use of a RAAS may be warranted to decrease the susceptibility to diabetic nephropathy in both normotensive and hypertensive patients.

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Combination of C-reactive Protein and High Molecular Weight (HMW)-Adiponectin Reflects Further Metabolic Abnormalities Compared with Each of Them Alone in Japanese Type 2 Diabetic Subjects

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Abstract. Objective: Serum C-reactive protein (CRP) and adiponectin levels predict atherosclerosis and coronary heart disease. However, the efficacy of the combination of both markers remains unknown. In the present study, we investigated whether the combination of CRP and adiponectin is associated with further metabolic abnormalities compared to each of them alone. Research design and methods: Eighty-three Japanese type 2 diabetic outpatients participated in this study. We measured serum high-sensitive CRP and high molecular weight (HMW)-adiponectin, and investigated their relationship with various metabolic parameters. Results: In univariate analysis, CRP was significantly correlated with diastolic blood pressure and HDL-cholesterol. On the other hand, HMW-adiponectin was significantly correlated with systolic (SBP) and diastolic blood pressure, plasma glucose, HDL-cholesterol, triglycerides and HOMA-IR, but not with CRP. We then classified the subjects into three groups: low CRP and high HMW-adiponectin levels (low risk group, 19%), high CRP and low HMW-adiponectin levels (high risk group, 22%), and others. In Spearman rank correlation coefficient analysis, this classification was significantly associated with a larger number of metabolic risk factors: SBP, glucose, HbA1c, LDL-cholesterol, HDL-cholesterol, triglycerides and HOMA-IR, compared with classification by CRP or HMW-adiponectin alone. Conclusion: These results suggest that combination of CRP and HMW-adiponectin reflects further metabolic abnormalities compared with each of them in type 2 diabetic subjects. The combined measurement of both markers may be useful to detect cardiovascular high risk patients.

Key words: C-reactive protein, Adiponectin, Coronary risk factor, Type 2 diabetes mellitus

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BOTH type 2 diabetes and atherosclerosis are characterized by low-grade inflammation and insulin resistance [1, 2]. Patients with type 2 diabetes show a 2- to 5-fold increase in coronary heart disease (CHD)-related death [3], thus it is very important to precisely evaluate the risk of CHD in diabetic patients in clinical settings. Serum CRP and adiponectin reflect low-grade inflammation and insulin resistance, respectively, and are known as the important markers of atherosclerosis and CHD [4, 5].

Serum CRP level is increased in obese [6] and diabetic [7–9] subjects, and predicts the onset of cardiovascular disease [4, 10–12]. On the other hand, serum adiponectin level is negatively associated with visceral fat [13] and insulin resistance [13–15]. It has been reported that adiponectin level is decreased in obese [16] and diabetic subjects [17] and subjects with cardiovas-

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Abbreviations: CRP, C-reactive protein; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HOMA-IR, homeostasis model assessment insulin resistance index; CHD, coronary heart disease; SBP, systolic blood pressure; DBP, diastolic blood pressure; HMW, high molecular weight; ELISA, enzyme-linked immunosorbent assay; SU, sulfonylureas; TZD, thiazolidinedione; IMT, intima-media thickness; PWV, pulse-wave velocity

cular disease [17]. It has also been reported that a low serum adiponectin level is associated with increased risk of myocardial infarction [18] and predicts the progression of coronary artery calcification [19]. Recently, high molecular weight (HMW)-adiponectin and HMW-adiponectin/total adiponectin ratio rather than total adiponectin have been reported to be useful for evaluation of coronary artery disease [20] or insulin resistance [21] in the subjects with type 2 diabetes. It has also been reported that HMW-, but not low molecular weight, adiponectin selectively decreases in the subjects with type 2 diabetes [22], suggesting that HMW-adiponectin plays an important role in the development of cardiovascular disease.

A negative correlation between serum CRP and adiponectin levels in non-diabetic [18, 23–27] and diabetic subjects [9, 20, 28–31] has been reported, and it is well-recognized that obesity is associated with both insulin resistance and inflammation, leading to atherosclerosis [32]. However, the precise mechanism of this correlation between CRP and adiponectin remains unclear. Others have shown no significant relation between CRP and adiponectin [33–35]. Furthermore, recent studies have shown that CRP is associated with obesity rather than insulin resistance or metabolic syndrome [36–38]. These results indicate a possibility of some different aspects between low-grade inflammation and insulin resistance, and suggest a synergistic effect of these two factors on atherosclerosis. Nonetheless, no report has examined the efficacy of the combination of serum CRP and adiponectin compared to each of them alone. In the present study, we investigated whether or not the combination of CRP and HMW-adiponectin is associated with further metabolic abnormality in type 2 diabetic subjects compared to each of them alone.

Patients and Methods

Subjects

We measured serum levels of CRP, HMW-adiponectin and other metabolic parameters in 83 (51 men and 32 post-menopausal women, age 63 ± 8 years, duration of diabetes 7.8 ± 5.4 years, Table 1) Japanese type 2 diabetic patients in the outpatient clinic of Keio University Hospital, Tokyo, Japan. Patients were treated with dietary therapy and/or oral hypoglycemic agents, but not with insulin. We recruited the subjects who had not been changed any diet or medication therapy for at least 3 months and glycemic control was relatively stable for this period (mean HbA1c 6.4 ± 0.9%, Table 1). The present study was conducted according to the principles expressed in the Declaration of Helsinki. Informed consent was obtained from each patient after full explanation of the purpose, nature and risk of all procedures used. The protocol was approved by the ethical review committee of the Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan.

Table 1. Clinical and laboratory characteristics of the patients

N (M/F)		83 (51/32)
Age	(years)	63 ± 8
Duration of diabetes	(years)	7.8 ± 5.4
Current smoking	(%)	28.9
BMI	(kg/m^2)	23.6 ± 3.2
Systolic blood pressure	(mmHg)	129 ± 17
Diastolic blood pressure	(mmHg)	77 ± 11
Glucose	(mmol/L)	7.7 ± 1.5
HbA1c	(%)	6.4 ± 0.9
LDL-cholesterol	(mg/dL)	121 ± 25
HDL-cholesterol	(mg/dL)	56 ± 13
Triglyceride	(mg/dL)	107 ± 55
Insulin	(μU/mL)	7.0 ± 5.9
HOMA-IR	—	2.43 ± 2.12
CRP	(mg/dL)	0.075 ± 0.090
HMW-adiponectin	(μg/mL)	4.7 ± 3.2

Values are means ± SD.

Thirty-eight (46%) patients were treated with dietary therapy only, and the others were treated with one or more oral hypoglycemic agents. Forty (48%) patients were treated with sulfonylureas (SU), 30 (36%) with alpha-glucosidase inhibitors, and 6 (7%) with biguanides. Patients treated with thiazolidinediones (TZD) were excluded from the study. The duration of diabetes and smoking habit were obtained from medical records or questionnaires to the patients.

Fifteen patients (18%) had diabetic retinopathy documented by ophthalmologists, and 9 patients (11%) had diabetic nephropathy with micro-albuminuria or proteinuria. Thirty-three patients (40%) had hypertension, and 21 patients (25%) were being treated with antihypertensive medicine. Seventeen patients (20%) were being treated with a statin for hyperlipidemia. Eight patients (10%) had macroangiopathy; 7 with myocardial infarction and 1 with cerebral infarction. Patients who had hepatitis or liver cirrhosis, renal

failure (Cr >1.9 mg/dL), inflammatory disease (or CRP >1 mg/dL) or malignancy were excluded from the study.

Measurements

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured with subjects in the sitting position after resting for at least 5 min. Blood samples were collected in the morning after an overnight fast.

Serum glucose was measured by glucose oxidase method. Serum insulin was measured as immunoreactive insulin by enzyme immunoassay. Low-density lipoprotein (LDL)-cholesterol, triglycerides and high-density lipoprotein (HDL)-cholesterol levels were determined using standard laboratory methods [14, 15].

Serum CRP level was measured by nephelometry, a latex particle-enhanced immunoassay (N Latex CRP II, Dade Behring, Tokyo, Japan) with both intra- and inter-assay coefficients of variation of <5.0%. The assay could detect 0.005 mg/dL of CRP.

Insulin resistance index was assessed using homeostasis model assessment (HOMA-IR), which was calculated as follows: HOMA-IR = (insulin (μU/mL) × plasma glucose (mmol/L))/22.5.

Measurement of HMW-adiponectin by ELISA

HMW-adiponectin was measured using a commercially available kit (HMW Adiponectin ELISA Kit, Fujirebio Co. Inc., Tokyo, Japan). This ELISA system does not need a denaturing step, and the antibody reacts specifically with the HMW form of adiponectin [39]. The dilution curve was parallel to the standard curve. Intra- and inter-assay coefficients were 2.4–3.0% and 4.2–5.1%, respectively.

Statistical analysis

All statistical analyses were performed using the StatView program for Windows (version 5.0-J; SAS Institute Inc., Cary, NC, USA). Mann-Whitney U-test and Kruskal-Wallis test were used for comparison of metabolic parameters among two or three groups, respectively. Relationships between serum CRP or HMW-adiponectin level and other parameters were analyzed by simple correlation and multiple linear regressions. Spearman rank correlation coefficient was used to analyze the correlation with metabolic parameters among the three classified groups. Because serum triglycerides, insulin, HOMA-IR, CRP and HMW-adiponectin were normally distributed after logarithmic transformation, the logarithms of these parameters were used for the analyses. All data are expressed as mean ± S.D., and values of $P<0.05$ were considered statistically significant.

Results

Characteristics of patients

Characteristics of the patients are shown in Table 1. Mean CRP and HMW-adiponectin concentrations were 0.075 ± 0.090 mg/dL and 4.7 ± 3.2 μg/mL, respectively. CRP and HMW-adiponectin tended to be lower and higher, respectively, in female patients, while the differences between male and female patients were not significant (CRP; 0.083 ± 0.106 vs. 0.063 ± 0.054 mg/dL, $P = 0.52$, HMW-adiponectin; 4.1 ± 2.7 vs. 5.6 ± 3.8 μg/mL, $P = 0.07$ in male vs. female, respectively). There was no significant difference in both CRP and HMW-adiponectin between the patients with and without statin treatment (0.056 ± 0.034 vs. 0.080 ± 0.099 mg/dL, $P = 0.80$ and 5.0 ± 3.3 vs. 4.6 ± 3.3 μg/mL, $P = 0.66$ in CRP and HMW-adiponectin, respectively).

Correlations of CRP and HMW-adiponectin with metabolic parameters

The correlations of serum CRP and HMW-adiponectin levels with other parameters in all patients are shown in Table 2. In univariate analysis, CRP showed a significant positive correlation with DBP ($r = 0.245$), and a significant negative correlation with HDL-cholesterol ($r = -0.290$). CRP also showed positive correlations with BMI ($r = 0.184$), SBP ($r = 0.182$) and triglycerides ($r = 0.187$), while the correlations did not reach statistical significance ($P = 0.1$). But there was no correlation between CRP and HOMA-IR ($r = 0.092$). Even after adjustment for age and sex, the correlations of CRP with DBP and HDL-cholesterol remained significant.

On the other hand, HMW-adiponectin showed significant negative correlations with SBP ($r = -0.266$), DBP ($r = -0.264$), glucose ($r = -0.270$), triglycerides

Table 2. Correlations of CRP and HMW-adiponectin with other parameters

	CRP (log)				HMW-adiponectin (log)			
			after adjustment[a]				after adjustment[a]	
	r	*P*	r	*P*	r	*P*	r	*P*
BMI	0.184	0.10	0.179	0.11	−0.093	0.40	−0.079	0.47
Systolic blood pressure	0.182	0.10	0.182	0.11	**−0.266**	**0.02**	**−0.228**	**0.04**
Diastolic blood pressure	**0.245**	**0.03**	**0.253**	**0.03**	**−0.264**	**0.02**	**−0.227**	**0.05**
Glucose	0.163	0.14	0.167	0.15	**−0.270**	**0.01**	**−0.236**	**0.03**
HbA1c	0.090	0.42	0.112	0.34	−0.196	0.08	−0.215	0.06
LDL-cholesterol	−0.005	0.96	0.006	0.96	−0.164	0.14	−0.193	0.08
HDL-cholesterol	**−0.290**	**0.008**	**−0.306**	**0.009**	**0.293**	**0.007**	**0.245**	**0.03**
Triglyceride (log)	0.187	0.09	0.190	0.09	**−0.239**	**0.03**	**−0.225**	**0.04**
Insulin (log)	0.047	0.68	0.051	0.66	−0.189	0.10	−0.196	0.08
HOMA-IR (log)	0.092	0.42	0.091	0.43	**−0.269**	**0.02**	**−0.266**	**0.02**
CRP (log)	—	—	—	—	−0.033	0.77	−0.021	0.85

Pearson's correlation coefficient. [a] Adjustment for age and sex.

(r = −0.239) and HOMA-IR (r = −0.269), but not BMI (r = −0.093), and a significant positive correlation with HDL-cholesterol (r = 0.293). These correlations, except that for DBP, remained significant even after adjustment for age and sex. There was no significant correlation between CRP and adiponectin (r = −0.033, P = 0.77, Fig. 1). Even when we excluded the subjects treated with either statins or SU, there was no correlation between CRP and HMW-adiponectin (r = −0.019, P = 0.88 and r = 0.090, P = 0.56, respectively).

Classification by combination of CRP and HMW-adiponectin

We then divided the patients into each quartile of CRP and HMW-adiponectin levels, respectively, and scored the values as shown in Table 3. The classification by the combination of CRP and HMW-adiponectin was defined as follows: low risk group, the sum of the scores was 2 or 3; moderate risk group, 4–6; and high risk group, 7 or 8. The characteristics of the three classification groups are shown in Table 4. The low risk group and high risk group comprised 19% and 22% of the total patients, respectively. Among the three groups, HbA1c, LDL-cholesterol, triglycerides and HOMA-IR as well as CRP were significantly higher in the high risk group. HDL-cholesterol as well as HMW-adiponectin was significantly lower in the high risk group. Other metabolic parameters, such as BMI, blood pressure, glucose and insulin, were also higher in the high risk group than in the low risk group, while the differences did not reach statistical significance.

Comparison among the classification by CRP, HMW-adiponectin and a combination of both is shown in Table 5. Classification by CRP and HMW-adi-

Table 3. Classification by CRP and HMW-adiponectin

CRP (mg/dL)	HMW-adiponectin (μg/mL)	Score
0.082~	~2.5	4
0.049~0.081	2.6~3.8	3
0.032~0.048	3.9~6.2	2
~0.031	6.3~	1

CRP and HMW-adiponectin were divided into four groups at 25th, 50th and 75th percentile levels.
Classification by a combination of CRP and HMW-adiponectin was defined as the sum of scores.

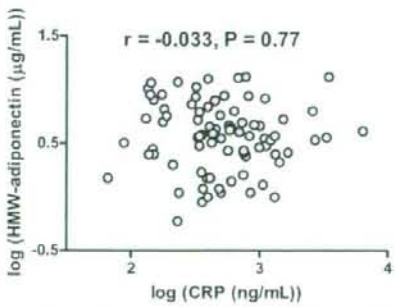

Fig. 1. Correlation between CRP and HMW-adiponectin.

Table 4. Clinical and laboratory characteristics of three classified groups by a combination of CRP and HMW-adiponectin

		Low risk group	Moderate risk group	High risk group	P^a
N		16 (19%)	49 (59%)	18 (22%)	
Male	(%)	37.5	69.4	61.1	0.08
Age	(years)	61 ± 7	63 ± 9	64 ± 7	0.51
Duration of diabetes	(years)	7.5 ± 6.7	7.8 ± 5.0	8.2 ± 5.7	0.66
Current smoking	(%)	25.0	24.5	44.4	0.26
BMI	(kg/m^2)	22.6 ± 3.6	23.7 ± 3.2	24.3 ± 2.5	0.21
Systolic blood pressure	(mmHg)	122 ± 13	129 ± 17	134 ± 18	0.11
Diastolic blood pressure	(mmHg)	72 ± 11	77 ± 10	80 ± 12	0.11
Glucose	(mmol/L)	7.2 ± 1.4	7.7 ± 1.5	8.2 ± 1.5	0.10
HbA1c	(%)	6.2 ± 1.1	6.3 ± 0.8	6.8 ± 0.9	**0.04**
LDL-cholesterol	(mg/dL)	119 ± 27	117 ± 24	136 ± 24	**0.03**
HDL-cholesterol	(mg/dL)	65 ± 11	55 ± 12	50 ± 13	**0.005**
Triglyceride	(mg/dL)	106 ± 80	99 ± 46	131 ± 45	**0.01**
Insulin	(μU/mL)	4.6 ± 2.5	7.8 ± 6.8	6.9 ± 4.5	0.05
HOMA-IR	—	1.50 ± 1.10	2.67 ± 2.41	2.59 ± 1.75	**0.01**
CRP	(mg/dL)	0.024 ± 0.011	0.074 ± 0.101	0.124 ± 0.072	**<0.0001**
HMW-adiponectin	(μg/mL)	8.5 ± 2.4	4.3 ± 3.0	2.4 ± 0.9	**<0.0001**

Low risk group: low CRP and high HMW-adiponectin group, High risk group: high CRP and low HMW-adiponectin group. Values are means ± SD. [a] Kruskal-Wallis test was used in analysis among three groups.

ponectin was defined as follows: low risk group, score 1; moderate risk group, score 2 or 3; and high risk group, score 4 in Table 3. Classification by CRP was significantly associated with BMI, DBP, glucose, HDL-cholesterol and triglycerides, but neither HOMA-IR nor HMW-adiponectin. Classification by HMW-adiponectin was significantly associated with glucose, HDL-cholesterol, triglycerides, insulin and HOMA-IR, but neither blood pressure nor CRP. On the other hand, classification by a combination of CRP and HMW-adiponectin was significantly associated with a larger number of metabolic parameters; *i.e.*, SBP, glucose, HbA1c, LDL-cholesterol, HDL-cholesterol, triglycerides and HOMA-IR, as well as CRP and HMW-adiponectin.

Table 5. Comparison among classification by CRP, HMW-adiponectin and combination of both

	CRP		HMW-adiponectin		Combination	
	r^a	P	r^a	P	r^a	P
Age	0.034	0.76	−0.012	0.91	0.110	0.32
Sex	0.085	0.44	0.197	0.07	0.144	0.19
Current smoking	0.102	0.35	0.163	0.14	0.143	0.19
BMI	**0.250**	**0.02**	0.117	0.29	0.194	0.08
Systolic blood pressure	0.189	0.09	0.130	0.24	**0.225**	**0.04**
Diastolic blood pressure	**0.271**	**0.01**	0.124	0.26	0.214	0.05
Glucose	**0.231**	**0.04**	**0.224**	**0.04**	**0.239**	**0.03**
HbA1c	0.173	0.12	0.139	0.21	**0.267**	**0.02**
LDL-cholesterol	0.154	0.16	0.164	0.14	**0.225**	**0.04**
HDL-cholesterol	**−0.332**	**0.003**	**−0.318**	**0.004**	**−0.354**	**0.001**
Triglyceride	**0.268**	**0.02**	**0.246**	**0.03**	**0.292**	**0.008**
Insulin	0.055	0.63	**0.227**	**0.04**	0.179	0.11
HOMA-IR	0.155	0.17	**0.324**	**0.004**	**0.284**	**0.01**
CRP	—		0.070	0.53	0.679	<0.0001
HMW-adiponectin	−0.166	0.13	—		−0.630	<0.0001

[a] Spearman rank correlation coefficient.

Discussion

In the present study, we investigated the efficacy of combination of CRP and HMW-adiponectin to evaluate the metabolic abnormalities in type 2 diabetic subjects. Both serum CRP and HMW-adiponectin levels were associated with several metabolic parameters in this study. However, the parameters associated with each of CRP and HMW-adiponectin were not completely consistent. Furthermore, CRP did not correlate with HMW-adiponectin. An association between CRP and adiponectin has been reported in non-diabetic [18, 23–27] and diabetic subjects [9, 20, 28–31], although others have reported no relation between CRP and adiponectin [33–35]. The lack of association between CRP and adiponectin in this study might have been due to medications affecting CRP and/or adiponectin, such as statins [12, 40]. There was, however, no significant difference in both CRP and HMW-adiponectin levels between patients with and without statin treatment. In addition, we did not include patients treated with TZD which affects adiponectin level [41] in this study. While it has been reported that the association between CRP and adiponectin is due to an anti-inflammatory action of adiponectin itself [5], the precise mechanism of the relation between CRP and adiponectin remains to be elucidated. Shetty *et al.* have reported that the significant correlation of adiponectin with CRP disappeared after adjustment for sex and BMI [29]. Putz *et al.* have reported that although CRP was significantly associated with percent fat and total fat mass, adiponectin was associated with neither of them [9]. Moreover, Matsushita *et al.* have reported that adiponectin showed a stronger correlation with metabolic syndrome than CRP in Japanese men [42], consistent with our finding that HMW-adiponectin rather than CRP was associated with the components of metabolic syndrome. Furthermore, recent studies have shown that CRP is associated with obesity rather than insulin resistance or metabolic syndrome [37, 38], and emerging evidence suggests a hypothesis that CRP has no causal association with metabolic syndrome [36]. Although there is little doubt about the fact that CRP and adiponectin reflect some common pathway between inflammation and insulin resistance [32], this and recent studies also suggest some different aspects between CRP and adiponectin. The different production sites between CRP and adiponectin, *i.e.*, liver and adipose tissue, might result in some different aspects between these two markers. It will be important to characterize the subjects with discrepancies between CRP and HMW-adiponectin (*i.e.*, high CRP/high HMW-adiponectin or low CRP/low HMW-adiponectin) to understand the difference between these two markers.

Additionally, we extended this concept by showing that classification by a combination of CRP and HMW-adiponectin was associated with a larger number of metabolic parameters, compared with classification by CRP or HMW-adiponectin alone, indicating that CRP and HMW-adiponectin reflect different aspects of metabolic abnormality. Interestingly, this classification was associated with not only markers of metabolic syndrome, *i.e.*, blood pressure, glucose, HDL-cholesterol, triglycerides and HOMA-IR, which are also well-known to be a predictor of coronary disease [43], but also classical coronary risk factors, *i.e.*, LDL-cholesterol. It has been reported that the combination of CRP and total or LDL-cholesterol highly predicted the onset of CHD [10–12]. Furthermore, the frequency of other classical risk factors such as age and the proportion of males and current smokers were also higher in the high risk group, although the differences were not significant. We did not check other coronary risk factors such as intima-media thickness (IMT) of the carotid artery or pulse-wave velocity (PWV) in this study. However, our findings suggest the possibility that the combination of CRP and HMW-adiponectin is a highly predictive marker for patients at high risk of coronary disease in clinical settings. While the small sample size is a major limitation of our study, we could confirm similar results even when we analyzed either a subgroup of the subjects not treated with SU (N = 43) or a subgroup not treated with statins (N = 66); *i.e.*, (1) there was no correlation between CRP and HMW-adiponectin, (2) the combination of CRP and HMW-adiponectin was associated with a larger number of metabolic parameters compared to each of them alone (data not shown). Because we did not examine any correlation between these markers and cardiovascular events in this study, it remains unclear whether the combination of CRP and HMW-adiponectin is more useful to predict cardiovascular events than each of them alone. The results of this study should be verified in a study with a larger sample size and a prospective study design.

In conclusion, we demonstrated that the combination of CRP and HMW-adiponectin was associated with further metabolic abnormalities in type 2 diabetic subjects compared with each of them alone. The com-

bined measurement of both markers may be useful to detect cardiovascular high risk patients.

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cGMP rescues mitochondrial dysfunction induced by glucose and insulin in myocytes

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Abstract

Mitochondrial dysfunction in the skeletal muscle has been implicated in a wide variety of pathological processes including insulin resistance in type 2 diabetes. A recent report indicates that calorie restriction can modulate mitochondrial function through the nitric oxide/cGMP-dependent pathway. Following up on these findings, we examined whether cGMP could rescue mitochondrial dysfunction in C2C12 myotubular cells induced by conditions of high-glucose and high-insulin. Treatment of the cells with cGMP promoted mitochondrial biogenesis and ATP synthesis without enhancing production of reactive oxygen species (ROS) in association with up-regulation of the genes involved in oxidative phosphorylation and ROS reduction. The increased mitochondria were revealed to have lower membrane potential, which is similar to the effect of calorie restriction, and reversed mitochondrial dysfunction caused by high-glucose and high-insulin. These results indicated that augmented cGMP-dependent cascades in the skeletal muscle may attenuate insulin resistance observed in patients with type 2 diabetes and metabolic syndrome.

Keywords: cGMP; Mitochondria; Diabetes; Insulin resistance; Skeletal muscle; Mitochondrial biogenesis; Reactive oxygen species; Calorie restriction; PGC1

Mitochondria are energy-producing organelles that generate ATP by means of oxidative phosphorylation (OXPHOS). Impairment of ATP production causes cellular dysfunction, particularly in tissues with higher energy expenditure, including heart, skeletal muscle, and nervous system, and has been implicated in a wide variety of pathological processes including neuro-degeneration, myopathy, obesity, and insulin resistance [1,2].

The number of mitochondria and the rate of ATP synthesis are known to decrease concomitantly in the skeletal muscle of patients with type 2 diabetes mellitus [3,4]. Moreover, offspring of diabetic patients show a reduction in mitochondrial density and ATP production even in the pre-diabetic state [5,6]. Inherited defects in the mitochondrial OXPHOS system in the skeletal muscle are therefore assumed to cause insulin resistance. However, no treatment has been aimed to modulate mitochondrial function to date.

Reactive oxygen species (ROS), which can rapidly impair a wide variety of intracellular molecules including lipids, proteins, and nucleic acids, and thus cause mitochondrial and cellular dysfunction, are also generated as a by-product of ATP [7]. Cumulative cellular damage caused by ROS has been shown to play a central role in the pathogenesis of cardiovascular diseases, cancer and aging [8]. Inappropriate synthesis of ATP with excessive ROS may therefore be harmful for living organisms.

Abbreviations: cGMP, guanosine-3′,5′-cyclic monophosphate; NO, nitric oxide; ATP, adenosine triphosphate; ROS, reactive oxygen species; OXPHOS, oxidative phosphorylation; ATPsyn, ATP synthase; COX1V, cytochrome *c* oxidase complex IV; UCP3, uncoupling protein 3; SOD2, superoxide dismutase 2; PGC1, PPAR gamma coactivator 1; NRF1, nuclear respiratory factor 1; mtTFA, mitochondrial transcription factor A; PPARδ, peroxisome proliferator-activated receptor delta; NFκB, nuclear factor-kappa B; CREB1, CCAAT enhancer-binding protein 1.

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A recent report has proposed the notion of "efficient mitochondria" which possess an organized electron transport system that can generate enough ATP, while at the same time maintaining lower oxygen consumption [9]. This study demonstrated that ROS production was reduced in the cells incubated with serum from rats submitted to long term calorie restriction by inducing "efficient mitochondria". Another study found that nitric oxide/cGMP-dependent pathways participate in the modulation of mitochondrial function induced by calorie restriction [10]. However, the exact mechanism for the regulation of mitochondrial function by cGMP has not been identified.

In light of these findings, we explored the roles of cGMP in the regulation of mitochondrial function by using C2C12 myotubular cells and investigated whether cGMP could rescue mitochondrial dysfunction induced by high-glucose and high-insulin, which are generally observed in type 2 diabetes and metabolic syndrome.

Materials and methods

Cell culture. C2C12 cells (RIKEN BioResource Center, Tsukuba, Japan), which were derived from murine skeletal myoblast cells, were grown to confluence in DMEM (Dulbecco's modified Eagle's medium, Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) and differentiated into myotubes by means of incubation with 0.1% FBS for 7 days. The C2C12-derived myotubes were then treated for 48 h with or without a potent membrane-permeable agonist of cGMP, 8-*para*-chlorophenylthio-guanosine-3',5'-monophosphate (8-*p*CPT-cGMP, 10^{-4}–10^{-3} mol/l, Calbiochem, La Jolla, CA); a competitive antagonist of cGMP, Rp-8-*p*CPT-cGMP (10^{-3} mol/l, Calbiochem); or a nitric oxide (NO)-donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP, 10^{-6} mol/l, Sigma, St. Louis, MO). When we used low-glucose (100 mg/dl) DMEM, the osmolarity was adjusted by mannitol (Sigma) to that of high-glucose (450 mg/dl) DMEM.

Quantification of mitochondrial DNA copy number. Total DNA was extracted with the aid of Qiamp DNA mini kit (Qiagen, Tokyo, Japan) from the C2C12-derived myotubes incubated with or without the agents specified above for 48 h. The mitochondrial DNA copy number was determined by means of quantitative PCR analysis (ABI7500 Real-Time PCR System, Applied Biosystems, Foster City, CA), using specific primers for the mitochondrial DNA encoded 16S ribosomal RNA gene and the nuclear DNA encoded hexokinase 2 gene, as described previously [11]. Results were estimated from the difference in threshold cycle values (delta-C_t) between the mitochondrial gene and the diploid nuclear gene.

Quantification of mitochondrial mass, ROS production, membrane potential, and ATP content. Mitochondrial mass, mitochondrial ROS production, and membrane potential of the C2C12 cells were determined with the aid of fluorescent dyes, MitoTracker Green FM, MitoSOX Red, which can selectively detect mitochondrial ROS, which is a superoxide derived from mitochondria, and Rhodamine 123 (Molecular Probes, Eugene, OR), with the same procedures as described elsewhere [9,12]. To normalize the data, we used Hoechst 33342 (Molecular Probes) for nuclear staining. The value for mitochondrial mass was normalized by that for nuclei, and the value for mitochondrial ROS and membrane potential was normalized by that for mitochondrial density. After the cells were cultured with or without the aforementioned agents for 48 h, they were treated with the dyes for 10 min, and washed twice with warm PBS. The fluorescent intensity was measured with a Wallac ARVO SX multiplate reader (Perkin-Elmer, Norwalk, CT). ATP content of the cells was determined with the chemi-luminescence method (ATP bioluminescence Assay Kit HS II, Roche Diagnostics, Mannheim, Germany).

Microscopic analysis of mitochondria. Sparsely disseminated C2C12 cells (10^3 cells/cm^2) were stained with fluorescent probes as described above, and visualized with a confocal microscope (LSM510; Carl Zeiss, Tokyo, Japan). Images were acquired with a 20× objective lens.

Estimation of gene expressions by real-time PCR. C2C12 myotubes were incubated for 48 h after addition of 10^{-3} mol/l cGMP. Total RNA was extracted with the aid of an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Reverse transcription with an ExScript RT reagent kit (TaKaRa Bio, Otsu, Japan) was performed using 100 ng of the total RNA with oligo-dT as a primer. The level of gene expression was determined by means of quantitative PCRs (ABI 7500) in the presence of a fluorescent dye (SYBR Premix Ex Taq; TaKaRa Bio). The relative quantity of mRNA was calculated after normalization to that of the internal control, 18S gene. Details of the primers (TaKaRa-Bio) used in this study are available on the manufacturer's homepage (http://www.takara-bio.com).

Quantification of protein levels by Western blotting. Total protein (10 μg) from whole cell extracts were stained with antibodies against ATP synthase (1:2000, subunit α, A21350; Molecular Probes), COXIV (1:1000, subunit IV, A21348; Molecular Probes), SOD2 (1:2000, sc-30080; Santa Cruz Biotechnology, Santa Cruz, CA), UCP3 (1:1000, ab3477; Abcam plc, Cambridge, UK) and beta-actin (1:1000, #4967; Cell Signaling, Danvers, MA). Proteins were separated by means of SDS–PAGE (Bio-Rad Laboratories, Hercules, CA), transferred to nitrocellulose membranes after electrophoresis, and incubated with the corresponding antibodies for 12–48 h at 4 °C. Membranes were probed with their secondary antibodies labeled with horseradish peroxidase (1:3000–1:10,000). Immuno-labeled proteins were detected by using a chemi-luminescence kit (ECL Plus; GE Healthcare, Tokyo, Japan) and a lumino-image analyzer (LAS-3000; FujiFilm, Tokyo, Japan). The density of the blot for each protein relative to that for the internal control, beta-actin, was also estimated by means of imaging software (MultiGauge; FujiFilm).

Statistical analysis. All data were expressed as means ± standard error. Comparison of means between two groups was performed with Student's *t* test. When more than two groups were compared, analysis of variance was used to evaluate significant differences among groups, and if significant differences were confirmed, each difference was further examined by means of multiple comparisons. p value < 0.05 was considered to be statistically significant.

Results

cGMP promotes mitochondrial biogenesis and ATP synthesis without enhancing ROS generation in C2C12 myotubes

To investigate the effects of cGMP on mitochondrial biogenesis and ROS production, we incubated C2C12 myotubular cells for 48 h with or without 10^{-4}–10^{-3} mol/l cGMP. Mitochondrial DNA copy number estimated by means of quantitative PCR analysis showed a significant increase (55% increase at 10^{-3} mol/l, $n = 6$, $p < 0.01$, Fig. 1A) in parallel with the increase in cellular ATP content (Fig. 1B). Mitochondrial density estimated by means of fluorescent staining demonstrated a dose-dependent increase induced by cGMP (Fig. 1C). In spite of the increase in mitochondrial mass, mitochondrial ROS showed a significant decrease (17% decrease at 10^{-3} mol/l, $n = 12$, $p < 0.01$, Fig. 1C) when the value was normalized by the mitochondrial mass. The increase in mitochondria induced by a representative NO-donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP, 10^{-6} mol/l) was similar to that induced by cGMP; however, ROS production per mitochondria remained unchanged at least 48 h after the treatment (Fig. 1C). Microscopic analysis with a confocal

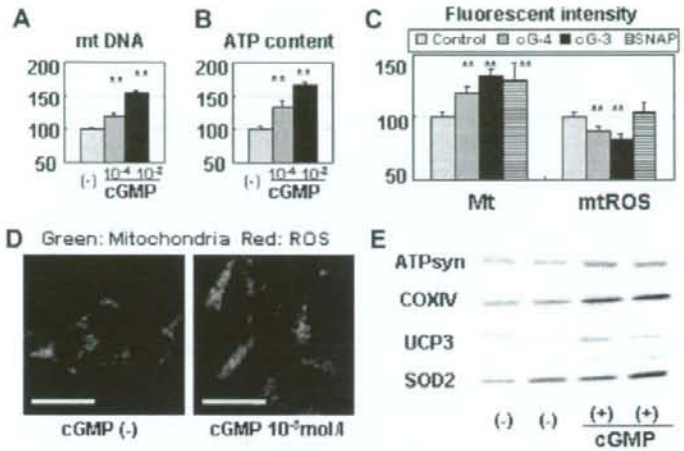

Fig. 1. cGMP promotes mitochondrial biogenesis and ATP synthesis without enhancing ROS production in C2C12 myotubes. C2C12 cells were incubated with or without cGMP (8-*p*CPT-cGMP, at the concentrations indicated: 10^{-4}–10^{-3} mol/l) or SNAP (10^{-6} mol/l) for 48 h. (A) Mitochondrial DNA copy number (mtDNA) estimated by quantitative PCR analysis ($n = 6$ per group). (B) ATP content determined with the chemi-luminescence method ($n = 6$). (C) Mitochondrial density (Mt) and mitochondrial ROS production (mtROS) estimated with the aids of fluorescent probes ($n = 12$). cG-4, treatment with cGMP 10^{-4} mol/l; cG-3, with cGMP 10^{-3} mol/l; SNAP, with SNAP 10^{-6} mol/l. (D) Mitochondrial mass (green) and mitochondrial ROS (red) were visualized by means of fluorescent probes and confocal microscopy after incubation for 48 h with or without cGMP (10^{-3} mol/l). Scale bar: 100 μm. (E) Western blot analysis of protein levels that play significant roles in ATP production and ROS reduction. C2C12 myotubes were treated with or without cGMP (10^{-3} mol/l) for 48 h. ATPsyn, ATP synthase; COXIV, cytochrome *c* oxidase complex IV; UCP3, uncoupling protein 3; SOD 2, superoxide dismutase 2. The values were standardized to those of control. $^{**}p < 0.01$ compared to control. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

microscope confirmed the result by visualization of the C2C12 cells treated with cGMP that had accumulated more mitochondrial (green) and fewer ROS (red) probes (Fig. 1D). Promotion of ATP production by cGMP was further confirmed by Western blot, which showed increases in ATP synthase and COXIV protein levels when cGMP was added to the culture medium (Fig. 1E). In addition, UCP3 and SOD2, which are known to contribute to ROS reduction, also increased as a result of the addition of cGMP (Fig. 1E).

cGMP augments expressions of genes participating in mitochondrial biogenesis, ATP production and ROS reduction

Mitochondrial DNA copy number after the treatment with cGMP (10^{-3} mol/l) started to increase at 6 h and continued to increase at least for 48 h (Fig. 2A). After the addition of cGMP; we examined the time course of expressions of genes that were known to have been involved in mitochondrial biogenesis (PGC1α, PGC1β, NRF1, and mtTFA), oxidative phosphorylation (ATP synthase and COXIV) and ROS reduction (UCP3 and SOD2) at various time points (6, 24, and 48 h), and found that gene expressions of ATP synthase, COXIV, UCP3, and SOD2 had significantly increased after 6 h (Fig. 2B). These increases were maintained for 24 h, but 48 h after the treatment, most of the expression levels had returned to basal values. In subsequent experiments, we estimated the effect of cGMP on the up-stream genes for mitochondrial biogenesis, namely, PGC1α, PGC1β, NRF1, and mtTFA, which positively regulate it. These gene expressions had increased synchronously increased 6 h after the addition of cGMP and returned to basal levels after 48 h, so that the time course was parallel to that for ATP synthase and COXIV (Fig. 2C). We also checked the effect of cGMP on the gene expressions of positive regulator for UCP3 (MyoD and PPARδ) and SOD2 (NFκB and CREB1). These expressions of PPARδ and CREB1 were up-regulated by cGMP and returned to basal level 48 h after the addition (Fig. 2D). However, the gene expressions of MyoD and NFκB showed no significant increase in response to cGMP (Fig. 2D).

cGMP rescues mitochondrial dysfunction caused by high-glucose and high-insulin in C2C12 myotubes

To examine the influence on mitochondrial function of high-glucose and high-insulin exposure that is generally seen in type 2 diabetic patients, we checked the effect of glucose and insulin on mitochondrial density, ROS production and membrane potential in C2C12 cells. High-glucose (450 mg/dl) significantly reduced mitochondria content in comparison with the reduction induced by low-glucose (100 mg/dl), while significant increase was observed in mitochondrial ROS production and higher membrane

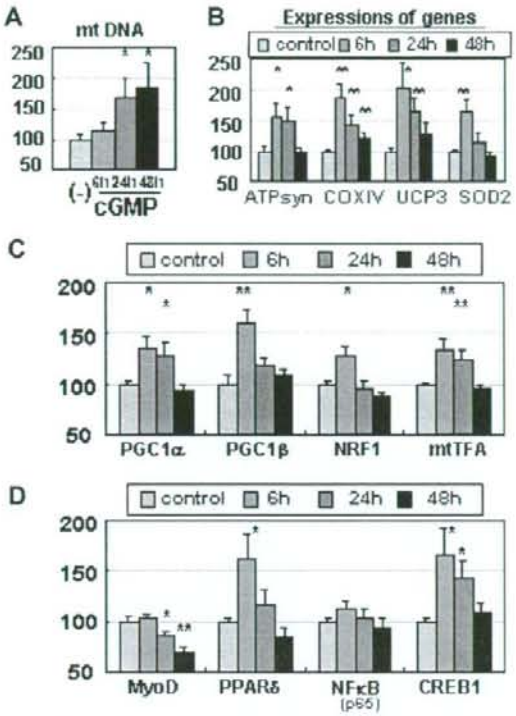

Fig. 2. cGMP augments expressions of genes involved in mitochondrial biogenesis, ATP production and ROS reduction. C2C12 cells were incubated with cGMP (10^{-3} mol/l) and harvested at various time points (6, 24, and 48 h after the addition of cGMP) for gene expression analysis by quantitative PCR. (A) Time course of augmentation of mitochondrial DNA copy number (mtDNA) after addition of cGMP ($n = 6$ per group). (B) Quantitative PCR analysis of gene expressions involved in ATP production and ROS reduction ($n = 8$). (C) Expression of genes involved in mitochondrial biogenesis ($n = 8$). (D) Expression of genes involved in the regulation of UCP3 (MyoD and PPARδ) or SOD2 (NFκB and CREB1) ($n = 8$). PGC1, PPAR gamma coactivator 1; NRF1, nuclear respiratory factor 1; mtTFA, mitochondrial transcription factor A; PPARδ, peroxisome proliferator-activated receptor delta; NFκB, nuclear factor-kappa B (subunit p65); CREB1, CCAAT enhancer-binding protein 1. The gene expressions were normalized by that of the internal control (18S gene). The values were standardized to those of control. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control.

potential (Fig. 3A). On the other hand, treatment with insulin significantly increased mitochondrial ROS production and membrane potential, without causing significant change in mitochondrial mass (Fig. 3B). The combination of high-glucose and high-insulin reduced the mitochondrial mass in association with a significant increase in mitochondrial ROS production and membrane potential (Fig. 3C). Treatment with cGMP significantly increased mitochondrial density which had decreased under conditions of high-glucose and high-insulin condition, accompanied by a reduction in mitochondrial ROS production and membrane potential (Fig. 3C). These effects of cGMP were almost completely nullified by the cGMP antagonist (Fig. 3C). Western blot analysis disclosed that protein levels for ATP synthase, COXIV, UCP3, and SOD2 had concurrently decreased under high-glucose and high-insulin conditions (Fig. 3D). As with mitochondrial density, cGMP reversed the decrease in protein levels and the cGMP antagonist nullified the effect (Fig. 3D). These results were confirmed by the densitometry of Western blots (Fig. 3E).

Discussion

In the study presented here, we found that cGMP increased mitochondrial density in cultured C2C12 myotubes combined with lower membrane potential and ROS production, which was similar to the result that obtained with calorie restriction. cGMP also reversed the mitochondrial dysfunction caused by high-glucose and high-insulin. The quantitative PCR analysis demonstrated that the treatment with cGMP induced expression of genes participating in mitochondrial biogenesis and oxidative phosphorylation, including PGC1α, NRF1, ATP synthase, and COX-IV, accompanied with those contributing to ROS reduction, including UCP3 and SOD2, and their up-stream regulator, PPARδ and CREB1.

A recent study found that calorie restriction induces a large number of efficient mitochondria that could generate enough ATP keeping lower oxygen consumption and ROS production [9]. It was suggested that these functional changes in mitochondrial bioenergetics, which were brought by calorie restriction, are closely related to a reduction in aging and age-related disorders including atherosclerosis, cancer, and diabetes. A characteristic feature of these efficient mitochondria was a reduction in mitochondrial membrane potential, which is the principal parameter regulating generation of mitochondrial ROS [13]. In our study, the treatment of C2C12 myotubes with cGMP produced similar changes in mitochondria, that is, it led to an increase in the number of low-potential mitochondria and thus attained enhancement of ATP content with relative reduction of ROS. On the other hand, a NO-donor SNAP increased mitochondrial density; however, ROS production was not reduced.

We therefore wondered whether cGMP could recover mitochondrial dysfunction caused by high-glucose and high-insulin, a condition which is often seen in diabetic patients. Among a number of mechanisms that have been implicated in functional and morphological changes in mitochondria in diabetic muscle, it has been indicated that a reduction in the expression of key enzymes in mitochondrial biogenesis and oxidative metabolism, including PGC1α, NRF1, ATP synthase, and COXIV, and a subsequent decrease in mitochondrial number and ATP production are essential for the pathogenesis of insulin resistance [14–16]. In addition, a decrease in the antioxidant system, including UCP3 and SOD2, and subsequent augmentation of mitochondrial ROS

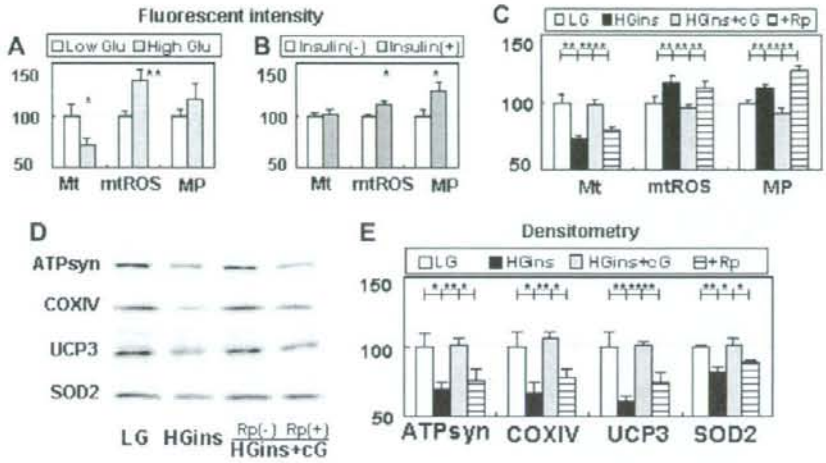

Fig. 3. cGMP rescues mitochondrial dysfunction caused by high-glucose and high-insulin. C2C12 cells were incubated in low-glucose (100 mg/dl), high-glucose (450 mg/dl) or high-glucose plus insulin (100 ng/ml) for 48 h; with or without cGMP (10^{-3} mol/l) or cGMP plus the cGMP-antagonist, Rp-8-*p*CPT-cGMP (10^{-3} mol/l). (A–C) Mitochondrial density (Mt), mitochondrial ROS production (mtROS), and mitochondrial membrane potential (MP) were estimated with the aid of fluorescent probes after incubation for 48 h under the conditions indicated. (A) Low glu, cells were incubated in low-glucose (100 mg/dl) medium; high glu, high-glucose (450 mg/dl) medium ($n = 8$). (B) Insulin (−), cells were incubated in high-glucose medium without insulin; insulin (+), high-glucose medium with insulin ($n = 8$). (C) LG, cells were incubated in low-glucose medium; HGins, high-glucose medium with insulin; HGins+cG, high-glucose medium with insulin plus cGMP (10^{-3} mol/l); Rp, the cGMP antagonist; Rp-8-*p*CPT-cGMP (10^{-3} mol/l) was added to HGins+cG ($n = 12$). (D) Western blot analysis of protein levels of ATP synthase (ATPsyn), cytochrome *c* oxidase complex IV (COXIV), uncoupling protein 3 (UCP3) and superoxide dismutase 2 (SOD2) in the cells incubated under the conditions indicated that were identical to those described for (C). (E) Densitometric analysis of Western blot. The density of the blot for each protein was estimated relative to that for the internal control (beta-actin) ($n = 4$). The values were standardized to those of control. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control.

are considered to be related to insulin resistance [17]. A reduction in the UCP3 content of the skeletal muscle of pre-diabetic and diabetic patients has been reported and this may account for an increase in mitochondrial membrane potential and subsequent ROS production in diabetic muscle because UCP3 reduces the membrane potential and mitochondrial ROS production [18,19]. Moreover, it has been demonstrated that SOD2, which is a representative molecule of the mitochondrial antioxidant system, is also down-regulated in diabetes [20].

In the study reported here, the combination of high-glucose and high-insulin reduced the mitochondrial mass in association with a significant increase in mitochondrial ROS and membrane potential. These changes were compatible with previously reported findings described above. Concomitant reductions in the protein levels of ATP synthase, COXIV, UCP3, and SOD2 indicated that high-glucose and high-insulin condition had a diminishing effect on not only mitochondrial biogenesis but also the ROS reduction system in mitochondria. In addition, we found that the response of mitochondria to glucose and insulin was similar, but showed certain differences. Glucose strongly reduced mitochondrial mass and insulin significantly increased mitochondrial potential, while both stimuli produced an increase in mitochondrial ROS. High-glucose and high-insulin thus reduced the number of mitochondria and increased membrane potential and ROS production. However, the treatment of C2C12 cells with cGMP increased the number of mitochondria with lower membrane potential but without enhancing ROS production. In accordance with these changes, the protein levels of ATP synthase, COXIV, UCP3, and SOD2 were concomitantly recovered by cGMP and this effect was nullified by cGMP antagonist. These findings suggest that cGMP can rescue mitochondrial dysfunction associated with over-nutrition, a result similar to that induced by calorie restriction.

In summary, the findings in our study demonstrate that cGMP can enhance mitochondrial biogenesis and ATP synthesis in cultured C2C12 myotubes while maintaining lower ROS production in a manner similar to that observed in calorie restriction. The treatment that augments cGMP-dependent signal cascades in the skeletal muscle may therefore attenuate the mitochondrial dysfunction that is observed in type 2 diabetes and metabolic syndrome.

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Significance of leptin and high–molecular weight adiponectin in the general population of Japanese male adolescents

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Abstract

Adipokines play crucial roles in obesity-related insulin resistance in adults, but little is known in the general adolescent population. This study was designed to investigate the relationships between adipokines and metabolic parameters, the insulin resistance index, and proinflammatory cytokines in the general population of Japanese male adolescents. We studied 662 Japanese male high school students aged 16 to 17 years and 282 healthy Japanese male adults aged 30 to 61 years who received annual health checkups. High–molecular weight (HMW) adiponectin levels were significantly lower in adolescents (4.18 ± 2.24 μg/mL) than in adults (4.84 ± 3.20 μg/mL), despite body mass index (BMI) being significantly lower in adolescents. The HMW adiponectin levels correlated negatively with BMI and the homeostasis model assessment of insulin resistance index (HOMA-IR) in adults. In adolescents, HMW adiponectin correlated negatively with BMI and waist circumference, but not with HOMA-IR or other metabolic parameters except high-density lipoprotein cholesterol. Leptin levels correlated positively with HOMA-IR, triglycerides, tumor necrosis factor α, interleukin 6, and monocyte chemoattractant protein 1 and negatively with high-density lipoprotein cholesterol even after adjustment for BMI. These findings suggest that serum leptin is a more useful biomarker of fat accumulation–related insulin resistance, inflammation, and metabolic abnormalities than HMW adiponectin in the general population of male adolescents. The inverse correlation between adiponectin and insulin resistance may manifest in the later phase of obesity development.

1. Introduction

Obesity is the most common cause of metabolic syndrome, type 2 diabetes mellitus, and long-term vascular complications. In recent years, the prevalence of obesity has increased dramatically and has become a major health problem worldwide. Accumulating evidence has revealed that adipose tissue is not only an energy storage organ, but also a highly active endocrine organ [1]. Moreover, it is well established that adipokines, a variety of biologically active peptides secreted from adipose tissue, play crucial roles in the pathophysiology of obesity-related insulin resistance and complications.

Among the many known adipokines, adiponectin and leptin have attracted considerable attention. Adiponectin was shown to be an antidiabetic, anti-inflammatory, and antiatherogenic cytokine. We [2] and other groups [3,4] have reported that serum adiponectin levels correlate inversely with adiposity variables and insulin resistance. Prospective studies have shown that low adiponectin levels predict the progression to type 2 diabetes mellitus [5,6] and cardiovascular diseases [7]. In addition, adiponectin exists in a variety of multimer complexes in circulating blood, that is, low–molecular weight trimer, middle–molecular weight hexamer, and high–molecular weight (HMW) 12- to 18-mer. Recent studies have revealed that the HMW form is the active form of adiponectin and the useful biomarker for insulin resistance [6,8].

Leptin is elevated in obesity and controls food intake and energy expenditure. High leptin levels are usually accompanied by leptin resistance, and some epidemiological studies have demonstrated high leptin levels to be associated

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