equilibrium. Because the prevalence of TT homozygosity in this cohort was only 0.3%, we analyzed it in combination with *CT* and labeled this group *CT/TT*. Logistic regression was performed to calculate the odds ratio (OR) for the *CT/TT* genotypes compared with the *CC* allele homozygote, and MetS was defined according to the IDF and modified NCEP criteria as described above—ie, it included hypertension, hypertriglyceridemia, low HDL cholesterol, elevated fasting glucose, and central obesity—or by specifying a number of components of MetS with the use of age as a covariate. A general linear model was applied to control for age. $P < 0.05$ was considered significant. The data were analyzed by using SAS software (version 8.2; SAS Inc, Cary, NC).

## RESULTS

The genotype frequencies for GPX1 Pro198Leu polymorphism were 0.846, 0.151, and 0.003 for *CC*, *CT*, and *TT*, respectively, and the *T* allele frequency was 0.078 among this cohort (**Table 1**). These frequencies are consistent with those expected under Hardy-Weinberg equilibrium. There were no significant differences in the genotype distributions of GPX1 Pro198Leu polymorphism between men and women (Table 1).

The means (and SEs) of the anthropometric variables, lipid or glucose metabolic variables, or blood pressure tested in the *CC* and *CT/TT* genotypes are shown in **Table 2**. In men, there was a significant difference in WHR and a marginal difference in WC between genotypes (these variables were higher in the *CT/TT* genotypes), but no significant differences were observed in other anthropometric measurements. In women, higher body fat mass was detected in the *CT/TT* genotypes than in the *CC* genotype, but there were no significant differences in other anthropometric measurements. Although there were no significant differences in lipid metabolic variables between the genotypes in women, significant differences in triacylglycerol concentrations were detected in men, who had higher triacylglycerol concentrations in the *CT/TT* genotypes. No significant differences in fasting glucose and glycated hemoglobin concentrations were observed between the genotypes, and no difference was seen in the rate of participants of either sex who were previously diagnosed with diabetes mellitus (control group men: 86.1% and 13.9% in the *CC* and *CC/TT* genotypes, respectively; men with diabetes: 85.2% and 14.8% in the *CC* and *CT/TT* genotypes, respectively; $P = 0.7966$, chi-square test; control group women: 82.9% and 17.1% in the *CC* and *CC/TT* genotypes, respectively; women with diabetes: 88.3% and 11.8% in the *CC* and *CC/TT* genotypes, respectively; $P = 0.2763$). However, significantly higher IRI concentrations were observed in the *CT/TT* genotypes than in the *CC* genotype in both men and women. In addition, higher HOMA-IR and HOMA-$\beta$ concentrations were observed in the *CT/TT* genotypes in both sexes, but the differences were significant only for HOMA-IR in women and for HOMA-$\beta$ in men. There were significant differences in systolic and diastolic blood pressure between the genotypes only in men, with higher blood pressure in the *CT/TT* genotypes.

The prevalence of MetS and each of the MetS components in the study population is shown in **Table 3**. Compared with the wild type (*CC*), the variant GPX1 (*CT/TT*) was associated with a higher frequency of MetS according to both the IDF and modified NCEP definitions in men but not in women. These variants were also associated with central obesity and hypertriglyceridemia in men but not in women. There were no associations between the genotypes and low HDL-cholesterol concentration, elevated blood pressure or hypertension, or elevated fasting glucose concentrations or diabetes mellitus.

When logistic regression was performed to calculate the OR for the *CT/TT* genotypes as compared with the CC homozygote, with the prevalence of MetS defined according to the 2 different criteria of the IDF definition and the modified NCEP definition that uses age as a covariate, the *CT/TT* genotypes showed a significant association with a higher prevalence of MetS as defined by both criteria in men (OR: 2.02; 95% CI: 1.30, 3.15 for IDF; OR:1.49; 95% CI: 1.02, 2.18 for modified NCEP) but not in women (Table 3). When we examined whether this variant was associated with the prevalence of each component of MetS, no association between the *CT/TT* genotypes and the prevalence of low HDL cholesterol, elevated glucose concentrations or diabetes mellitus, or elevated blood pressure or hypertension was found in either sex. However, when the *CT/TT* genotypes were compared with the *CC* genotype, the OR for the prevalence of central obesity (WC > 90 cm and > 80 cm in men and women, respectively) was 1.93 (95% CI: 1.31, 2.85) for men. The OR for hypertriglyceridemia was 1.52 (95% CI: 1.08, 2.15) for men. However, no association was observed between the *CT/TT* genotypes and the prevalence of central obesity and hypertriglyceridemia among women.

The OR for the risk of showing different numbers of features of MetS for the *CT/TT* genotypes is shown in **Figure 1**. Significantly higher ORs for clusters of 2 to 4 risks of MetS (OR: 1.88; 95% CI: 1.07, 3.31 for 2 risks; OR: 1.97; 95% CI: 1.04, 3.74 for 3 risks; and OR: 2.66; 95% CI: 1.27, 5.56 for 4 risks) were observed in men with the *CT/TT* genotypes. In contrast, in women the only significant OR for this variant was observed at the 3-risk accumulation (OR: 2.05; 95% CI: 1.16, 3.64). In this cohort, only 60 participants (2.7%) showed a 5-risk accumulation.

## DISCUSSION

In the present study, we observed that GPX1 Pro198Leu variant is associated with higher WHRs, triacylglycerol concentrations, IRI, HOMA-IR and HOMA-$\beta$, and blood pressure levels than is the *CC* genotype in men. In women, however, those with

**TABLE 1**
Genotype frequency of GPX1 Pro198Leu[1]

| | *CC* | *CT* | *TT* | *CT/TT* |
|---|---|---|---|---|
| Total ($n$ = 2183) | 1846 (84.6) | 330 (15.1) | 7 (0.3) | 337 (15.4) |
| Men ($n$ = 1105) | 948 (85.8) | 154 (13.9) | 3 (0.3) | 157 (14.2) |
| Women ($n$ = 1078) | 898 (83.3) | 176 (16.3) | 4 (0.4) | 180 (16.7) |

[1] All values are $n$; percentage in parentheses. GPX1, glutathione peroxidase 1. *CC*, *CT*, and *TT* in men vs women: $\chi^2 = 2.63$, $P = 0.268$ (chi-square test); *CC* and *CT/TT* in men vs women: $\chi^2 = 2.59$, $P = 0.108$ (chi-square test).

**TABLE 2**
Anthropometric and metabolic variables according to *GPX1 Pro 198Leu* genotypes[1]

| | Men | | | | Women | | | |
|---|---|---|---|---|---|---|---|---|
| | Subject | *CC* | *CT/TT* | *P* | Subject | *CC* | *CT/TT* | *P* |
| | *n* | | | | *n* | | | |
| Age (y) | 1105 | 59.3 ± 0.4[2] | 58.7 ± 0.9 | 0.566 | 1078 | 59.1 ± 0.4 | 59.6 ± 0.8 | 0.636 |
| Waist (cm) | 1115 | 82.2 ± 0.3 | 83.5 ± 0.7 | 0.072 | 1078 | 75.1 ± 0.3[3] | 75.8 ± 0.7[3] | 0.380 |
| Waist-hip ratio | 1105 | 0.901 ± 0.002 | 0.911 ± 0.005 | 0.045 | 1078 | 0.827 ± 0.002[3] | 0.832 ± 0.005[3] | 0.326 |
| BMI (kg/m$^2$) | 1105 | 22.9 ± 0.1 | 23.2 ± 0.2 | 0.165 | 1078 | 22.9 ± 0.1 | 23.1 ± 0.2 | 0.364 |
| Weight (kg) | 1105 | 62.0 ± 0.3 | 62.9 ± 0.7 | 0.293 | 1078 | 52.5 ± 0.3[3] | 52.6 ± 0.6[3] | 0.872 |
| Height (cm) | 1105 | 164.6 ± 0.2 | 164.5 ± 0.5 | 0.840 | 1078 | 151.4 ± 0.2[3] | 150.7 ± 0.4[3] | 0.188 |
| Body fat mass (kg) | 1103 | 21.3 ± 0.1 | 21.6 ± 0.4 | 0.406 | 1071 | 31.5 ± 0.2[3] | 32.4 ± 0.4[3] | 0.027 |
| Total cholesterol (mmol/L)[4] | 1025 | 5.47 ± 0.03 | 5.55 ± 0.07 | 0.290 | 963 | 5.86 ± 0.03[3] | 5.80 ± 0.07[3] | 0.460 |
| LDL cholesterol (mmol/L)[4] | 1017 | 3.40 ± 0.03 | 3.40 ± 0.07 | 0.978 | 948 | 3.59 ± 0.03[3] | 3.56 ± 0.07 | 0.714 |
| HDL cholesterol (mmol/L)[4] | 1025 | 1.48 ± 0.01 | 1.51 ± 0.03 | 0.493 | 963 | 1.72 ± 0.01[3] | 1.68 ± 0.03[3] | 0.208 |
| Triglyceride (mmol/L)[4] | 1025 | 1.46 ± 0.03 | 1.68 ± 0.08 | 0.012 | 963 | 1.19 ± 0.02[3] | 1.25 ± 0.05[3] | 0.390 |
| Fasting glucose (mmol/L)[5] | 1036 | 5.73 ± 0.03 | 5.78 ± 0.07 | 0.544 | 1040 | 5.53 ± 0.03[3] | 5.52 ± 0.06[3] | 0.873 |
| $HbA_{1c}$ (%)[5] | 1035 | 5.20 ± 0.02 | 5.25 ± 0.05 | 0.300 | 1040 | 5.14 ± 0.02[3] | 5.13 ± 0.04[3] | 0.735 |
| IRI (μU/mL)[5] | 1036 | 8.0 ± 0.2 | 9.2 ± 0.5 | 0.019 | 1040 | 8.1 ± 0.2 | 9.1 ± 0.4 | 0.010 |
| HOMA-IR[5] | 1036 | 2.11 ± 0.07 | 2.45 ± 0.17 | 0.065 | 1040 | 2.03 ± 0.06 | 2.40 ± 0.13 | 0.008 |
| HOMA-β[5] | 1036 | 76.6 ± 1.4 | 86.4 ± 3.5 | 0.011 | 1040 | 87.7 ± 1.6[3] | 92.4 ± 3.6 | 0.231 |
| Systolic blood pressure (mm Hg)[6] | 846 | 120.1 ± 0.6 | 124.8 ± 1.5 | 0.004 | 844 | 119.4 ± 0.7 | 121.7 ± 1.6 | 0.176 |
| Diastolic blood pressure (mm Hg) | 846 | 74.8 ± 0.4 | 77.8 ± 0.9 | 0.003 | 844 | 72.4 ± 0.4[3] | 74.0 ± 0.9[3] | 0.112 |

[1] GPX1, glutathione peroxidase 1; $HbA_{1c}$, glycated hemoglobin; IRI, immunoneative insulin; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, HOMA of β-cell function. Unpaired Student's *t* tests were used for all analyses.

[2] $\bar{x}$ ± SEM (all such values).

[3] Significantly different from men, $P < 0.05$.

[4] Subjects who received specific treatment for lipid abnormality were excluded.

[5] Subjects who were previously diagnosed with diabetes mellitus or who received hypoglycemia treatment were excluded.

[6] Subjects who received treatment for previously diagnosed hypertension were excluded.

the *CT/TT* genotypes had higher IRI, HOMA-IR, and body fat mass, but no differences were seen in triacylglycerol concentrations, WHRs, and blood pressure levels between genotypes. These results suggest that there is a sex difference in the association between the variant and these variables. In addition, higher body fat mass was observed in women in the *CT/TT* genotypes but not in men. The higher WHR and WC without any difference in body fat mass or BMI in men with the GPX1 Pro198Leu variant suggest that the *CT/TT* genotypes are associated with central obesity in men. On the basis of the role of GPX1 in the antioxidant defense system, it seems that subjects with the *T* allele have lower GPX1 activity, and thus those subjects have a weaker antioxidant defense system than do subjects with the *C* allele. The association between GPX1 198Leu variants and central obesity in men may suggest that a weaker antioxidant defense system or greater oxidative stress might be a causative factor for obesity. Further research will be required to elucidate the interactions between GPX1 and central obesity. It should be noted that the allelic distribution observed among Japanese persons in this study is different from those reported for other ethnicities. In fact, a greater frequency of the *T* allele was reported among other ethnicities—a *T* allele frequency of 0.326, 0.363, and 0.340 in African Americans (15), Finns (14), and Danes (29), respectively—than among Japanese, which indicates that there are ethnicity-specific variations at this position of GPX1.

We also showed that *CT/TT* genotypes were associated with the higher prevalence of MetS as defined by both the IDF and modified NCEP criteria in men but not in women. It should be noted that higher ORs were observed in MetS according to the IDF definition than in MetS according to the modified NCEP definition in men. It is obvious that this difference is attributable to the inclusion of central obesity (large waist) as an essential component of MetS in the IDF criteria. In fact, there is an association between *CT/TT* genotypes and central obesity in men. In addition, these variants are more likely to be associated with a higher prevalence of hypertriglyceridemia in men but not in women. In contrast, there was no association between the *CT/TT* genotypes and a higher prevalence of low HDL cholesterol, elevated blood pressure or hypertension, or elevated fasting glucose concentrations or diabetes mellitus in either sex. The possible explanation for the lack of the association with other components of MetS except for central obesity and hypertriglyceridemia may be a lack of statistical power or the low frequency of the *T* allele in this population. The men with *CT/TT* genotypes were associated with a greater number of MetS components, but no apparent association was observed in women. These results indicate that the GPX1 Pro198Leu variant is associated with the prevalence of MetS and the cluster of risks of MetS in men but not in women, which suggests that GPX1 genetic susceptibility to MetS is dependent on sex.

As described above, we showed that the *CT/TT* genotypes had higher HOMA-IR, as well as higher IRI and HOMA-β, although there was no significant difference in HOMA- IR in men and HOMA-β in women between the *CC* and *CT/TT* genotypes. We also observed no difference in the prevalence of diabetes mellitus between the *CC* and *CT/TT* genotypes and no association between the *CT/TT* genotypes and a greater prevalence of elevated fasting glucose concentrations or diabetes mellitus in either sex.

**TABLE 3**
The odds ratio (OR) for the metabolic syndrome (MetS) and each of the MetS components[1]

| | OR[2] (95% CI) |
|---|---|
| MetS by IDF | |
| Men | |
| Control ($n$ = 970) | |
| Case ($n$ = 135) | 2.02 (1.30, 3.15) |
| Women | |
| Control ($n$ = 901) | |
| Case ($n$ = 177) | 1.14 (0.75, 1.75) |
| MetS by modified NCEP | |
| Men | |
| Control ($n$ = 970) | |
| Case ($n$ = 135) | 1.49 (1.02, 2.18) |
| Women | |
| Control ($n$ = 901) | |
| Case ($n$ = 177) | 1.40 (0.97, 2.03) |
| Central obesity | |
| Men | |
| Control ($n$ = 900) | |
| Case ($n$ = 205) | 1.93 (1.31, 2.85) |
| Women | |
| Control ($n$ = 790) | |
| Case ($n$ = 288) | 1.20 (0.84, 1.72) |
| High triglyceride concentration | |
| Men | |
| Control ($n$ = 746) | |
| Case ($n$ = 359) | 1.52 (1.08, 2.15) |
| Women | |
| Control ($n$ = 806) | |
| Case ($n$ = 270) | 1.11 (0.76, 1.62) |
| Low HDL cholesterol | |
| Men | |
| Control ($n$ = 942) | |
| Case ($n$ = 162) | 1.01 (0.63, 1.63) |
| Women | |
| Control ($n$ = 842) | |
| Case ($n$ = 234) | 1.22 (0.82, 1.80) |
| Elevated blood pressure or hypertension | |
| Men | |
| Control ($n$ = 612) | |
| Case ($n$ = 493) | 1.25 (0.88, 1.77) |
| Women | |
| Control ($n$ = 608) | |
| Case ($n$ = 469) | 1.22 (0.87, 1.73) |
| Elevated glucose or diabetes mellitus | |
| Men | |
| Control ($n$ = 519) | |
| Case ($n$ = 586) | 0.99 (0.70, 1.39) |
| Women | |
| Control ($n$ = 667) | |
| Case ($n$ = 411) | 1.17 (0.83, 1.65) |

[1] IDF, International Diabetes Federation; NCEP, National Cholesterol Education Program. Logistic regression analysis among men and women was adjusted for age.
[2] The OR for the *CT/TT* genotypes compared to the *CC* homozygote.

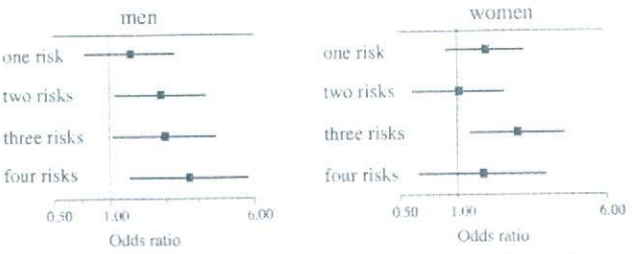


**FIGURE 1.** Odds ratio (and 95% CIs) of the GPX1 Pro198Leu polymorphism for the cluster of risks of the metabolic syndrome (MetS). The $y$-axis represents the odds ratio on a log scale for the risks associated with the various numbers of features of MetS for *CT/TT* genotypes (reference group: *CC* genotype). ■, Point estimates from a logistic regression model adjusted for age; error bars represent 95% CIs. Men: 1 risk ($n$ = 353), 2 risks ($n$ = 283), 3 risks ($n$ = 161), and 4 risks ($n$ = 76); women: 1 risk ($n$ = 261), 2 risks ($n$ = 203), 3 risks ($n$ = 154), and 4 risks ($n$ = 84).

The insulin resistance observed in the *CT/TT* genotypes may be compensated for by the elevated $\beta$-cell function (insulin secretion). Therefore, there appear to be no apparent differences in the prevalence of diabetes mellitus or of elevated glucose in this cohort.

The reasons for these sex differences are not known. However, the result is consistence with a number of prior reports (30–33). Among coronary heart disease patients, the polymorphism in the LDL receptor–related protein gene, *LRPAP1*, is associated with MetS in women but not in men (30). Regulator of G-protein signaling-2 polymorphism is associated with MetS in men but not in women in European populations (31). The beta2-adrenergic receptor gene (Arg16Gly, Gln27Glu) is associated with MetS only in men in the World Health Organization–Monitoring Trends in Cardiovascular Disease population survey (33). These observations and those of the present study indicate that genetic risk factors for MetS may differ between men and women. Although the biological basis of gene-sex interactions in the etiology of MetS remains to be elucidated, the clustering of the traits making up MetS may be due to pleiotropy, when the same gene or genes influence several traits, or to common environmental determinants. It has been shown that erythrocyte GPX activity was significantly higher in premenopausal than in postmenopausal women and higher in premenopausal women than in age-matched men (34). Estrogen seems to be responsible for the sex-related differences in GPX activity. This sexual dimorphism potentially may be conditioned by the activity of sex hormones, differences in lifestyle, or exposure to various environmental factors. Another possibility is that there may be sex differences in free radical homeostasis (35).

The present study has various strengths and limitations. It was conducted in a representative sample of the population, and therefore a possible bias due to the selection of participants was avoided. These findings may not be generalizable to other populations, given that differences in racial and ethnic attitudes toward lifestyle may influence these results. Limitations include the lack of the lifestyle and dietary data for the participants in the analysis, which may affect the prevalence of MetS.

In the present study, we observed that GPX1 Pro198Leu variants are associated with the prevalence of MetS in Japanese men but not in Japanese women. This result may support the hypothesis that oxidative stress is involved in the pathogenesis of MetS. However, further research is needed to establish whether the GPX1 Pro198Leu polymorphism is also associated with MetS in other populations and ethnicities and whether the functional variants of the potential antioxidative enzymes besides GPX1 may be associated with the prevalence of MetS.

The authors' responsibilities were as follows—MK: contributed to the study concept and design and manuscript writing and editing; AF: contributed to data collection and statistical analysis; AI: contributed to the study concept and design; and HS: helped obtain funding and contributed to the study concept and design, data analysis, and statistical support. None of the authors had a personal or financial conflict of interest.

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Original Article

# Having More Healthy Practice was Associated with Low White Blood Cell Counts in Middle-aged Japanese Male and Female Workers

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**Abstract:** White blood cell (WBC) count is well known to be an independent risk marker for cardiovascular disease. The aim of this study is to examine the relationships of WBC counts to seven health practices including obesity, eating habits, smoking, alcohol intake, sleeping, physical activity, and perceived mental stress, and then clustering the relevant healthy practices. The subjects were 1,492 male and 316 female Japanese workers aged 40 yr and over in 2002. Each of seven health practices from a self-administered questionnaire was categorized as a 'healthy' or 'unhealthy' practice, and WBC counts from fasting blood samples were determined by automated particle counters. The means of age and WBC counts were 49.5 yr and 5,375 cells/$\mu$l in men, and 48.6 yr and 4,890 cells/$\mu$l in women, respectively. After multivariate adjustments for all health practices and age, the estimated WBC counts were significantly lower in normal weight subjects and never or former smokers ($p<0.01$). Age-adjusted WBC counts decreased significantly by 204.9 ± 23.7 cells/$\mu$l (means ± SE) and 117.6 ± 53.2 cells/$\mu$l for each increase in one healthy practice ($p<0.05$), respectively, suggesting that cultivating healthier practices would lead to lower WBC counts. This study recommends modifying unhealthy practice one by one and maintaining healthy practices as an effective strategy for the prevention of atherosclerotic diseases, in addition, to quit smoking or abstain from heavy smoking especially in men is important to prevent the low-grade inflammation.

**Key words:** White blood cell, Healthy practice, Cross-sectional study, Questionnaire, Epidemiology, Japanese

## Introduction

The white blood cell (WBC) count is an objective marker of acute infection, tissue damage, and other inflammatory, immunological, or hematological conditions[1, 2]. Many epidemiological studies have implicated WBC counts as an independent risk marker for cardiovascular disease (CVD) and have suggested that WBC may be important in the development and progression of atherosclerosis and cardiovascular disease[3–6].

From the viewpoint of primary prevention of atherosclerotic disease, it is important to examine the associations between WBC counts and some lifestyles among apparently healthy men since such lifestyles have been

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thought to be related to an increase in WBC counts[7–11]. Previous epidemiological studies have suggested an association between elevated WBC counts and low physical activity[7, 8], poor sleep[9], and smoking[10, 11]. However, the evidence of an association between WBC and other basic lifestyles is sparse[12, 13]. In addition, it is not clear whether the clustering of healthy practices within individuals as measured by the number of healthy practices is associated with WBC counts.

Therefore, we analyzed the relationships of WBC counts with seven health practices including obesity, eating habits, smoking, alcohol intake, sleeping, physical activity, and perceived mental stress, and examined the clustering the relevant healthy practices in a large sample of Japanese men and women in the workplace.

## Materials and Methods

*Study subjects*

A survey was conducted in 2002 of local government offices in Aichi Prefecture, central Japan. The survey included a self-administered questionnaire and a physical examination including height and weight. WBC counts were available only among participants in health check-ups. Among them, those who expressed their written consent to the use of information on lifestyle and from physical examinations numbered 1,515 men and 317 women.

Six health practices including obesity, eating habits, smoking status, alcohol intake, sleeping hours, and physical activity were selected as study variables from physical examinations and self-administered questionnaires with reference to seven health practices proposed by Breslow[14], in addition to which we added perceived mental stress. All health practices except for obesity were asked about the past month.

We excluded participants with WBC counts of 10,000≥cells/$\mu$l (23 men and 1 woman), indicating a clinically relevant inflammatory condition. Finally, the current analysis was restricted to 1,492 men and 316 women with complete data on all the lifestyle components and WBC counts. All subjects signed an informed consent statement, and this study was approved by the Ethics Review Committee of the Nagoya University Graduate School of Medicine, Nagoya, Japan.

*Biochemical analysis and anthropometric measurements*

Venous whole blood samples were drawn from an antecubital vein after the subjects fasted for 8 h or overnight. WBC counts were determined by automated particle counters within 24 h after venipuncture in a commercial laboratory.

Height and weight were measured when subjects were dressed in light indoor clothing and without footwear. Height in cm was measured to the nearest 0.1 cm, and weight in kg was measured to the nearest 0.1 kg. Body mass index (BMI) was calculated as weight in kilograms divided by the square of the height in meters.

*Statistical analysis*

All analyses were performed separately for men and women. Each of seven health practices was categorized into two or three groups, and then divided into 'healthy' and 'unhealthy' practices with reference to seven health practices proposed by Breslow[14] as follows:

1. Obesity: 'normal weight (<25 kg/m$^2$)' and 'obese (≥25 kg/m$^2$)' according to the classification of the Japan Society for the Study of Obesity[15].
2. Eating habits: Subjects were to reply either 'Yes' or 'No' to the question: 'Do you usually pay attention to your eating habits?'
3. Smoking status: 'former and never smoker' or 'current smoker'.
4. Alcohol intake: '<23 g' and '≥23 g' ethanol per day. Alcohol intake evaluated as a subject's usual weekly intake of alcohol was converted to grams of ethanol per day.
5. Sleep: '≤7–<9 h' and '<7 or ≤9 h' per day.
6. Physical activity: Subjects were to reply either 'Yes' or 'No' to the question: 'Do you usually try to have physical activity?'
7. Perceived mental stress: 'very much or much' and 'ordinary or little' to the question: 'Do you have much stress in your life?'

Comparisons of WBC counts among each health practice were conducted by *t*-test or analysis of variance (ANOVA). One-way analysis of covariance (ANCOVA) was used to test differences in WBC counts across each health practice after adjustment for covariates including age as a continuous variable and other health practices. These variables with three categories were entered into the model using dummy variables (Table 1). Differences in proportions were tested using $\chi^2$ test. The total number of healthy practices was obtained by adding each of the current healthy practices. A linear trend test was also performed by a polynomial contrast procedure in a general linear model (Table 2).

Multiple regression analysis was performed to estimate the contribution of healthy practice to WBC counts, which were taken as the dependent variable, while the independent variables included age and the total number of healthy practices (0–7) (Table 3). All subjects were classified into four groups by the total number of healthy practices, i.e. 0 to 3, 4, 5, and 6 to 7, since only a few subjects had a lower or higher total number of healthy practices.

*p* values of less than 0.05 were considered statistically

**Table 1. White blood cell counts (cells/$\mu$l) according to age and health practices**

| | Crude mean | | | | Multivariate adjusted mean | |
|---|---|---|---|---|---|---|
| | n | mean | SD | $p$-value[a] | estimated mean[b] | $p$-value[c] |
| **Men (n=1,492)** | | | | | | |
| Age (yr) | | | | | | |
| 35–44 | 490 | 5,287 | 1,343 | 0.02 | — | — |
| 45–54 | 550 | 5,337 | 1,330 | | | |
| 55–64 | 452 | 5,517 | 1,337 | | | |
| Body mass index (kg/m$^2$) | | | | | | |
| <25 | 1,112 | 5,266 | 1,320 | <0.001 | 5,276 | <0.001 |
| ≥25 | 380 | 5,695 | 1,345 | | 5,666 | |
| Pay attention to eating habits | | | | | | |
| yes | 842 | 5,267 | 1,285 | <0.001 | 5,353 | 0.48 |
| no | 650 | 5,516 | 1,394 | | 5,404 | |
| Smoking | | | | | | |
| Never/Former smoker | 989 | 5,073 | 1,197 | <0.001 | 5,080 | <0.001 |
| Current smoker (<20 cigarettes/d) | 130 | 5,686 | 1,373 | | 5,690 | |
| Current smoker (≥20 cigarettes/d) | 373 | 6,070 | 1,402 | | 6,049 | |
| Alcohol intake (g/day of ethanol) | | | | | | |
| none | 235 | 5,640 | 1,401 | 0.004 | 5,582 | 0.005 |
| 0< – <23 | 796 | 5,320 | 1,325 | | 5,383 | |
| ≥23 | 461 | 5,336 | 1,317 | | 5,258 | |
| Sleeping (hours/day) | | | | | | |
| <7 | 761 | 5,361 | 1,353 | 0.91 | 5,357 | 0.73 |
| ≤7 – <9 | 721 | 5,391 | 1,329 | | 5,397 | |
| ≥9 | 10 | 5,342 | 906 | | 5,174 | |
| Try to have physical activity | | | | | | |
| yes | 971 | 5,227 | 1,313 | <0.001 | 5,331 | 0.09 |
| no | 521 | 5,559 | 1,368 | | 5,459 | |
| Perceived mental stress | | | | | | |
| little/ordinary | 718 | 5,345 | 1,281 | 0.40 | 5,340 | 0.29 |
| much/very much | 774 | 5,404 | 1,390 | | 5,409 | |
| **Women (n=316)** | | | | | | |
| Age (yr) | | | | | | |
| 35–44 | 126 | 4,989 | 1,248 | 0.20 | — | — |
| 45–54 | 105 | 4,927 | 1,240 | | | |
| 55–64 | 85 | 4,697 | 1,035 | | | |
| Body mass index (kg/m$^2$) | | | | | | |
| <25 | 269 | 4,789 | 1,151 | <0.001 | 4,801 | 0.002 |
| ≥25 | 47 | 5,469 | 1,281 | | 5,399 | |
| Pay attention to eating habits | | | | | | |
| yes | 217 | 4,880 | 1,139 | 0.83 | 4,896 | 0.90 |
| no | 99 | 4,911 | 1,314 | | 4,877 | |
| Smoking | | | | | | |
| Never/Former smoker | 295 | 4,818 | 1,148 | <0.001 | 4,827 | 0.002 |
| Current smoker (<20 cigarettes/d) | 14 | 5,937 | 1,296 | | 5,866 | |
| Current smoker (≥20 cigarettes/d) | 7 | 5,824 | 1,694 | | 5,590 | |
| Alcohol intake (g/day of ethanol) | | | | | | |
| none | 127 | 5,059 | 1,277 | 0.03 | 5,083 | 0.04 |
| 0< – <23 | 162 | 4,714 | 1,074 | | 4,729 | |
| ≥23 | 27 | 5,153 | 1,355 | | 4,947 | |
| Sleeping (hours/d) | | | | | | |
| <7 | 204 | 4,935 | 1,194 | 0.37 | 4,936 | 0.34 |
| ≤7 – <9 | 112 | 4,808 | 1,196 | | 4,805 | |
| ≥9 | 0 | — | — | | — | |
| Try to have physical activity | | | | | | |
| yes | 192 | 4,908 | 1,159 | 0.74 | 4,952 | 0.27 |
| no | 124 | 4,862 | 1,251 | | 4,793 | |
| Perceived mental stress | | | | | | |
| little/ordinary | 128 | 4,884 | 1,217 | 0.95 | 4,912 | 0.78 |
| much/very much | 188 | 4,894 | 1,182 | | 4,875 | |

SD: Standard deviations, [a]$t$-test (2 groups) or one way analyses of variance (3 groups). [b]Adjusted for age and other health practices, [c]One way analyses of covariance.

**Table 2. White blood cell counts (cells/$\mu$l) according to the total number of seven healthy practices**

| | | | Current smoker | | | Obese[a] | | | Crude mean | | | | Age adjusted mean | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | *p*-value[b] | n | % | *p*-value[b] | mean | SE | *p*-value[c] | Trend *p* | estimated mean | SE | *p*-value[d] | Trend *p* |
| Total number of healthy practices | | | | | | | | | Men (n=1,492) | | | | | | | |
| 0 | 5 | 0.3 | 5 | 100 | | 5 | 100 | | 6,712 | 586 | | | 6,714 | 584 | | |
| 1 | 34 | 2.3 | 26 | 76.5 | | 27 | 79.4 | | 5,781 | 225 | | | 5,812 | 224 | | |
| 2 | 136 | 9.1 | 90 | 66.2 | | 76 | 55.9 | | 5,855 | 112 | | | 5,876 | 112 | | |
| 3 | 263 | 17.6 | 155 | 58.9 | <0.001 | 97 | 36.9 | <0.001 | 5,700 | 81 | <0.001 | <0.001 | 5,711 | 81 | <0.001 | <0.001 |
| 4 | 364 | 24.4 | 132 | 36.3 | | 111 | 30.5 | | 5,394 | 69 | | | 5,397 | 68 | | |
| 5 | 381 | 25.5 | 77 | 20.2 | | 48 | 12.6 | | 5,186 | 67 | | | 5,182 | 67 | | |
| 6 | 235 | 15.8 | 18 | 7.7 | | 16 | 6.8 | | 5,066 | 85 | | | 5,042 | 85 | | |
| 7 | 74 | 5.0 | 0 | 0.0 | | 0 | 0.0 | | 4,933 | 152 | | | 4,918 | 152 | | |
| | | | | | | | | | Women (n=316) | | | | | | | |
| 0 | 0 | — | — | — | | — | — | | — | — | | | — | — | | |
| 1 | 2 | 0.6 | 1 | 50.0 | | 2 | 100 | | 7,360 | 823 | | | 7,295 | 825 | | |
| 2 | 16 | 5.1 | 9 | 56.3 | | 9 | 56.3 | | 5,677 | 291 | | | 5,643 | 293 | | |
| 3 | 29 | 9.2 | 2 | 6.9 | <0.001 | 10 | 34.5 | <0.001 | 4,730 | 216 | 0.001 | <0.001 | 4,733 | 216 | 0.002 | <0.001 |
| 4 | 76 | 24.1 | 3 | 3.9 | | 13 | 17.1 | | 4,763 | 134 | | | 4,755 | 134 | | |
| 5 | 103 | 32.6 | 6 | 5.8 | | 11 | 10.7 | | 5,018 | 115 | | | 5,020 | 115 | | |
| 6 | 68 | 21.5 | 0 | 0.0 | | 2 | 2.9 | | 4,780 | 141 | | | 4,790 | 142 | | |
| 7 | 22 | 7.0 | 0 | 0.0 | | 0 | 0.0 | | 4,484 | 248 | | | 4,495 | 248 | | |

SE: Standard error, [a]Obese: Body mass index ≥25 (kg/m$^2$), [b]$\chi^2$ test, [c]One way analyses of variance, [d]One way analyses of covariance.

**Table 3. Age-adjusted white blood cell counts (cells/$\mu$l) according to the total number of seven healthy practices and multiple linear regression analysis of WBC counts**

| | Age-adjusted white blood cell counts | | | | | | Regression coefficients[a] | | |
|---|---|---|---|---|---|---|---|---|---|
| | n | % | estimated mean | SE | *p*-value[b] | Trend *p* | Age-adjusted means | SE | *p*-value |
| Total number of healthy practices | | | | | | Men (n=1,492) | | | |
| 0–3 | 438 | 29.4 | 5,782 | 63 | | | | | |
| 4 | 364 | 24.4 | 5,397 | 68 | <0.001 | <0.001 | −204.9 | 23.7 | <0.001 |
| 5 | 381 | 25.5 | 5,182 | 67 | | | | | |
| 6–7 | 309 | 20.7 | 5,013 | 75 | | | | | |
| | | | | | | Women (n=316) | | | |
| 0–3 | 47 | 14.9 | 5,147 | 173 | | | | | |
| 4 | 76 | 24.1 | 4,752 | 136 | 0.10 | 0.13 | −117.6 | 53.2 | 0.03 |
| 5 | 103 | 32.6 | 5,021 | 117 | | | | | |
| 6–7 | 90 | 28.5 | 4,722 | 125 | | | | | |

SE: Standard error, [a]total number of healthy practices was used as a continuous variable, [b]One way analyses of covariance.

significant. All statistical analyses were conducted using the SPSS statistical package for Windows, version 12.0 (SPSS Inc., Chicago, IL, USA).

## Results

The means (standard deviations: SD) of age, current BMI and WBC counts were 49.5 (6.7) yr, 23.4 (2.8) kg/m$^2$ and 5,375 (1339) cells/$\mu$l, respectively, in men, and 48.6 (6.6) yr and 22.2 (2.8) kg/m$^2$ and 4,890 (1194) cells/$\mu$l, in women.

Table 1 shows the means and differences in WBC counts according to age and health practices. Crude WBC counts were lower in normal weight subjects and never or former smokers among both men and women. Current smokers with more than 20 cigarettes per day had higher WBC counts than those with less than 20 cigarettes per day. The men who paid attention to eating habits also had significantly lower WBC counts ($p$<0.001, $t$-test). After multivariate adjustment for all these factors including age, the estimated WBC counts remained significantly lower in normal weight subjects (<25 kg/m$^2$) and never

or former smoker than in obese subjects (≥25 kg/m$^2$) and current smokers, respectively, both in men and women. On the other hand, WBC counts were lower in men with a daily ethanol intake of 23 g and over than in men with less than 23 g per day (ANCOVA, $p$=0.005).

Table 2 shows WBC counts according to the total number of seven healthy practices, the distribution of which differed significantly between men and women ($p$=0.001). The category occupied by the largest number was '5 healthy practices' in men (381/1,492, 25.5%) and in women (103/316, 32.6%). Each proportion of current smoker and obese were decreased along with the increase total number of seven healthy practices. Crude and age-adjusted WBC counts decreased significantly along with an increase in healthy practices among both men and women ($p$<0.001, Trend test).

All subjects were classified into four groups by the total number of healthy practices, that is, 0 to 3, 4, 5, and 6 to 7, since only a few subjects had a lower or higher total number of healthy practices (Table3). In men, age-adjusted WBC counts decreased along with the progress of categories in the order of the increase in healthy practices, which were 5,782, 5,397, 5,182, and 5,013 in '0–3', '4', '5', and '6–7' healthy practices, respectively ($p$<0.001, Trend test). In women, the respective value was 5,147, 4,752, 5,021, and 4,722 ($p$=0.13, Trend test), respectively.

When the total number of healthy practices was used as a continuous variable in the linear regression model to estimate the magnitude of their effect on WBC counts, after adjustment for age, WBC counts decreased significantly by 204.9 ± 23.7 cells/$\mu$l (means ± SE) and 117.6 ± 53.2 cells/$\mu$l for an increase in one healthy practice among men and women ($p$<0.05), respectively. In addition, to exclude the effect of smoking status and obesity, we entered the total number of healthy practices except for smoking and obesity (0–5) as a continuous variable in the linear regression model. After adjustment for age, WBC counts decreased significantly by 101.3 ± 30.0 cells/$\mu$l ($p$=0.001) in men and 27.0 ± 60.5 cells/$\mu$l ($p$=0.66) in women for an increase in one healthy practice, respectively. In sub analyses, the association between WBC counts and unhealthy practices (obese and smoking) was assessed by multiple linear regression analysis, age adjusted WBC counts was positively associated with obese and current smoking (standardized $\beta$ for obese and current smoking: 0.13, $p$<0.001 and 0.31 $p$<0.001, Total $R^2$: 0.35 in men, 0.18, $p$<0.001 and 0.20 $p$<0.001, Total $R^2$: 0.30 in women, respectively).

Additional statistical analyses were done by excluding individuals (146 men, 20 women) with a medical history of heart disease, stroke, diabetes or cancer did not alter the results (data not shown).

## Discussion

We found an independent negative association between WBC counts and healthy practices such as normal weight (BMI of <25 kg/m$^2$), and current non-smoking. On the other hand, men with a daily ethanol intake of 23 g and over had significantly lower WBC counts. The total number of healthy practices was significantly and negatively associated with WBC counts among both men and women. To our knowledge, this is the first report suggesting that cultivating healthier practices would lead to lower WBC counts, or the prevention of low-grade inflammation.

Obesity and current smoking were found to be independently associated with elevated WBC counts, both of which are consistent with previous reports[9–11, 15–17]. In addition, higher cigarette consumption (more than 20 cigarettes per day) was positively associated with WBC counts in men. Although the precise mechanisms underlying the association between these two health practices and WBC counts have not been clearly defined, changes in secreted amount of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-$\alpha$) and interleukin-6 (IL-6), which are known to increase WBC counts[1], may be related to our findings. These cytokines are secreted by adipocytes and expressed at higher levels in the adipose tissue of obese subjects[18]. Moreover, a direct injury to epithelial and endothelial surfaces known to have an inflammatory impact on lung tissue and/or changes in the level of cytokines such as IL-6 caused by the constituents of cigarette smoke may induce elevations in the peripheral WBC count and modifications in leukocyte function[19].

On the contrary, ethanol intakes of 23 g and over per day were associated with reduced WBC counts than those with none or less than 23 g in men, and non-drinkers had higher WBC counts than those with drinkers. As for the effect of alcohol intake on systemic markers of inflammation, Nakanishi *et al.*[20] found a negative dose-response relationship between alcohol consumption and WBC counts after adjustment for BMI and cardiovascular risk factors such as systolic blood pressure, total cholesterol, and fasting plasma glucose. Imhof *et al.*[21] also reported a U-shaped association between alcohol intake and C-reactive protein (CRP) with a negative peak at alcohol intake of 40–60 g ethanol per day. The suppression of production of the TNF-$\alpha$ and IL-6 and/or the inhibition of their action caused by alcohol consumption are proposed as possible mechanisms. Although we could not evaluate the effect of heavy alcohol intake on WBC counts because of the small number of heavy drinkers, moderate or appropriate level of alcohol intake may have some favorable effects in this regard.

The other four health practices such as perceived mental stress, paying attention to eating habits, sleep, and physical activity showed no significant association with individual WBC counts. Although not all good healthy practices were associated with lower WBC counts, the clustering of good practices within individuals as measured by the number of healthy practices was significantly and linearly associated with low WBC counts in both men and women. An increase in one healthy practice decreased WBC counts by $204.9 \pm 23.7$ cells/$\mu$l and $117.6 \pm 53.2$ cells/$\mu$l among men and women, respectively. This finding indicates that the total number of selected 7 health practices may reflect the magnitude of low-grade inflammation accurately beyond their mere addition. This phenomenon is maybe due to other health practices accompanying each of 7 health practices.

It was considered that smokers or obese were probably to have smaller healthy practices, indeed, the proportion of current smoker and obese were decreased along with the increase with total number of healthy practices. Although, the total number of healthy practices except for smoking and obesity was also negatively associated with WBC counts in men, this may imply not only smoking status and obesity but also other five health practices was associated with WBC counts.

Several limitations of this study deserve mention. First, the directionality of associations could not be conclusively established because the study is cross-sectional. Second, only one WBC count measurement was performed, whereas multiple WBC measurements over time and their changes may provide more accurate and detailed information on the association between lifestyle and WBC counts. Third, we used self-reported questionnaires to obtain data on individual health practices. In terms of the validity of study variables, self-reported health practices may influence the observed results, thus causing us to underestimate the true association. Finally, the subjects in this study were comprised of middle-aged Japanese men and women, and thus the results may not be applicable to Westerners with different lifestyles and body sizes. Breslow *et al.*[14] proposed seven health practices including "not eating between meals" and "having breakfast" as preventive factors for higher mortality, which were somewhat different from those we selected. We chose one variable as eating behavior whether subjects usually pay attention to eating habits or not, since we could not obtain data about "eating between meals or not", and more than 90% of men and women who pay attention to eating habits had breakfast almost every day. Their observation may be in part explained by elevated WBC count.

In summary, this cross-sectional study on the association of health practices with WBC counts confirmed previously reported studies and provided additional insights to prevent the development of low-grade inflammation. Among fundamental health practices studied, obesity and cigarette smoking were more closely associated with elevated WBC counts. On the other hand, we found a strong association between reduced WBC counts and a number of healthy practices. We strongly recommended efforts to modify unhealthy practices one by one and to maintain healthy ones as primary elements in any strategy for the prevention of atherosclerotic diseases, in addition, to quit smoking or abstain from heavy smoking especially in men was important to prevent the low-grade inflammation.

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# Impaired Basal Thermal Homeostasis in Rats Lacking Capsaicin-sensitive Peripheral Small Sensory Neurons

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**We studied the effects of selective loss of capsaicin-sensitive primary sensory neurons on thermosensation and thermoregulation in rats. Neonatal capsaicin treatment in rats caused a remarkable decrease in the number of small-diameter neurons in the dorsal root ganglion (DRG) compared with their number in the control rats. Gene expression analysis for various thermo-sensitive transient receptor potential (TRP) channels indicated marked reductions in the mRNA levels of TRPV1 (70%), TRPM8 (46%) and TRPA1 (64%), but not of TRPV2, in the DRG of capsaicin-treated rats compared with those in the control rats. In addition to the heat and cold insensitivity, capsaicin-treated rats showed lower rectal core temperature, higher skin temperature and decreased sensitivity to ambient temperature alteration under normal housing at room temperature, suggesting impaired thermosensation and change in thermoregulation in the rats. Uncoupling protein 1 (UCP1) expression and the thermogenic ability in brown adipose tissues were attenuated in the capsaicin-treated rats. These results indicate a critical role of capsaicin-sensitive sensory neurons in both heat and cool sensation and hence in basal thermal homeostasis, which is balanced by heat release and production including UCP1 thermogenesis, following sensation of the ambient temperature.**

**Key words: dorsal root ganglion, sensory neuron, thermoregulation, transient receptor potential channel, uncoupling protein 1.**

Abbreviations: BAT, brown adipose tissue; DRG, dorsal root ganglia; LE, living environment; TE, test environment; Trectal, rectal temperature; TRP, transient receptor potential; Tskin, tail skin temperature; UCP, uncoupling protein.

Body temperature in homeothermic animals is controlled at a constant level by the balance between heat release and production. Animals sense the ambient temperature at all times, presumably through thermoreceptors belonging to the transient receptor potential (TRP) family of ion channels (temperature-sensitive channels) in primary sensory neurons of the periphery; and, based on the thermosensation, the central nervous system (CNS) regulates the responses to maintain body temperature (*1–3*). Several physiological mechanisms such as cutaneous vasoreaction or insulation are involved in the control of heat loss in animals. In particular, cutaneous vasodilation is the major mechanism for heat loss from the skin surface (*4–6*). Effective heat loss is achieved through various body parts, *e.g.* the hand in humans and the tail in rats, both of which have a high surface-to-volume ratio, absence of hair or fur and a high density of arteriovenous anastomoses. In rats, ~25% of the basic metabolic heat can be dissipated at the tail (*7*).

Likewise, heat production by thermogenic organs, such as muscle and brown adipose tissue (BAT), controlled *via* CNS, is indispensable (*8*). To date, BAT-specific uncoupling protein (UCP1) is known to be the most potent thermogenic protein (*9, 10*); and the critical role of UCP1 in adaptive non-shivering thermogenesis in a cold environment has been verified by studies using UCP1-deficient mice (*11, 12*). UCP2 and UCP3, homologues of UCP1, were discovered in 1997, but their roles in thermogenesis seem to be low (*8–10*). With respect to the connection between thermosensation and thermoregulation, Jancso-Gabor *et al.* (*13*) were the first to show that capsaicin treatment of adult rodents causes desensitization of assumed warmth detectors and impaired regulation of rectal core temperature, suggesting the involvement of capsaicin-sensitive neurons in thermoregulation. However, the roles of these sensory neurons

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and their thermoreceptors in the mechanism of thermal homeostasis still remain to be understood.

Recent advances towards understanding the molecular mechanism of thermosensation including that in the noxious range have been made since the cloning of the capsaicin receptor (vanillloid receptor 1, VR1/TRPV1) by Julius' group in 1997 (*14*). TRPV1 is located in small-diameter neurons with unmyelinated C-fibres and thinly myelinated Aδ-fibres in sensory ganglia such as the dorsal root and trigeminal ganglia. After the discovery of TRPV1, a series of other thermoreceptors sensing different ranges of ambient temperature were identified by using the techniques of expression cloning and electrophysiology *in vitro* (*15–23*). Thermoreceptors are gated by noxious heat (>43°C, TRPV1; >52°C, VRL1/TRPV2), warmth to hot (about 25–40°C, TRPV3 and TRPV4), cold to cool (about 15–25°C, TRPM8) and cold (<18°C, ANKTM1/TRPA1). In addition, recent studies using gene knockout mice have clarified the physiological and pathological roles of these receptors. For example, mice lacking TRPV1 show a deficiency in response to noxious heat and in the development of heat hyperalgesia (*24*), but the mutant mice maintain a normal resting body temperature (*25*). TRPV4-knockout mice exhibit an impaired pressure sensation (*26*), whereas circadian body temperature and thermoregulation in the mutant mice were normal at warm environmental temperatures of 25–35°C (*27*). Two studies using TRPA1-deficient mice demonstrated that TRPA1 is essential for transduction of chemical stimuli in nociceptor sensory neurons, although the role of TRPA1 in sensing noxious cold is controversial (*28*, *29*). Most recently, three groups have reported a predominant role of TRPM8 in cold sensation by using mice lacking this receptor (*30–32*). Nevertheless, the contribution of these receptors to the regulation of thermal homeostasis *in vivo* remains to be fully understood. To extend our understanding about the roles of primary sensory neurons in thermosensation and basal thermal homeostasis and to assess the involvement of thermoreceptors in the mechanisms, we investigated the thermal responses and heat production in conscious animals with selective loss of capsaicin-sensitive primary afferent neurons in the present study.

## MATERIALS AND METHODS

*Animals*—F344/N pregnant rats were obtained from Japan SLC. On Day 1 after birth, the neonatal rats were injected or not intraperitoneally with capsaicin (Cap, 50 mg/kg of BW; Wako, Japan) dissolved in a solvent of 10% ethanol, 10% Tween-80, and 80% saline, as described earlier (*33*). The newborn rats in both groups were weaned at the age of 4 weeks and reared until they were 9–10 weeks old, during which time they were given a regular chow diet (Labo MR stock, Nihon Nousan Co., Japan) and tap water *ad libitum* under a 12-h:12-h light–dark cycle at 23±1°C. In the present study, five independent experiments were conducted; and a total of 35 (19 females and 16 males) and 33 (13 females and 20 males) animals were used in control and Cap groups, respectively. The physiological analyses were conducted after maturation of the rats (after 8 weeks of age) under the conscious condition, because anaesthetization greatly affects the regulation of body temperature (*34*). After a series of analyses, the rats were killed by decapitation; and tissues including BAT and skeletal muscles were then dissected for RNA and/or protein analyses. The lumbar dorsal root ganglia (DRG, level 1–5) were removed and frozen on dry ice. The average body weights of 9-week-old rats in the control and capsaicin groups were 124.4±2.0 and 119.1±3.3 g, respectively, for females and 187.4±3.9 and 186.1±4.1 g, respectively for males. All experiments were carried out according to the institutional guidelines for animal care and the principles in the Helsinki Declaration.

*Physiological Analysis*—The hot-plate test was performed in an aluminium cage (D165 mm × W250 mm × H100 mm), the floor temperature of which was controlled at 52±1°C by a hot plate (MODEL PC-420, Corning) beneath it. The floor temperature of the test cage was monitored with a thermal sensor (TD-300, Shibaura Electronics, Tokyo). The latency until rats showed the first signs of discomfort (paw-lifting, -licking or -shaking) was recorded with a cutoff time of 60 s. The cold-plate test was designated to assess any difference in thermal sensation to cold temperature. For this purpose, we used an experimental setup similar to that for the hot-plate test, in which the aluminium cage, which had been placed in a polystyrene box, was surrounded by small ice cubes. The floor temperature of the test cage, monitored as in the hot-plate test, was ~1°C. Rats showed similar signs of discomfort to those in the hot-plate test, although the responses to the cold stress took a longer time compared with those to the hot stress. The shortest and longest latency times in the cold-plate test were 34.6 s in the control group and 216.7 s in the capsaicin group, respectively. In the cold tolerance test, rats were maintained in a cold room (5°C) individually for 5 h. Rectal temperature (Trectal) of the animals was measured every hour with an electronic thermistor equipped with a rectal probe (Mon-a-therm 4070TM, Mallinckrodt Medical Inc., St. Louis, MO). Changes in the skin-surface temperature (Tskin) of conscious rats were recorded at 10-s intervals by use of an infrared thermographic device, Thermotracer (TH5100, NEC San-ei, Tokyo Ltd., Tokyo) or Thermo-Viewer (JTG-5200, JEOL Ltd., Tokyo), as described (*6*), which is a good tool to evaluate heat release in conscious, unrestrained animals non-invasively and successively. We determined the Tskin in the area of the tail, because it has no fur, thus facilitating accurate analysis, and is a crucial site for regulation of heat release in rodents (*4*, *35*). After the Tskin of rats in their regular cage (living environment, LE: 23.5–24.3°C) had been recorded for several minutes, the rats were transferred singly to a new cage without wooden chips (test environment, TE: 21.5–22.4°C). This transfer gave the rats a drop of ~1–2°C in the ambient temperature around them. Two cages were set in the field of the device, and the recording for two rats from each group was done at the same time for 5 min after transferring the rats to the TE. The highest skin temperature in a fixed area of the tail was measured by using image analysing software (TH51-701, NEC San-ei, or TG-5000CNTA, JEOL Datum., Tokyo).

*Histological Analysis*—Horizontal sections (15-μm thickness) of the lumbar DRGs were cut on a cryostat and thaw-mounted on Superfrost slides. The slides were stained with cresyl violet and thionine and observed with OptiPhoto2 (Nikon). The microscopic images were scanned by a digital camera (HC-2000, Fujix). The contours of thionine-stained neurons in the images were traced, and the cell diameter was measured by using a digital caliper (Mitutoyo, Japan).

*RNA Analysis*—For northern blot analysis, total RNA (10 or 20 μg), isolated from the DRG, BAT or gastrocnemius muscles by use of TRIzol reagent (GIBCO BRL), was electrophoresed on 1.25% formaldehyde–agarose gels. The separated RNA was transferred onto a GeneScreen membrane (NEN™ Life Science Products Inc.; Boston, MA) in 10 × saline sodium citrate (SSC) by capillary blotting and was immobilized by exposure to ultraviolet light (0.35 J). Blots were hybridized with probes (labelled with [$^{32}$P] dCTP) for the mRNAs of TRPV1, TRPV2, TRPM8, UCP1 and 18S rRNA, as previously described (*36*). The cDNA probes for TRPV1, TRPV2 and TRPM8 mRNAs were produced from positions 81 to 580 of the rat VR1 sequence (GenBank accession No. AF029310), from positions 208 to 676 of the rat VRL1 sequence (GenBank accession No. AF129113), and from positions 446 to 3765 of the rat TRPM8 sequence (GenBank accession No. AY072788), respectively, by using rat DRG total RNA and the reverse transcription PCR technique. The PCR products were sequenced after subcloning into the pCRII or pCR2.1 vector (Invitogen, CA, USA). The blots were hybridized sequentially with the probes after having stripped away the previous probe. Each probe was confirmed to react with the specific mRNA. Hybridization signals were quantified with a Fuji Bioimage Analyzer. Gene expression of TRPA1 was evaluated by the technique of real-time quantitative PCR. After first-strand cDNAs for TRPA1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs had been synthesized with 1 μg total RNA and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA), the cDNAs were analysed by using Light Cycler FastStrat DNA Master$^{PLUS}$ SYBR Green I and Light Cycler 2.0 system (Roche Diagnostics, Mannheim, Germany). The gene expression of TRPA1 was normalized to the level of that of GAPDH. The sequences of primers used were the following: TRPA1, GAAAACTAAGCAAGTACGAG (forward) and CTCCCAC TGAAATTAGGTAG (reverse); GAPDH, forward primer, ACCACAGTCCATGCCATCAC (forward) and TCCACCA CCCTGTTGCTGTA (reverse).

*Biochemical Analysis*—Immunodetection of UCP1 and cytochrome oxidase subunit IV (COX IV) was performed by using the mitochondrial fraction isolated from BAT, as described previously (*11*). The protein concentration of the fraction was measured with a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of mitochondrial protein (2 μg) were separated on 12.5% gels (Daiichi Pure Chemicals; Tokyo, Japan) and transferred onto Immobilon polyvinylidene difluoride membranes (Millipore Corporation; Bedford, MA, USA). The membranes were incubated with affinity-purified rabbit polyclonal antibodies specific for UCP1 (STRATA GENE, USA) or monoclonal antibody specific for COX IV (Molecular Probes, Inc., USA). After the secondary antibody reaction for 1 h at room temperature, the specific signals were detected by using an ECL kit (Amersham Pharmacia Biotech). The resulting images were quantified with NIH Image (version 1.63). Thermogenic activity was evaluated by using the mitochondrial fraction isolated from BAT, as previously described (*37*). Briefly, [$^{3}$H] GDP-binding was measured by incubation of 1 mg/ml mitochondria with 1 μM [8, 5′-$^{3}$H] GDP (specific radioactivity 32.5 Ci/mmol, PerkinElmer Life Sciences, Boston, MA, USA) in the presence or absence of unlabelled 1 mM GDP at 37°C for 15 min in an assay buffer of 100 mM sucrose, 0.1 mg/ml fatty acid-free bovine serum albumin, 2 μM rotenone and 10 mM sodium Na–Tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid (pH 7.2). After the reaction mixture had been centrifuged at 8,500*g* for 5 min, the pellets were recovered, dissolved in 20 μl of 5% SDS, and then transferred into vials for scintillation counting. The mitochondrial samples pooled from six rats were used for Scatchard plot analysis.

*Statistical Analysis*—Data were expressed as means ± SE. The statistical significance of the data was assessed by using the unpaired Student's *t*-test or repeated measure analysis of variance (ANOVA).

## RESULTS

*Reduction in Small-diameter Neurons and Expression of TRPV1, TRPM8 and TRPA1 in DRG of Capsaicin-treated Rats*—Capsaicin treatment of neonatal rats resulted in a marked reduction in the small-diameter DRG neurons (Fig. 1). The number of neurons with the diameter <20 μm in Cap rats reduced to 23% of the control rats. The number of small-diameter neurons (20–<30 μm) in Cap rats was about a half compared to that in the control rats. These results indicated a marked loss of small-diameter neurons in the DRG of Cap rats. On the other hand, there was no significant difference in the number of medium-diameter neurons (30–<40 μm) between the control and Cap groups. The numbers of medium- to large-diameter neurons (40–<50 and 50–<60 μm) were rather greater in Cap rats than in the control rats. When the effect of the capsaicin treatment on the expression of TRP channels in the DRG was examined by northern blot analysis (Fig. 2A), an ~70% decrease in the TRPV1 mRNA level in the DRG was found in Cap rats. The results also indicated a significant reduction (about 46%) in the TRPM8 mRNA level in the DRG of Cap rats, compared with that of the control rats; however, there was no difference in the TRPV2 mRNA level between the two groups. Moreover, a marked reduction (about 64%) in the TRPA1 mRNA level was detected in Cap rats (Fig. 2B).

*Distinct Thermosensation and Thermoregulation in Rats Lacking Capsaicin-sensitive Neurons*—To ascertain the functional defect expected for the rats lacking capsaicin-sensitive small sensory neurons in their DRGs, we first examined the response of rats to noxious heat. In the hot-plate test, Cap rats exhibited a significantly longer response latency than the control

rats (Fig. 3A). We then performed the cold-plate test to examine whether the reduction in small sensory neurons affected the sensation of cold temperature as well (Fig. 3B). When the rats were put on a cold plate (1°C), they showed several signs of discomfort such as paw-lifting, -licking and -shaking, which were quite similar to those in the hot-plate test. There was a tendency for a longer latency in the Cap group than in the control group ($P=0.0895$), although the latency time was not significantly different between the two groups.

We also performed a cold tolerance test to examine whether the reduction in small sensory neurons affected the regulation of body temperature (Trectal) in the cold (Fig. 3C). The core temperature was slightly but significantly lower in Cap rats (37.3±0.1°C) than in the control rats (37.6±0.1°C) at the room temperature of 23°C (Time 0). When the rats were exposed to the cold at 5°C, Cap rats retained tolerance against the cold just like the control rats did; whereas the decrease in the Trectal of rats in the first 1 h seemed to be slow in Cap group (Δ 0.2°C) compared with that in the control group (Δ 0.6°C). The Trectal of rats was reduced 0.8 and 0.6°C by 5 h of cold exposure in the control and Cap group, respectively. We then measured the Tskin, an index of heat release, by using an infrared thermographic device. In contrast to the Trectal (Fig. 3C), the Tskin in the regular cage was slightly higher in Cap group than in the control group (27.1±0.2 and 26.7±0.1°C, respectively, $P<0.01$; Fig. 3D). To further test the sensitivity to a small change in ambient temperature, the rats were transferred from their LE (LE, 23.5–24.3°C) to the TE (TE, 21.5–22.4°C), a drop of ~1–2°C. The Tskin of rats immediately decreased just after the change in ambient temperature in both groups (at 0 time), though the initial fall in Tskin was considerably smaller (~1.7°C) in Cap rats than in the control rats (~2.7°C). After that, the Tskin in the control rats was kept at a constant level, while the Tskin in Cap rats further decreased gradually and became close to the level in the control rats by 5 min (Fig. 3D).

*Decrease in UCP1 Thermogenic Ability in the BAT of Capsaicin-treated Rats*—To determine whether the lack of capsaicin-sensitive neurons affected the thermogenic ability, we examined the expression of UCPs in major thermogenic tissues; *i.e.* BAT and skeletal muscles. As shown in Fig. 4A, we detected a decrease in the steady-state level of UCP1 mRNA in the BAT of Cap group (about 80% of the control). There were no

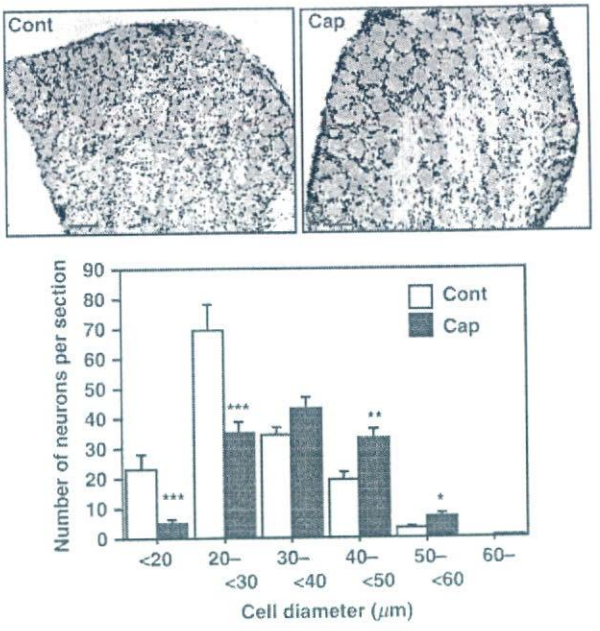


Fig. 1. **Histological analysis of the DRG in rats with neonatal treatment of capsaicin.** Tissue sections of the lumbar DRGs in the control (Cont) and capsaicin-treated (Cap) rats were stained with cresyl violet and thionine. Representative images are shown (scale bar, 100 μm). Cell soma diameter of the DRG neurons was measured and the data are expressed as mean±SE. The numbers of sections analysed were 14 for the control group and 18 for capsaicin group. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus control group.

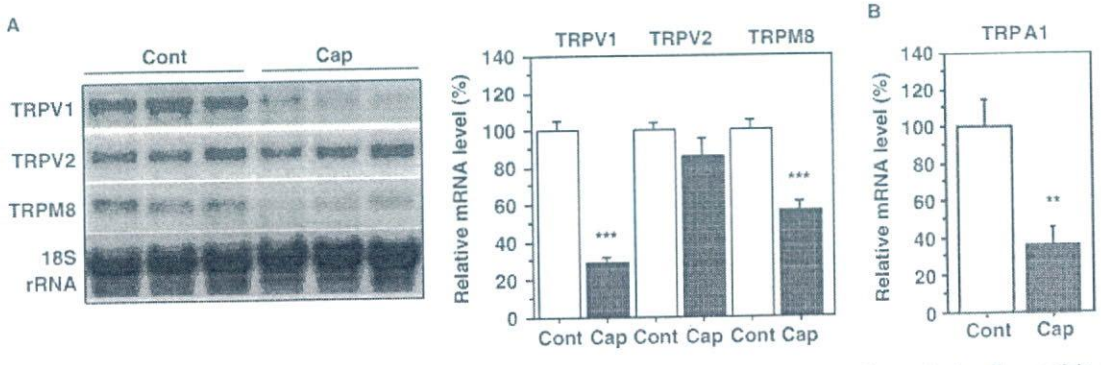


Fig. 2. **mRNA levels of TRPs in the DRG of rats.** (A) mRNA of TRPV1, TRPV2 and TRPM8 was analysed by northern blots using total RNA (10 μg) from DRGs of the control (Cont) and capsaicin-treated (Cap) rats, as described in MATERIALS AND METHODS section. The relative levels of mRNA for TRPs are expressed as means±SE after normalization by 18S rRNA levels. The numbers of rats were 28 and 32 (for TRPV1 and TRPM8), and 12 and 11 (for TRPV2), in the control and capsaicin groups, respectively. $^{***}P<0.001$ versus control group. (B) mRNA of TRPA1 was measured by real-time PCR and expressed as relative to GAPDH mRNA. There was no significant difference in the GAPDH mRNA levels between the groups. Data are expressed as means±SE ($n=8$ for control group, $n=9$ for capsaicin group). $^{**}P<0.01$ versus control group.

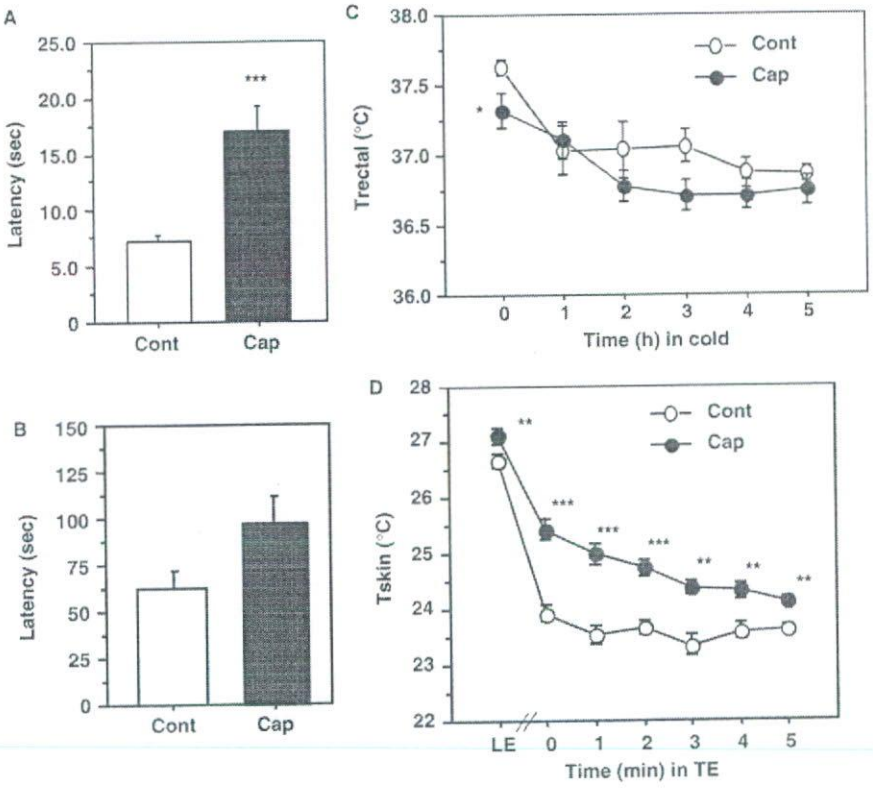


Fig. 3. **Thermal responses to various stimuli in the control (Cont) and capsaicin-treated (Cap) rats.** (A) Hot-plate test at 52 ± 1°C ($n = 24$ for the control group, $n = 22$ for capsaicin group). (B) Cold-plate test at 1°C ($n = 8$ for the control group, $n = 10$ for capsaicin group). (C) Cold tolerance test. Rectal temperature (Trectal) of the rats exposed to a cold temperature of 5°C was measured as described in MATERIALS AND METHODS section. The data for time 0 are those obtained at 23°C ($n = 13$ for the control group, $n = 16$ for capsaicin group). (D) Regulation of heat release in response to a small change in ambient temperature. Tail skin temperature (Tskin, an index of heat release) of the unrestrained rat ($n = 6$ for each groups) was recorded for 5 min by an infrared thermographic device before and after the rats had been transferred from their living environment (LE: 23.5–24.3°C) to the test environment (TE: 21.5–22.4°C). Data are expressed as means ± SE. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control group.

significant differences in UCP2 and UCP3 levels in the BAT or skeletal muscles between the two groups (data not shown). The decreased expression of UCP1 in the BAT of Cap rats was confirmed by the decrease in protein level, which was 67% of the control (Fig. 4B). In contrast, the COX IV level in Cap group was 30% higher than that in the control group. The GDP-binding activity, a marker of thermogenic activity, was significantly lower in the BAT of Cap rats than in that of the control ones (Fig. 4C). In addition, Scatchard plot analysis showed a considerable decrease in total GDP-binding sites (Bmax), but not in binding affinity (Kd; Fig. 4D), in Cap rats compared with those in the control rats.

## DISCUSSION

To understand the mechanism of thermal homeostasis, many studies have been performed previously. Jancso-Gabor *et al.* (*13*) for the first time reported the effect of capsaicin treatment of adult rodents on thermoregulation. After this, capsaicin has been used as a tool to study the mechanism of thermoregulation as well as that of nociception, leading to the discovery of the capsaicin receptor, TRPV1 (*14*). Later Osaka *et al.* (*38*, *39*) demonstrated the role of capsaicin-sensitive nerve fibres in the regulation of BAT thermogenesis and thermal homeostasis by using anaesthetized rats. However, the role of capsaicin-sensitive neurons in the sensation of ambient temperature in conscious animals remained unknown. Likewise, the degree of desensitization by capsaicin treatment of mature rodents in previous studies is unclear. In the present study, therefore, we used neonatal capsaicin treatment, which is different from the desensitized rat model, as it is well established that a single capsaicin administration selectively destroys small sensory neurons in the peripheral ganglia in newborn rats, but not in mature rats (*33*, *40*).

Indeed, Mezey *et al.* (*41*) showed that the capsaicin treatment of neonatal rats significantly decreased the number of TRPV1-positive cells in their DRG, whereas the treatment did not affect the expression and/or distribution of the mRNA in the CNS except in the spinal trigeminal nucleus. Our results of the histological and RNA analyses of DRG also suggested a marked

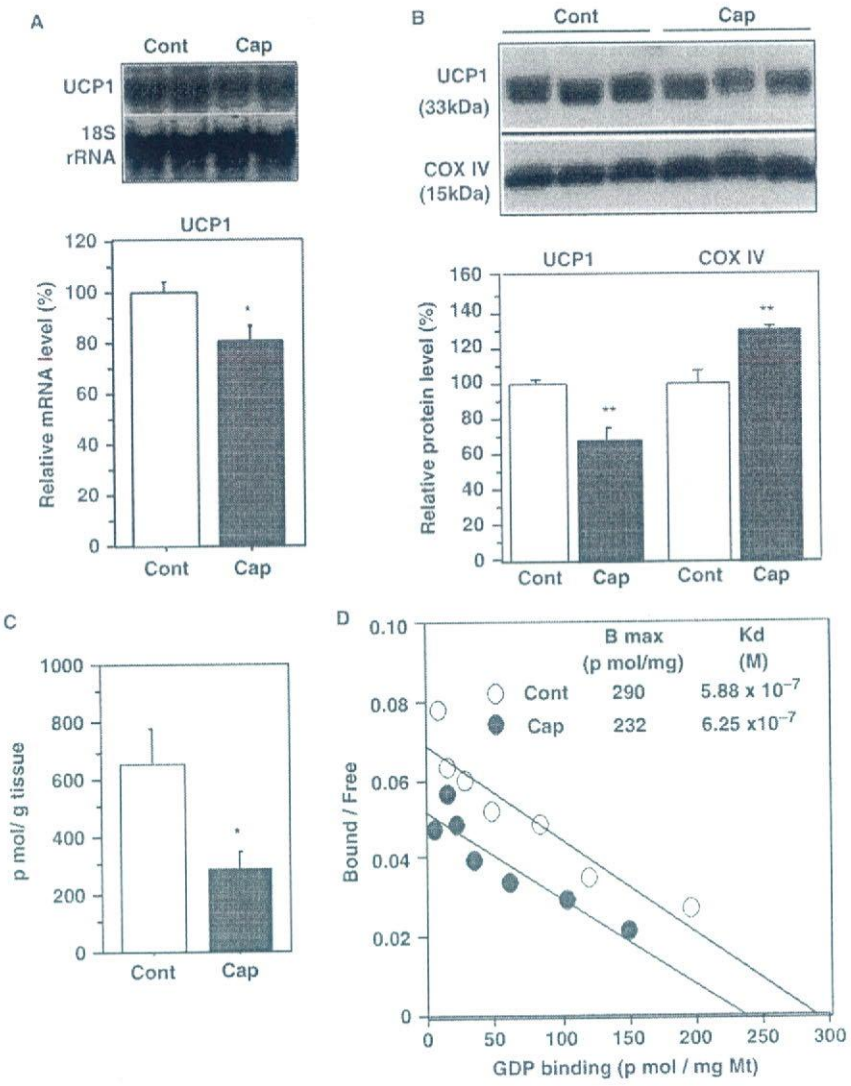


Fig. 4. **Reduction in UCP1 expression and thermogenic ability in the BAT of capsaicin-treated rats.** (A) Northern blots using total RNA (20 μg) of BAT from the control (Cont) and capsaicin-treated (Cap) rats were hybridized with the probes for UCP1 or 18S rRNA as described in MATERIALS AND METHODS section. The numbers of rats were 23 and 26 in the control and capsaicin groups, respectively. (B) Immunodetection of UCP1 and COX IV. (C) GDP-binding activity. (D) Scatchard plot analysis. Western blot analysis and GDP-binding assay using mitochondrial proteins isolated from the BAT of rats were performed as described in MATERIALS AND METHODS section. Data are expressed as means ± SE ($n = 6$ for each group). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group.

reduction in the number of small sensory neurons expressing TRPV1 in the DRG of Cap rats. On the other hand, an increase in the numbers of medium- to large-diameter neurons was detected in the DRG of Cap rats. We presently do not know the reason; however, there may be a compensatory mechanism for the loss of small-diameter neurons in Cap rats. In addition, we found that the loss of capsaicin-sensitive small-diameter neurons was associated with a marked decrease in the mRNA level of TRPA1. This result may be reasonable, because TRPA1 is co-expressed with TRPV1 in a subset of nociceptive sensory neurons (*42*). We also found a significant reduction in the mRNA level of TRPM8 in the DRG of Cap rats. The present data suggest that TRPM8 was expressed in a subpopulation of TRPV1-expressing neurons in the rat DRG, which is consistent with the report of McKemy *et al.* (*18*), although the co-expression of these receptors in same subpopulation was controversial (*19*, *42*). Story *et al.* (*22*) have demonstrated that NGF treatment of DRG neurons isolated from adult rats elicited co-expression of TRPM8 and TRPV1 in culture. One may consider another possibility that the death of capsaicin-sensitive neurons caused a decrease in TRPM8-expressing neurons by some

unknown mechanism. Although the expression of TRPV3 in DRGs of monkeys and humans was reported previously (*21*, *23*), we could not detect a clear signal for TRPV3 in the rat DRG, in agreement with Peier *et al.* (*20*). These observations led us to the experiments to examine the effects of loss of the small sensory neurons expressing TRPV1, TRPA1 and/or TRPM8 on thermoregulation in rats.

To assess the responsiveness of rats to the ambient temperature, we used four different methods, *i.e.* the hot-plate test for the sensation of high temperature (~52°C), cold-plate test for the sensation of low temperature (~1°C), cold tolerance test for the regulation of body temperature in the cold (~5°C) and infrared thermography for the sensation of mild temperature (~23°C). As a result of these methods, rats lacking capsaicin-sensitive small sensory neurons displayed not only the heat-insensitivity but also a tendency to be insensitive to noxious cold, suggesting that heat and cool sensors are in the same subset of small sensory neurons. A reduction in the neurons expressing TRPM8 and/or TRPA1 might cause this insensitivity of cold sensation in rats, as suggested by knockout studies (*29–32*). Moreover, we found novel phenotypes indicating changes in thermal homeostasis in Cap rats. Particularly, the thermographic analysis revealed greater heat release in Cap rats than in the control ones. This finding may indicate an adaptive response to dissipate excess heat for the control of body temperature, which was significantly lower in Cap rats than in the control rats; because heat release from the body surface to the environment is huge and profoundly affects the regulation of body temperature (*6*, *39*, *43*). In addition, Cap rats were significantly insensitive to a small change in ambient temperature around 23°C as well as to noxious temperature, suggesting that capsaicin-sensitive small sensory neurons in DRG is responsible to sense the mild temperature. This may be related to a considerable decrease in the mRNA level of TRPM8, which functional range covers the ambient temperature in a normal animal facility (*18*, *19*). In the cold tolerance test, the change in Trectal after 1 h of cold exposure was smaller in Cap rats than in the control rats. Since TRPA1 senses the lowest temperature (<18°C) among the members of the thermosensitive TRP channel family (*22*), the blunt response of Trectal to acute cold exposure in Cap rats could be associated with the decrease in the number of TRPA1 and an impaired sensation of cold temperature.

Adaptive thermogenesis is an important response in mammals to environmental alterations such as cold and excessive food intake in order to maintain body temperature or energy homeostasis (*8–10*). BAT and skeletal muscle are the principal thermogenic organs and dominantly express UCP1 and UCP3, respectively. The critical role of UCP1 in adaptive thermogenesis in the cold was verified by studies using UCP1-knockout mice (*11*, *12*). With respect to the effect of capsaicin on the regulation of body fat, Cui and Himms-Hagen (*44*) reported an atrophy of and decrease in the UCP1 level in the BAT of the capsaicin-desensitized rats; however, the functional change in thermosensation was not determined. Our results showed a significant decrease in BAT theromogenic ability in Cap rats, which displayed a lowered core temperature and an increased heat release under normal housing conditions. Interestingly, these phenotypes were contrast to those in UCP1-knockout mice, in which thermosensation seems to be normal but the core temperature tended to be higher than that in wild-type mice (*45*). Several groups including our group have demonstrated that induction of UCP1-independent thermogenesis and strong vasoconstrictor response contributed to the regulation of body temperature in mice lacking UCP1 under the thermal conditions of ~20–25°C in the normal animal facilities (*6*, *12*, *45*). The difference in thermoregulation between Cap rats and UCP1-knockout mice may be related to that of the target tissue in these animal models. Namely, capsaicin-sensitive afferent neurons were destroyed and hence several types of thermo-sensitive channels were markedly reduced in Cap rats, which caused abnormal integration of thermosensation and affected the mechanisms to maintain body temperature such as thermogenesis in BAT (an effector of thermoregulation). In UCP1-knockout mice, however, BAT thermogenesis was eliminated by the target manipulation of *Ucp1* gene but thermosensation was normal, in which UCP1-independent thermogenesis was induced and heat conservation was strengthened for homeothermic regulation. Taken together, the results in the two animal models suggest that normal recognition of ambient temperature *via* thermosensory neurons is a crucial factor to elicit proper thermal responses in homeothermic regulation. Furthermore, the changes in thermoregulation in Cap rats could be related to a decline in sympathetic nervous activity, because norepinephrine stimulates UCP1 expression and BAT thermogenesis and simultaneously has a vasoconstriction effect, which decreases peripheral blood flow and suppresses heat loss from the body (*12*, *43*). Scatchard plot analysis indicated a decrease in BAT thermogenic capacity and a reduced demand for UCP1 thermogenesis in Cap rats. The present data also suggest a role of UCP1 in the regulation of basal thermal homeostasis. If there is an intimate interplay between thermosensation and thermoregulation, how is the thermoregulation in mice lacking TRP channels such as TRPM8 and TRPA1? Unfortunately, the studies using knockout mice did not look into the consequences of loss of cold sensitivity on thermoregulation and core temperature, as pointed out by Nilius and Voets (*46*). Because body temperature is controlled by the balance between heat loss and production, the changes in thermosensation by gene knockout of TRP channels may affect thermal responses such as thermogenesis through regulation of UCP1 expression.

Thus, the present study indicated a critical role of capsaicin-sensitive small sensory neurons in thermosensation not only of noxious heat but also of mild room temperature. The change in thermosensation by loss of the capsaicin-sensitive neurons profoundly affected basal thermal homeostasis, which is balanced by heat release and production. It is plausible that the decreased sensitivity of Cap rats to a change in the ambient temperature in the LE was originated, at least in part, from the marked reduction in the numbers of TRPM8- and/or TRPA1-expressing neurons, coincident with the reduction in TRPV1-expressing small-diameter neurons

relating to another phenotype of heat insensitivity. In addition, UCP1 thermogenesis appears to be involved in basal thermal homeostasis in rats. However, further studies such as those using double-knockout animals are required to understand the precise mechanisms of the cross-talk between thermosensation and thermoregulation and their contribution to basal thermal homeostasis *in vivo*.

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