ciency *in vivo*. The D442G mutation was first identified in two probands, although heterozygous for this mutation, which had a 3-fold increase in HDL cholesterol and markedly decreased plasma CETP activity and mass (17). These data suggested that the D442G mutation might have some dominant effects on CETP and HDL *in vivo*. This was confirmed *in vitro* by the expression of wild type and mutant proteins in COS-7 cells. Cellular expression of mutant cDNA results in secretion at only 30% of wild type CETP activity. Moreover, co-expression of wild type and mutant cDNA leads to inhibition of the secretion of wild type CETP and activity. However, in the latter studies, the D442G heterozygous subjects had relatively high CETP activity and mass and a wide range of HDL cholesterol levels (18). Moreover, the specific activity of plasma CETP in the homozygous subjects with the D442G mutation was similar to that of the wild type (21). Therefore, as the authors explained in the discussion, other unknown factors contributed to the increased HDL in the two probands. The R282C mutation was found in only one compound heterozygote with the D442G mutation, whose plasma CETP levels were significantly lower when compared with those in D442G heterozygous sub-

jects (21). The mutant CETP showed a marked reduction in the secretion of CETP protein into media from transfected COS-7 cells (39% of wild type). Arginine at codon 282, a positively charged residue, is thought to be in the vicinity of the C-terminal pocket and interacts with the phosphate group of a bound phospholipid (31). Arginine at codon 282 is likely to be structurally rather than functionally important because the specific activity of the R282C mutant was similar to that of the wild type in the transfection experiment.

Association of plasma CETP levels and lipid profiles with CETP mutations

Table 2 shows the lipid profiles and plasma CETP levels of the HALP subjects with the common CETP gene mutations (HDL cholesterol $\geq$ 2.07 mmol/l = 80 mg/dl) (20, 21). The plasma CETP concentrations determined by sandwich ELISA in 211 normolipidemic male subjects and female subjects without the known CETP gene mutations were 1.93 $\pm$ 0.50 μg/ml (mean $\pm$ SD) and 2.36 $\pm$ 0.66 μg/ml, respectively. The plasma CETP mass and activity were not detected in the homozygous subjects with the Int14 + 1 G → A mutation. The subjects with the

Table 2. Lipid profiles and plasma CETP levels in HALP subjects with the common mutations of the CETP gene.

Mutation	Int14 + 1 G → A HE (n = 58)	Int14 + 1 G → A HO (n = 29)	D442G HE (n = 84)	D442G HO (n = 12)	Int14 + 1 G → A HE / D442G HE (n = 24)	Controls (n = 90)
Sex	M/F 22/36	M/F 17/12	M/F 25/59	M/F 3/9	M/F 10/14	M/F 53/37
Age, years	41 ± 15*	37 ± 20	57 ± 11**	55 ± 16**	52 ± 10**	32 ± 6
Total cholesterol, mmol/l	5.92 ± 1.01**	7.01 ± 1.42**	6.15 ± 1.16**	5.74 ± 0.57**	5.92 ± 1.03**	4.42 ± 0.59
HDL cholesterol, mmol/l	2.72 ± 0.52**	4.03 ± 0.83**	2.51 ± 0.36**	2.74 ± 0.49**	3.21 ± 0.80**	1.55 ± 0.34
Triglyceride, mmol/l	1.01 ± 0.60	1.29 ± 0.66*	0.89 ± 0.51	0.82 ± 0.37	0.94 ± 0.45	1.03 ± 0.67
LDL cholesterol, mmol/l	2.74 ± 1.06	2.35 ± 0.78	3.23 ± 1.14**	2.66 ± 0.70	2.56 ± 0.98	2.48 ± 0.62
Apo A-I, mg/dl	195 ± 32**	224 ± 56**	190 ± 37**	205 ± 26**	206 ± 52**	123 ± 16
Apo A-II, mg/dl	36.6 ± 8.2	40.9 ± 10.3**	35.4 ± 7.3	38.0 ± 9.8*	40.7 ± 10.4**	33.6 ± 5.1
Apo B, mg/dl	85 ± 22	77 ± 29	93 ± 27**	77 ± 18	81 ± 25	79 ± 17
Apo C-II, mg/dl	5.0 ± 1.4**	7.5 ± 2.3**	4.6 ± 1.3**	4.5 ± 1.3*	5.4 ± 1.6**	3.7 ± 1.2
Apo C-III, mg/dl	12.2 ± 3.4**	22.2 ± 10.0**	12.0 ± 3.2**	11.5 ± 2.1*	15.0 ± 5.8**	8.9 ± 4.0
Apo E, mg/dl	5.3 ± 1.6*	9.9 ± 4.3**	5.9 ± 2.2**	6.6 ± 2.2**	7.5 ± 2.9**	4.6 ± 0.9
CETP mass, μg/ml	1.4 ± 0.5**	0.0 ± 0.0**	2.0 ± 0.6**	1.2 ± 0.3**	0.6 ± 0.1**	2.4 ± 0.6
CETP activity, %	61 ± 20**	2 ± 3**	86 ± 19**	61 ± 8**	29 ± 3**	100 ± 19

HE indicates heterozygote, HO: homozygote, Int14 + 1 G → A HE/D442G HE, compound heterozygote for Int14 + 1 G → A and D442G. Values are expressed as mean ± SD. * p <0.01, ** p <0.001 compared with control subjects.

heterozygous Int14 + 1 G → A mutation had about half the CETP mass of the normal controls. On the other hand, the CETP concentration in the subjects with the homozygous D442G mutation was 0.72 ± 0.16 µg/ml and 1.23 ± 0.27 µg/ml, for the males and females, respectively, but in the heterozygous subjects was 1.56 ± 0.42 µg/ml and 2.09 ± 0.56 µg/ml, for the males and females, respectively, showing only a mild change (a 17% decrease). The specific activity of plasma CETP in the subjects with the D442G mutation was similar to that in the normolipidemic controls without CETP gene mutations. In each genotype with the common CETP mutations except for the homozygous Int14 + 1 G → A mutation, the mean plasma CETP levels in the male subjects were significantly lower than those of the female subjects.

Among the completely CETP-deficient subjects, the homozygous subjects with the Int14 + 1 G → A mutation had markedly elevated levels of HDL cholesterol (3–6-fold increase), while the heterozygous subjects had moderately increased HDL cholesterol levels (12, 13, 15, 16, 18, 20, 21, 35). The TG levels of the subjects were slightly increased compared with those of the controls. The concentrations of apo A-I, C-II, C-III and E in CETP deficiency were also markedly increased, while the apo B level was normal or slightly decreased. These increases in apo C-III and E are derived from the increment of HDL particles containing apo C-III and E. In subjects with other genotypes of common CETP gene mutations, the plasma HDL cholesterol levels and the concentrations of apo A-I, A-II, C-II, C-III and E were significantly increased, although the difference was not so marked compared with that for the homozygous Int14 + 1 G → A mutation. Among the subjects with two common CETP gene mutations, CETP activity and mass concentration were negatively correlated with plasma HDL cholesterol, apo A-I, A-II, C-II, C-III and E, and positively with plasma LDL cholesterol.

Effect of CETP gene polymorphisms on plasma CETP mass and HDL cholesterol in the heterozygous subjects with common CETP gene mutations

Several common polymorphisms have been reported in the human CETP gene including – 629 A/C in promoter region (36), TaqIB polymorphism in intron 1 (37), MspI in intron 8 (37) and I405V in exon 14 (11). Among these polymorphisms, TaqIB polymorphism has been intensely studied, although the polymorphism is not expected to directly influence CETP transcriptional regulation or RNA splicing but rather to affect CETP gene expression, due to its location in intron 1 (+ 279 G/A). In normolipidemic subjects, the B2 allele (absence of the TaqIB restriction site) at this polymorphism site has been associated with decreased CETP levels and increased HDL cholesterol levels (38–42), thus resembling a mild phenotype of CETP

deficiency. Moreover, it has been shown that the TaqIB-B2 allele had a dose-dependent association with CHD risk in many studies including the Framingham Offspring Study (43), although this association may be population-specific and highly influenced by environmental factors, such as alcohol consumption and smoking.

Our results suggested that the TaqIB-B2 allele is associated with both lower plasma CETP concentrations and higher HDL cholesterol levels in subjects not only without CETP gene mutations, but also with heterozygous CETP deficiency; this association was also observed in the heterozygous subjects with the Int14 + 1 G → A mutation, but not significantly (Table 3). The linkage disequilibrium that we have observed among 4 different polymorphisms and 2 common mutations in the Japanese, the CETP gene splitting into a 5' haplotype block and a 3' haplotype block, is similar to that observed previously in Caucasians (44, 45). Table 4 summarizes the haplotype based on 4 polymorphisms in homozygous subjects with Int14 + 1 G → A or D442G mutations. The 4 polymorphisms generated 8 main haplotypes in normolipidemic subjects without CETP gene mutations. On the other hand, in the homozygous subjects with the Int14 + 1 G → A mutation, all had 1 haplotype, and the homozygous subjects for D442G had 2 main haplotypes. These results seem to be supported by studies investigating the association between common CETP gene mutations and polymorphism(s) (15, 47, 48). Thus, we determined the haplotypes of the normal allele in the heterozygous subjects with common CETP gene mutations when haplotype analysis was performed. Moreover, there are some haplotypes in the D442G mutation of the CETP gene, and this mutation is frequent in West Asian populations. It is suggested that the D442G mutation originated earlier than the Int14 + 1 G → A mutation.

Recently, Lu et al. investigated in detail the association between promoter polymorphisms and CETP/HDL cholesterol in a sample of 357 elderly Japanese men (49). Their haplotype analyses indicated that the – 2,505 C/A polymorphism (50) and 1,946 VNTR [gaaa]n (51) might explain the variations in the CETP concentrations, and may independently determine the variations in HDL cholesterol levels, whereas the – 629 A/C and TaqIB polymorphisms were not instrumental in determining CETP concentrations or HDL cholesterol levels. The importance of 1,946 VNTR for plasma HDL cholesterol was also described by Thompson et al (45). 1,946 VNTR seems to be significantly associated with CETP/HDL cholesterol, even in heterozygous subjects with common CETP gene mutations.

Lipoprotein abnormalities in homozygous CETP deficiency

In 1984, we described two patients with marked HALP who suffered from angina pectoris and premature cor-

Table 3. Relationship of the CETP *Taq*IB polymorphism to plasma CETP mass and HDL cholesterol levels in Japanese subjects who had the common CETP mutations.

(A) Normolipidemic subjects without CETP gene mutations

		Male			Female	
	n	CETP mass (μg/ml)	HDL-C (mmol/l)	n	CETP mass (μg/ml)	HDL-C (mmol/l)
B1B1	42	2.05 ± 0.50	1.06 ± 0.28	25	2.61 ± 0.61	1.26 ± 0.36
B1B2	77	1.95 ± 0.49	1.19 ± 0.30	26	2.30 ± 0.58	1.29 ± 0.32
B2B2	28	1.72 ± 0.45	1.23 ± 0.35	13	1.95 ± 0.72	1.52 ± 0.45
p		0.023	0.035		0.013	0.133

(B) Heterozygous subjects with Int14 +1 G → A mutation

		Male			Female	
	n	CETP mass (μg/ml)	HDL-C (mmol/l)	n	CETP mass (μg/ml)	HDL-C (mmol/l)
B1B1	13	1.22 ± 0.46	2.20 ± 0.70	17	1.66 ± 0.41	2.64 ± 0.65
B1B2	13	1.03 ± 0.30	2.48 ± 0.83	22	1.24 ± 0.27	2.61 ± 0.41
B2B2	0	–	–	0	–	–
p		0.159	0.360		0.003	0.893

(C) Heterozygous subjects with D442G mutation

		Male			Female	
	n	CETP mass (μg/ml)	HDL-C (mmol/l)	n	CETP mass (μg/ml)	HDL-C (mmol/l)
B1B1	23	1.85 ± 0.47	1.53 ± 0.52	36	2.08 ± 0.45	1.91 ± 0.49
B1B2	35	1.59 ± 0.44	1.71 ± 0.65	48	2.06 ± 0.65	2.02 ± 0.54
B2B2	13	1.36 ± 0.37	2.09 ± 0.41	20	1.66 ± 0.50	2.40 ± 0.54
p		0.037	0.024		0.044	0.004

Table 4. Reconstructed haplotype frequencies for – 629 A/C, *Taq*IB, Int7 + 8 C/T and I405V polymorphisms of the CETP gene.

	Haplotype				Frequency		
	– 629 A/C	*Taq*IB	Int7 + 8 C/T	I405V (A/G)	Int14 + 1 G → A HO (n = 38)	D442G HO (n = 17)	Controls (n = 227)
1	C	B1	C	A	0.000	0.349	0.315
2	A	B2	T	G	0.000	0.000	0.290
3	C	B1	C	G	0.000	0.000	0.130
4	A	B2	C	G	0.000	0.000	0.096
5	A	B1	C	A	1.000	0.033	0.064
6	A	B1	C	G	0.000	0.000	0.049
7	A	B2	T	A	0.000	0.616	0.026
8	A	B2	C	A	0.000	0.000	0.024
9	C	B1	T	A	0.000	0.000	0.003
10	C	B2	C	A	0.000	0.000	0.002
11	C	B2	T	A	0.000	0.001	0.000

HO indicates homozygote. Control subjects had no known mutations of the CETP gene. Haplotypes were reconstructed using the PHASE program (version 1.0) (Ref. 46).

neal opacity. We hypothesized that marked HALP is not always beneficial, but can be athergenic (52). We extensively analyzed lipoproteins from CETP-deficient subjects, and demonstrated that LDL and HDL were abnormal biochemically and biologically. Fig. 2 summarizes the characteristics of plasma lipids and lipoproteins in CETP-deficient subjects homozygous for the Int14 + 1 G → A mutation. The subjects showed extremely high HDL cholesterol and relatively low LDL cholesterol levels. The increase in HDL cholesterol was attributed to the increase in HDL_2 cholesterol, while HDL_3 cholesterol was not increased. Large and apolipoprotein E-rich HDL particles accumulated in the plasma of the CETP-deficient subjects (53). HDL particles in CETP deficiency, which are more enriched with CE but poorer in TG than control HDL, had a reduced capacity for inhibiting acetyl LDL-induced accumulation of CE in mouse peritoneal macrophages, and less capacity than normal HDL_2 for cholesterol efflux from macrophages preloaded with CE by acetylated LDL (54). This suggests that large CE-rich HDL_2 particles from CETP-deficient subjects do not have an anti-atherogenic function. Two types of HDL particles exist in human plasma: HDL particles containing only apo A-I (LpA-I) and those containing both apo A-I and A-II (LpA-I/A-II). The LpA-I level is low in subjects with CETP deficiency, resulting in a significant decrease in the efflux and LCAT-mediated esterification of cholesterol (55). Furthermore, Oliveira et al. demonstrated that the esterification of cholesterol was reduced in subjects with both heterozygous and homozygous CETP deficiency (56).

A stable isotope study demonstrated that the fractional catabolic rates (FCRs) of apo A-I and A-II were significantly reduced in CETP-deficient subjects, whereas the rates of apo A-I and apo A-II production were normal (57). Thus, homozygous CETP deficiency causes a marked delay in the catabolism of apo A-I and A-II. In contrast, the FCRs of apo B were significantly increased in the subjects, while the production rate of apo B was slightly decreased (58). However, the LDL particles from CETP-deficient subjects are small and very heterogeneous (polydisperse LDL) and apo B-rich (59, 60). These particles have a reduced affinity to the LDL receptor of fibroblasts (61), which may be susceptible to in vivo oxidation (Fig. 2). In fact, plasma oxidized-LDL is increased in CETP-deficient subjects (62). Taken together, homozygous CETP deficiency causes functional abnormalities as well as compositional changes in both HDL and LDL. Further analysis is essential to investigate whether heterozygous CETP-deficient subjects have lipoprotein abnormalities similar to those of homozygous CETP-deficient subjects, and to determine the other HDL functions, such as anti-oxidant, anti-thrombotic and anti-inflammatory properties.

Relationship between CETP deficiency and atherosclerosis

Several epidemiological studies in the Japanese have investigated the atherogenicity of CETP deficiency. We found a unique area in the northern part of Japan called Omagari (Akita Prefecture), where a null type mutation, Int14 + 1 G → A, of the CETP gene was present in high frequency (63). The prevalence of marked HALP and the Int14 + 1 G → A mutation were 10-fold and 20-fold higher in this area than in other areas of Japan, respectively. This discovery provided us with an opportunity to ascertain whether or not marked HALP caused by CETP gene mutation is associated with longevity. The prevalence of both the Int14 + 1 G → A mutation and marked HALP declined with aging. Furthermore, we examined the relationship between HDL cholesterol levels and ischemic ECG changes, showing that the negative correlations which have been demonstrated by numerous epidemiologic studies were observed only in the range of HDL
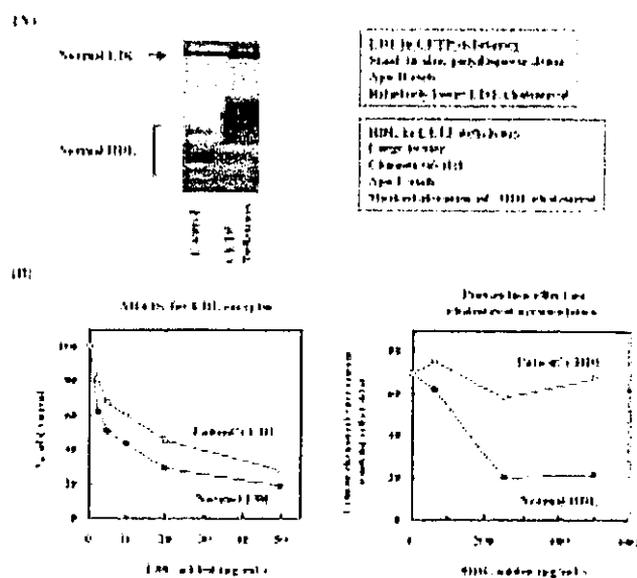

Fig. 2. Lipoprotein abnormalities in CETP deficient subjects homozygous for Int14 + 1 G → A, null mutation. (A) Biochemical characteristics of lipoproteins in CETP deficiency. Left panel: Plasma lipoproteins (d < 1.210 g/ml) were run on 4–30% of nondenaturing polyacrylamide gradient gel electrophoresis. Right panels: Summary of biochemical characteristics. (B) Atherogenic properties of LDL (Left panel) as well as HDL (Right panel) in CETP deficiency. Left panel: The affinity of LDL for LDL receptor was examined. LDL from CETP deficiency had reduced binding ability to LDL receptor on human skin fibroblasts. Right panel: Preventive effect of HDL on the accumulation of cholesterol in mouse peritoneal macrophages was examined. HDL from CETP deficiency had no preventive effect.

cholesterol < 1.81 mmol/l = 70 mg/dl. In the range of HDL cholesterol ≥ 1.81 mmol/l, the incidence of ischemia was, rather, increased (Fig. 3). These results show that CETP deficiency is not associated with longevity, but instead may be atherogenic. Recently, we performed the quantification of atherosclerotic areas in CETP deficiency with HDL cholesterol ≥ 2.59 mmol/l. Subjects with any known major risk factors including hypertension, diabetes mellitus, high LDL cholesterol, smoking and obesity were excluded from this study. Parameters tested were plaque score (PS) in the carotid artery and pulse wave velocity (PWV) from the aortic root to the femoral artery. Both PS and PWV were higher in subjects with CETP deficiency, compared with those of age- and sex-matched controls, suggesting that CETP deficiency is substantially atherogenic.

Two other epidemiological studies were reported in populations with the D442G missense mutation. One of them was performed in Japanese-American men living in Hawaii (Honolulu Heart Program) (64), the other in the Japanese population living in Kochi Prefecture, Japan (65). In 3,496 Japanese-American men living in Hawaii, the incidence of CHD was higher in subjects with the D442G mutation than in those without mutations, although the difference was significant only in subjects whose HDL cholesterol level was between 1.06 and 1.55 mmol/l (40–60 mg/dl). However, men with increased HDL levels (>1.55 mmol/l) in this population had a low risk of CHD, irrespective of the presence or absence of the CETP

gene mutation, appearing to be protected against atherosclerosis. The other study in the Kochi Prefecture demonstrated results similar to those in the Japanese-American men living in Hawaii.

The above data show that CETP deficiency is atherogenic, but that atherogenicity in CETP deficiency is not always correlated with the level of plasma HDL cholesterol. Thus, further analysis is essential for the complete understanding of the atherogenicity of CETP deficiency.

Conclusion

CETP deficiency is the most important and common cause of HALP in the Japanese. Further analysis is essential for the complete understanding of the atherogenicity of human CETP deficiency, especially the heterozygous deficiency.

References

(1) Gordon T, Castelli WP, Hjortland MC, Kannel KB, and Dawber TR: High density lipoproteins as a protective factor against coronary heart disease. Am J Med, 62: 707–714, 1977

(2) Breslow JL: Familial disorders of high-density lipoprotein metabolism. In: The Metabolic and Molecular Bases of Inherited Disease. 7th Ed, ed by Scriver CR, Beaudet AL, Sly WS, and Valle V, pp 2031–2052, McGraw-Hill, New York, 1995

(3) Tall AR: An overview of reverse cholesterol transport. Eur Heart J, SupplA: A31–A35, 1998

(4) Bruce C, Chouinard RA, and Tall AR: Plasma lipid transfer protein, high-density lipoproteins, and reverse cholesterol transport. Annu Rev Nutr, 18: 297–330, 1998

(5) Tall AR: Plasma lipid transfer protein. Annu Rev Biochem, 64: 235–257, 1995

(6) Yamashita S, Sakai N, Hirano K, Arai T, Ishigami M, Maruyama T, and Matsuzawa Y: Molecular genetics of plasma cholesteryl ester transfer protein. Curr Opin Lipidol, 8: 101–110, 1997

(7) Yamashita S, Hirano K, Sakai N, and Matsuzawa Y: Molecular biology and pathophysiological aspects of plasma cholesteryl ester transfer protein. Biochim Biophys Acta, 1529: 257–275, 2000

(8) Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, and Krieger M: Identified of scavenger receptor SR-BI as a high density lipoprotein receptor. Science, 271: 518–520, 1996

(9) Drayna D, Jarnagin AS, McLean J, Henzel W, Kohr W, Fielding CJ, and Lawn R: Cloning and sequenc-
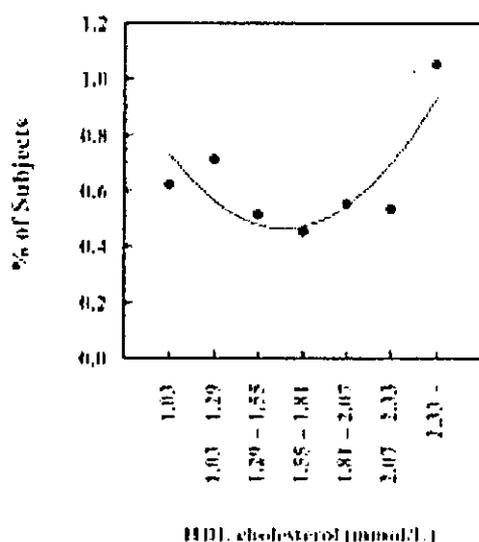

Fig. 3. Relationship between plasma high density lipoprotein (HDL) cholesterol levels and the incidence of ischemic ECG changes in Omagari (modified from Ref. 61). •, denotes the incidence of an ST-segment depression of > 1 mm in the subjects in each HDL cholesterol range.

ing of the human cholesteryl ester transfer protein cDNA. Nature, 327: 632–634, 1987

(10) Lagrost L: Regulation of cholesteryl ester transfer protein (CETP) activity; review of in vitro and in vivo studies. Biochim Biophys Acta, 1215: 209–236, 1994

(11) Agellon L, Quinet E, Gillette T, Drayna D, Brown M, and Tall AR: Organization of the human cholesteryl ester transfer protein gene. Biochemistry, 29: 1372–1376, 1990

(12) Kurasawa T, Yokoyama S, Miyake Y, Yamamura T, and Yamamoto A: Deficiency of serum cholesteryl ester transfer between high and low density lipoproteins in human serum and a case with decreased transfer rate in association with hyperalphalipoproteinemia. J Biochem, 98: 1499–1508, 1985

(13) Koizumi J, Mabuchi H, Yoshimura A, Michishita I, Takeda M, Itoh H, Sakai Y, Sakai T, Ueda K, and Takeda R: Deficiency of serum cholesteryl-ester transfer activity in patients with familial hyper-alphalipoproteinemia. Atherosclerosis, 58: 175–186, 1985

(14) Brown ML, Inazu A, Hesler CB, Agellon LB, Mann C, Whitlock ME, Marcel YL, Milne RW, Koizumi J, Mabuchi H, Takeda R, and Tall AR: Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoproteins. Nature, 342: 448–451, 1989

(15) Inazu A, Brown ML, Hesler CB, Agellon LB, Koizumi J, Takata K, Maruhama Y, Mabuchi H, and Tall AR: Increased high-density lipoprotein levels caused by a common cholesteryl-ester transfer protein gene mutation. N Engl J Med, 323: 1234–1238, 1990

(16) Yamashita S, Hui, DY, Sprecher DL, Matsuzawa Y, Sakai N, Tarui S, Kaplan D, Wetterau JR, and Harmony JA: Total deficiency of plasma cholesteryl ester transfer protein in subjects homozygous and heterozygous for the intron 14 splicing defect. Biochem Biophys Res Commun, 170: 1346–1351, 1990

(17) Takahashi K, Jiang XC, Sakai N, Yamashita S, Hirano K, Bujo H, Yamazaki H, Kusunoki J, Miura T, Kussie P, Matsuzawa Y, Saito Y, and Tall AR: A missense mutation in the cholesteryl ester transfer protein gene with possible dominant effects on plasma high density lipoproteins. J Clin Invest, 92: 2060–2064, 1993

(18) Inazu A, Jiang XC, Haraki T, Yagi K, Kamon N, Koizumi J, Mabuchi H, Takeda R, Takata K, Moriyama Y, Doi M, and Tall AR: Genetic cholesteryl ester transfer protein deficiency caused by two prevalent mutations as a major determinant of increased levels of high density lipoprotein cholesterol. J Clin Invest, 94: 1872–1882, 1994

(19) Sakai N, Yamashita S, Hirano K, Menju M, Arai T, Kobayashi K, Ishigami M, Yoshida Y, Hoshino T, Nakajima N, Kameda-Takemura K, and Matsuzawa Y: Frequency of exon 15 missense mutation (442D:G) in cholesteryl ester transfer protein gene in hyperalphalipoproteinemic Japanese subjects. Atherosclerosis, 114: 139–145, 1995

(20) Nagano M, Yamashita S, Hirano K, Kujiraoka T, Ito M, Sagehashi Y, Hattori H, Nakajima N, Maruyama T, Sakai N, Egashira T, and Matsuzawa Y: Point mutation (– 69 G → A) in the promoter region of cholesteryl ester transfer protein gene in Japanese hyperalphalipoproteinemic subjects. Arterioscler Thromb Vasc Biol, 21: 985–990, 2001

(21) Nagano M, Yamashita S, Hirano K, Ito M, Maruyama T, Ishihara M, Sagehashi Y, Oka T, Kujiraoka T, Hattori H, Nakajima N, Egashira T, Kondo M, Sakai N, and Matsuzawa Y: Two novel missense mutations in the CETP gene in Japanese hyper-alpha-lipoproteinemic subjects: high-throughput assay by Invader* assay. J Lipid Res, 43: 1011–1018, 2002

(22) Arai T, Yamashita S, Sakai N, Hirano K, Okada S, Ishigami M, Maruyama T, Yamane M, Kobayashi H, Nozaki S, Funahashi T, Kameda-Takemura K, Nakajima N, and Matsuzawa Y: A novel nonsense mutation (G181X) in the human cholesteryl ester transfer protein gene in Japanese hyperalphalipoproteinemic subjects. J Lipid Res, 37: 2145–2154, 1996

(23) Takata K, Mishima Y, Shirahata H, Umemoto S, Toyota Y, and Hiraoka A: A novel nonsense mutation of cholesteryl ester transfer protein gene and the clinical characterization of Japanese primary hyperalphalipoproteinemias (Abstract). Atherosclerosis, 4: 301, 2003

(24) Gotoda T, Kinoshita M, Shimano H, Harada K, Shimada M, Ohsuga J, Teramoto T, Yazaki Y, and Yamada N: Cholesteryl ester transfer protein deficiency caused by a nonsense mutation detected in the patient's macrophage mRNA. Biochem Biophys Res Commun, 194: 519–524, 1993

(25) Sakai N, Santamarina-Fojo S, Yamashita S, Matsuzawa Y, and Brewer HB Jr: Exon skipping caused by intron 10 splicing donor site mutation in cholesteryl ester transfer protein gene results in abnormal downstream splice site selection. J Lipid Res, 37: 2065–2073, 1996

(26) Hirano K, Yamashita S, Funahashi T, Sakai N, Menju M, Ishigami M, Hiraoka H, Kameda-Takemura K, Tokunaga K, Hoshino T, Kumasaka K, and Matsuzawa Y: Frequency of intron 14 splicing defect of cholesteryl ester transfer protein gene in the Japanese general population; relation between the mutation and hyperalphalipoproteinemia. Atherosclerosis, 100: 85–90, 1993

(27) Inazu A, Koizumi J, Haraki T, Yagi K, Wakasugi T, Takegoshi T, Mabuchi H, and Takeda R: Rapid detection and prevalence of cholesteryl ester transfer protein deficiency caused by an intron 14 splicing defect in hyperalpha-lipoproteinemia. Hum Genet, 91: 13–16, 1993

(28) Fors L, Lieder KW, Vavra SH, Kwiatkowski RW: Large-scale SNP scoring from unamplified genomic DNA. Pharmacogenomics, 1: 219–229, 2000

(29) Gotoda T, Kinoshita M, Ishibashi S, Inaba T, Harada K, Shimada M, Osuga J, Teramoto T, Yazaki Y, and Yamada N: Skipping of exon 14 and possible instability of both the mRNA and the resultant truncated protein underlie a common cholesteryl ester transfer protein deficiency in Japan. Arterioscler Thromb Vasc Biol 17: 1376–1381, 1997

(30) Beamer LJ, Carroll SF, and Eisenberg D: Crystal structure of human BPI and two bound phospholipids at 2.4 angstrom resolution. Science, 276: 1861–1864, 1997

(31) Bruce C, Beamer LJ, and Tall AR: The implications of the structure of the bactericidal/permeability-increasing protein on the lipid-transfer function of the cholesteryl ester transfer protein. Curr Opin Struct Biol, 8: 426–434, 1998

(32) Desrumaux C, Labeur C, Verhee A, Tavernier J, Vandekerckhove J, Rosseneu M, and Peelman F: A hydrophobic cluster at the surface of the human plasma phospholipids transfer protein is critical for activity on high density lipoproteins. J Biol Chem, 276: 5908–5915, 2000

(33) Oliveira HCF, Chouinard RA, Agellon LB, Bruce C, Ma L, Walsh A, Breslow JL, and Tall AR: Human cholesteryl ester transfer protein gene proximal promoter contains dietary cholesterol positive responsive elements and mediates expression in small intestine and periphery while predominant liver and spleen expression is controlled by 5'-distal sequences. J Biol Chem, 271: 31831–31838, 1996

(34) Gaudet F and Ginsburg GS: Transcriptional regulation of the cholesteryl ester transfer protein gene by the orphan nuclear hormone receptor apolipoprotein AI regulatory protein-1. J Biol Chem, 270: 29916–29922, 1995

(35) Yamashita S, Hui DY, Wetterau JR, Sprecher DL, Harmony JA, Sakai N, Matsuzawa Y, and Tarui S: Characterization of plasma lipoproteins in patients heterozygous for human plasma cholesteryl ester transfer protein (CETP) deficiency: Plasma CETP regulates high-density lipoprotein concentration and composition. Metabolism, 40: 756–763, 1991

(36) Dachet C, Poirier O, Cambien F, Chapman J, and Rouis M: New functional promoter polymorphism, CETP/- 629, in cholesteryl ester transfer protein (CETP) gene related to CETP mass and high density lipoprotein cholesterol levels; role of Sp1/Sp3 in transcriptional regulation. Arterioscler Thromb Vasc Biol, 20: 507–515, 2000

(37) Drayna D and Lawn R: Multiple RFLPs at the human cholesteryl ester transfer protein (CETP) gene. Nucleic Acids Res, 15: 4698, 1987

(38) Kondo I, Berg K, Drayna DT, and Lawn RM: DNA polymorphism at the locus for human cholesteryl ester transfer protein (CETP) is associated with high density lipoprotein cholesterol and apolipoprotein levels. Clin Genet, 35: 49–56, 1989

(39) Freeman DJ, Griffin BA, Holmes AP, Lindsay GM, Gaffney D, Packard CJ, and Shepherd J: Regulation of plasma HDL cholesterol and subfraction distribution by genetic and environmental factors: associations between the TaqIB RFLP in the CETP gene and smoking and obesity. Arterioscler Thromb, 14: 336–344, 1994

(40) Hannuksela ML, Liinamaa MJ, Kesaniemi YA, and Savolainen MJ: Relation of polymorphisms in the cholesteryl ester transfer protein gene to transfer protein activity and plasma lipoprotein levels in alcohol drinkers. Atherosclerosis, 110: 35–44, 1994

(41) Kuivenhoven JA, de Knijff P, Boer JMA, Smalheer HA, Botma GJ, Seidell JC, Kastelein JJ, and Pritchard PH: Heterogeneity at the CETP gene locus: influence on plasma CETP concentrations and HDL cholesterol levels. Arterioscler Thromb Vasc Biol, 17: 560–568, 1997

(42) Ikewaki K, Mabuchi H, Teramoto T, Yamada N, Oikawa S, Sasaki J, Takata K, and Saito Y; Japan CETP Study Group: Association of cholesteryl ester transfer protein activity and TaqIB polymorphism with lipoprotein variations in Japanese subjects. Metabolism, 52: 1564–1570, 2003

(43) Ordovas JM, Cupples LA, Corella D, Otvos JD, Osgood D, Martinez A, Lahoz C, Coltell O, Wilson PW, and Schaefer EJ: Association of cholesteryl ester transfer protein-TaqIB polymorphism with variations in lipoprotein subclasses and coronary heart disease risk: the Framingham study. Arterioscler Thromb Vasc Biol, 20: 1323–1329, 2000

(44) Corbex M, Poirier O, Fumeron F, Betouille D, Evans A, Ruidavets JB, Arveiler D, Luc G, Tiret L, and Cambien F: Extensive association analysis between the CETP gene and coronary heart disease phenotypes reveals putative functional polymorphisms and gene-environment interaction. Genet Epidemiol, 19: 64–80, 2000

(45) Thompson JF, Lira ME, Durham LK, Clark RW, Bamberger MJ, and Milos PM: Polymorphisms in the CETP gene and association with CETP mass and HDL levels. Atherosclerosis, 167: 195–204, 2003

(46) Stephens M, Smith NJ, and Donnelly P: A new sta-

tistical method for haplotype reconstruction from population data. Am J Hum Genet, 68: 978–989, 2001

(47) Bruce C, Sharp DS, and Tall AR: Relationship of HDL and coronary heart disease to a common amino acid polymorphism in the cholesteryl ester transfer protein in men with and without hyper-triglyceridemia. J Lipid Res, 39: 1071–1078, 1998

(48) Hsu LA, Ko YL, Hsu KH, Ko YH, and Lee YS: Genetic variations in the cholesteryl ester transfer protein gene and high density lipoprotein cholesterol levels in Taiwanese Chinese. Hum Genet, 110: 57–63, 2002

(49) Lu H, Inazu A, Moriyama Y, Higashikata T, Kawashiri MA, Yu W, Huang Z, Okamura T, and Mabuchi H: Haplotype analyses of cholesteryl ester transfer protein gene promoter: a clue to an unsolved mystery of TaqIB polymorphism. J Mol Med, 81: 246–255, 2003

(50) Klerkx AH, Tanck MW, Kastelein JJ, Molhuizen HO, Jukema JW, Zwinderman AH, and Kuivenhoven JA: Haplotype analysis of the CETP gene: not TaqIB, but the closely linked – 629 C → A polymorphism and a novel promoter variant are independently associated with CETP concentration. Hum Mol Genet, 12: 111–123, 2003

(51) Williams S, Hayes L, Elsenboss L, Williams A, Andre C, Abramson R, Thompson JF, and Milos PM: Sequencing of the cholesteryl ester transfer protein 5' regulatory region using artificial transposons. Gene, 197: 101–107, 1997

(52) Matsuzawa Y, Yamashita S, Kameda K, Kubo M, Tarui S, and Hara I: Marked hyper-HDL2-cholesterolemia associated with premature corneal opacity; a case report. Atherosclerosis, 62: 117–121, 1984

(53) Yamashita S, Sprecher DL, Sakai N, Matsuzawa Y, Tarui S, and Hui DY: Accumulation of apolipoprotein E-rich high density lipoproteins in hyper-alphalipoproteinemic human subjects with plasma cholesteryl ester transfer protein deficiency. J Clin Invest, 86: 688–695, 1990

(54) Ishigami M, Yamashita S, Sakai N, Arai T, Hirano K, Hiraoka H, Kameda-Takemura K, and Matsuzawa Y: Large and cholesteryl ester-rich high-density lipoproteins in cholesteryl ester transfer protein (CETP) deficiency can not protect macrophages from cholesterol accumulation induced by acetylated low-density lipoproteins. J Biochem, 116: 257–262, 1994

(55) Ohta T, Nakamura R, Takata K, Saito Y, Yamashita S, Horiuchi S, and Matsuda I: Structural and functional differences of subspecies of apoA-I-containing lipoprotein in patients with plasma cholesteryl ester transfer protein deficiency. J Lipid Res, 36:

696–704, 1995

(56) Oliveria HCF, Ma L, Milne R, Marcovina SM, Inazu A, Mabuchi H, and Tall AR: Cholesteryl ester transfer protein (CETP) activity enhances plasma cholesteryl ester formation: studies in CETP transgenic mice and human genetic CETP deficiency. Arterioscler Thromb Vasc Biol, 17: 1045–1052, 1997

(57) Ikewaki K, Rader DJ, Sakamoto T, Nishiwaki M, Wakimoto N, Schaefer JR, Ishikawa T, Fairwell T, Zech LA, Nakamura H, and Brewer HB Jr: Delayed catabolism of high density lipoprotein apolipoproteins A-I and A-II in human cholesteryl ester transfer protein deficiency. J Clin Invest, 92: 1650–1658, 1993

(58) Ikewaki K, Nishiwaki M, Sakamoto T, Ishikawa T, Fairwell T, Zech LA, Nagano M, Nakamura H, Brewer HB Jr, and Rader DJ: Increased catabolic rate of low density lipoproteins in humans with cholesteryl ester transfer protein deficiency. J Clin Invest, 96: 1573–1581, 1995

(59) Yamashita S, Matsuzawa Y, Okazaki M, Kako H, Yasugi T, Akioka H, Hirano K, and Tarui: Small poly-disperse low density lipoproteins in familial hyperalphalipoproteinemia with complete deficiency of cholesteryl ester transfer activity. Atherosclerosis, 70: 7–12, 1988

(60) Sakai N, Matsuzawa Y, Hirano K, Yamashita S, Nozaki S, Ueyama Y, Kubo M, and Tarui S: Detection of two species of low density lipoprotein particles in cholesteryl ester transfer protein deficiency. Arterioscler Thromb, 11: 71–79, 1991

(61) Sakai N, Yamashita S, Hirano K, Ishigami M, Arai T, Kobayashi K, Funahashi T, and Matsuzawa Y: Decreased affinity of low density lipoprotein (LDL) particles for LDL receptors in patients with cholesteryl ester transfer protein deficiency. Eur J Clin Invest, 25: 332–339, 1995

(62) Chiba H, Akita H, Kotani K, Kanno T, and Manabe M: Complete cholesteryl ester transfer protein deficiency increases oxidized-LDL in plasma. Jpn J Clin Pathol, 45: 55–57, 1997 (in Japanese)

(63) Hirano K, Yamashita S, Nakajima N, Arai T, Maruyama T, Yoshida Y, Ishigami M, Sakai N, Kameda-Takemura K, and Matsuzawa Y: Genetic cholesteryl ester transfer protein deficiency is extremely frequent in the Omagari area of Japan. Arterioscler Thromb Vasc Biol, 17: 1053–1059, 1997

(64) Zhong S, Sharp DS, Grove JS, Bruce C, Yano K, Curb JD, and Tall AR: Increased coronary heart disease in Japanese-American men with mutation in the cholesteryl ester transfer protein gene despite increased HDL levels. J Clin Invest, 97: 2917–2923, 1996

(65) Moriyama Y, Okamura T, Inazu A, Doi M, Iso H,

Mouri Y, Ishikawa Y, Suzuki H, Iida M, Koizumi J, Mabuchi H, and Komachi Y: A low prevalence of cornary heart disease among subjects with increased high-density lipoprotein cholesterol levels, including those with plasma cholesteryl ester transfer protein deficiency. Prev Med, 27: 659–667, 1998

Effects of fenofibrate on lipoproteins, vasomotor function, and serological markers of inflammation, plaque stabilization, and hemostasis

Kwang Kon Koh [a,*], Jeong Yeal Ahn [b], Seung Hwan Han [a], Dong Kyu Jin [a], Hyung Sik Kim [c], Kyung Cheon Lee [d], Eak Kyun Shin [a], Ichiro Sakuma [e]

[a] *Vascular Medicine and Atherosclerosis Unit, Department of Cardiology, Gil Heart Center, Gachon Medical School, 1198 Kuwol-dong, Namdong-gu, Incheon 405-760, Republic of Korea*
[b] *Department of Clinical Pathology, Gachon Medical School, 1198 Kuwol-dong, Namdong-gu, Incheon 405-760, Republic of Korea*
[c] *Department of Radiology, Gachon Medical School, 1198 Kuwol-dong, Namdong-gu, Incheon 405-760, Republic of Korea*
[d] *Department of Anesthesiology and Pain Medicine, Gachon Medical School, 1198 Kuwol-dong, Namdong-gu, Incheon 405-760, Republic of Korea*
[e] *Department of Cardiovascular Medicine, Graduate School of Medicine, Hokkaido University, Sapporo, Japan*

Received 4 June 2003; received in revised form 12 January 2004; accepted 22 January 2004

Available online 28 March 2004

Abstract

We investigated the effects of fenofibrate, peroxisome proliferator-activated receptors (PPARs) agonist, on endothelial function in patients with hypertriglyceridemia. We administered placebo or fenofibrate 200 mg daily to 25 patients with hypertriglyceridemia for 8 weeks. This study was randomized, double-blind, placebo-controlled, crossover in design. Compared with placebo, fenofibrate significantly changed lipoprotein levels including non-HDL cholesterol and significantly improved the percent flow-mediated dilator response to hyperemia by $33 \pm 6\%$ ($P < 0.001$) and lowered plasma levels of tumor necrosis factor-α by $13 \pm 3\%$ ($P = 0.002$). Fenofibrate reduced fibrinogen and plasminogen activator inhibitor type 1 antigen levels by 17 ± 3 and $10 \pm 3\%$, respectively ($P < 0.001$ and $P = 0.014$, respectively). However, fenofibrate did not significantly change plasma levels of nitrate, malondialdehyde, tissue factor activity, and serological markers of plaque stabilization. Fenofibrate significantly changed lipoprotein levels and improved the percent flow-mediated dilator response to hyperemia as well as lowered levels of tumor necrosis factor-α (TNF-α), fibrinogen, and plasminogen activator inhibitor type 1 antigen.
© 2004 Elsevier Ireland Ltd. All rights reserved.

Keywords: Fibrate; Hyperlipidemia; Endothelial function; Atherosclerosis

1. Introduction

Endothelial dysfunction of epicardial coronary arteries precedes development of atherosclerotic disease that is either angiographically apparent or of sufficient obstructive severity to cause myocardial ischemia and angina pectoris [1]. Patients with coronary heart disease or risk factors for coronary heart disease have been associated with impaired functions of the endothelium [2]. The vessel wall in these conditions may promote inflammation, smooth muscle proliferation, extracellular matrix deposition or lysis, and thrombus formation. All of these consequences of endothelial dysfunction contribute to development and clinical expression of atherosclerosis. Nitric oxide (NO) plays

a pivotal role in maintaining vascular health and protecting from vascular injury under these pathological conditions.

Plaque disruption and thrombosis remains an important cause of acute coronary syndrome. High-risk lesions are not necessarily the angiographically severe stenosis. Rather, unstable vulnerable lesions have large lipid cores and thin fibrous caps. Plaque instability relates closely to the development of inflammation within the intima. Acute coronary syndromes usually result from rupture of a vulnerable atherosclerotic plaque mechanistically linked to the inflammatory process. Matrix metalloproteinase (MMP), tissue inhibitor of matrix metalloproteinase (TIMP), tissue factor (TF), and plasminogen activator inhibitor type-1 (PAI-1) within the plaque are the major components in determining the plaque instability and thrombogenicity [2,3].

Clinical trials of fibric acid derivatives therapy demonstrate an improvement in cardiovascular end points and

* Corresponding author. Tel.: +82-32-460-3683;
fax: +82-32-460-3117/467-9302.
E-mail address: kwangk@ghil.com (K. Kon Koh).

coronary stenosis [4]. Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors activated by fatty acids and derivatives. PPARα mediates the hypolipidemic action of fibrates and is highly expressed in tissues such as heart, where it stimulates the β-oxidative degradation of fatty acids. PPARα controls plasma levels of cholesterol and triglycerides, which constitute major risk factors for coronary heart disease. Furthermore, experimental studies have shown that PPARα regulates the expression of key proteins involved in all stages of atherogenesis, such as vascular inflammation, plaque stability, and thrombosis, suggesting that PPARα exerts direct anti-atherogenic actions at the level of the vascular wall [5]. These direct vascular effects of fibrates may contribute to the cardiovascular event reduction and explain the clinical benefit in these clinical trials.

Cholesterol level lowering in experimental models was accompanied by a reduction of extracellular matrix and TF within atherosclerotic plaque [6,7]. Accordingly, the mechanism of fibrates therapy on the reduction of cardiovascular risk may be mediated by inhibiting vascular inflammation and thrombosis and stabilizing plaque. Thus, we investigated the vascular effects of fenofibrate such as vasomotor function, inflammation, plaque stability and hemostasis in patients with hypertriglyceridemia and further, the mechanism of regulation suggested by experimental studies [8–13].

2. Methods

2.1. Study population and design

This study was randomized, double-blind, placebo-controlled, crossover in design. None were diabetic, smokers, or had previous angina. We administered placebo or fenofibrate 200 mg daily to 25 patients with hypertriglyceridemia (>200 mg/dl) for 8 weeks, with the second treatment period initiated upon completion of the first treatment period (without washout phase). Any medications were not allowed during the study period to avoid other drugs' effects. The study was approved by the Gil Hospital Institute Review Board and all participants gave written, informed consent.

2.2. Laboratory assays

Blood samples for laboratory assays were obtained at approximately 8:00 a.m. following overnight fasting at baseline and at the end of each treatment period, and immediately coded so that investigators performing laboratory assays were blinded to subject identity or study sequence. Assays for lipids, fibrinogen, plasma nitrate (using the Griess reaction), malondialdehyde (MDA), tumor necrosis factor (TNF)-α, MMP-9 activity (Fluorokine® E Active MMP-9 kit), TIMP-1, and serum C-reactive protein (CRP) were performed in duplicate by ELISA (R&D Systems Inc., Minneapolis, MN, USA; BIOXYTECH® LPO-586,

Oxis Research, Portland, OR, USA; rate nephelometry; IMMAGE®, Beckman Coulter, Brea, CA, USA) as previously described [14–18]. Assay for PAI-1 antigens and TF activity were measured in duplicates by ELISA (Biopool) and actichrome assays (American Diagnostica, Greenwich, CT) as previously described [16,17]. All samples from the same patient (batch samples) were measured in blinded pairs on the same ELISA kit to minimize run-to-run variability. The inter- and intra-assay coefficients of variation were <6%.

2.3. Vascular studies

Imaging studies of the right brachial artery were performed using a ATL HDI 3000 ultrasound machine equipped with a 10 MHz linear-array transducer, based on a previously published technique [15,16,18]. Measurements were performed by two independent investigators (D.K.J. and H.S.K.) blinded to the subject's identity and medication status. Measurements of maximum diameter and percent flow-mediated dilation were made in 10 studies selected at random. The inter- and intra-observer variability for repeated measurement of maximum diameter were 0.004±0.039 and 0.005±0.089 mm, respectively. The inter- and intra-observer variability for repeated measurement of

Table 1
Effects of Fenofibrate in hypertryglyceridemic patients

Variables	Placebo	Fenofibrate
Lipids (mg/dl)		
Total cholesterol	198 ± 5	191 ± 7
Triglycerides	354 ± 12	137 ± 11***
ApoB	109 ± 2	96 ± 3**
HDL cholesterol	42 ± 2	54 ± 2***
ApoA-I	146 ± 4	166 ± 5***
Non-HDL cholesterol	156 ± 5	137 ± 7**
Vasomotor function (%)		
Flow-mediated dilation	4.99 ± 0.33	6.33 ± 0.34***
Nitroglycerin dilation	13.70 ± 0.58	14.27 ± 0.64
Nitrate (μmol/l)	98 ± 9	98 ± 9
Malondialdehyde (μM)	0.98 ± 0.10	1.20 ± 0.12
Cytokines		
TNF-α (pg/ml)	1.64 ± 0.10	1.43 ± 0.11**
C-reactive protein (mg/dl)	0.17 (0.11–0.28)	0.11 (0.11–0.36)
Plaque stability		
MMP-9 activity (ng/ml)	90 ± 13	72 ± 9
TIMP-1 (ng/ml)	92 ± 4	98 ± 4
MMP-9 activity/TIMP-1	0.92 ± 0.13	0.80 ± 0.11
Hemostasis		
Fibrinogen (mg/dl)	288 ± 9	236 ± 9***
Tissue factor (nM)	0.96 ± 0.16	0.93 ± 0.14
PAI-1 (ng/ml)	94 ± 7	84 ± 7*

Data are expressed as means ± S.E.M. Non-HDL cholesterol = total cholesterol − HDL cholesterol [30].
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ vs. placebo.

·percent flow-mediated dilation were 0.07 ± 1.27 and 0.15 ± 1.24%, respectively.

2.4. Statistical analysis

Data are expressed as mean ± S.E.M. or median (range:25–75%). After testing data for normality, we used Student's paired t test or Wilcoxon Signed Rank test to compare values after placebo and fenofibrate therapies, as reported in Table 1. We calculated that 25 subjects will provide 80% power for detecting difference of absolute increase 1.3% or greater flow-mediated dilation of the brachial artery on fenofibrate compared with placebo, with $\alpha = 0.05$. The comparison of endothelium-dependent dilation between placebo and fenofibrate therapies was prospectively designated as the primary end-point of the study. For a conservative analysis, a P-value less than the Bonferroni-adjusted α of $0.05/12 = 0.004$ was deemed as statistically significant for each of the 12 parameters that underwent statistical comparison in the study. Pearson or Spearman correlation coefficient analysis was used to assess associations between measured parameters.

3. Results

Baseline total cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol, non-HDL cholesterol, apolipoprotein B, and apolipoprotein A-I levels were 201±6, 346 ± 11, 43 ± 2, 161 ± 6, 106 ± 3, and 148 ± 4 mg/dl, respectively. The mean age was 51 ± 2 years and 15 (60%) were male. Mean body mass index was 24.9 ± 0.6.

To assess the possibility of a carryover effect from the initial treatment periods to the next treatment period, we compared the percent changes of (1) the first treatment placebo and the second treatment placebo (2) the first treatment fenofibrate and the second treatment fenofibrate, relative to baseline values. There were no significant differences in baseline values, vascular function (diameter and flow) and serological markers between each group. No significant differences were found in above two comparisons. (data not shown).

3.1. Effects of therapies on lipids and vasomotor function

Compared with placebo, fenofibrate significantly changed lipoprotein levels. As expected, fenofibrate decreased total cholesterol, non-HDL cholesterol, apolipoprotein B, and triglyceride and increased HDL-C and apolipoprotein A-I. Fenofibrate significantly improved the percent flow-mediated dilator response to hyperemia by 33 ± 6% ($P < 0.001$, Fig. 1), however, the brachial artery dilator response to nitroglycerin was not significantly changed ($P = 0.200$). Fenofibrate did not change plasma levels of nitrate and MDA.
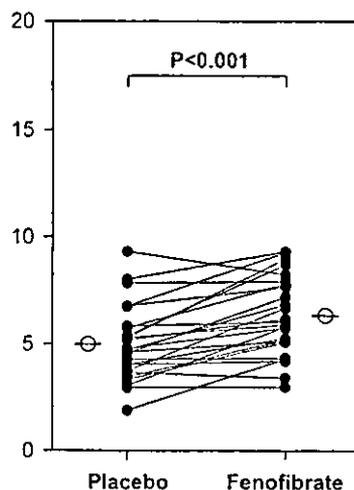

Fig. 1. Flow-mediated dilation on fenofibrate. Compared with placebo, fenofibrate significantly improved the percent flow-mediated dilator response to hyperemia. Mean values are identified by open circles.

3.2. Effects of therapies on TNF-α and markers of plaque stability

Compared with placebo, fenofibrate significantly lowered plasma levels of TNF-α by 13 ± 3% ($P = 0.002$). Fenofibrate lowered MMP-9 activity by 6 ± 10% ($P = 0.152$) and changed TIMP-1 by 9 ± 4% ($P = 0.180$) and lowered the ratio of MMP-9 activity over TIMP-1 (MMP-9 activity/TIMP-1) by 0 ± 14% ($P = 0.494$).

There were no significant inverse correlations between the degree of changes in flow-mediated dilation or HDL cholesterol and the degree of changes in MMP-9 activity ($r = -0.180$ and -0.144, respectively). However, a weak correlation between TNF-α levels and MMP-9 activity levels was determined ($r = 0.317$, $P = 0.123$).

3.3. Effects of therapies on CRP and markers of thrombosis

Compared with placebo, fenofibrate lowered serum levels of CRP from 0.17 to 0.11 mg/dl ($P = 0.424$). Fenofibrate reduced fibrinogen and PAI-1 antigen levels relative to placebo by 17 ± 3 and by 10 ± 3%, respectively ($P < 0.001$ and $P = 0.014$, respectively). However, fenofibrate did not lower plasma levels of TF activity relative to baseline measurements ($P = 0.903$).

There were significant inverse correlation between the degree of changes in TNF-α levels and the degree of changes in flow-mediated dilation ($r = -0.409$, $P = 0.042$). However, no significant correlations between the degree of changes in lipoproteins or CRP levels and the degree of changes in TF activity on fenofibrate were determined ($-0.237 \le r \le 0.154$).

4. Discussion

In the current study, we observed that compared with placebo, fenofibrate significantly decreased total cholesterol, non-HDL cholesterol, apolipoprotein B, and triglyceride and increased HDL-C and apolipoprotein A-I. Several studies have examined the effect of fibrates on vasomotor function, but results were controversial. Improved flow-mediated dilation after oral fat loading has been shown in type 2 diabetes after 12 weeks of ciprofibrate therapy [19], but similar benefits were not confirmed in healthy volunteers after 3 weeks of gemfibrozil [20]. In patients after coronary angioplasty, exercise-induced coronary artery dilation measured by quantitative coronary angiogram increased after bezafibrate therapy compared with placebo [21]. However, in patients with coroanry artery disease, Andrews et al. [22] reported that gemfibrozil alone or in combination with niacin did not significantly improve flow-mediated dilation. In the current study, we observed that fenofibrate significantly improved the percent flow-mediated dilator response to hyperemia. However, fenofibrate did not change plasma levels of nitrate and MDA. Fenofibrate may not have anti-oxidant effect in humans. We did not see any correlations between lipoprotein changes and flow-mediated dilation percent changes.

In order to gain insight as to mechanisms of potential vasculoprotective effects of fenofibrate, we measured vasomotor function, plasma TNF-α, and markers of plaque stability. Lowering blood LDL cholesterol levels may facilitate plaque stability either through a reduction in size or by an alteration of the physiochemical properties of lipid cores [2]. However, changes in plaque size by lipid lowering tend to occur over an extended period of time and are quite minimal, as assessed by angiography. Rather, the clinical benefits from lipid lowering are probably due to decreases in macrophage accumulation in atherosclerotic lesions and inhibition of MMP production by activated macrophages. In this regard, Aikawa et al. [6] demonstrated that intimal smooth muscle cells in the low cholesterol group displayed reduced expression of MMP-9 compared with the high cholesterol groups. Lipid-lowering therapies diminished accumulation of macrophages as well as macrophage expression of MMP-9 in animal studies [7,23]. Indeed, Xu et al. [9] demonstrated that oxidized LDL upregulated MMP-9 expression while reducing TIMP-1 in monocyte-derived macrophages. Furthermore, HDL abrogated oxidized LDL-induced MMP-9 expression. However, we did not observe significant correlations between lipoprotein levels and MMP-9 activity or TIMP-1 levels on fenofibrate. On the other hand, PPAR activator inhibited the expression of MMP-9 [11]. This PPAR-dependent inhibition may prevent the rupture of the atheroscleortic plaque and subsequent thrombosis. Despite the experimental observations, we observed that fenofibrate did not significantly change serological markers of plaque stability in the current study.

Moreover, endothelial NO synthase gene transfer significantly decreased MMP-2 and MMP-9 activities simultaneously with increase of TIMP-2 levels in the conditioned medium [10]. Furthermore, TNF-α, a proinflammatory cytokine, stimulated the synthesis and secretion of MMP-9 [8]. In the current study, we observed a weak correlation between the degree of changes in TNF-α and the degree of changes in MMP-9 activity on fenofibrate.

Two experimental studies demonstrated that native or oxidized LDL enhanced lipopolysaccharide-induced TF expression [24,25]. Furthermore, CRP, a proinflammatory cytokine, stimulated the synthesis of TF [26]. Meanwhile, PPARα also inhibits the expression of TF in human monocytes and macrophages [12]. However, we observed no effects of fenofibrate on TF activity and no correlations between the degree of changes in lipoproteins or CRP levels and the degree of changes in TF activity on fenofibrate.

Impaired fibrinolysis as measured by an elevation in PAI-1 and fibrinogen is predictive of ischemic heart disease [27,28]. Several lipid-lowering agents may potentiate fibrinolysis independent of alterations in plasma lipoproteins. In this regard, the gemfibrozil and fenofibrate reduced PAI-1 and fibrinogen plasma levels in hypercholesterolemic subjects [5,13,29]. Fibrates also modulate the secretion of the thrombosis inducer PAI-1 [13]. These actions of fenofibrate on fibrinogen and PAI-1 antigen levels may result in a decreased thrombogenic response.

Our current observations are consistent with the Adult Treatment Panel III Guidelines reported in 2001 [30]. Since metabolic syndrome is one of the most important clinical features underlying atherosclerosis, and since the Adult Treatment Panel III Guidelines use non-HDL cholesterol as one of the indexes to evaluate the dyslipidemic status including hypertriglyceridemia, our current observations showing the effects of fenofibrate on lipoproteins and endothelial function in patients with hypertriglyceridemia, may have important clinical implications to reduce cardiovascular events in these patients.

In conclusion, fenofibrate significantly changed lipoprotein levels including non-HDL cholesterol and improved the percent flow-mediated dilator response to hyperemia as well as lowered levels of TNF-α, fibrinogen, and PAI-1 antigen.

References

[1] Ludmer PL, Selwyn AP, Shook TL, et al. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. N Eng J Med 1986;315:1046–51.

[2] Koh KK. Effects of statins on vascular wall: vasomotor function, inflammation, and plaque stability. Cardiovasc Res 2000;47:648–57.

[3] Koh KK. Effects of HMG-CoA reductase inhibitor on hemostasis. Int J Cardiol 2000;76:21–30.

[4] Rubins HB, Robins SJ, Collins D, et al. Gemfibrozil for the secondary prevention of coronary heart disease in men with low levels of high-density lipoprotein cholesterol. Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial Study Group. N Engl J Med 1999;341:410–8.

[5] Barbier O, Torra IP, Duguay Y, et al. Pleiotropic actions of peroxisome proliferator-activated receptors in lipid metabolism and atherosclerosis. Arterioscler Thromb Vasc Biol 2002;22:717–26.

[6] Aikawa M, Rabkin E, Okada Y, et al. Lipid lowering by diet reduces matrix metalloproteinase activity and increases collagen content of rabbit atheroma. Circulation 1998;97:2433–44.

[7] Aikawa M, Rabkin E, Sugiyama S, et al. An HMG-CoA reductase inhibitor, cerivastatin, suppresses growth of macrophages expressing matrix metalloproteinases and tissue factor in vivo and in vitro. Circulation 2001;103:276–83.

[8] Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions human atherosclerotic plaques. J Clin Invest 1994;94:2493–503.

[9] Xu XP, Meisel SR, Ong JM, et al. Oxidized low-density lipoprotein regulates matrix metalloproteinase-9 and its tissue inhibitor in human monocyte-derived macrophages. Circulation 1999;99:993–8.

[10] Gurjar MV, Sharma RV, Bhalla RC. ENOS gene transfer inhibits smooth muscle cell migration and MMP-2 and MMP-9 activity. Arterioscler Thromb Vasc Biol 1999;19:2871–7.

[11] Marx N, Sukhova G, Murphy C, Libby P, Plutzky J. Macrophages in human atheroma contain PPARgamma: differentiation-dependent peroxisomal proliferator-activated receptor gamma(PPARgamma) expression and reduction of MMP-9 activity through PPARgamma activation in mononuclear phagocytes in vitro. Am J Pathol 1998;153:17–23.

[12] Neve BP, Corseaux D, Chinetti G, et al. PPARalpha agonists inhibit tissue factor expression in human monocytes and macrophages. Circulation 2001;103:207–12.

[13] Durrington PN, Mackness MI, Bhatnagar D, et al. Effects of two different fibric acid derivatives on lipoproteins, cholesteryl ester transfer, fibrinogen, plasminogen activator inhibitor and paraoxonase activity in type IIb hyperlipoproteinaemia. Atherosclerosis 1998;138:217–25.

[14] Koh KK, Son JW, Ahn JY, et al. Non-lipid effects of statin on hypercholesterolemic patients established to have coronary artery disease who remained hypercholesterolemic while eating a step-II diet. Coron Artery Dis 2001;12:305–11.

[15] Koh KK, Cardillo C, Bui MN, et al. The effect of estrogen and cholesterol-lowering therapies on vascular function in hypercholesterolemic postmenopausal women. Circulation 1999;99:354–60.

[16] Koh KK, Jin DK, Yang SH, et al. Vascular effects of synthetic or natural progestagen combined with conjugated equine estrogen in healthy postmenopausal women. Circulation 2001;103:1961–6.

[17] Koh KK, Mincemoyer R, Bui MN, et al. Effects of hormone-replacement therapy on fibrinolysis in postmenopausal women. N Engl J Med 1997;336:683–90.

[18] Koh KK, Son JW, Ahn JY, et al. Comparative effects of diet and statin on nitric oxide bioactivity and matrix metalloproteinases in hypercholesterolemic patients with coronary artery disease. Arterioscler Thromb Vasc Biol 2002;22:e19–23.

[19] Evans M, Anderson RA, Graham J, et al. Ciprofibrate therapy improves endothelial function and reduces postprandial lipemia and oxidative stress in type 2 diabetes mellitus. Circulation 2000;101:1773–9.

[20] Wilmink HW, Twickler MB, Banga JD, et al. Effect of statin versus fibrate on postprandial endothelial dysfunction: role of remnant-like particles. Cardiovasc Res 2001;50:577–82.

[21] Seiler C, Suter TM, Hess OM. Exercise-induced vasomotion of angiographically normal and stenotic coronary arteries improves after cholesterol-lowering drug therapy with bezafibrate. J Am Coll Cardiol 1995;26:1615–22.

[22] Andrews TC, Whitney EJ, Green G, Kalenian R, Personius BE. Effect of gemfibrozil ± niacin ± cholestyramine on endothelial function in patients with serum low-density lipoprotein cholesterol levels <160 mg/dl and high-density lipoprotein cholesterol levels <40 mg/dl. Am J Cardiol 1997;80:831–5.

[23] Fukumoto Y, Libby P, Rabkin E, et al. Statins alter smooth muscle cell accumulation and collagen content in established atheroma of Watanabe heritable hyperlipidemic rabbits. Circulation 2001;103:993–9.

[24] Brand K, Banka CL, Mackman N, et al. Oxidized LDL enhances lipopolysaccharide-induced tissue flictor expression in human adherent monocytes. Arterioscler Thromb 1994;14:790–7.

[25] Cui MZ, Penn MS, Chisolm GM. Native and oxidized low density lipoprotein induction of tissue factor gene expression in smooth muscle cells is mediated by both Egr-1 and Sp 1. J Biol Chem 1999;274:32795–802.

[26] Cermak J, Key NS, Bach RR, et al. C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. Blood 1993;82:513–20.

[27] Salomaa V, Stinson V, Kark JD, et al. Association of fibrinolytic parameters with early atherosclerosis: the ARIC Study. Circulation 1995;91:284–90.

[28] Acevedo M, Pearce GL, Kottke-Marchant K, Sprecher DL. Elevated fibrinogen and homocysteine levels enhance the risk of mortality in patients from a high-risk preventive cardiology clinic. Arterioscler Thromb Vasc Biol 2002;22:1042–5.

[29] Zambrana JL, Velasco F, Castro P, et al. Comparison of bezafibrate versus lovastatin for lowering plasma insulin, fibrinogen, and plasminogen activator inhibitor-1 concentrations in hyperlipemic heart transplant patients. Am J Cardiol 1997;80:836–40.

[30] Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III). JAMA 2001;285:2486–97.

nature
cell biology

SREBPs suppress IRS-2-mediated insulin signalling in the liver

Tomohiro Ide[1], Hitoshi Shimano[2,4], Naoya Yahagi[2], Takashi Matsuzaka[1], Masanori Nakakuki[1], Takashi Yamamoto[1], Yoshimi Nakagawa[2], Akimitsu Takahashi[1], Hiroaki Suzuki[1], Hirohito Sone[1], Hideo Toyoshima[1], Akiyoshi Fukamizu[2,3] and Nobuhiro Yamada[1]

Insulin receptor substrate 2 (IRS-2) is the main mediator of insulin signalling in the liver, controlling insulin sensitivity. Sterol regulatory element binding proteins (SREBPs) have been established as transcriptional regulators of lipid synthesis. Here, we show that SREBPs directly repress transcription of IRS-2 and inhibit hepatic insulin signalling. The IRS-2 promoter is activated by forkhead proteins through an insulin response element (IRE). Nuclear SREBPs effectively replace and interfere in the binding of these transactivators, resulting in inhibition of the downstream PI(3)K/Akt pathway, followed by decreased glycogen synthesis. These data suggest a molecular mechanism for the physiological switching from glycogen synthesis to lipogenesis and hepatic insulin resistance that is associated with hepatosteatosis.

Insulin has long been known to have a major role in the anabolic effects associated with carbohydrate and lipid metabolism. IRS proteins link insulin receptor binding to its final biological actions through a series of intermediate effectors in which the phosphatidylinositol-3-OH kinase (PI(3)K)/Akt cascade is the main pathway (reviewed in ref. 1). The roles of insulin signalling in the liver include activation of glycogen synthesis for energy storage, and suppression of hepatic glucose output by inhibiting phosphoenol pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). Gene targeting experiments have demonstrated that hepatic insulin signalling for these effects is mediated mainly through IRS-2, rather than IRS-1 (refs 2, 3). After these acute actions of insulin on glucose metabolism, the liver initiates lipogenesis through induction of lipogenic enzymes. SREBPs are membrane-bound transcription factors that regulate genes involved in lipid synthesis. After sterol-regulated cleavage, the amino-terminal basic helix-loop-helix leucine zipper domains (nuclear SREBPs) are translocated to the nucleus, where they activate their target-gene promoters containing SREs (reviewed in ref. 4). Whereas SREBP-2 is crucial for the regulation of cholesterol synthesis genes, SREBP-1c controls gene expression of lipogenic enzymes (reviewed in refs 5, 6). Insulin and glucose are well known to stimulate lipogenesis, and SREBP-1c expression is nutritionally regulated in

the liver and adipose tissue. Thus, SREBP-1c has been thought of as a mediator for insulin action on gene transcription.

Hepatic insulin resistance is an important pathophysiological feature of type-2 diabetes mellitus and metabolic syndrome. Decreased IRS-2 expression (and the resultant impairment of PI(3)K/Akt signalling) has been reported in the livers of animal models for insulin resistance, such as ob/ob mice and lipodystrophic mice[7,8]. These studies, in conjunction with the hepatic insulin resistance observed in IRS-2 knockout mice, led to the hypothesis that hepatic insulin resistance is mediated through inhibition of IRS-2. In these animals, SREBP-1c is highly expressed and is important for the formation of fatty liver[9].

In this study, hepatic SREBP-1 and IRS-2 gene expression were compared under various nutritional conditions. First, refeeding of normal mice with a high-sucrose/fat-free diet after fasting (a potent insulin-stimulating diet formula) markedly induced hepatic SREBP-1c expression from almost undetectable levels, as estimated by northern blotting (Fig. 1a). In contrast, IRS-2 mRNA levels were very high during fasting and markedly reduced by refeeding, whereas IRS-1 levels did not show a significant change. The suppression of hepatic IRS-2 gene expression in a refed state was accompanied by a concomitant decrease in IRS-2 protein levels (Fig. 1b). Second, the chronic effects of forced SREBP expression on IRS-2 gene expression were estimated in transgenic mice. Overproduction of nuclear-active SREBP-1a and -1c caused a marked reduction in IRS-2 mRNA levels, whereas IRS-1 mRNA levels were not much affected (Fig. 1c). Next, IRS-2 mRNA levels were examined in the livers of SREBP-1-deficient mice, which have been reported to show impaired nutritional induction of lipogenic genes[10]. IRS-2 mRNA was elevated in the livers of SREBP-1-deficient mice, compared with those of wild-type mice both at fasting and at refeeding (Fig. 1d). Increased IRS-2 RNA levels were also detected in SREBP-1c-knockout mice[11]. Together, these data consistently show an inverse correlation between hepatic SREBP-1 and IRS-2 expression in different nutritional states, and raise the possibility that SREBP-1 might regulate IRS-2 gene expression.

To test this hypothesis in a more acute and transient manner, the effects of SREBPs on the IRS-2 gene were examined by infection of rat primary hepatocytes with adenoviral vectors expressing either
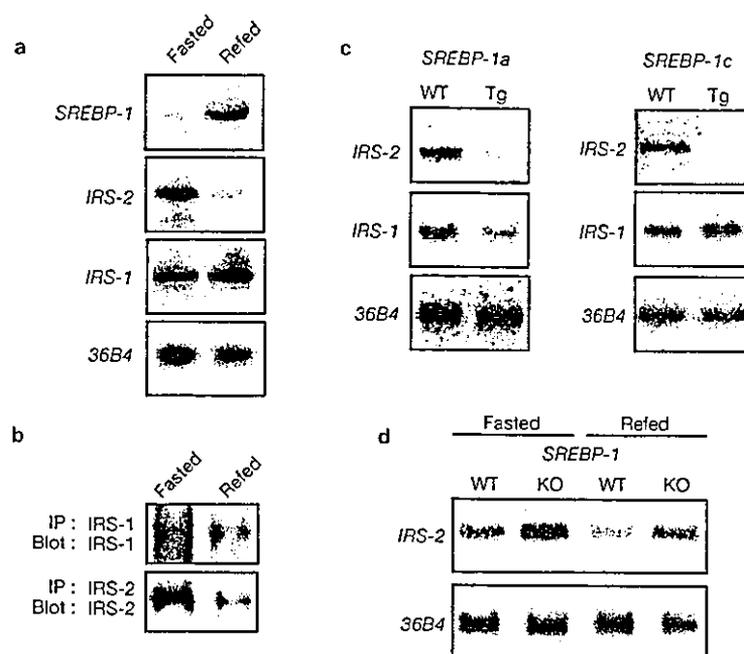
Figure 1 An inverse correlation between hepatic *IRS-2* and *SREBP-1* gene expression. Amounts of hepatic *IRS-2* and *SREBP-1* mRNA (a, c, d) and protein (b) were measured by northern blotting and by immunoblot analysis after immunoprecipitation, respectively, using the indicated animals. a, b, fasted or refed C57BL6 mice; c, fasted wild-type (WT) mice, and *SREBP-1a* or *SREBP-1c* transgenic (Tg) mice. d, Wild-type mice and *SREBP-1*-knockout (KO) mice in fasted and refed states.

SREBP-1a, -1c or -2. Transduction of cultured hepatocytes with increasing titres of the SREBP adenovirus resulted in dose-dependent expression of each SREBP isoform (Fig. 2a). The accumulation of nuclear SREBP-1c or SREBP-1a induced expression of *FAS* mRNA, a target gene for SREBP-1, while decreasing *IRS-2* mRNA levels in a dose-dependent manner. SREBP-2 also had a similar inhibitory action on *IRS-2* expression. The suppression of *IRS-2* mRNA levels through over-production of SREBP-1c, -1a and -2 was accompanied by a reduction in IRS-2 protein levels (Fig. 2b). Interestingly, the level of IRS-1 protein was also reduced. To determine whether the SREBP-induced reduction of IRS-2 protein levels affects insulin signalling, insulin-mediated stimulation of IRS-2 tyrosine phosphorylation, Akt-Ser 473 phosphorylation and glycogen synthesis were measured. As estimated by immunoblotting after immunoprecipitation in insulin-stimulated hepatocytes, tyrosine phosphorylation of IRS-2 and Ser 473 phosphorylation of Akt were reduced by expression of SREBPs in a dose-dependent manner (corresponding to reduced IRS-2 protein levels), whereas Akt protein levels were not affected (Fig. 2c). This inhibition caused a resultant suppression of glycogen synthesis under both basal and insulin-induced conditions (Fig. 2d). In contrast, but consistent with a well-established theory, SREBPs activated fatty acid synthesis, which is a late metabolic effect of insulin (Fig. 2e). Similar results were observed in SREBP-1a-expressing hepatocytes, but with a slightly more potent inhibition. These data indicate that activation of SREBPs markedly suppresses hepatic insulin signalling through repression of *IRS-2*. The diminished level of IRS-1 protein could also contribute to impaired insulin signalling caused by adenovirus-mediated overproduction of SREBPs in primary hepatocytes, although the mechanism is currently unknown.

To investigate mechanisms for SREBP-mediated suppression of *IRS-2* expression, promoter analysis of the 5′-flanking region of the human *IRS-2* gene was performed in transfection studies in rat primary hepatocytes. The *IRS-2* gene promoter contains an IRE through

which chronic insulin treatment down-regulates *IRS-2* expression[12]. Intriguingly, a highly probable SREBP-binding site was identified in a region that overlaps with the IRE[13] (Fig. 3a). In luciferase reporter assays, IRS-2 promoter activity was consistently suppressed by insulin and increased by dexamethasone, as previously described[14] (Fig. 3b). Transfection of nuclear SREBP-1a and -1c suppressed basal IRS-2 promoter activity, indicating that SREBPs repress IRS-2 at the transcriptional level (Fig. 3c). Similar consensus IRE sequences in the promoters of genes involved in hepatic glucose output, such as *PEPCK* and *G6Pase* genes, have been reported[14]. Forkhead (FKH) proteins such as FKHR and FKHRL1 (also known as FOXO1 and FOXO3a, respectively) have been shown to be crucial for the regulation of PEPCK and G6Pase through their IREs[15,16]. Insulin-mediated repression of these genes occurs through PI(3)K/Akt-mediated phosphorylation of the FKHR proteins, resulting in their translocation out of nucleus[17,18]. Therefore, it is highly conceivable that the FKH proteins could activate *IRS-2* gene expression through its IRE in a similar manner. Supporting this prediction, overexpression of FKHRL1 increased IRS-2 promoter activity (Fig. 3c). Even in this FKHRL1-induced condition, IRS-2 promoter activity was efficiently suppressed by co-transfection of SREBP-1a and -1c. Overproduction of SREBP did not affect FKHRL1 expression (Fig. 3c, inset). Similar results were obtained in HepG2 cells, where FKHRL1 and FKHR were shown to activate, and SREBPs were shown to inhibit, the IRS-2 promoter in a competitive manner (Fig. 3d, e). Competition between SREBP-1 and FKH was further confirmed with mutants of FKHR (Fig. 3f, g). FKHR3A, in which three Akt-mediated serine-phosphorylation sites were substituted for alanine, is resistant to nuclear exclusion by Akt and is therefore constitutively active[19]. The FKHR-mediated activation of the IRS-2 promoter was enhanced by this mutation, but still robustly suppressed by co-expression of SREBP-1. A mutation that disrupts binding to IRE (FKHR^{H212R}; ref. 20) abolished activation of IRS-2 by FKH and
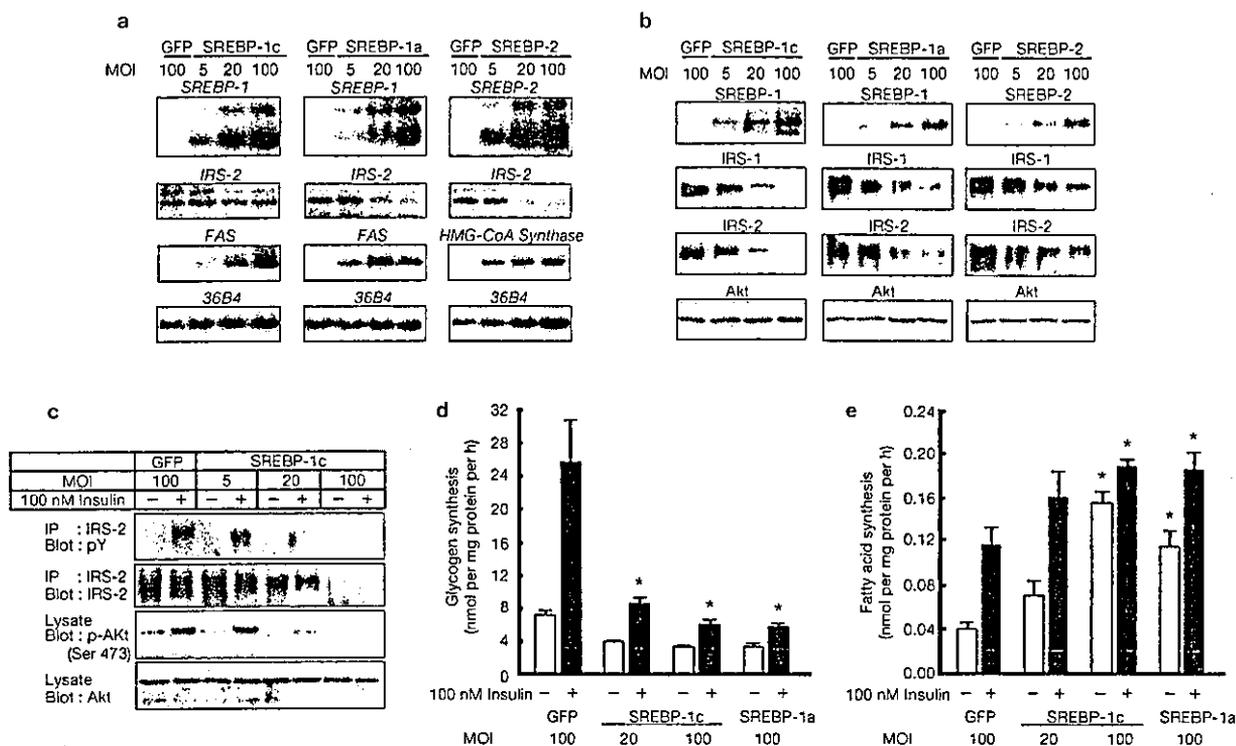
Figure 2 Nuclear SREBPs reduce *IRS-2* expression and insulin signalling in primary hepatocytes. (a) Expression of *IRS-2, FAS* and *HMG-CoA synthase* in adenovirus-mediated SREBP-overexpressing hepatocytes, as estimated by northern blotting. Hepatocytes were infected with green fluorescent protein (GFP), SREBP-1c, SREBP-1a and SREBP-2 adenovirus at the indicated multiplicity of infection (MOI). (b) Protein levels of IRS-1, IRS-2 and Akt were examined by immunoblotting of hepatocytes expressing SREBP-1c, -1a, and -2. (c) Insulin-induced tyrosine phosphorylation of IRS-2 and serine (Ser) phosphorylation of Akt in SREBP-1c-expressing hepatocytes. (d, e) Nuclear SREBPs suppress the hepatic insulin effect, as measured by glycogen synthesis. Insulin-induced glycogen (d) and fatty acid (e) synthesis from U-^{14}C-glucose were measured in SREBP-1c- and SREBP-1a-expressing hepatocytes. Error bars represent standard errors from three independent experiments. *P < 0.05, compared with GFP-treated cells.

FKH3A (Fig. 3f). PGC-1 (peroxisome proliferative activated receptor-γ co-activator 1) was recently established as a co-activator for FKH-mediated activation of gluconeogenic genes[21]. As shown in Fig. 3h, cotransfection of PGC-1 enhanced FKHRL1-mediated activation of the IRS-2 promoter, suggesting that PGC-1 is involved in transactivation of IRS-2 by FKHs. SREBP-1 suppressed this PGC-1-mediated activation.

On the basis of these results from luciferase assays, direct binding of SREBP to the IRS-2 promoter was examined. Using different probes covering the region neighbouring SRE/IRE (see Supplementary Information, Fig. S1a), the binding site was determined and tentatively designated as IRS-2-SRE (see Fig. 3a and Supplementary Information, Fig. S1b). Gel-shift assays demonstrated that *in-vitro*-translated SREBP-1a, -1c and -2 proteins bound specifically to this region with a higher affinity than they do to an authentic low-density lipoprotein receptor (LDLR)-SRE (see Supplementary Information, Fig. S1c). Both FKH proteins were shown to bind specifically to the IRE-containing region (see Supplementary Information, Fig. S1d, e), as well as FKHR (data not shown). Although IRS-2-SRE and IRS-2-IRE partially overlap, SREBPs and FKHs bind to this region in a different way, because the mutation that abolishes FKH binding did not affect SREBP binding (see Supplementary Information, Fig. S1e). Fig. 3i shows luciferase assays with the IRS-2 promoter construct in which this newly identified IRS-2-SRE was disrupted, but in which the IRS-2-IRE remained. The construct retained the activation by FKHRL1, but lost the suppression by SREBP-1c on the IRS-2 promoter activity.

These data demonstrate that IRS-2-SRE is responsible for the repression of IRS-2 by SREBP-1.

When both FKHRL1 and SREBP-1c proteins were added competitively to the IRS-2-SRE/IRE region for gel-shift assays, SREBP binding replaced FKHRL1 binding in a dose-dependent manner (Fig. 4a). This indicates that SREBP can prevent FKHRL1 from binding to the IRS-2 promoter. In a more physiologically relevant experiment, nuclear extracts from mouse livers were used to estimate binding to the IRS-2-SRE/IRE region (Fig. 4b, c). By the time of refeeding, nuclear SREBP-1c protein accumulated and FKHR proteins declined, as shown by immunoblotting (Fig. 4b). Nuclear extracts from refed mouse livers shifted the IRS-2-SRE probe more prominently than those from fasted mice (Fig. 4c). The strong signal was almost exclusively specific to SREBP-1c, as shown with the anti-SREBP antibody. Finally, to confirm direct binding of SREBP to the IRS-2 promoter *in vivo*, chromatin immunoprecipitation (ChIP) assays were performed on hepatic nuclei from fasted and refed mice (Fig. 4d). These results support the idea that SREBP-1 binds to the IRS-2-SRE in liver nuclei. Reflecting nutritional induction of nuclear SREBP-1c, the signal was highly enhanced by refeeding. In contrast, binding of FKH to the IRS-2-IRE exhibited a completely reciprocal change, suggesting that SREBP-1c prevents binding of FKH to the IRS-2 promoter. The ChIP assay with the PGC-1 antibody also detected the IRS-2 promoter, supporting our observations that this co-activator could be involved in the transcription of IRS-2 (Fig. 4d).
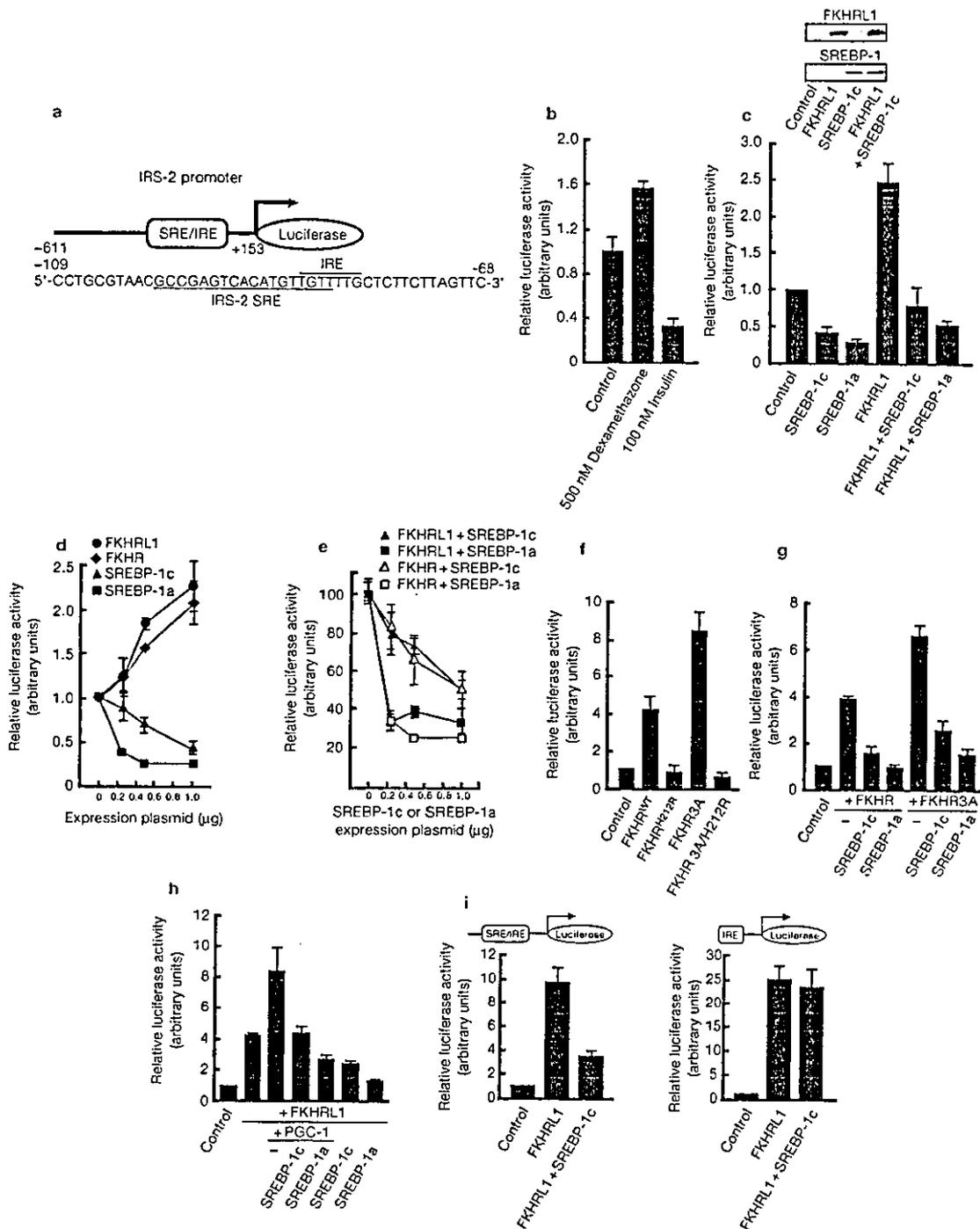
Figure 3 Nuclear SREBP-1c and SREBP-1a inhibit IRS-2 promoter activity competing with FKHs. (a) The human IRS-2 promoter-luciferase reporter plasmid was used for transfections. The SRE/IRE region contains an SREBP-binding site (SRE) and insulin response element (IRE).
(b) Dexamethasone-mediated induction and insulin-mediated inhibition of IRS-2 promoter activity in rat primary hepatocytes. (c) Effects of SREBP-1c, SREBP-1a and FKHRL1 on IRS-2 promoter activity in primary rat hepatocytes. Immunoblotting of the cotransfected SREBP-1c and FKHRL1 proteins is also shown (inset). (d) Dose-dependent effects of SREBP-1c, SREBP-1a, FKHRL1 and FKHR on IRS-2 promoter activity in HepG2 cells.
(e) Inhibition of FKHRL1- or FKHR-induced IRS-2 promoter activity by SREBP-1c and SREBP-1a in HepG2 cells. (f) Effects of wild-type and

mutant FKHR. FKHR^{H212R} (ref. 20), DNA-binding-defective mutant; FKHR3A (ref. 19), phosphorylation-deficient, and thus constitutively active, mutant. (g) Effects of FKHR3A mutants on IRS-2 promoter activity and SREBP inhibition. (h) Effects of PGC-1 on FKHRL1-induced IRS-2 promoter activity and SREBP-1 inhibition. (i) IRS-2-SRE is responsible for repression of SREBP. HepG2 cells were transfected with the indicated expression plasmids, IRS-2 promoter luciferase constructs and a renilla luciferase plasmid as a reference, before a 24-h incubation in the presence or absence of the indicated hormones. Luciferase assays were then performed. The firefly luciferase activity of transfectants was normalized to renilla luciferase activity as an internal reference. Error bars represent standard errors for three independent experiments.
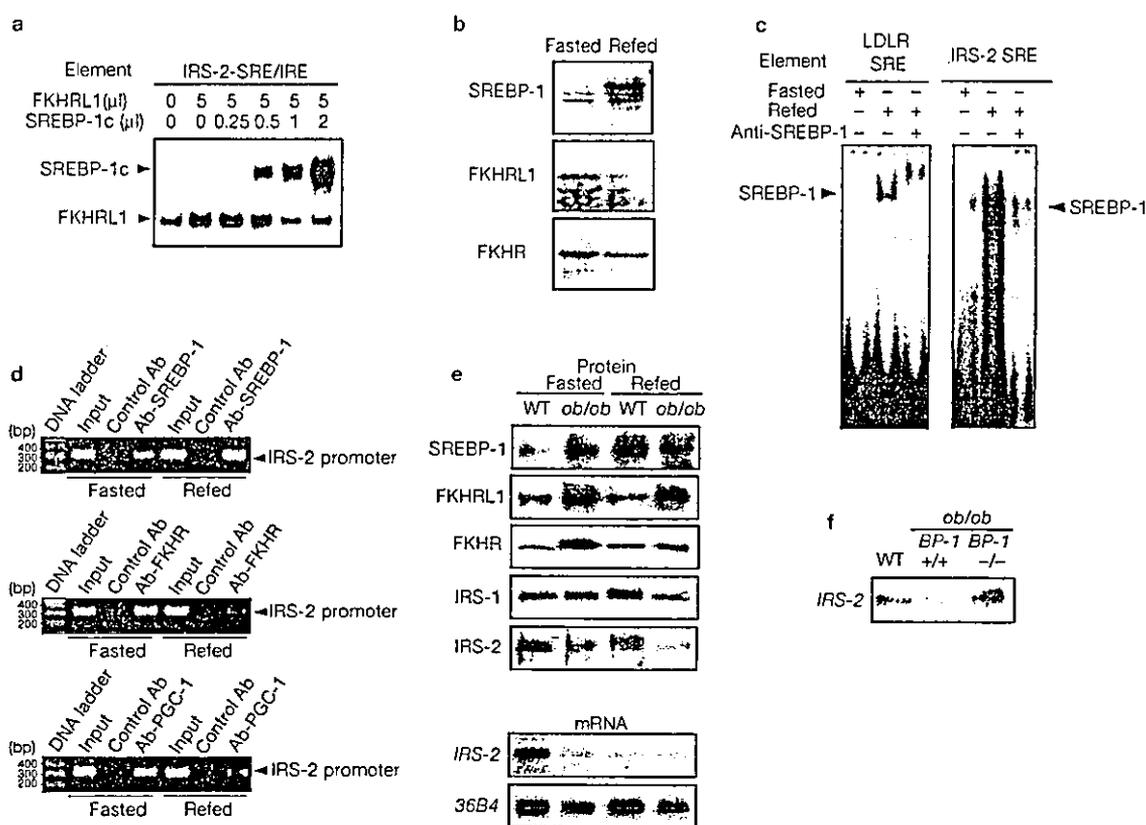
Figure 4 SREBP-1c in hepatic nuclear extracts dominates binding to the IRS-2-SRE. (a) Competition between SREBP-1c and FKHRL1 in binding to IRS-2-SRE/IRE. Both *in vitro*-translated proteins were used in the indicated amounts for an EMSA assay with the IRS-2-SRE/IRE probe. (b) The amounts of SREBP-1c and forkhead proteins in hepatic nuclei from fasted or refed normal mice. Liver nuclear extracts were subjected to immunoblotting with the indicated antibodies. (c) Endogenous SREBP-1c from hepatic nuclear extracts binds to IRS-2-SRE. Binding reactions were performed by incubation with hepatic nuclear extracts from fasted or refed mice using IRS-2-SRE (probe G in Supplementary Information, Fig. S2a) or LDLR-SRE

as probes. An anti-SREBP-antibody was used to confirm the specificity of the binding. (d) SREBP-1c and FKH proteins bind to the IRS-2 gene promoter *in vivo*. ChIP assays were performed for livers from mice in fasted and refed states using anti-SREBP-1 (top), anti-FKHR antibody (middle), anti-PGC-1 antibodies (bottom), or IgG as a negative control. PCR reactions were conducted with primers for the IRS-2 promoter region containing IRS-2-SRE/IRE. (e) SREBP-1, FKH and IRS protein levels in hepatic nuclei and *IRS-2* mRNA level from wild-type (WT) and leptin-deficient *ob/ob* mice in fasted and refed states. (f) Hepatic *IRS-2* mRNA level of wild-type, *ob/ob* and *ob/ob-SREBP-1* double-knockout mice.

In this study, we demonstrate that the competition between SREBP-1c and FKH proteins is observed *in vivo* in pathophysiological livers. In leptin-deficient *ob/ob* mice, extreme insulin resistance results in dysregulation of both SREBP-1c and FKH proteins in the liver. Hepatic nuclear SREBP-1c is unsuppressed at fasting, whereas nuclear FKH proteins are resistant to nuclear exclusion by insulin treatment and remain in the nucleus even in a fed state (Fig. 4e). Therefore, hepatic nuclear extracts from these insulin-resistant mice contained an abundance of both FKH and SREBP-1c proteins in either fasted or refed states. Nevertheless, hepatic *IRS-2* mRNA and protein were markedly decreased, supporting the observation that SREBP-1c dominates binding to the IRS-2 promoter. Furthermore, the low level of hepatic *IRS-2* mRNA in insulin-resistant *ob/ob* mice was restored by deletion of SREBP-1, as shown in *ob/ob SREBP-1* double-knockout mice[9] (Fig. 4f). In conjunction with the results from the luciferase assays, these data illustrate that SREBPs bind strongly to the IRS-2-SRE and efficiently inhibit binding of trans-activators (FKHs) to the IRS-2-IRE to repress *IRS-2* expression and sustain insulin resistance.

Here, we clearly demonstrate that SREBPs — traditionally characterized as transcriptional activators for lipid synthesis — also regulate expression of *IRS-2*, the major hepatic insulin signal mediator. An

abundance of nuclear SREBPs, as observed by a high enrichment of SREBP-1c in the liver nuclei of refed or insulin-resistant mice, causes efficient repression of *IRS-2* expression, resulting in impaired PI(3)K/Akt-mediated insulin signalling. The molecular mechanism for SREBP-mediated inhibition of IRS-2 is transcriptional repression through direct binding to the IRS-2-SRE in competition with putative trans-activators, including FKHRL1, FKHR and HNF3 (data not shown). Although it is possible that other factors could be involved in *IRS-2* expression through the IRS-2-IRE, the high affinity of SREBPs for the IRS-2-SRE seems to function in a dominant manner to remove their activators from the IRE. Our data also suggest that PGC-1 could be involved in transcription of IRS-2 by interacting with FKHs on the IRS-2-IRE, and that SREBP represses IRS-2 expression through inhibition of PGC-1 recruitment because SREBP-1 does not interact with PGC-1 (ref. 22).

Reciprocal effects of FKH and SREBP-1c on IRS-2 promoter activity could provide a mechanism for nutritional regulation of hepatic insulin sensitivity and expression of metabolic genes by these factors (see Supplementary Information, Fig. S2). In insulin-depleted or fasted states, high nuclear FKH and low nuclear SREBP-1c assure high expression of *IRS-2*, presumably to sensitize hepatocytes for

subsequent calorie intake and insulin influx (see Supplementary Information, Fig. S2a). After food intake, glucose consumption and glycogen synthesis proceed according to insulin signalling pathways. As feeding proceeds, insulin signalling stimulates phosphorylation of FKHs through PI(3)K/Akt activation and excludes them from the nucleus, reducing *PEPCK* and *G6Pase* gene expression (see Supplementary Information, Fig. S2b). Meanwhile, nuclear SREBP-1c is accumulated and lipogenesis is initiated. High SREBP-1c and low FKHs in the liver nuclei are prominent in the fully fed state, resulting in a further reduction of *IRS-2* expression and insulin sensitivity (see Supplementary Information, Fig. S2c). Thus, the physiological consequence of IRS-2 suppression by SREBP-1c is a transitional switch from glycogen synthesis to lipogenesis, and could be considered as a feedback response to prolonged insulin/glucose action. It has been reported that chronic exposure of hepatocytes to insulin represses IRS-2 promoter activity through the IRE, implying that prolonged hyperinsulinemia causes secondary insulin resistance[12]. Suppression of IRS-2 in the refed state mimics this situation *in vivo*.

This study also suggests that SREBP-1c may be involved in the pathogenesis of hepatic insulin resistance. High amounts of hepatic SREBP-1c were observed in numerous insulin-resistant animals[8,23]. Excess carbohydrate intake, an exacerbating factor for insulin resistance, also increases hepatic SREBP-1c expression. Our studies demonstrate that a high level of SREBP-1c results in further hepatic insulin resistance through suppression of *IRS-2* expression, as well as formation of fatty liver and production of triglyceride-rich VLDL that could be converted to atherogenic remnant particles. These are the main features of insulin-resistant animals, and clinical components of the metabolic syndrome. Concomitantly, nuclear FKHs sustain gluconeogenesis and could cause the onset of diabetes (Fig. 4e; also see Supplementary Information, Fig. S2d). Resultant hyperglycemia would also enhance further glucose-mediated induction of SREBP-1c, forming an unfavourable positive feedback loop of insulin resistance[24]. The current studies suggest that SREBP-1c could be central to the pathogenesis of hepatic insulin resistance and represent a potential therapeutic target. □

METHODS

Materials. Anti-IRS-1 and anti-IRS-2 antibodies were purchased from Upstate Biotechnology Institute (Lake Placid, NY), anti-FKHRL1 and anti-FKHR antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Affinity purified antibodies against Akt and phosphorylated Akt (Ser 473) were obtained from Cell Signaling Technology Inc. (Beverly, MA). The anti-phosphotyrosine monoclonal antibody (4G10) was a kind gift from T. Asano. The adenovirus expression vector kit (AdEasy system) was from B. Vogelstein and T.C. He.

Animals. Male mice (C57BL/6J) were obtained from CLEA Japan (Tokyo, Japan). For fasting and refeeding, mice were fasted for 24 h and then fed a high-sucrose/fat-free diet for 12 h, as previously described[10]. *SREBP-1* knockout mice and *ob/ob SREBP-1* double-knockout mice are previously described[9,10]. *SREBP-1a* and *SREBP-1c* transgenic mice, and the wild-type littermates, were put on a high-protein/low-carbohydrate diet for two weeks to induce transgene expression and then fasted for 12 h before sacrifice[25,26]. *ob/+* mice on a C57BL/6 background were purchased from Jackson Laboratories (West Grove, PA) and intercrossed to obtain leptin-deficient *ob/ob* mice and wild-type mice.

Isolation and culture of hepatocytes. Primary hepatocytes were isolated from male Sprague-Dawley rats (200–300 g, Japan Clea, Tokyo, Japan). Cells were resuspended in DMEM containing penicillin and streptomycin supplemented with 10% foetal bovine serum (FBS) and 30 nM dexamethasone before being seeded on 100-mm collagen-coated dishes at a final density of 4 × 10^{4} cells cm^{-2}. After incubation for 4 h to allow attachment, the medium was replaced with serum-free DMEM containing adenovirus.

Preparation of recombinant adenovirus. cDNAs encoding the active amino-terminal fragment of human SREBP-1c (amino acids 1–436), SREBP-1a (amino acids 1–460) and SREBP-2 (amino acids 1–460) were integrated into the adenovirus vector[27]. SREBP adenoviral vectors were propagated in 293 cells and purified by caesium chloride density centrifugation.

Glycogen and fatty acid synthesis. After adenoviruses infection, rat primary hepatocytes were incubated for 24 h in DMEM containing 10% FBS and 30 nM dexamethasone before a 4-h incubation in serum-free DMEM. Cells were then incubated for 3 h in DMEM containing 5.6 mM glucose and 2.5 µCi ml^{-1} U-^{14}C-glucose in the presence or absence of 100 nM insulin. Glycogen and fatty acid synthesis were determined by measuring the incorporation of U-^{14}C-glucose into glycogen and fatty acid, as described previously[28].

Plasmids. Human SREBP-1c, SREBP-1a, SREBP-2, FKHRL1 and FKHR cDNAs were cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) containing a CMV promoter. FKHR[H212R], FKHR3A and FKHR3A-H212R expression plasmids were previously described[19,20]. The human IRS-2 promoter (base pairs −611 to +153, relative to the transcriptional start site) was amplified by PCR[12,29] and sub-cloned into the pGL3-basic luciferase vector (Promega, Madison, WI).

Northern blotting. After adenovirus infection, rat primary hepatocytes were incubated for 24 h. The cDNA probes for SREBP-1, SREBP-2, FAS, HMG-CoA synthase and acidic ribosomal phosphoprotein PO (36B4) were prepared as previously described[10]. The cDNA probes for mouse IRS-1 and IRS-2 were prepared by RT-PCR using mouse liver total RNA as a template. The 5'- and 3'-PCR primers used to generate these probes were as follows: IRS-1, 5'-CGCCTGGAG-TATTATGAGAACGAG-3' and 5'-TGATGGGAAATGGTAGGAGATGTG-3' (GenBank accession number: X69722); IRS-2, 5'-CCTTAGGAGTGGTGGTCC-CAATAG-3' and 5'-TGACGGTGGTGGTAGAGGAAAAG-3' (GenBank accession number: AF090738).

Transfections and luciferase assays. HepG2 cells were grown in serum-free DMEM supplemented with 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin at 37 °C in 12-well plates for 6 h before transfection. Cells were transfected with 50 ng each of a pIRS-2 luciferase plasmid and a pRL-SV40 plasmid (Promega) using the SuperFect reagent (Qiagen, Valencia, CA). Rat primary hepatocytes were placed in serum-free DMEM and transfected with 1.5 µg of pIRS-2-Luciferase and 0.5 µg of pRL-SV40 using the Lipofectin reagent (Invitrogen). An SREBP-1c or SREBP-1a (2 µg) expression plasmid was cotransfected with or without an FKHRL1 or FKHR expression plasmid (2 µg). After a 24-h incubation, the amount of firefly luciferase activity in transfectants was measured and normalized to the amount of renilla luciferase activity.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from livers as previously described[10]. Nuclear SREBP-1c, SREBP-1a, SREBP-2 and FKHRL1 proteins were generated from expression vectors using a coupled *in vitro* reticulocyte transcription-translation system (Promega). Double-stranded oligonucleotides used in gel mobility shift assays were directed against the SRE of the LDLR promoter, 5'-TTTGAAAATCACCCCACTGCA-3' and IRS-2 SRE/IRE promoter, 5'-CCTGCGTAACGCCGAGTCACATGTTGTTTTGCTCTTC-3' (G probe in Supplementary Information, Fig. S1) or 5'-GCCGAGTCACAT-GTTGTTTTGCTCTTCTTAGTTC-3' (C probe in Supplementary Information, Fig. S1). *In vitro* synthetic protein lysate (0.25–5-µl) or nuclear extracts (1.0 µg) were incubated in a reaction mixture as previously described. DNA–protein complexes were resolved on a 4.0% polyacrylamide gel.

Immunoblotting. Proteins were extracted from rat primary hepatocytes or immunoprecipitated from livers of fasted or refed mice as described previously[10].

ChIP assay. Hepatic nuclei were prepared from livers as previously described[10]. ChIP assays were performed as described by the manufacturer (Upstate Biotechnology, Lake Placid, NY) with some modifications. Cross-linking between transcription factors and chromatin was achieved by adding formaldehyde (final concentration 1%) to hepatic nuclei for 15 min for 37 °C. Chromatin solutions were sonicated and incubated with 15 µg of anti-PGC-1 antibody (H-300; Santa Cruz), anti-SREBP-1 antibody (H-160, Santa Cruz),