**Fig. 1.** Comparison of the age-adjusted %dBPs in four subgroups categorized according to the gain or loss of %dWC and %dBMI values. p values were from the result of the Tukey's post-hoc analysis following analyses of variance. Mean ± 95% confidence interval is shown in each group.
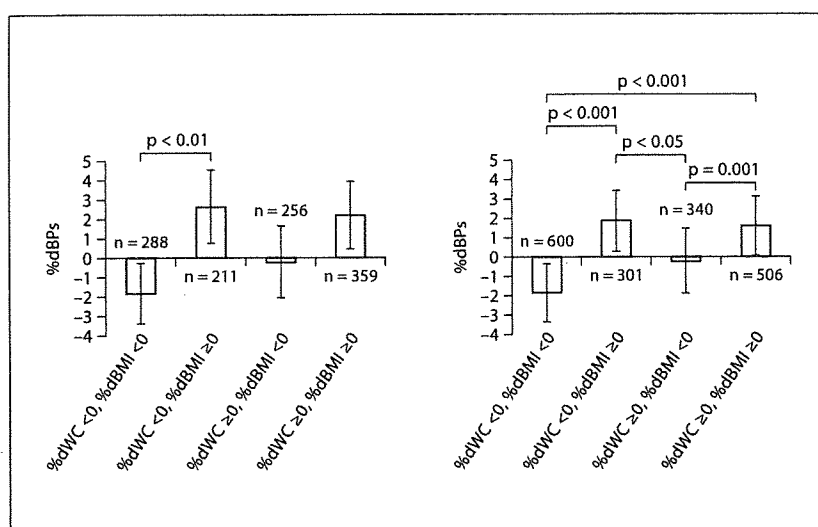
**Table 1.** Clinical characteristics and laboratory data at the first visit

| Variables | Whole | %dBPs | | | | p value |
|---|---|---|---|---|---|---|
| | | first (range: −40 ~ −7) | second (range: −7 ~ 0) | third (range: +1 ~ +6) | fourth (range: +6 ~ +52) | |
| Number | 2,861 | 714 | 809 | 639 | 699 | |
| Women/men | 1,114/1,747 | 288/426 | 314/495 | 251/388 | 261/438 | 0.712 |
| Age, years | 52.0 ± 10.1 | 52.8 ± 10.1 | 51.4 ± 9.9 | 51.8 ± 10.0 | 52.2 ± 10.2 | 0.047 |
| Height, cm | 164.8 ± 8.4 | 164.5 ± 8.3 | 165.2 ± 8.5 | 164.7 ± 8.5 | 164.7 ± 8.6 | 0.379 |
| Weight, kg | 61.8 ± 11.5 | 61.8 ± 11.4 | 62.0 ± 11.6 | 61.5 ± 11.3 | 61.8 ± 11.7 | 0.883 |
| BMI, kg/m² | 22.6 ± 3.0 | 22.7 ± 3.0 | 22.6 ± 3.1 | 22.5 ± 3.0 | 22.6 ± 3.1 | 0.781 |
| WC, cm | 81.8 ± 9.1 | 82.0 ± 9.1 | 81.8 ± 9.3 | 81.5 ± 9.0 | 81.9 ± 9.0 | 0.851 |
| Systolic BP, mm Hg | 120.9 ± 18.0 | 128.7 ± 18.3 | 121.8 ± 17.0 | 118.5 ± 16.7 | 114.2 ± 16.8 | <0.001 |
| Diastolic BP, mm Hg | 76.4 ± 11.4 | 79.3 ± 11.3 | 76.8 ± 10.9 | 75.5 ± 11.0 | 73.7 ± 11.5 | <0.001 |
| LDL cholesterol, mg/dl | 129.2 ± 31.1 | 131.4 ± 31.5 | 128.3 ± 29.5 | 127.1 ± 30.9 | 130.1 ± 32.4 | 0.051 |
| HDL cholesterol, mg/dl | 61.2 ± 15.3 | 60.8 ± 15.0 | 61.8 ± 15.7 | 61.4 ± 15.6 | 60.7 ± 15.0 | 0.465 |
| Triglyceride, mg/dl | 109.9 ± 71.4 | 115.7 ± 69.9 | 104.7 ± 61.8 | 109.8 ± 81.0 | 110.1 ± 73.4 | 0.030 |
| Uric acid, mg/dl | 5.4 ± 1.3 | 5.4 ± 1.3 | 5.5 ± 1.3 | 5.4 ± 1.4 | 5.5 ± 1.4 | 0.688 |
| Fasting glucose, mg/dl | 95.2 ± 20.0 | 96.8 ± 20.4 | 95.1 ± 21.1 | 94.2 ± 18.0 | 94.7 ± 20.0 | 0.072 |
| Hemoglobin A1C, % | 5.3 ± 0.7 | 5.3 ± 0.7 | 5.3 ± 0.7 | 5.3 ± 0.7 | 5.3 ± 0.7 | 0.506 |
| HOMA-IR | 1.5 ± 1.1 | 1.6 ± 1.1 | 1.5 ± 1.1 | 1.4 ± 1.0 | 1.5 ± 1.0 | 0.066 |
| Blood urea nitrogen, mg/dl | 14.0 ± 3.4 | 13.8 ± 3.7 | 14.0 ± 3.2 | 14.2 ± 3.4 | 14.1 ± 3.5 | 0.245 |
| Serum creatinine, mg/dl | 0.8 ± 0.3 | 0.8 ± 0.4 | 0.8 ± 0.2 | 0.8 ± 0.2 | 0.8 ± 0.2 | 0.764 |
| Estimated glomerular filtration rate | 68.6 ± 11.8 | 68.3 ± 11.4 | 69.3 ± 12.0 | 68.4 ± 11.8 | 68.1 ± 11.8 | 0.177 |
| Antidiabetic medication, n (%) | 51 (1.8) | 12 (1.7) | 20 (2.5) | 10 (1.6) | 9 (1.3) | 0.335 |
| Current smoker, n (%) | 680 (23.8) | 179 (25.0) | 184 (22.7) | 139 (21.8) | 178 (25.5) | 0.298 |

Data are means ± SD, unless stated otherwise. BMI = Body mass index; WC = waist circumference; HOMA-IR = homeostasis model assessment of insulin resistance. %dBPs was calculated by the following equation: (BPs at the second visit − BP1 at the second visit)/(BP1 at the second visit) × 100 (%). p value is for trend.

Changes in WC and BMI in Association with BP Change

**Table 2.** Pearson's correlation coefficient of obesity indices and blood pressure parameters

|  | %dWC | %dBMI | %dBPs | WC1 | BMI1 | BPs1 |
|---|---|---|---|---|---|---|
| *Women* |  |  |  |  |  |  |
| %dWC |  |  |  |  |  |  |
| r | – |  |  |  |  |  |
| p value | – |  |  |  |  |  |
| %dBMI |  |  |  |  |  |  |
| r | 0.241 | – |  |  |  |  |
| p value | <0.001 | – |  |  |  |  |
| %dBPs |  |  |  |  |  |  |
| r | –0.014 | 0.097 | – |  |  |  |
| p value | 0.635 | 0.001 | – |  |  |  |
| WC1 |  |  |  |  |  |  |
| r | –0.317 | –0.053 | –0.028 | – |  |  |
| p value | <0.001 | 0.078 | 0.350 | – |  |  |
| BMI1 |  |  |  |  |  |  |
| r | –0.026 | –0.087 | –0.029 | 0.787 | – |  |
| p value | 0.393 | 0.004 | 0.331 | <0.001 | – |  |
| BPs1 |  |  |  |  |  |  |
| r | –0.025 | –0.055 | –0.325 | 0.365 | 0.409 | – |
| p value | 0.396 | 0.064 | <0.001 | <0.001 | <0.001 | – |
| *Men* |  |  |  |  |  |  |
| %dWC |  |  |  |  |  |  |
| r | – |  |  |  |  |  |
| p value | – |  |  |  |  |  |
| %dBMI |  |  |  |  |  |  |
| r | 0.476 | – |  |  |  |  |
| p value | <0.001 | – |  |  |  |  |
| %dBPs |  |  |  |  |  |  |
| r | 0.116 | 0.232 | – |  |  |  |
| p value | <0.001 | <0.001 | – |  |  |  |
| WC1 |  |  |  |  |  |  |
| r | –0.268 | –0.089 | –0.031 | – |  |  |
| p value | <0.001 | <0.001 | 0.189 | – |  |  |
| BMI1 |  |  |  |  |  |  |
| r | –0.054 | –0.071 | –0.026 | 0.830 | – |  |
| p value | 0.023 | 0.003 | 0.286 | <0.001 | – |  |
| BPs1 |  |  |  |  |  |  |
| r | –0.090 | –0.077 | –0.327 | 0.308 | 0.322 | – |
| p value | <0.001 | 0.001 | <0.001 | <0.001 | <0.001 | – |

BPs = Systolic blood pressure; WC = waist circumference; BMI = body mass index. BPs at visit 1 and visit 2 were designated BPs1 and BPs2, respectively. BMI at visit 1 and visit 2 were designated BMI1 and BMI2, respectively, and WC at visit 1 and visit 2 were designated WC1 and WC2, respectively. %dBMI, %dWC, and %dBPs were calculated by the equation (BMI2 – BMI1)/BMI1 × 100 (%), (WC2 – WC1)/WC1 × 100 (%), and (BPs2 – BPs1)/BPs1 × 100 (%), respectively.

**Table 3.** Multiple regression analysis between %dBPs and age1, WC1, BMI1, %dWC, and %dBMI

|  | β | 95% CI | Standardized β | p value |
|---|---|---|---|---|
| *Women* |  |  |  |  |
| Model 1 |  |  |  |  |
| BPs1 | –0.23 | –0.27 to –0.20 | –0.38 | <0.001 |
| Age1 | 0.11 | 0.05 to 0.18 | 0.10 | 0.001 |
| WC1 | 0.11 | 0.03 to 0.19 | 0.09 | 0.005 |
| %dWC | 0.01 | –0.06 to 0.09 | 0.01 | 0.733 |
| Model 2 |  |  |  |  |
| BPs1 | –0.24 | –0.28 to –0.21 | –0.40 | <0.001 |
| BMI1 | 0.47 | 0.25 to 0.70 | 0.13 | <0.001 |
| Age1 | 0.13 | 0.07 to 0.19 | 0.12 | <0.001 |
| %dBMI | 0.34 | 0.15 to 0.53 | 0.10 | 0.001 |
| Model 3 |  |  |  |  |
| BPs1 | –0.24 | –0.28 to –0.21 | –0.40 | <0.001 |
| BMI1 | 0.65 | 0.28 to 1.03 | 0.17 | 0.001 |
| Age1 | 0.14 | 0.07 to 0.20 | 0.13 | <0.001 |
| %dBMI | 0.39 | 0.19 to 0.60 | 0.11 | <0.001 |
| WC1 | –0.08 | –0.21 to 0.05 | –0.06 | 0.244 |
| %dWC | –0.08 | –0.17 to 0.01 | –0.06 | 0.071 |
| *Men* |  |  |  |  |
| Model 1 |  |  |  |  |
| BPs1 | –0.22 | –0.25 to –0.19 | –0.35 | <0.001 |
| WC1 | 0.15 | 0.08 to 0.22 | 0.11 | <0.001 |
| %dWC | 0.28 | 0.17 to 0.39 | 0.11 | <0.001 |
| Age1 | 0.02 | –0.03 to 0.07 | 0.02 | 0.467 |
| Model 2 |  |  |  |  |
| BPs1 | –0.22 | –0.25 to –0.19 | –0.35 | <0.001 |
| %dBMI | 0.80 | 0.64 to 0.96 | 0.22 | <0.001 |
| BMI1 | 0.41 | 0.23 to 0.59 | 0.10 | <0.001 |
| Age1 | 0.05 | 0.00 to 0.10 | 0.05 | 0.035 |
| Model 3 |  |  |  |  |
| BPs1 | –0.22 | –0.25 to –0.19 | –0.35 | <0.001 |
| %dBMI | 0.82 | 0.63 to 1.00 | 0.22 | <0.001 |
| BMI1 | 0.38 | 0.04 to 0.72 | 0.10 | 0.027 |
| Age1 | 0.05 | 0.00 to 0.10 | 0.05 | 0.046 |
| WC1 | 0.01 | –0.11 to 0.14 | 0.01 | 0.845 |
| %dWC | –0.03 | –0.16 to 0.11 | –0.01 | 0.705 |

BPs = Systolic blood pressure; WC = waist circumference; BMI = body mass index. Standardized β values are the estimates resulting from an analysis performed on variables that were standardized. BPs at visit 1 and visit 2 were designated BPs1 and BPs2, respectively. BMI at visit 1 and visit 2 were designated BMI1 and BMI2, respectively, and WC at visit 1 and visit 2 were designated WC1 and WC2, respectively. %dBMI, %dWC, and %dBPs were calculated by the equation of (BMI2 – BMI1)/BMI1 × 100 (%), (WC2 – WC1)/WC1 × 100 (%), and (BPs2 – BPs1)/BPs1 × 100 (%), respectively.

Model 1 = Independent variables include age, BPs1, WC1, and %dWC; model 2 = independent variables include age, BPs1, BMI1, and %dBMI; model 3 = independent variables include model 1 + BMI1, and %dBMI.

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*Multiple Linear Regression Analysis*

In multiple regression analysis, in which age1, WC1, BPs1, and %dWC were used as independent variables (model 1), %dWC was found to be an independent predictive value for %dBPs in men, but not in women (table 3). In a model where age1, BMI1, BPs1, and %dBMI were used as independent variables (model 2), %dBMI was found to be an independent predictive value for %dBPs in the both genders. After including all of the age1, BPs1, WC1, BMI1, %dWC, and %dBMI in a model as independent variables (model 3), %dBMI remained to be a predictor for %dBPs in both genders. In model 3, the variance inflation factor scores of all applied independent variables were <10 (data not shown)

*Comparison between Individuals with BMI Gain or Loss together with WC Gain or Loss*

We then compared the %BPS values between individuals with both WC loss (%dWC <0) and BMI loss (%dBMI <0), those with both WC loss and BMI gain (%dBMI ≥0), both WC gain and BMI loss, and those with both WC gain and BMI gain during a 1-year period (fig. 1). Age-adjusted %dBPs was significantly greater in individuals with both WC loss and BMI gain compared with those with both WC loss and BMI loss. On the other hand, age-adjusted %dBPs did not significantly differ between individuals with both WC loss and BMI loss and those with WC gain and BMI loss in both genders. When the same analysis was performed after excluding 154 subjects who experienced WC change of −10 cm or less or +10 cm or more, the results obtained were not essentially changed (data not shown).

## Discussion

By analyzing data from individuals who underwent general health screening for 2 consecutive years, we showed that a percent difference in BMI (%dBMI) was a statistically significant predictor for a percent difference in BPs (%dBPs) in both genders. A percent difference in WC (%dWC) was also found to be a predictor for %dBPs in men; however, it lost statistical significance after further adjustment for BMI at the first visit and %dBMI, and it was not significant in women before and after such further adjustment.

A body of evidence indicates an association between obesity parameters and blood pressure levels [15, 16]. A reduction in body weight may result in a lowering of blood pressure in overweight or obese subjects [17, 18],

although the results may not be always uniform. Moore et al. [19] showed that modest weight loss over a 4-year period substantially lowered the long-term risk of hypertension in overweight adults in Framingham. Haung et al. [20] showed that weight loss occurring after 18 years of age was related to a significantly lower risk, whereas weight gain was related to greater risk of hypertension in middle-aged women. In addition, Yang et al. [21] showed that in men aged between 40 and 74 years, weight gain occurring after 20 years of age was significantly associated with prehypertension. Most of the reports studying the potential association between changes in obesity parameters and changes in blood pressure were carried over a follow-up period longer than that in the current study. Furthermore, Truesdale et al. [22] have more recently shown that weight change over a 3-year period resulted in change in blood pressure levels; men who had experienced a 10% weight gain over the previous 3 years had BPs that was 2.6 mm Hg higher. They found, however, that the impact of weight change was, albeit present, less prominent in women. Women who had experienced a 10% weight gain over the previous 3 years had BPs that was only 0.9 mm Hg higher, suggesting the presence of gender difference in the extent of association between weight change and blood pressure change. We also showed here that the magnitude of the effect of changes in obesity parameters on blood pressure changes may vary by gender (table 3).

As compared to changes in weight, and thus in BMI, fewer analyses have focused on the relationship between changes in WC and blood pressure alterations. Considering that reductions in WC have been recommended more strongly than before for the purpose of prophylaxis and/or resolution of metabolic syndrome by the government in our country [23], the impact of WC reduction (gain) in terms of alterations of atherogenic risk factors, including blood pressure and levels of glucose and lipids, is becoming a more important issue to be investigated. Therefore, we also assessed whether changes in WC were reflected by the BPs change, and whether this relationship, if present, was independent of BMI change. We found that WC change was predictive of BPs change in men but not in women. In addition, the association between %dWC and %dBPs in men lost statistical significance after controlling for BMI1 and %dBMI (table 3). In contrast, %dBMI was a predictor for %dBPs in both genders regardless of the control of %dWC, suggesting that a reduction in BMI may represent a more essential target than WC reduction in terms of blood pressure control. This concept may be further supported by our finding that mean %dBPs did

not differ significantly between individuals with %dWC <0 and those with %dWC ≥0 among individuals with %dBMI <0. In reverse, %dBPs reduction was significantly greater in individuals with %dBMI <0 than in those with %dBMI ≥0 among individuals with %dWC <0 (fig. 1).

It has been reported that, in individuals with a mean BMI of 31, change in BMI was significantly correlated with change in BPs in both genders, even after adjusting for change in waist-hip ratio [24]. In the same study, it was reported that change in waist-hip ratio was not significantly correlated with change in BPs after adjusting for BMI change in men, and that the relationship between change in waist-hip ratio and BPs change was not significant before any adjustment in women. The results of Wing et al. [24] can be said to be similar to our current observation although there is a difference between WC and waist-hip ratio.

The current study has several limitations. First, we retrospectively analyzed data on individuals who underwent general health screening at our institute for 2 consecutive years; as a result, individuals who did not visit our institute the second year for unknown reasons were not enrolled in the current study, which may cause some biases. Second, we could not specify the reasons for weight gain or loss in individuals, however, very few individuals would have been taking antiobesity medications because only one individual in each gender had a BMI of 35 kg/m$^2$ or more at the first visit. Third, this study population included many non-obese subjects; a BMI1 ≥30 was found only in 1.1% of women and 1.9% of men. Fourth, we excluded those subjects who were taking antihypertensive drugs at either visit. We found that BMI was significantly greater in these excluded subjects than in the study population for both genders. Lastly, although

change in BMI may seem to be superior for predicting BPs change than changes in abdominal obesity, abdominal fat volume should be measured by more reliable methods, such as computed tomography, before conclusion. In addition, we have to follow the subjects for a longer period, as a recent study has shown that surrogate measures of abdominal obesity are stronger predictors of all-cause and cardiovascular death than BMI in the general population [25].

In conclusion, in individuals who underwent general health screening for consecutive years, percent change in WC was significantly associated with percent change in BPs in men, but not in women; although this association in men lost statistical significance after controlling for percent change in BMI. By contrast, percent change in BMI was significantly associated with percent change in BPs regardless of controlling for percent change in WC. Our data suggest that controlling BMI, and thus controlling body weight, may represent a more essential goal than a reduction in WC in terms of blood pressure lowering among Japanese individuals who are not taking antihypertensive medication.

### References

1 Dustan HP: Obesity and hypertension. Diabetes Care 1991;14:488–504.
2 Faria AN, Ribeiro Filho FF, Gouveia Ferreira SR, Zanella MT: Impact of visceral fat on blood pressure and insulin sensitivity in hypertensive obese women. Obes Res 2002;10: 1203–1206.
3 Sung KC, Ryu SH: Insulin resistance, body mass index, waist circumference are independent risk factor for high blood pressure. Clin Exp Hypertens 2004;26:547–556.
4 Harsha DW, Bray GA: Weight loss and blood pressure control (Pro). Hypertension 2008; 51:1420–1425.

5 Yap S, Yang Z, Wang J, Bacon SL, Campbell TS: Waist circumference, not body mass index, is associated with blood pressure in a sample of young Chinese adults. J Hum Hypertens 2006;20:904–906.
6 Feldstein AC, Nichols GA, Smith DH, Stevens VJ, Bachman K, Rosales AG, Perrin N: Weight change in diabetes and glycemic and blood pressure control. Diabetes Care 2008; 31:1960–1965.

7 Itoh K, Imai K, Masuda T, Abe S, Tanaka M, Koga R, Itoh H, Nakamura M: Association between blood pressure and insulin resistance in obese females during weight loss and weight rebound phenomenon. Hypertens Res 2001;24:481–487.
8 He J, Klag MJ, Whelton PK, Chen JY, Qian MC, He GQ: Body mass and blood pressure in a lean population in southwestern China. Am J Epidemiol 1994;139:380–389.
9 He Q, Ding ZY, Fong DY, Karlberg J: Blood pressure is associated with body mass index in both normal and obese children. Hypertension 2000;36:165–170.

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10  Ishizaka Y, Ishizaka N, Tani M, Toda A, Toda E, Koike K, Yamakado M: Association between changes in obesity parameters and incidence of chronic kidney disease in Japanese individuals. Kidney Blood Press Res 2009;32:141–149.

11  Sakurai M, Miura K, Takamura T, Ota T, Ishizaki M, Morikawa Y, Kido T, Naruse Y, Nakagawa H: Gender differences in the association between anthropometric indices of obesity and blood pressure in Japanese. Hypertens Res 2006;29:75–80.

12  Kokubo Y, Okamura T, Yoshimasa Y, Miyamoto Y, Kawanishi K, Kotani Y, Okayama A, Tomoike H: Impact of metabolic syndrome components on the incidence of cardiovascular disease in a general urban Japanese population: the Suita study. Hypertens Res 2008;31:2027–2035.

13  Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K, Nitta K, Yamagata K, Tomino Y, Yokoyama H, Hishida A: Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis 2009;53:982–992.

14  Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412–419.

15  Benetou V, Bamia C, Trichopoulos D, Mountokalakis T, Psaltopoulou T, Trichopoulou A: The association of body mass index and waist circumference with blood pressure depends on age and gender: a study of 10,928 non-smoking adults in the Greek EPIC cohort. Eur J Epidemiol 2004;19:803–809.

16  Majane OH, Norton GR, Maseko MJ, Makaula S, Crowther N, Paiker J, Thijs L, Brooksbank R, Sareli P, Staessen JA, Woodiwiss AJ: The association of waist circumference with ambulatory blood pressure is independent of alternative adiposity indices. J Hypertens 2007;25:1798–1806.

17  Haynes RB: Is weight loss an effective treatment for hypertension? The evidence against. Can J Physiol Pharmacol 1986;64:825–830.

18  Neter JE, Stam BE, Kok FJ, Grobbee DE, Geleijnse JM: Influence of weight reduction on blood pressure: a meta-analysis of randomized controlled trials. Hypertension 2003;42:878–884.

19  Moore LL, Visioni AJ, Qureshi MM, Bradlee ML, Ellison RC, D'Agostino R: Weight loss in overweight adults and the long-term risk of hypertension: the Framingham study. Arch Intern Med 2005;165:1298–1303.

20  Huang Z, Willett WC, Manson JE, Rosner B, Stampfer MJ, Speizer FE, Colditz GA: Body weight, weight change, and risk for hypertension in women. Ann Intern Med 1998;128:81–88.

21  Yang G, Xiang YB, Zheng W, Xu WH, Zhang X, Li HL, Shu XO: Body weight and weight change in relation to blood pressure in normotensive men. J Hum Hypertens 2007;21:45–52.

22  Truesdale KP, Stevens J, Cai J: Effect of 3-year weight history on blood pressure: the atherosclerosis risk in communities study. Obesity (Silver Spring) 2008;16:1112–1119.

23  Matsuzawa Y: Metabolic syndrome – definition and diagnostic criteria in Japan. J Atheroscler Thromb 2005;12:301.

24  Wing RR, Jeffery RW, Burton LR, Thorson C, Kuller LH, Folsom AR: Change in waist-hip ratio with weight loss and its association with change in cardiovascular risk factors. Am J Clin Nutr 1992;55:1086–1092.

25  Yusuf S, Hawken S, Ounpuu S, Bautista L, Franzosi MG, Commerford P, Lang CC, Rumboldt Z, Onen CL, Lisheng L, Tanomsup S, Wangai P Jr, Razak F, Sharma AM, Anand SS: Obesity and the risk of myocardial infarction in 27,000 participants from 52 countries: a case-control study. Lancet 2005;366:1640–1649.

Immunopathology and Infectious Diseases

# Tacrolimus Ameliorates Metabolic Disturbance and Oxidative Stress Caused by Hepatitis C Virus Core Protein

## Analysis Using Mouse Model and Cultured Cells

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Hepatic steatosis and insulin resistance are factors that aggravate the progression of liver disease caused by hepatitis C virus (HCV) infection. In the pathogenesis of liver disease and metabolic disorders in HCV infection, oxidative stress due to mitochondrial respiratory chain dysfunction plays a pivotal role. Tacrolimus (FK506) is supposed to protect mitochondrial respiratory function. We studied whether tacrolimus affects the development of HCV-associated liver disease using *HCV core gene* transgenic mice, which develop hepatic steatosis, insulin resistance, and hepatocellular carcinoma. Administration of tacrolimus to *HCV core gene* transgenic mice three times per week for 3 months led to a significant reduction in the amounts of lipid in the liver as well as in serum insulin. Tacrolimus treatment also ameliorated oxidative stress and DNA damage in the liver of the *core gene* transgenic mice. Tacrolimus administration reproduced these effects in a dose-dependent manner in HepG2 cells expressing the core protein. The intrahepatic level of tumor necrosis factor-$\alpha$, which may be a key molecule for the pathogenesis in HCV infection, was significantly decreased in tacrolimus-treated core gene transgenic mice. Tacrolimus thus reversed the effect of the core protein in the patho-
genesis of HCV-associated liver disease. These results may provide new therapeutic tools for chronic hepatitis C, in which oxidative stress and abnormalities in lipid and glucose metabolism contribute to liver pathogenesis. *(Am J Pathol 2009, 175:1515–1524; DOI: 10.2353/ajpath.2009.090102)*

Hepatitis C virus (HCV) is a major cause of liver disease; approximately 170 million people are chronically infected worldwide. Persistent HCV infection leads to the development of chronic hepatitis, cirrhosis, and, eventually, hepatocellular carcinoma (HCC), thereby being a serious problem from both medical and socioeconomic viewpoints.[1,2] Recently, a growing amount of evidence showing that HCV infection induces alteration in lipid[3–7] and glucose metabolism has accumulated.[8,9] Augmentation of oxidative stress is also substantiated in HCV infection by a number of clinical and basic studies.[10–13]

We demonstrated previously that the core protein of HCV induces HCC in transgenic mice that have marked hepatic steatosis in the absence of inflammation.[14] In this animal model for HCV-associated HCC, there is augmentation of oxidative stress in the liver during the incubation period.[10] Also noted is an accumulation of lipid droplets that are rich with carbon 18 monounsaturated fatty acids such as oleic and vaccenic acids, which is also observed in liver tissues of patients with chronic hepatitis C com-

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pared with those in patients with fatty liver due to simple obesity.[15] Recently, we have also shown, using the HCV transgenic mouse model, that the ability of insulin to lower plasma glucose levels is impaired in association with HCV infection,[16] which would be the basis for the frequent development of type 2 diabetes in patients with chronic hepatitis C.[8,9]

Disturbances in lipid and glucose metabolism are notable features of HCV infection and may be profoundly involved in the pathogenesis of liver diseases. Although the mechanism underlying these phenomena is not yet well understood, the development of clues to correct these metabolic disturbances occurring in HCV infection, which have been recently connected to the poor prognosis of patients with chronic hepatitis C, is awaited. Moreover, a key role for oxidative stress in the pathogenesis of hepatitis C,[11,12] which may be closely associated with the aforementioned metabolic disorders, has been identified. The association of oxidative stress augmentation in HCV infection with mitochondrial respiratory dysfunction[10,13,17] suggests that one possibility to ameliorate such a condition is the use of agents that can protect the mitochondrial respiratory function.

We have conducted information retrieval and screening for agents that can protect the mitochondrial respiratory function. Tacrolimus (FK506), which is widely used in organ transplantation, is one such agent with evidence showing protection of the mitochondrial respiratory function,[18–21] although it shows no antiviral effect. We explored, using transgenic mouse and cultured cell models that express the HCV core protein, whether tacrolimus improves metabolic disturbances including lipid and glucose homeostases as well as oxidative stress augmentation through a possible involvement of mitochondrial function.

## Materials and Methods

### Transgenic Mouse and Cultured Cells

The production of *HCV core gene* transgenic mice has been described previously.[6] Mice were cared for according to institutional guidelines with the approval by the institutional review board of the animal care committee, fed an ordinary chow diet (Oriental Yeast Co., Ltd., Tokyo, Japan), and maintained in a specific pathogen-free state. Because there is a sex preference in the development of liver lesions in the transgenic mice, we used only male mice. At least five mice were used in each experiment, and the data were subjected to statistical analysis. HepG2 cell lines expressing the HCV core protein under the control of the CAG promoter (Hep39J, Hep396, and Hep397) or a control HepG2 line (Hepswx) carrying the empty vector were described previously.[22,23] Bulk HepG2 cells were also used as a control.

### Reagents

Cholesterol esters and lipid standards were purchased from Sigma-Aldrich (St. Louis, MO), and glycogen and amyloglucosidase were obtained from Seikagaku Kogyo (Tokyo, Japan). Other chemicals were of analytical grade and were purchased from Wako Chemicals (Tokyo, Japan). Tacrolimus (FK506) was kindly provided by Astellas Pharma Inc. (Tokyo, Japan). Cyclosporine A (CyA) was purchased from Sigma-Aldrich.

### Administration of Tacrolimus and Cyclosporine A

Tacrolimus (0.1 mg/kg b.wt., suspended in mannitol and hydroxychlorinated caster oil [HCO-60]), or vehicle only was administered to the core gene transgenic or control mice i.p., three times per week for 3 months beginning at 3 months of age. For *in vitro* experiments, tacrolimus was added to the culture medium at the final concentration of 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 $\mu$mol/L. CyA was also added to the culture medium at the same concentrations.

### Assessment of Glucose Homeostasis

Blood was drawn at different time points from the tail vein, and plasma glucose concentrations were measured using an automatic biochemical analyzer (DRI-CHEM 3000V, Fuji Film, Tokyo, Japan). The levels of serum insulin were determined by radioimmunoassay (Biotrak, Amersham Pharmacia Biotech, Piscataway, NJ) using rat insulin as a standard. For the determination of the fasting plasma glucose level, the mice were fasted for >16 hours before the study. An insulin tolerance test was performed as described previously.[16]

### Lipid Extraction, Measurement of Triglyceride Content, and Analysis of Fatty Acid Compositions

Lipid extraction from the mouse liver tissues or cultured cells was performed as described previously.[15,24] For the analysis of fatty acid compositions, the residue was methanolysed by the modified Morrison and Smith method with boron trifluoride as a catalyst.[25] Fatty acid methyl esters were analyzed using a Shimadzu GC-7A gas chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with a 30-m-long × 0.3-mm diameter support coated with ethylene glycol succinate.[24]

### Evaluation of Oxidative and Antioxidative System

Lipid peroxidation was estimated spectrophotometrically using thiobarbituric acid-reactive substances and is expressed in terms of malondialdehyde formed per milligram protein. Reduced glutathione and oxidized glutathione levels were measured as described previously.[10] The total amount of glutathione was calculated by adding the amounts obtained for glutathione and oxidized glutathione. For the evaluation of DNA damage in cells, apurinic/apyrimidinic sites were determined using a DNA Damage Quantification Kit (Dojindo Molecular Technolo-

gies, Inc., Tokyo, Japan) following the manufacturer's protocol.

## Determination of Reactive Oxygen Species

Cells were plated onto glass coverslips and examined for reactive oxygen species (ROS) production as a marker for oxidative stress. They were loaded for 2 hours with chloromethyl 2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes Inc., Eugene, OR) at a final concentration of 10 $\mu$mol/L.[26] Results were expressed as relative fluorescence intensity and normalized to the control cells. In some experiments, ROS was measured after the incubation with tacrolimus or CyA.

## Measurement of Ketone Body Ratio

For the determination of ketone body ratio (KBR), cells were cultured to confluence on a 3.5-cm dish, and the medium was replaced with 700 $\mu$l of fresh medium. For arterial KBR, the mice were fasted for >16 hours, followed by the drawing of arterial blood. After a 24-hour incubation, acetoacetate and $\beta$-hydroxybutyrate in the medium were measured by monitoring the production or consumption of NADH with a Ketorex kit (Sanwa Chemical, Nagoya, Japan).[27] The KBR was calculated as the acetoacetate/$\beta$-hydroxybutyrate ratio.

## Microarray Analysis

An Affymetrix GeneChip analysis cDNA array system (Mouse Genome 430A 2.0, Kurabo, Osaka, Japan) was used for the analysis. Two thousand species of mouse DNA fragments were spotted on the filter. Genes that were 1.5-fold increased or decreased in both of the two tacrolimus-treated mice compared with mice treated with vehicle were defined as up-regulated or down-regulated, respectively.

## Real-Time PCR and Western Blotting

RNA was prepared from mouse liver tissues using TRIzol LS (Invitrogen, Carlsbad, CA). The first-strand cDNAs were synthesized with a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Franklin Lakes, NJ). The fluorescent signal was measured with an ABI Prism 7000 system (Applied Biosystems, Tokyo, Japan).

The genes encoding mouse *tumor necrosis factor (TNF)-$\alpha$, sterol regulatory element binding protein (SREBP)-1c, resistin, stearoyl-CoA desaturase (SCD)-1, and hypoxanthine phosphoribosyltransferase* were amplified with the primer pairs 5'-GACAAGGTGGGCTACGGGCTTG-3' and 5'-TCCCAAATGGGCTCCCTCT-3', 5'-ACGGAGCCATGG-ATTGCACATTTG-3' and 5'-TACATCTTTAAAGCAGCGG-GTGCCGATGGT-3', 5'-GAAGGCACAGCAGTCTTGA-3' and 5'-GCGACCTGCAGCTTACAG-3', 5'-TTCCCTCCTG-CAAGCTCTAC-3' and 5'-CGCAAGAAGGTGCTAAC-GAAC-3', and 5'-CCAGCAAGCTTGCAACCTTAACCA-3' and 5'-GTAATGATCAGTCAACGGGGGAC-3', respec-

tively. The sense and antisense primers were located in different exons to avoid false-positive amplification from contaminated genomic DNA. Each PCR product was confirmed as a single band of the correct size by agarose gel electrophoresis (data not shown).

## Reporter Assay for SREBP-1c Promoter Activity

A plasmid encoding firefly luciferase under the control of the *SREBP-1c* promoter (pGL3-srebp-1cPro) and a control plasmid encoding *Renilla* luciferase (Promega, Madison, WI) were transfected into 293T cells. Tacrolimus was added at a final concentration of 100 nmol/L to the culture medium of 293T cells transfected with pGL3-srebp-1cPro with or without an expression plasmid of HCV core protein at 24 hours after transfection. Cells were harvested 24 hours after treatment. Luciferase activity was measured by using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was standardized with that of *Renilla* luciferase, and the results are expressed as the fold-increase in relative luciferase units.

## Statistical Analysis

Data are presented as the mean $\pm$ SE. The significance of the difference in means was determined by a Mann-Whitney $U$ test wherever appropriate. $P < 0.05$ was considered significant.

## Results

### Effect of Tacrolimus on Insulin Resistance Induced by HCV

The *core gene* transgenic mice exhibit insulin resistance in the absence of obesity from the age of 2 months.[16] In tacrolimus-treated mice, there was a slight, but not significant, reduction in body weight compared with control mice at the end of tacrolimus administration at 6 months of age (Figure 1A). Tacrolimus administration to the *core gene* transgenic mice restored the plasma glucose levels to within normal limit (Figure 1B) ($P < 0.05$), whereas it caused no significant reduction in the control mice. The plasma glucose levels in the vehicle-treated core gene transgenic mice were higher than those in the core gene transgenic mice reported previously,[16] probably owing to the older age of mice in the current study than in the previous one. The levels of serum insulin were also significantly reduced by the treatment with tacrolimus for 3 months in the core gene transgenic mice, whereas there was no significant change in the control mice (Figure 1C). The reduction in both plasma glucose and serum insulin levels indicates that the administration of tacrolimus restored the resistance to insulin action, which is attributed to the suppression of insulin action in the liver by the core protein.[16] Actually, an insulin tolerance test (1 U/kg b.wt.) demonstrated the improvement of insulin action in the tacrolimus-treated core gene transgenic mice (Figure 1D).
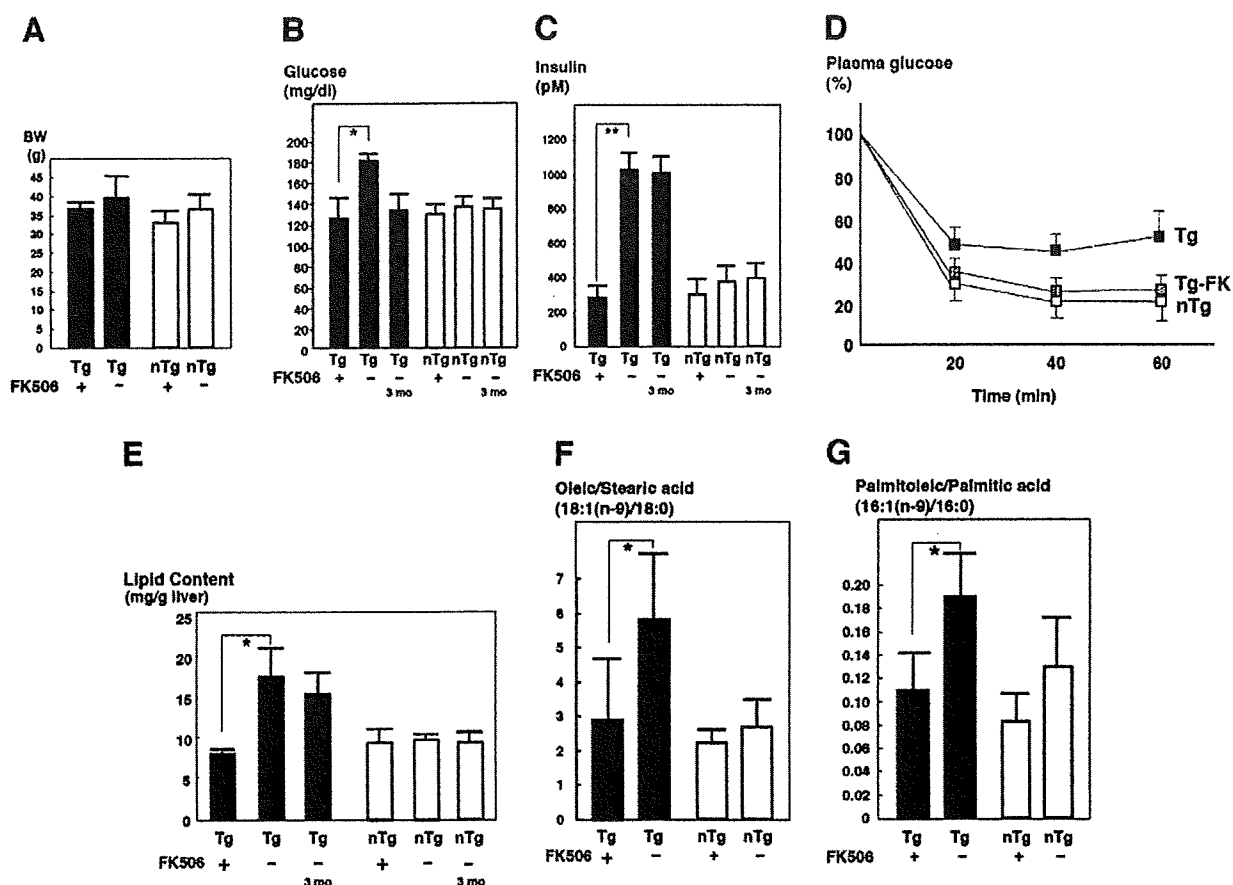
**Figure 1.** Effect of tacrolimus (FK506) on glucose and lipid metabolism in the core gene transgenic mice. Tacrolimus (0.1 mg/kg b.wt.) or vehicle was administered to core gene transgenic or control mice i.p., three times weekly for 3 months beginning at 3 months of age. **A:** Body weight at the baseline and end of treatment. **B:** Plasma glucose level. **C:** Serum insulin level. **D:** Insulin tolerance test. Black boxes represent core gene transgenic mice; white boxes represent control mice; gray boxes represent core gene transgenic mice treated with tacrolimus (Tg-FK). **E:** Total lipid content in the liver. **F:** Ratio of oleic/stearic acid [18:1(n-9)/18:0]. **G:** Ratio of palmitoleic/palmitic acid [16:1(n-9)/16:0]. black bars represent transgenic mice; white bars represent control mice. Tg 3 mo indicates 3-month-old transgenic mice showing the baseline state just before FK treatment, and Tg indicates 6-month-old transgenic mice, either with or without tacrolimus treatment for 3 months. Values represent the mean $\pm$ SE, $n = 5$ in each group. $*P < 0.05$. Tg, transgenic mice; nTg, nontransgenic control mice. $**P < 0.01$.

## Tacrolimus Improves Lipid Metabolism Disorders in Mice

We then studied whether tacrolimus administration affects lipid metabolism in the mice. The core gene transgenic mice developed a marked hepatic steatosis.[6,14] In addition, the composition of accumulated lipid was different from that in the fatty liver as a result of simple overnutrition: carbon 18 or 16 monounsaturated fatty acid levels were significantly increased.[15] As shown in Figure 1E, the tacrolimus treatment significantly reduced the lipid content in liver tissues compared with the vehicle treatment of the core gene transgenic mice ($P < 0.05$, $n = 5$ each), whereas there was no change in the control mice. The increased ratios of oleic to stearic acid [18:1 (n-9)/18:0] and palmitoleic to palmitic acid [16:1(n-9)/16: 0] in the core gene transgenic mice returned to levels similar to those in control mice (Figure 1, F and G) ($P < 0.05$). Thus, the administration of tacrolimus for 3 months restored the abnormalities in lipid metabolism that were induced by the core protein of HCV. Histologically, tacrolimus significantly improved steatosis in the liver of core gene transgenic mice, in which micro- and macrovesicular lipid droplets were accumulated in hepatocytes, chiefly around the central veins of the liver (Figure 2A). There was no sign of inflammation in the liver with or without the tacrolimus treatment.

## Effect of Tacrolimus on Lipid Metabolism in HepG2 Cells Expressing HCV Core Protein

To further prove the ameliorating effect of tacrolimus on lipid metabolism, we then performed experiments using HepG2 cells that express the core protein.[22,23] HepG2 cells, the lipid metabolism of which is somewhat different from that in normal hepatocytes,[28] show a significant increase in the level of 5,8,11-eicosatrienoic acid [20:3 (n-9)], as a result of activations of the fatty acid enzymes, $\Delta^9$-, $\Delta^6$-, and $\Delta^5$-desaturases, by the core protein (H. Miyoshi and K. Koike, unpublished data). Incubation of the core-expressing HepG2 cells with tacrolimus at 100 nmol/L and 1 $\mu$mol/L for 48 hours significantly reduced the accumulation of 20:3(n-9), whereas CyA treat-

## A

**Vehicle          FK506**

## B
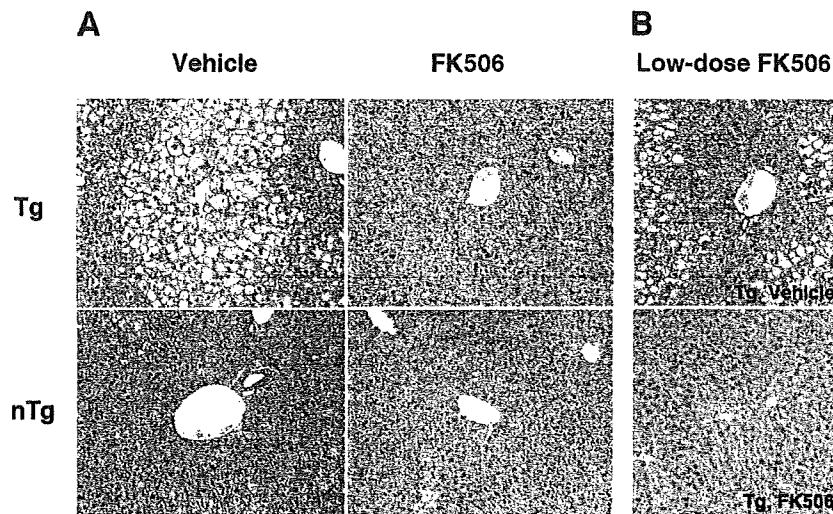
**Low-dose FK506**

**Tg**

**nTg**

Figure 2. Morphological analysis of the liver of the core gene transgenic mice. Representative cases are shown either treated with tacrolimus (FK506) or vehicle (H&E staining). A: There is a prominent improvement of steatosis in the 3-month tacrolimus-treated core gene transgenic mice compared with the vehicle-treated mice. B: A prominent improvement in steatosis was also obtained by the administration of one-fifth dose of tacrolimus for 1 month beginning at 3 months of age. For histological analysis, two independent researchers evaluated 40 microscopic fields each, and a representative picture is shown for each category. Original magnification, ×125. Tg, transgenic mice; nTg, nontransgenic control mice.

ment increased the level of 20:3(n-9) in a dose-dependent manner in the core-expressing HepG2 cells (Figure 3, A and B). Neither tacrolimus nor CyA changed the 20:3(n-9) content in HepG2 cells that do not express the core protein.

### Low Dose of Tacrolimus Also Ameliorates Steatosis and Insulin Resistance

Because the usual dose of tacrolimus for liver transplantation naturally induces an immunosuppressed state in patients, we conducted a mouse study with a tacrolimus dose lower than that in the aforementioned study. In this low-dose experiment, tacrolimus at 0.02 mg/kg b.wt. (one-fifth of the previous one) was administered to mice for 1 month from the age of 3 months. Similar to the results with the dose of 0.1 mg/kg b.wt., there were significant decreases in the lipid content in the liver (9.5 ± 0.8 [0.02 mg/kg b.wt. tacrolimus] versus 18.7 ± 4.4 [vehicle only] mg/g liver; $P < 0.05$) and serum insulin concentration (96.6 ± 16.9 [0.02 mg/kg b.wt. tacrolimus] versus 1137.1 ± 88.0 [vehicle only] pmol/L; $P < 0.05$) in

the core gene transgenic mice treated with tacrolimus. Histological changes are shown in Figure 2B.

### Effect of Tacrolimus on Oxidative Stress and Antioxidative System in Mice

We next examined whether the 3-month administration of tacrolimus affects the redox state in the core gene transgenic mice. In the liver of the core gene transgenic mice, the ROS level was higher than that in the liver of control mice as determined by lipid peroxidation.[10] Treatment with tacrolimus significantly reduced the level of thiobarbituric acid-reactive substances in the liver of the core gene transgenic mice (Figure 4A) ($P < 0.05$). As a result of oxidative stress overproduction, there was damage in the DNA of hepatocytes of the core gene transgenic mice from a young age.[10] To evaluate the effect of tacrolimus on the nuclear DNA damage, the apurinic/apyrimidinic site index was determined in liver tissues from the core gene transgenic mice. As shown in Figure 4B, the apurinic/apyrimidinic site index in the liver of the core gene transgenic mice, which was significantly higher

## A

**20:3 (n-9)**
**(%)**

FK506   0 nM   100 nM 1 μM        0 nM   100 nM 1 μM
                core(+)                     core(-)

## B

**C20:3 (n-9)**
**(%)**

CyA   0 nM  100 nM 1 μM        0 nM   100nM 1 μM
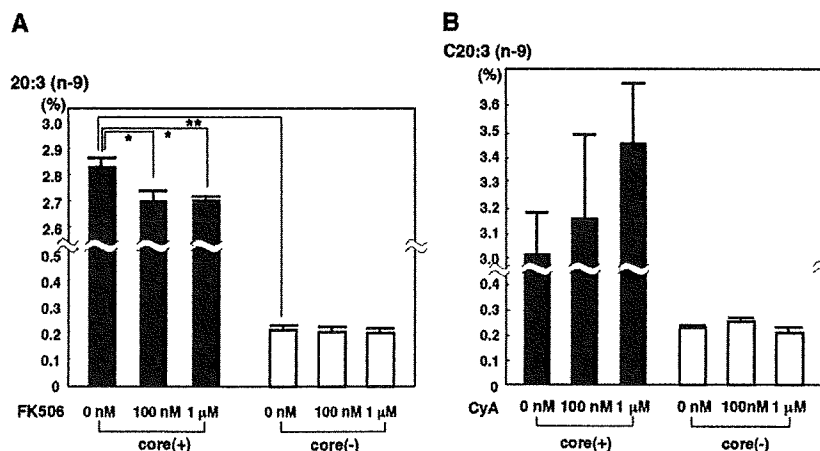              core(+)                   core(-)

Figure 3. Effect of tacrolimus (FK506) or CyA on fatty acid compositions in HepG2 cells expressing the core protein. The fatty acid compositions of the total cell lipids were analyzed, and the percentage of 5,8,11-eicosatrienoic acid [20:3(n-9)] in the core-expressing and control HepG2 cells was calculated. A: Treatment with tacrolimus at 0 nmol/L, 100 nmol/L, or 1 μmol/L. B: Treatment with CyA at 0 nmol/L, 100 nmol/L, or 1 μmol/L. Black bars represent core-expressing cells; white bars represent control cells. Because similar results were obtained by using Hep39J, Hep396, and Hep397 cell lines, representative results using the Hep39J cell line are shown. Values represent the mean ± SE; $n = 5$ in each group. *$P < 0.05$ and **$P < 0.01$.

**A**

TBARS
(nmol/g liver)



FK506

**B**

AP site
(index)



FK506

**C**

Glutathione
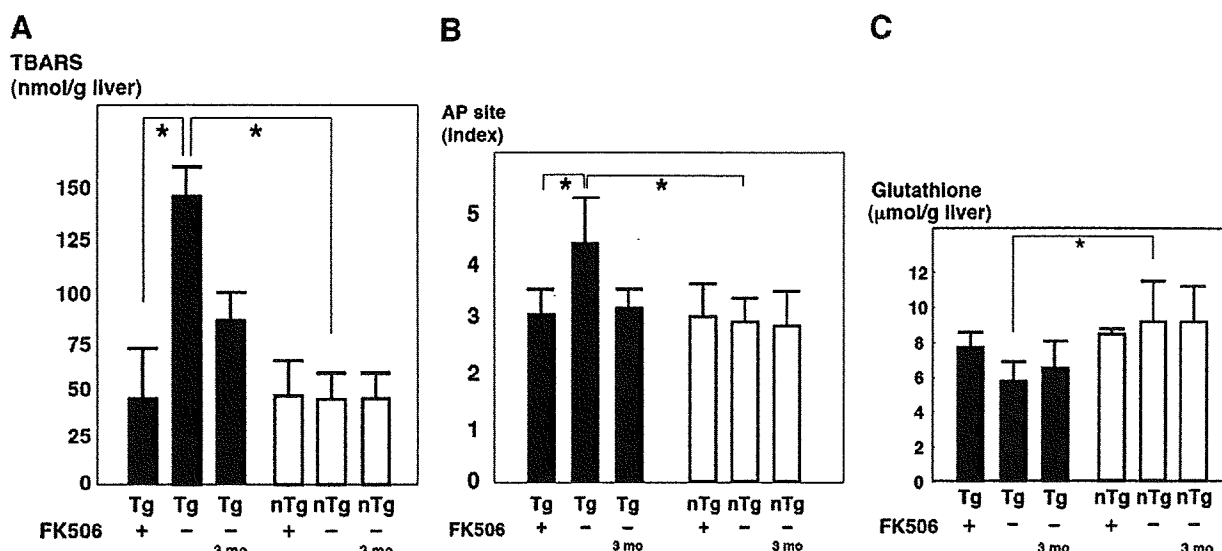(µmol/g liver)



FK506

**Figure 4.** Effect of tacrolimus (FK506) on oxidative stress in the core gene transgenic mice. Tacrolimus (0.1 mg/kg b.wt.) or vehicle only was administered to the core gene transgenic or control mice for 3 months. **A:** Lipid peroxidation in the liver. **B:** apurinic/apyrimidinic (AP) site in the liver as a marker of nuclear DNA damage; **C:** Total glutathione level in the liver. Black bars represent transgenic mice; white bars represent control mice. Tg 3 mo indicates 3-month-old transgenic mice, showing the baseline state just before tacrolimus treatment, and Tg indicates 6-month-old transgenic mice, either with or without 3 months of tacrolimus treatment. Values represent the mean ± SE; $n = 5$ in each group. *$P < 0.05$. Tg, transgenic mice; nTg, nontransgenic control mice. TBARS, thiobarbituric acid-reactive substances.

than that in the control mice, was significantly decreased by the tacrolimus treatment to a level similar to that in the control mice ($P < 0.05$).

The level of glutathione, one of the antioxidant systems, was significantly decreased in the liver of the core gene transgenic mice presumably as a result of oxidative stress overproduction but returned to a level similar to that in the control mice after the 3-month administration of tacrolimus, although the difference was not statistically significant ($P = 0.063$) (Figure 4C). Thus, the oxidative stress augmentation induced by the core protein of HCV was reduced by tacrolimus.

### Effect of Tacrolimus on Oxidative Stress in Core-Expressing HepG2 Cells

Evidence for scavenging ROS by the administration of tacrolimus to the mice prompted us to validate this finding using cultured cells. For this purpose, tacrolimus or CyA was added to the culture medium of HepG2 cells that express or do not express the core protein. After 24 hours of incubation, tacrolimus decreased the ROS production level in the core-expressing HepG2 cells in a dose-dependent manner (Figure 5A). In contrast, no decrease but rather an augmentation of ROS production was observed by the treatment with CyA at various concentrations (Figure 5B).

Because dysfunction of the mitochondrial respiratory chain complex 1 is suspected to be the reason for the ROS production associated with HCV infection (H. Miyoshi and K. Koike, unpublished data),[12,13,17] an increase in the NADH/NAD$^+$ ratio, which is caused by the repression of the complex 1 NADH dehydrogenase activity, would be a good marker for the mitochondrial complex 1 dys-

function. Therefore, we evaluated the effect of tacrolimus on the accumulation of NADH in the core-expressing HepG2 cells. The NADH/NAD$^+$ ratio, which is strictly estimated from a reciprocal of KBR,[26,29] was significantly higher in the core gene transgenic mice than in control mice (1/atrial KBR) and in HepG2 cells expressing the core protein than in control cells (1/KBR) (Figure 6A). By the treatment with 1 µmol/L tacrolimus, the ratio significantly decreased compared with the baseline (Figure 6B), whereas CyA treatment caused no effect in the core-expressing HepG2 cells (Figure 6C), as was the
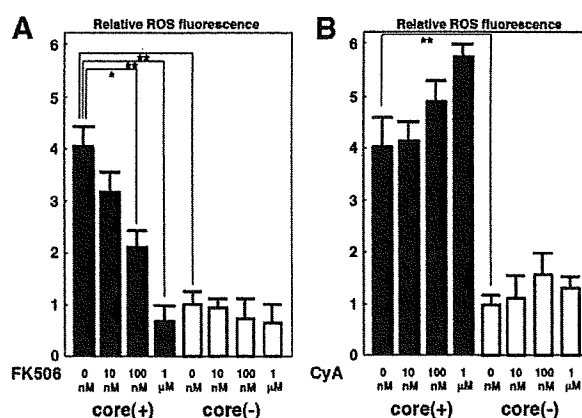
**A**    Relative ROS fluorescence



FK506

core(+)    core(-)

**B**    Relative ROS fluorescence



CyA

core(+)    core(-)

**Figure 5.** Effect of tacrolimus (FK506) or CyA on ROS production in HepG2 cells expressing the core protein. Results are expressed as relative brightness and normalized to control cells. **A:** Treatment with tacrolimus at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 µmol/L. **B:** Treatment with CyA at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 µmol/L. Black bars represent transgenic mice; white bars represent control cells. Because similar results were obtained by using Hep39J, Hep396, and Hep397 cell lines, representative results using the Hep39J cell line are shown. Values represent the mean ± SE; $n = 5$ in each group. *$P < 0.05$; **$P < 0.01$.

case in the determination of ROS by chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate.

## Changes in Gene Expression by Tacrolimus Treatment of Mice

We then performed a comprehensive microarray analysis of gene expression in the liver, which was up- or down-regulated by tacrolimus. For this analysis, the tacrolimus-treated mice were compared with the vehicle-treated mice, in two pairs of the core gene transgenic and control mice, respectively. Genes that were 1.5-fold increased or decreased in both of the two tacrolimus-treated mice



compared with those treated with vehicle were defined as up-regulated or down-regulated, respectively. As shown in Table 1, several genes were found to be up-regulated or down-regulated in both the core gene transgenic and control mice after the treatment with tacrolimus for 3 months. A number of genes including that for TNF-α were up- or down-regulated both in the core gene transgenic and control mice. In contrast, the expressions of some genes including that for resistin were differentially regulated between the core gene transgenic and control mice. The expressions of these genes were confirmed by real-time PCR analysis.

Then, to explore the mechanism by which tacrolimus reverses the pathological effect of the core protein in the liver, we examined, by real-time PCR analysis, the expression of some cellular genes including TNF-α, SREBP-1c, SCD-1, and proteasome activator 28-γ. These genes or gene products have been suggested to play a pivotal role in the pathogenesis of HCV-associated liver disease.[30,31] TNF-α and SREBP-1c genes have been shown to be up-regulated in the liver of the core gene transgenic mice and considered to play a role in the development of insulin resistance and steatosis.[30,31] By the treatment of the core gene transgenic mice with tacrolimus for 3 months, there was a significant decrease in the mRNA level of both TNF-α and SREBP-1c (Figure 7, A–C) ($P <$ 0.05). The SCD-1 mRNA level was also reduced in the tacrolimus-treated core gene transgenic mice. Because down-regulation of SREBP-1c expression by tacrolimus was observed only in the core gene transgenic mice but not in control mice, it is estimated that tacrolimus antagonizes the action of core protein in its transactivating function of the SREBP-1c promoter. The down-regulation of SREBP-1c, then, would lead to the suppression of SCD-1 expression and amelioration of steatosis. We confirmed this by conducting luciferase assays using cultured cells. As shown in Figure 7D, tacrolimus cancelled the effect of the core protein on the activation of SREBP-1c gene promoter. The level of the proteasome activator 28-γ protein, which is indispensable for the action of the core protein in the pathogenesis of HCV-associated liver lesion,[31] was determined by Western blotting, but there was no change caused by the tacrolimus treatment (data not shown).
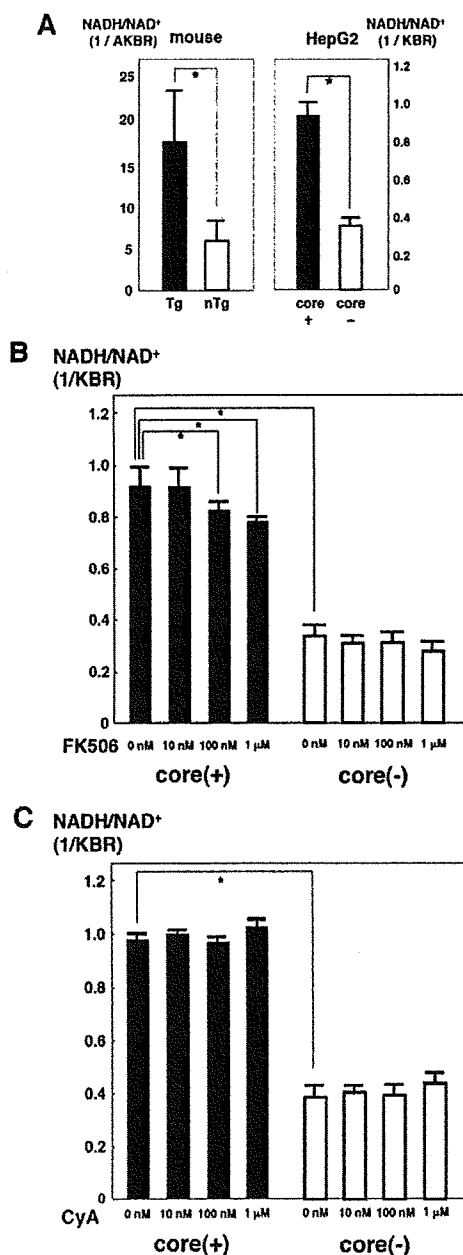
## Discussion

Antiviral treatment for chronic hepatitis C has advanced markedly. Nearly 50% of patients with chronic hepatitis C

**Figure 6.** Effect of tacrolimus (FK506) or CyA on NADH accumulation in HepG2 cells expressing the core protein. **A:** NADH/NAD⁺ was determined in mice (left) or HepG2 cells (right) with or without the core protein. **B:** The ketone body ratio was determined in HepG2 cells with or without the core protein after incubation with tacrolimus for 24 hours at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μmol/L. **C:** The ketone body ratio was determined in HepG2 cells with or without the core protein after incubation with CyA for 24 hours at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μmol/L. Black bars represent transgenic mice; white bars represent control cells. Because similar results were obtained by using Hep39J, Hep396 and Hep397 cell lines, representative results using the Hep39J cell line are shown. Values represent the mean ± SE; $n = 5$ in each group. *$P < 0.05$. AKBR, arterial KBR; Tg, transgenic mice; nTg, nontransgenic mice.

**Table 1.**    Genes Whose Expression Levels in the Mouse Liver Were Altered by the Treatment with FK506

|  | Up-regulated in Tg | Down-regulated in Tg |
|---|---|---|
| Up-regulated in nTg | Nuclear factor, erythroid derived 2<br>DNA segment, human D6S2654E<br>Fatty acid binding protein 5<br>  epidermal squalene epoxidase<br>Zinc finger protein 69 | Resistin<br>Resistin like alpha<br>Nuclear receptor subfamily 4, group A, member<br>  insulin-like growth factor binding protein 1<br>  calcium and integrin binding family member 3 |
| Down-regulated in nTg | X-linked lymphocyte-regulated 4<br>Cytochrome P450, family 2,<br>  subfamily b, polypeptide 9<br>X-linked lymphocyte-regulated 3a<br>Signal sequence receptor, delta | Tumor necrosis factor alpha<br>Cytochrome P450, family 17, subfamily a,<br>  polypeptide 1<br>B-cell leukemia/lymphoma 6 |

Genes with altered expression in Tg (columns) or in nTg (rows) are described in a 4 × 4 table. Genes that were 1.5-fold increased or decreased in both of the two FK506-treated mice compared with those treated with placebo were defined as up-regulated or down-regulated, respectively. Tg, core gene transgenic mouse; nTg, nontransgenic control mouse.

with HCV genotype 1 and high viral loads achieve a sustained virological response as a result of ribavirin/peginterferon combination therapy.[32,33] However, the remaining patients who could not achieve sustained virological response continue to experience progression of chronic hepatitis and have a high probability for development of HCC. Although therapies with new agents such as viral protease or RNA polymerase inhibitors are being developed, there is hope for development of the means to retard the progression of chronic hepatitis.

Recently, evidence showing that hepatic steatosis and insulin resistance are crucial determinants of the progression of liver fibrosis has accumulated.[34-37] Moreover, the importance of oxidative stress, which is closely associated with metabolic disorders such as insulin resistance and steatosis, is implicated in the pathogenesis of HCV-associated liver disease. Given the suggested association of oxidative stress augmentation with the dysfunction of mitochondrial respiration in HCV infection,[12,13,17] one possibility to ameliorate such a condition is the use of agents that can protect the mitochondrial respiratory function. Tacrolimus is one such agent with evidence of providing protection of the mitochondrial respiratory function,[18-21] although it does not show an antiviral effect.

In the current study, the administration of tacrolimus significantly improved the disturbances in lipid and glucose metabolism both *in vivo* and *in vitro*. As disorders of

lipid metabolism associated with HCV infection, hepatic steatosis and increases in monounsaturated fatty acid levels have been demonstrated.[3,4,6,7,15] The latter is caused by the activation of fatty acid enzymes such as $\Delta^9$- or $\Delta^6$-desaturase, resulting in increases in 18:1(n-9)/18:0 and 16:1(n-9)/16:0 ratios (H. Miyoshi and K. Koike, unpublished data).[15] Tacrolimus ameliorated these lipid alterations associated with HCV infection with no impact on mouse body weight. Tacrolimus also improved the insulin resistance in the HCV mouse model, in which tyrosine phosphorylation of insulin receptor substrate-1 is impaired by the HCV core protein.[16]

Moreover, tacrolimus treatment ameliorated oxidative stress augmentation, which is considered to play a pivotal role in the progression of liver disease or the development of HCC in HCV infection.[10-13] In mice transgenic for the HCV core gene, in which DNA damage develops because of oxidative stress augmentation,[13] tacrolimus decreased the levels of peroxylipid and DNA damage formations. Dysfunction of the mitochondrial respiratory chain complex 1 is suspected to be a source of ROS overproduction in HCV infection.[12,13,17] To assess changes in mitochondrial complex 1 function caused by tacrolimus, the NADH/NAD$^+$ ratio, which reflects the complex 1 NADH dehydrogenase activity, was determined in HepG2 cells expressing the core protein. The NADH/NAD$^+$ ratio, which is strictly estimated from a reciprocal of KBR (1/atrial KBR),[26,29] was significantly re-
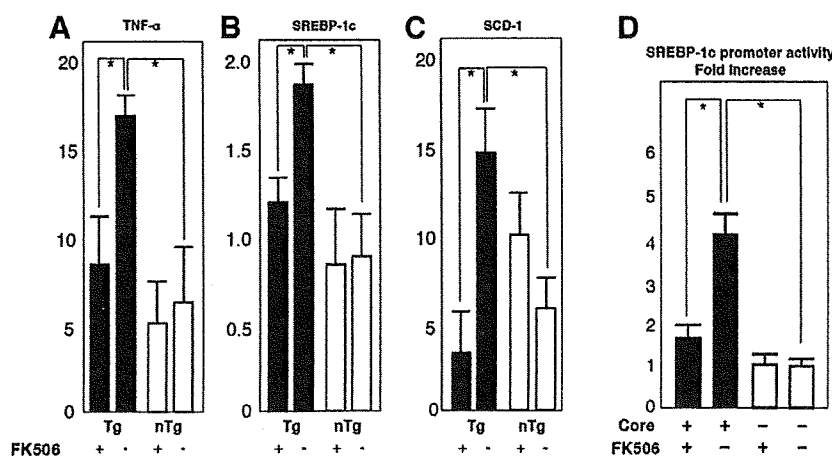


Figure 7. A–C: Effect of tacrolimus (FK506) on mRNA levels of cellular genes. The mRNA levels of TNF-α (A), SREBP-1c (B), and SCD-1 (C) genes were determined by real-time PCR analysis in the tacrolimus- or vehicle-treated mouse livers. The transcriptions of the genes were normalized with that of hypoxanthine phosphoribosyltransferase, and the values are expressed as relative activities. D: Effect of tacrolimus on the transactivating function of the core protein on the SREBP-1c promoter. A luciferase assay was performed using a plasmid encoding firefly luciferase under the control of the SREBP-1c promoter with or without the expression of HCV core protein. Tacrolimus was added at a final concentration of 100 nmol/L to the culture medium. Black bars represent transgenic mice; white bars represent control cells. Values represent the mean ± SE; $n = 5$ in each group. *$P < 0.05$. Tg, transgenic mice; nTg, nontransgenic mice.

duced by the addition of tacrolimus but not CyA. Thus, tacrolimus protected the mitochondrial respiratory chain complex 1 function from the impact of the core protein, decreased oxidative stress, and improved steatosis and insulin resistance.

Some of features induced by the core protein including steatosis, insulin, and DNA damage were already present in the core gene transgenic mice at 3 months of age as the baseline, and those were improved by tacrolimus treatment. This fact indicates that tacrolimus is not only preventing the development of core-induced features but also reversing such changes in the mouse liver.

The tacrolimus dose used in the current study was 0.1 mg/kg b.wt. This is the same dose as that used in recipients of liver or kidney transplantation. The result of a subexperiment with a lower tacrolimus dose of 0.02 mg/kg b.wt. was similar to that with the dose of 0.1 mg/kg b.wt. This finding is promising because it indicates that the "anti-core protein effect" may be achievable at such a low dose of tacrolimus without provoking strong immunosuppression. The tacrolimus concentration (100 nmol/L) that caused the anti-core protein effect in the cultured cell study is similar to that in the blood of recipients of liver transplantation and much lower than those used in previous studies.[19,38] In the current study, tacrolimus was administered only i.p., although it tacrolimus is administered i.v. or p.o. in humans. Therefore, a concern may arise regarding the administration route. Because the bioavailability of tacrolimus is approximately 25% (range from 5 to 93%) in human patients,[39] a difference in the concentrations of tacrolimus may be possible between i.p. and p.o. administration. However, in human patients, target levels of tacrolimus concentration are generally achieved by p.o. administration as the maintenance therapy. Therefore, the target concentration would be achieved in mouse models by p.o. administration for 3 months as it is in human patients. Our current results strongly support the notion that tacrolimus can protect the mitochondrial respiratory function, resulting in a reduction of ROS production.

There is also a controversy concerning the effect of tacrolimus on glucose homeostasis. Post-transplantation diabetes is a complication in kidney or liver transplantation.[40,41] In vivo and in vitro studies have shown that tacrolimus may inhibit insulin secretion from the pancreatic $\beta$-cells.[40] Thus, tacrolimus may have a potential to induce diabetes. However, there have been no well designed studies on this specific point: in one study, corticosteroid withdrawal from tacrolimus-based immunosuppression reduced insulin resistance without changing insulin secretion.[41] In our study using the HCV mouse model, tacrolimus administration at the dose similar to those in organ transplant recipients decreased serum insulin levels without increasing plasma glucose levels. These results point toward the future use of tacrolimus in vivo for the amendment of metabolic abnormalities, such as steatosis and insulin resistance, associated with HCV infection. However, it should be noted that there is a difference between our mouse model and human patients. Organ transplant recipients generally have injury to other bodily organs after a prolonged course of illness,

whereas the mouse model we have exploited does not. In addition, our mouse model originally has insulin resistance with the presence of hyperplasia of Langerhans islands.[16] Therefore, the effect of tacrolimus on glucose homeostasis in the current mouse study may not be exactly applicable to human patients.

The results of the gene expression analysis by microarray and subsequent real-time PCR were of considerable interest. Tacrolimus reduced the mRNA levels of TNF-$\alpha$, SCD-1, and SREBP-1c genes, which are elevated in both patients with chronic hepatitis C and HCV core gene transgenic mice.[30,31] The elevation in the TNF-$\alpha$ level causes insulin resistance in vivo, which is also observed in HCV core gene transgenic mice.[16] The elevations in SREBP-1c and SCD-1 gene mRNA levels cause the overproduction of triglycerides, leading to the development of steatosis. The reductions in the expression levels of these genes may explain the effect of tacrolimus on the improvement of steatosis, insulin resistance, and oxidative stress in these HCV models. Although recent investigations have shown that the immunosuppressive drugs tacrolimus and rapamycin inhibit the expression of different inflammatory mediators,[42,43] the anti-inflammatory functions of these drugs are not well established. Our in vitro and in vivo experiments confirmed that tacrolimus inhibited the induction of ROS generation, which is mediated by the core protein. Our data indicate that the inhibition of ROS formation may explain part of the favorable effect of immunosuppressive agents on inflammatory conditions.

In conclusion, our results demonstrate that tacrolimus has protective potential against damage caused by the HCV core protein including the induction of steatosis, insulin resistance, and oxidative stress, both in mice and cultured cells. Although more studies are required to elucidate the precise mechanism underlying the potential of tacrolimus in reversing the pathogenesis in HCV infection, these results may provide new therapeutic tools for chronic hepatitis C, in which oxidative stress and abnormalities in lipid and glucose metabolism contribute to liver pathogenesis.

## References

1. Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, Watanabe Y, Koi S, Onji M, Ohta Y, Choo Q, Houghton M, Kuo G: Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc Natl Acad Sci USA 1990, 87:6547–6549
2. Simonetti RG, Camma C, Fiorello F, Cottone M, Rapicetta M, Marino L, Fiorentino G, Craxì A, Ciccaglione A, Giuseppetti R, Stroffolini T, Pagliaro L: Hepatitis C virus infection as a risk factor for hepatocellular carcinoma in patients with cirrhosis. Ann Intern Med 1992, 116:97–102
3. Scheuer PJ, Ashrafzadeh P, Sherlock S, Brown D, Dusheiko GM: The pathology of chronic hepatitis C. Hepatology 1992, 15:567–571
4. Bach N, Thung SN, Schaffner F: The histological features of chronic hepatitis C and autoimmune chronic hepatitis: a comparative analysis. Hepatology 1992, 15:572–577
5. Barba G, Harper F, Harada T, Kohara M, Goulinet S, Matsuura Y, Eder G, Schaff Z, Chapman MJ, Miyamura T, Bréchot C: Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc Natl Acad Sci USA 1997, 94:1200–1205
6. Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, Miyamura T, Koike K: Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol 1997, 78:1527–1531

7. Lerat H, Honda M, Beard MR, Loesch K, Sun J, Yang Y, Okuda M, Gosert R, Xiao SY, Weinman SA, Lemon SM: Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology 2002, 122:352–365

8. Caronia S, Taylor K, Pagliaro L, Carr C, Palazzo U, Petrik J, O'Rahilly S, Shore S, Tom BD, Alexander GJ: Further evidence for an association between non-insulin-dependent diabetes mellitus and chronic hepatitis C virus infection. Hepatology 1999, 30:1059–1063

9. Mehta SH, Brancati FL, Sulkowski MS, Strathdee SA, Szklo M, Thomas DL: Prevalence of type 2 diabetes mellitus among persons with hepatitis C virus infection in the United States. Ann Intern Med 2000, 133:592–599

10. Choi J, Ou JH: Mechanisms of liver injury. III. Oxidative stress in the pathogenesis of hepatitis C virus. Am J Physiol Gastrointest Liver Physiol 2006, 290:G847–G851

11. Koike K, Miyoshi H: Oxidative stress and hepatitis C viral infection. Hepatol Res 2006, 34:65–76

12. Korenaga M, Wang T, Li Y, Showalter LA, Chan T, Sun J, Weinman SA: Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. J Biol Chem 2005, 280:37481–37488

13. Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Miyazawa T, Ishibashi K, Horie H, Imai K, Miyamura T, Kimura S, Koike K: Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. Cancer Res 2001, 61:4365–4370

14. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Matsuura Y, Kimura S, Miyamura T, Koike K: The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998, 4:1065–1067

15. Moriya K, Todoroki T, Tsutsumi T, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Takayama T, Makuuchi M, Watanabe K, Miyamura T, Kimura S, Koike K: Increase in the concentration of carbon 18 monounsaturated fatty acids in the liver with hepatitis C: analysis in transgenic mice and humans. Biophys Biochem Res Commun 2001, 281:1207–1212

16. Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Kimura S, Moriya K, Koike K: Hepatitis C virus and diabetes: direct involvement of the virus in the development of insulin resistance. Gastroenterology 2004, 126:840–848

17. Piccoli C, Scrima R, Quarato G, D'Aprile A, Ripoli M, Lecce L, Boffoli D, Moradpour D, Capitanio N: Hepatitis C virus protein expression causes calcium-mediated mitochondrial bioenergetic dysfunction and nitro-oxidative stress. Hepatology 2007, 46:58–65

18. Cetinkale O, Konukoğlu D, Senel O, Kemerli GD, Yazar S: Modulating the functions of neutrophils and lipid peroxidation by FK506 in a rat model of thermal injury. Burns 1999, 25:105–112

19. Kaibori M, Inoue T, Tu W, Oda M, Kwon AH, Kamiyama Y, Okumura T: FK506, but not cyclosporin A, prevents mitochondrial dysfunction during hypoxia in rat hepatocytes. Life Sci 2001, 69:17–26

20. Keswani SC, Chander B, Hasan C, Griffin JW, McArthur JC, Hoke A: FK506 is neuroprotective in a model of antiretroviral toxic neuropathy. Ann Neurol 2003, 53:57–64

21. Kaymaz M, Emmez H, Bukan N, Dursun A, Kurt G, Paçsaoğlu H, Paçsaoğlu A: Effectiveness of FK506 on lipid peroxidation in the spinal cord following experimental traumatic injury. Spinal Cord 2005, 43:22–26

22. Ruggieri A, Murdolo M, Harada T, Miyamura T, Rapicetta M: Cell cycle perturbation in a human hepatoblastoma cell line constitutively expressing hepatitis C virus core protein. Arch Virol 2004, 149:61–74

23. Aizaki H, Harada T, Otsuka M, Seki N, Matsuda M, Li YW, Kawakami H, Matsuura Y, Miyamura T, Suzuki T: Expression profiling of liver cell lines expressing entire or parts of hepatitis C virus open reading frame. Hepatology 2002, 36:1431–1438

24. Todoroki T, Imai K, Matsumoto K, Kano S: Initial deactivation of Florisil adsorbent for column chromatographic separation of lipids. Analyst 1983, 108:1267–1269

25. Morrison WR, Smith LM: Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. J Lipid Res 1964, 5:600–608

26. Gelasco AK, Raymond JR: Indoxyl sulfate induces complex redox alterations in mesangial cells. Am J Physiol Renal Physiol 2006, 290:F1551–F1558

27. Williamson DH, Mellanby J, Krebs HA: Enzymic determination of $D(-)$-$\beta$-hydroxybutyric acid and acetoacetic acid in blood. Biochem J 1962, 82:90–96

28. Choi Y, Park Y, Pariza MW, Ntambi JM: Regulation of stearoyl-CoA desaturase activity by the *trans*-10,*cis*-12 isomer of conjugated linoleic acid in HepG2 cells. Biochem Biophys Res Commun 2001, 284:689–693

29. Williamson DH, Lund P, Krebs HA: The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. Biochem J 1967, 103:514–527

30. Tsutsumi T, Suzuki T, Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Matsuura Y, Kimura S, Koike K, Miyamura T: Intrahepatic cytokine expression and AP-1 activation in mice transgenic for hepatitis C virus core protein. Virology 2002, 304:415–424

31. Moriishi K, Mochizuki R, Moriya K, Miyamoto H, Mori Y, Abe T, Murata S, Tanaka K, Suzuki T, Miyamura T, Koike K, Matsuura Y: Critical role of PA28γ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. Proc Natl Acad Sci USA 2007, 104:1661–1666

32. Hoofnagle JH, Seeff LB: Peginterferon and ribavirin for chronic hepatitis C. N Engl J Med 2006, 355:2444–2451

33. Koike K: Antiviral treatment of hepatitis C: present status and future prospects. J Infect Chemother 2006, 12:227–232

34. Adinolfi LE, Gambardella M, Andreana A, Tripodi MF, Utili R, Ruggiero G: Steatosis accelerates the progression of liver damage of chronic hepatitis C patients and correlates with specific HCV genotype and visceral obesity. Hepatology 2001, 33:1358–1364

35. Patton HM, Patel K, Behling C, Tripodi MF, Utili R, Ruggiero G: The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients. J Hepatol 2004, 40:484–490

36. Hui JM, Sud A, Farrell GC, Bandara P, Byth K, Kench JG, McCaughan GW, George J: Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression. Gastroenterology 2003, 125:1695–1704

37. Hickman IJ, Powell EE, Prins JB, Clouston AD, Ash S, Purdie DM, Jonsson JR: Insulin resistance is associated with chronic hepatitis C and virus infection fibrosis progression. J Hepatol 2003, 39:1042–1048

38. Han SY, Chang EJ, Choi HJ, Kwak CS, Suh SI, Bae JH, Park SB, Kim HC, Mun KC: Effect of tacrolimus on the production of oxygen free radicals in hepatic mitochondria. Transplant Proc 2006, 38:2242–2243

39. Staatz CE, Tett SE: Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplantation. Clin Pharmacokinet 2004, 43:623–653

40. Penfornis A, Kury-Paulin S: Immunosuppressive drug-induced diabetes. Diabetes Metab 2006, 32:539–546

41. van Hooff JP, Christiaans MH, van Duijnhoven EM: Evaluating mechanisms of post-transplant diabetes mellitus: Nephrol Dial Transplant 2004, 19(Suppl 6):vi8–vi12

42. Vigil SV, de Liz R, Medeiros YS, Fröde TS: Efficacy of tacrolimus in inhibiting inflammation caused by carrageenan in a murine model of air pouch. Transpl Immunol 2008, 19:25–29

43. Pereira R, Medeiros YS, Fröde TS: Antiinflammatory effects of tacrolimus in a mouse model of pleurisy. Transpl Immunol 2006, 16:105–111

# Proteomics Analysis of Mitochondrial Proteins Reveals Overexpression of a Mitochondrial Protein Chaperon, Prohibitin, in Cells Expressing Hepatitis C Virus Core Protein

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The hepatitis C virus (HCV) core protein is involved in viral pathogenesis such as oxidative stress induction and lipid metabolism disturbance, and is primarily located in the cytoplasm and endoplasmic reticulum in association with lipid droplets as well as in the mitochondria. To clarify the impact of the core protein on mitochondria, we analyzed the expression pattern of mitochondrial proteins in core protein-expressing cells by two-dimensional polyacrylamide gel electrophoresis. Several proteins related to the mitochondrial respiratory chain or protein chaperons were identified by mass spectrometry. Among the identified proteins with consistently different expressions, prohibitin, a mitochondrial protein chaperon, was up-regulated not only in core-expressing cells but also in full-genomic replicon cells and livers of core-gene transgenic mice. The stability of prohibitin was increased through interaction with the core protein. Further analysis demonstrated that interaction of prohibitin with mitochondrial DNA-encoded subunits of cytochrome c oxidase (COX) was disturbed by the core protein, resulting in a significant decrease in COX activity. *Conclusion:* The HCV core protein affects the steady-state levels of a subset of mitochondrial proteins including prohibitin, which may lead to an impaired function of the mitochondrial respiratory chain with the overproduction of oxidative stress. (HEPATOLOGY 2009;50:378-386.)

The hepatitis C virus (HCV) is a causative agent of chronic hepatitis, which often leads to cirrhosis and, eventually, to the development of hepatocellular carcinoma (HCC). However, the mechanism of hepatocarcinogenesis in HCV infection is not yet fully elucidated. The HCV core protein forms the viral nucleocapsid protein and has various properties that modulate cellular processes in numerous ways. The core protein binds to cellular proteins, suppresses or enhances apoptosis, and modulates the transcription of some host genes.[1] In addition, transgenic mice expressing the core protein develop HCC,[2-4] indicating a direct contribution of the core protein to the pathogenesis of hepatitis C.

The core protein is mostly localized to the endoplasmic reticulum (ER), but we and other groups have shown its localization to the mitochondria in cultured cells and transgenic mice.[2,5,6] In addition, the double structure of mitochondrial membranes is disrupted in hepatocytes of core-gene transgenic mice.[2-4] Evidence suggests that the core protein modulates some mitochondrial functions, including fatty acid β-oxidation, the impairment of which may induce lipid abnormalities and hepatic steatosis. In addition, the mitochondrion is an important source of reactive oxygen species (ROS). In livers of transgenic

mice harboring the core gene, increased ROS production has been observed.[7-9] A recent study found, by the proteomic profiling of biopsy specimens, that an impairment in key mitochondrial processes, including fatty acid oxidation and oxidative phosphorylation, and in the response to oxidative stress occurs in HCV-infected human liver with advanced fibrosis.[10] Therefore, it is probable that the HCV core protein affects mitochondrial functions because such pathogenesis is observed in both HCV core-transgenic mice and HCV-infected patients.[11-13]

The recent progress in proteomics has opened new avenues for disease-related biomarker discovery. Among proteomics approaches, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a technique for the separation and identification of proteins in a sample by displacement in two dimensions oriented at right angles to one another. This method is generally used as a component of proteomics and is the step used for the isolation of proteins for further characterization by mass spectrometry. 2D-PAGE is particularly useful when comparing two related samples such as healthy and diseased tissue. For example, proteins that are more abundant in diseased tissue may represent novel drug targets or diagnostic markers. In fact, several candidate biomarkers for many human cancers have been identified by this approach.[14] There are, however, tens of thousands of proteins in a cell, differing in abundance over six orders of magnitude. 2D-PAGE is not sensitive enough to detect rare proteins, and hence many proteins are not resolved. Therefore, splitting a sample into different fractions is often necessary to reduce the complexity of protein mixtures prior to 2D-PAGE. For this advantage, Lescuyer et al.[15] performed a 2D-PAGE of human mitochondrial proteins derived from the placenta and identified proteins mainly by peptide mass fingerprinting.

In this study, we performed a 2D-PAGE of mitochondria isolated from HepG2 cells stably expressing the HCV core protein and identified several proteins of different expressions when compared with control HepG2 cells. Among up-regulated proteins in the core-expressing cells, we focused on prohibitin, which functions as a mitochondrial protein chaperon, and found that the core protein interacts with prohibitin and represses the interaction between prohibitin and subunit proteins of cytochrome c oxidase (COX), which may lead to decreases in the expression level of the proteins and in COX activity. These results may explain the pathogenesis of liver disease in HCV infection including ROS induction.

## Materials and Methods

*Cells and Purification of Mitochondria.* Hep39 cells,[16] which stably express the HCV core protein, and control HepG2 cells (Hepswx) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1 mg/mL G418. Mitochondria were purified using Nycodenz (Nycomed Pharma, Zürich, Switzerland) according to the protocols reported by Okado-Matsumoto et al.[17] For transient transfection experiments, HepG2 cells were transfected with a core-expression plasmid using TransIT-LT1 (Mirus Bio, Madison, WI). Huh7 cells harboring HCV genotype 1b full-genomic (RCYM1)[18] or subgenomic replicon (5-15), and livers of 3-month-old core-gene transgenic mice[2] were also used for the analysis.

*2D-PAGE.* Gel electrophoresis in the first dimension was performed using an immobilized pH gradient gel (Immobiline Dry Strip gel, pH 4-7 linear, 13 cm; GE Healthcare, Uppsala, Sweden). The two-dimensional separation was performed on 12.5%, 14 × 16 cm², SDS polyacrylamide gels. After the electrophoresis, gels were silver-stained using a silver staining kit (GE Healthcare) according to the manufacturer's protocols. The stained gels were scanned and electronic images of the gels were analyzed using ImageMaster 2D Elite software (GE Healthcare).

*In-Gel Digestion and Matrix-Assisted Laser Desorption Ionization, Time-of-Flight Mass Spectrometry (MALDI-TOF-MS).* Protein spots on the gels were excised and a "control" piece was cut from a blank region of the gel and processed in parallel with the sample. In-gel digestion with trypsin was performed as reported.[19] The resulting peptides were concentrated using Zip-Tip C18 (Millipore, Bedford, MA). The peptide mixtures were eluted from Zip-Tip with 75% acetonitrile in 0.1% trifluoroacetic acid (TFA). The matrix (α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile, 0.1% TFA) was deposited on a dried sample target. Then 0.5-$\mu$L aliquots of the analyte solution were deposited onto matrix surfaces and the solvent was allowed to evaporate at ambient temperature. The digests were analyzed with a TOF mass spectrometer, PE Biosystems Voyager DE STR MALDI (Foster City, CA).

*Database Analysis.* For protein identification the measured monoisotopic masses of the peptides were analyzed using MS-Fit provided by UCSF (http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm).

*Immunoblotting and Immunoprecipitation.* Purified mitochondria were lysed and sonicated in RIPA buffer, then centrifuged at 16,000 rpm for 10 minutes. Protein concentration was determined using a BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). The samples were separated by sodium dodecyl sulfate (SDS)-PAGE and electrotransferred onto a polyvinylidene fluoride membrane (Immobilon; Millipore, Japan), then blocked with BlockAce (Snow Brand, To-
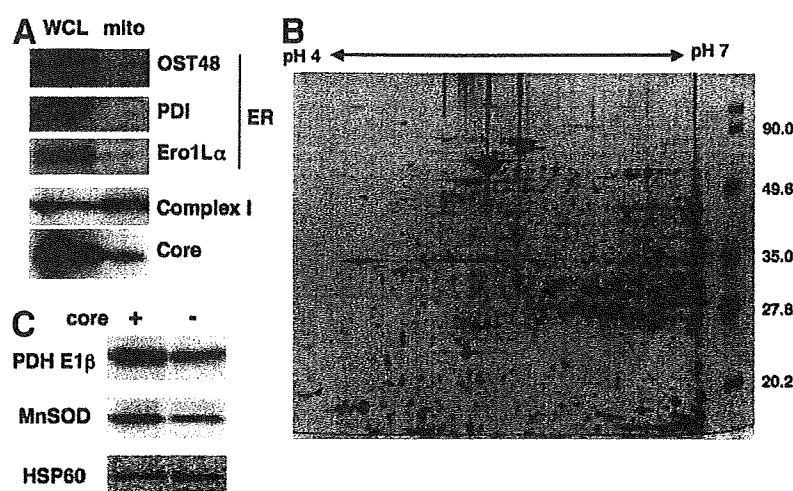
Fig. 1. 2D-PAGE of mitochondria purified from core-expressing cells. (A) Whole-cell lysates (WCL) and purified mitochondria (mito) derived from core-expressing cells were subjected to SDS-PAGE and immunoblotted with anti-core, anti-subunit of complex I (mitochondrial protein), or anti-OST48, PDI, Ero1La (ER proteins) antibodies. (B) Purified mitochondria of core-expressing cells were subjected to 2D-PAGE and the gel was stained with silver. The numbers shown on the right are molecular weights. (C) Purified mitochondria of core-expressing and control cells were subjected to SDS-PAGE and blotted with an anti-E1$\beta$ subunit of PDH (PDH E1$\beta$), anti-MnSOD, or anti-HSP60 antibody.

kyo, Japan). The membrane was subsequently incubated with specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Antibodies against the core protein (Anogen, Mississauga, Canada), manganese superoxide dismutase (MnSOD) (BD Biosciences, San Jose, CA), prohibitin (Neomarkers, Fremont, CA), oligosaccharyl-transferase-48 (OST48), heat shock protein (HSP) 60 (Santa-Cruz Biotechnology, Santa Cruz, CA), pyruvate dehydrogenase (PDH), ubiquinol-cytochrome c oxidoreductase, COX (Molecular Probes, Eugene, OR), protein disulfide isomerase (PDI), ER protein endoplasmic oxidoreduction-1 (Ero1)-L$\alpha$, and I$\kappa$B$\alpha$ (Cell Signaling Technology, Danvers, MA), were used as primary antibodies. For immunoprecipitation experiments, cells were lysed in NET-N buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) and the lysates were incubated with anti-prohibitin overnight followed by the addition of protein Sepharose 4B (GE Healthcare), then washed with the same buffer five times. Immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with specific antibodies.

*Determination of COX Activity.* COX activity was determined with a MitoProfile Rapid Microplate Assay Kit (MitoSciences, Eugene, OR) using 10 $\mu$g of purified mitochondria. The assay was performed three times independently.

*Statistical Analysis.* Results are expressed as means $\pm$ SE. The significance of the difference in means was determined by Student's *t* test or Mann-Whitney's *U* test.

## Results

*Presence of HCV Core Protein in Purified Mitochondria.* Increasing evidence suggests that the HCV

core protein is localized to mitochondria as well as to ER and the nucleus. Therefore, we first investigated whether the core protein is expressed in the mitochondria of core-expressing (Hep39) cells used in this study. We used Nycodenz discontinuous gradients to extract mitochondria as described.[17] In the mitochondria derived from core-expressing HepG2 cells, the core protein was detected by immunoblotting, whereas ER resident proteins such as an ER-specific type I transmembrane protein OST48, ER-resident molecular chaperon PDI, and ER membrane-associated N-glycoprotein Ero1-L$\alpha$, were not (Fig. 1A). In this fraction, reduced nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase, complex I of mitochondrial oxidative phosphorylation system, was more strongly expressed than that in the whole cell. These results indicate that the purified mitochondria fraction was free of ER, and that a portion of the core protein was localized to the mitochondria in core-expressing cells.

*Proteomics Analysis of Mitochondria by 2D-PAGE.* For proteomics analysis, purified mitochondrial proteins derived from core-expressing cells were subjected to 2D-PAGE followed by silver-staining of the gel. In this study we analyzed only acidic proteins using IPG strips covering pH 4 to pH 7 because the analysis of acidic proteins by 2D-PAGE is relatively easy. The mitochondrial fraction was also extracted from Hepswx, a control cell line resistant to G418 but does not express the core protein, then similarly subjected to 2D-PAGE and used for comparing the expression pattern. We repeated the above procedure (purification of mitochondria, 2D-PAGE, and silver-staining) five times, and confirmed a similar expression pattern in core-expressing cells. The representative gel image is shown in Fig. 1B. ImageMaster 2D Elite software detected about 1100 spots on the silver-stained acidic gel, i.e., at pH 4-7 and Mrs of 20-100 kDa. The number of

**Table 1. Proteins of Differential Expression in Mitochondria of Core-Expressing Cells**

| Protein Name | Fold Change (Mean ± SD) |
|---|---|
| Increased | |
| Succinyl-CoA:ketoacid CoA transferase | 10.43 ± 1.29 |
| NADH-specific isocitrate dehydrogenase a subunit precursor | 9.64 ± 4.66 |
| Unknown | 8.65 ± 2.40 |
| GrpE-like protein co-chaperon | 5.71 ± 0.49 |
| Leucine aminopeptidase | 4.26 ± 1.14 |
| Pyruvate dehydrogenase E1 component b subunit | 3.79 ± 1.34 |
| CGO15alt2 | 3.18 ± 0.80 |
| HSP70 | 3.11 ± 1.39 |
| Prohibitin | 2.60 ± 0.24 |
| 3-Hydroxyisobutyrate dehydrogenase | 2.47 ± 0.77 |
| HSPC108 | 2.46 ± 0.69 |
| MnSOD | 2.35 ± 0.65 |
| Ubiquinol-cytochrome c oxidoreductase core I protein | 2.00 ± 0.23 |
| Decreased | |
| Aldehyde dehydrogenase 2 | 0.12 ± 0.02 |
| Aldehyde dehydrogenase 5 precursor | 0.25 ± 0.03 |
| ATP synthase a subunit isoform 1 | 0.50 ± 0.09 |
| Reference protein | |
| HSP60 | 1.02 ± 0.02 |

protein spots was smaller than those reported in a recent study investigating the human placental mitochondrial proteome.[15]

We then compared the intensity of the spots between core-expressing and control cells. Analysis of repeated experiments by Student's $t$ test revealed 13 increased and three decreased spots in intensity in core-expressing cells. These spots were excised and digested with trypsin, then proteins were identified by mass spectrometry. The names of the identified proteins are listed in Table 1. Among them were proteins related to mitochondrial respiratory chain, protein chaperons, and lipid metabolism. Because antibodies to some of these proteins are commercially available, expression levels of the proteins were examined by immunoblotting. The expression levels of the PDH-E1$\beta$ subunit and MnSOD, which were identified as increased proteins, were higher in core-expressing cells than in control cells (Fig. 1C), whereas that of HSP60, which was identified as having a similar expression, was unchanged.

*Up-regulation of Prohibitin by the Core Protein.* Among the identified proteins, we focused on prohibitin, an up-regulated protein in mitochondria of core-expressing cells (Fig. 2A). Prohibitin is a mitochondrial protein associated with cell proliferation.[20] It also works as a chaperon of mitochondrial proteins.[21,22] We confirmed an increased prohibitin expression level in core-expressing cells
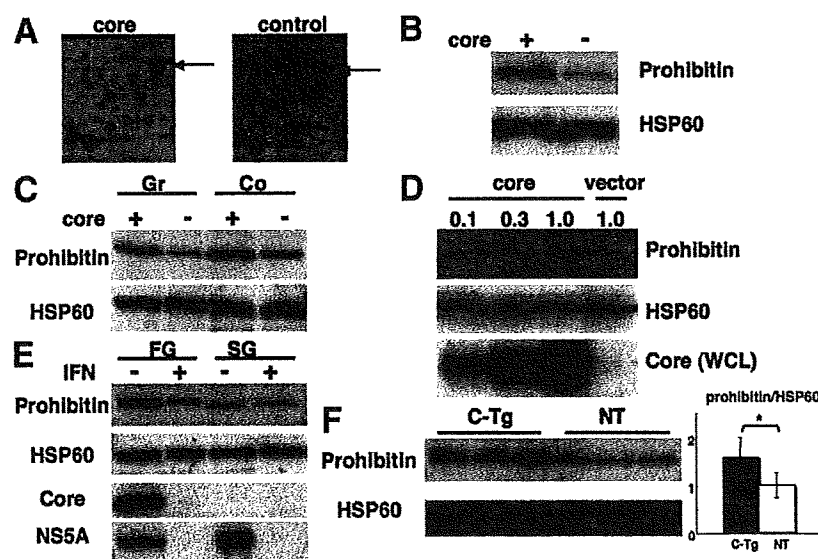


Fig. 2. Up-regulation of prohibitin in core-expressing cells. (A) Protein spot corresponding to prohibitin (arrow) in 2D-PAGE. (B) Purified mitochondria from core-expressing or control cells were subjected to SDS-PAGE and immunoblotted with anti-prohibitin or anti-HSP60 antibody. (C) Mitochondria were purified from growing (Gr) or confluent (Co) cells in 100-mm dishes and subjected to SDS-PAGE, then immunoblotted with an anti-prohibitin or anti-HSP60 antibody. (D) HepG2 cells in six-well plates were transfected with different amounts ($\mu$g) of core-expressing plasmid and mitochondrial proteins were analyzed by immunoblotting with anti-prohibitin or anti-HSP60 antibody. The expression levels of the core protein in whole-cell lysates (WCL) were also determined. (E) Cells harboring HCV replicon were untreated or treated with IFN and expression levels of prohibitin in mitochondria were determined. Expression of HCV core and NS5A proteins was also examined. FG, full-genomic replicon cells; SG, subgenomic replicon cells. (F) Expression levels of prohibitin in mitochondria were determined in liver tissues HCV core-gene transgenic and nontransgenic mice. Prohibitin/HSP60 expression levels were determined by densitometry. C-Tg, core-gene transgenic mouse; NT, nontransgenic littermate (n = 3) *$P < 0.05$.

A

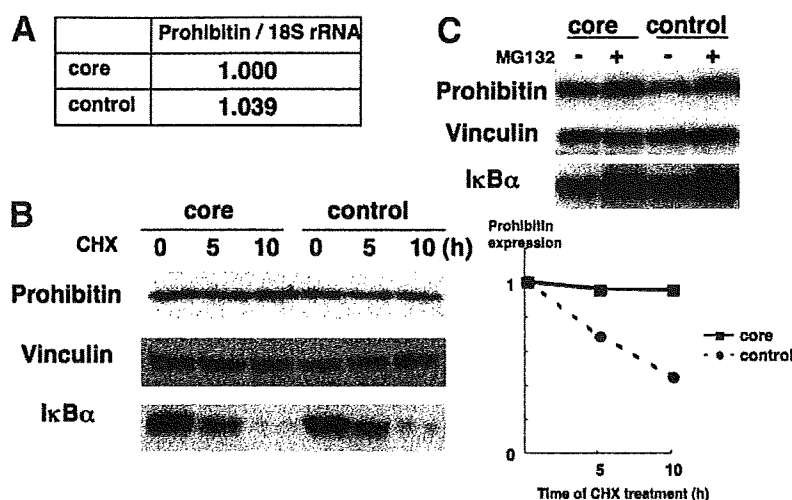| | Prohibitin / 18S rRNA |
| --- | --- |
| core | 1.000 |
| control | 1.039 |

B



C



Fig. 3. Increased protein stability of prohibitin in core-expressing cells. (A) RNA was extracted from core-expressing and control cells, and the amount of specific mRNA was determined by real-time PCR with specific primers/probe against prohibitin. The amount of prohibitin mRNA was standardized by that of 18S ribosomal RNA (18S rRNA). (B) Cells were incubated with 100 ng/mL cycloheximide and harvested at the timepoints indicated above the lanes (numbers are hours of cycloheximide treatment). Whole-cell lysates were subjected to SDS-PAGE and immunoblotted with anti-prohibitin, anti-IκBα, or anti-vinculin (as an internal standard) antibody. The intensity of each band was measured by densitometry, and expression levels (prohibitin/vinculin) are shown in the right panel. (C) Cells were harvested after incubation with 20 μM MG132 for 8 hours and subjected to immunoblotting with anti-prohibitin, anti-IκBα, or anti-vinculin antibody.

by immunoblotting (Fig. 2B). Because prohibitin is associated with cell proliferation, it is possible that prohibitin expression changed according to the cell proliferative status. As shown in Fig. 2C, core-expressing cells had high prohibitin expression levels in the cells in both confluent growth and growing statuses compared with control cells. We also determined the expression levels in cells synchronized with aphidicolin followed by l-mimosine treatment and found an increased expression level in core-expressing cells (data not shown). To exclude the possibility that the increased prohibitin expression level is due to the expansion of limited cell clones, not specific to the core protein expression, we examined prohibitin expression in cells transiently expressing the core protein and found that prohibitin expression level increased dose-dependently in core-expressing cells (Fig. 2D). We also examined the prohibitin expression levels in Huh7 cells harboring full- or subgenomic HCV replicon. For this purpose, we used interferon (IFN)-treated replicon cells (cured cells) as a control. Core and nonstructural (NS)5A proteins were not detected after treatment of full-genomic replicon cells with IFN, suggesting a successful elimination of replicon. Prohibitin expression levels in cells with full-genomic replicon were increased compared with those in IFN-treated cured cells, whereas levels of prohibitin expression were low in subgenomic replicon cells regardless of IFN-treatment (Fig. 2E). In addition, prohibitin expression levels were also increased in livers of 3-month-old transgenic mice expressing the core protein compared with those in nontransgenic littermates (Fig. 2F).

We next sought to determine the mechanism of the increased steady-state level of prohibitin in core-expressing cells. To determine prohibitin messenger RNA (mRNA) expression, we performed a real-time polymerase chain reaction (PCR) using specific primers/probe.

No difference in prohibitin mRNA was observed between core-expressing and control cells (Fig. 3A). We next determined the stability of prohibitin in these cells. By treating the cells with cycloheximide, the expression levels of prohibitin gradually decreased in control cells (Fig. 3B). On the other hand, in core-expressing cells prohibitin was hardly degraded by cycloheximide treatment for 10 hours, whereas IκBα was equally degraded in both cells. This result suggests that prohibitin was stabilized in the presence of the core protein. Because prohibitin has been shown to be degraded by proteasome,[23] we examined expression levels of prohibitin in the presence of proteasome inhibitor MG132. By treatment with MG132, prohibitin expression was increased to the similar level in core-expressing and control cells. These results suggest that the core protein may inhibit proteasomal degradation of prohibitin by some mechanism, including the prevention of degradation by interaction with the core protein. Then, core-expressing cells were lysed and subjected to immunoprecipitation with an anti-prohibitin antibody. As shown in Fig. 4, the core protein was coimmunoprecipitated with an anti-prohibitin antibody. To exclude a nonspecific interaction with the antibody or Sepharose beads, cells expressing a small amount of prohibitin by transfection with small interfering RNA (siRNA) against prohibitin were also examined. In these cells the amount of the coimmunoprecipitated core protein decreased. In addition, the core protein was not coimmunoprecipitated by control immunoglobulin G (IgG), indicating a specific interaction of prohibitin with the core protein. These results suggest that prohibitin expression increased in core-expressing cells owing to the increased stability presumably by interaction with the core protein.

**Impaired Chaperon Function of Prohibitin in Core-Expressing Cells.** We next examined the effect of