# Author's Reply

## Cardiovascular Risk Clustering With Obesity: A Good Target to Reduce Medical Expenditures as a First Step of High-Risk Approach in Communities and Worksites

We appreciate the letter by Dr Oda concerning about our recently published paper entitled "Effect of combined cardiovascular risk factors on individual and population medical expenditures: a 10-year cohort study of national health insurance in a Japanese population"! Cardiovascular risk factors are often clustered, which has resulted in a high incidence of cardiovascular disease accounted for by metabolic syndrome, recognized as visceral fat accumulation[2] Metabolic syndrome is diagnosed according to several criteria, some of which require obesity for the diagnosis. The definition proposed by the Examination Committee of Criteria for Metabolic Syndrome also requires abdominal obesity measured by waist circumference[3] From April 2008, the Ministry of Health, Labour and Welfare has decided to start a new health service system, which gives an opportunity for all citizens to prevent cardiovascular disease and diabetes by both screening for metabolic syndrome and lifestyle modification[4] This system requires the measurement of waist circumference and its main purpose is to ensure appropriate medical expenditure. As pointed out, the excess medical expenditure by cardiovascular risk clustering in normal weight categories (16.5%) was higher than those in overweight categories (7.1%) because more participants were of normal weight. Accordingly, intervention for obese persons only may not work well to hold back the increase in medical expenditure. However, we did not have values for fasting blood glucose, triglycerides or high-density lipoprotein-cholesterol, which are major components of metabolic syndrome. We may also have misclassified abdominal obesity because we used body mass index instead of waist circumference.

On the other hand, it is an important message that individual medical expenditure is highest for overweight persons with cardiovascular risk factor clustering. Such people are the main target for a high-risk approach to suppress the increase in medical expenditure. High-risk strategies, such as comprehensive health guidance by public health nurses, dieticians or physicians, can be readily understood and strongly motivate a person to change lifestyle to control cardiovascular risk factors. I believe it is feasible to spread a systematic way of health guidance to prevent metabolic syndrome in communities and worksites, because high-risk status due to obesity is easy to detect and easy to give a way to manage it. At least, as the first step in health guidance, public health nurses in local municipalities or nurses in factories do not need to have accurate evaluation of salt intake, the balance of polyunsaturated and saturated fatty acids, mental stress and so on. It is a kind of 'Selection and Concentration' in health service business.

However, another approach for the non-obese majority with cardiovascular risk factor clustering could be made, because they show a high-risk of cardiovascular disease[5] and account for a greater proportion of the excess medical expenditure than the obese minority. Effective and low-cost individual intervention methods are needed. Another way to solve this problem may be a 'population approach', which is useful for shifting the distribution of cardiovascular risk levels towards the low-risk side, even if this shift is minimal[6,7] For example, ingredient labeling for foods in supermarkets which shows accurate amounts of salt and details of fatty acids, is an effective method of giving information to many citizens. The contents of dishes in the restaurant and box lunches delivered by caterers should be evaluated, followed by a health professional's recommendations for improving the amount of sodium and potassium intake, nutritional balance, and caloric intake from fat. For physical activity, walking paths should be constructed for every area. Anyway, the criteria for metabolic syndrome and lifestyle modifications will be improved after the development of ongoing clinical and epidemiologic studies.

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# Medical costs of obese Japanese: a 10-year follow-up study of National Health Insurance in Shiga, Japan

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**Background:** For the Japanese population, a body mass index (BMI) of 25.0–29.9 is classified as obesity and is a risk factor for cardiovascular disorders such as hypertension. **Methods:** A cohort study to clarify obesity costs for a Japanese population was conducted utilizing baseline BMI and medical costs over a 10-year follow-up period. The participants were 4502 community dwelling Japanese National Health Insurance (NHI) beneficiaries aged 40–69 years. According to their baseline BMI values (kg/m$^2$), participants were classified into the following three categories: BMI < 18.5, 18.5 ≤ BMI < 25.0 and 25.0 ≤ BMI. Medical costs per person per month were compared among the three categories. Excess medical costs attributable to the 25.0 ≤ BMI category compared to the 18.5 ≤ BMI < 25.0 category were estimated. **Results:** Approximately 20% of the Japanese population studied had a BMI of 25.0 or over. A J-shaped relationship between BMI and personal total medical costs was observed. Personal total medical costs per month determined from the 10-year follow-up in each category were 189 Euros (BMI < 18.5), 134 Euros (18.5 ≤ BMI < 25.0) and 155 Euros (25.0 ≤ BMI). A J-shaped pattern was observed after adjusting for age, sex, smoking and drinking habits, and excluding early deceased participants. Furthermore, smoking habit did not modify the J-shaped pattern of total medical costs. The estimated excess medical costs for the 25.0 ≤ BMI category represented 3.1% of the total medical costs for the entire study population (634 105 Euros). **Conclusion:** The Japanese NHI beneficiaries with a BMI of 25.0 or over showed increased medical costs compared to those with a BMI of 18.5–24.9.

**Keywords:** obesity, body mass index, medical costs, Japan

## Introduction

Obesity is an important public health problem, and a cause of excess death[1–5] and medical costs[6–12] in many developed countries. In the United States, the impact of obesity on medical economics has been a major burden which has been examined by many studies.[6–10] However, the mean body mass index (BMI) in Asian populations is quite different from that found in Western populations[13,14], and the results of studies in the United States may not be directly relevant or adaptable to the Japanese population. Furthermore, no long-term cohort studies investigating obesity costs have been conducted for Asian populations. Therefore, we attempted to measure the effect of obesity, evaluated by BMI, on medical economics, using a 10-year follow-up study in a community-based population in Japan.

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## Methods

### *Medical costs*

In Japan, medical costs are based on the original medical insurance institution[15,16] which is under control of the National Government. This official medical insurance institution consists of two insurance systems, and everyone living in Japan is required to enroll in one of these insurance systems. There is no private medical insurance. The eligibility for each insurance system is as follows: the first system is for employees and their dependants and covers 65.3% of the overall population, while the other system is for self-employed individuals such as farmers and fishermen, as well as retirees and their dependants, and covers the remaining 34.7% of the population. All eligible beneficiaries in both insurance systems must pay an annual fee to help fund the system. In principle, both insurance systems guarantee that each beneficiary can have access to medical services for any condition at any clinic or hospital throughout Japan. Medical costs depend upon the medical services which a beneficiary receives at a clinic or hospital. No taxes are imposed on the medical costs. The clinic or hospital requests medical costs from both the insurance system and the beneficiary, with insurance paying 70% and the beneficiary paying 30% of the total costs. In the present study, total medical costs were divided into outpatient and inpatient medical costs.

### *Study design and participants*

The cohort in the present study comprised 4535 Japanese beneficiaries of the National Health Insurance (NHI), the insurance system for self-employed individuals. The details of the present cohort have been reported previously.[17]

The participants, aged 40–69 years, lived in seven rural towns and one village in Shiga Prefecture, West Japan and had undergone a voluntary baseline survey in 1989–1991. In 1990, the study area had 82 155 residents, including 31 564 individuals aged 40–69 years old, of whom 11 900 were NHI beneficiaries.[18] Therefore, the participants in the present study represented approximately 38% of all NHI beneficiaries aged 40–69 years living in this area. Of the 4535 participants, 33 were excluded because of missing information at the time of the baseline survey. The remaining 4502 participants were included in the analysis. Monthly NHI claim history files of the Shiga NHI Organizations were linked with the baseline survey data files at the organizations. In order to protect the participants' privacy their names were deleted from the linked data at the organizations. Therefore, the data were analysed without knowledge of the participants' identity. The present study was approved by the Institutional Review Board of Shiga University of Medical Science for ethical issues (No. 16–15).

### *Data collection*

A baseline survey was performed in the period 1989–1991 using standardized methods according to the Manual for Health Check-ups under the Medical Service Law for the Aged, issued by the Japan Public Health Association in 1987.[19] Body height and weight were measured, and BMI was calculated as body weight (kg) divided by the square of body height (m). Referring to the obesity classification of the World Health Organization[20] and that of the Japan Society for the Study of Obesity[21], the participants were classified into the following three categories: BMI $< 18.5\,kg/m^2$, $18.5 \leq$ BMI $< 25.0\,kg/m^2$ and $25.0 \leq$ BMI $kg/m^2$. Obesity is defined as a BMI of 25.0 or over for the Japanese population[21], although it is defined as a BMI of 30.0 or over for Western populations.[20] Thus, we also defined obesity as a BMI $\geq 25.0$ in the present study. Smoking and drinking habits, and medication status for hypertension or a history of diabetes mellitus were evaluated from interviews performed by well-trained public health nurses. Blood pressure was measured using a standard mercury sphygmomanometer on the right arm of each participant in the sitting position after at least a five-minute rest. Serum total cholesterol levels were measured by an enzymatic method. Hypertension was defined as a systolic blood pressure $\geq 140$ mmHg, a diastolic blood pressure $\geq 90$ mmHg or taking anti-hypertensive medication. Hypercholesterolemia was defined as a serum total cholesterol level $\geq 5.69$ mmol/l (220 mg/dl). Diabetes was defined as having a history of diabetes.

We evaluated medical costs per person after a 10-year follow-up, as well as all-cause mortality for each BMI category. We used the $18.5 \leq$ BMI $< 25.0$ category as a reference in the evaluation.[20,21] Medical costs per person in the two sub-categories of obesity ($25.0\,kg/m^2 \leq$ BMI $< 30.0\,kg/m^2$ and $30.0\,kg/m^2 \leq$ BMI) were also evaluated. Information on medical costs for each participant, as well as on participants who withdrew from the NHI or those who died, were obtained from monthly NHI-claim history files, beginning from April in the year following their initial health check-up until March 2001. Costs were expressed in Euros (i.e. 1 Euro = 143 Japanese Yen or 1.21 US Dollars, at the foreign exchange rate on 1 April 2006). Data on medical costs for each participant differed depending upon the period of subscription to the NHI. Therefore, medical costs for each participant were divided by the period of subscription and expressed as costs per month of follow-up. If a beneficiary withdrew from the NHI or died, the follow-up was stopped at that point, but was restarted for beneficiaries who withdrew and then re-enrolled in the NHI. Reasons for withdrawal from the NHI included moving to regions outside of Shiga Prefecture or transfer to the other insurance system.

### *Data analysis*

Because the distribution of real medical costs was positively skewed, the data were logarithmically transformed in order to normalize the distribution, and the results were expressed as geometric means. For participants with 0 Euros (per month) in costs, the logarithmic transformations were performed by replacing 0 Euros with 0.01 Euros. There were 15 participants with total medical costs of 0 Euros and 16 participants with outpatient medical costs of 0 Euros. For comparisons of total and outpatient medical costs per person in each BMI category, we performed an analysis of covariance with the Bonferroni correction to adjust the *P*-value for multiple *post-hoc* comparisons. The analysis of covariance incorporated the following variables as covariates: age, sex, smoking habit (non-smoker or current smoker), and drinking habit (non-, current occasional or current daily drinker, using two dummy variables with the non-drinker as a reference). Because 2589 participants (57.5%) had inpatient medical costs of 0 Euros, logarithmic transformations were not performed, and the Wilcoxon's rank sum test was used to compare medical costs in each BMI category.

A Cox proportional hazards model for all-cause mortality was used to calculate the hazard ratio in each BMI category compared to the $18.5 \leq$ BMI $< 25.0$ category. This model also incorporated the same covariates previously listed.

Initially, the significance of an interaction for total medical costs and for all-cause mortality between BMI and sex was tested using an interaction term for the categorical variables in each multivariate-adjusted model. Next, medical costs per person and the hazard ratio for all-cause mortality in each of the three BMI categories were evaluated.

Smoking habit or poor health status is significantly associated with unintentional weight loss.[22] This may affect the relationship between BMI and medical costs, especially the medical costs of underweight people. Therefore, similar analyses were performed after taking into account smoking habit—i.e. non-smoking, including ex-smoking, or current smoking—for the three BMI categories. In addition, similar analyses were performed after excluding participants who had died in the first 5 years of follow-up.

Finally, we examined excess medical costs attributable to the $25.0 \leq$ BMI category compared to the $18.5 \leq$ BMI $< 25.0$ category by using the arithmetic means of total medical costs, when a significant difference in medical costs between the two BMI categories was observed. The excess medical costs attributable to the $25.0 \leq$ BMI category were calculated as follows: (total medical costs in the $25.0 \leq$ BMI category—total medical costs in the $18.5 \leq$ BMI $< 25.0$ category) $\times$ number of the participants in the $25.0 \leq$ BMI category.

The statistical package SPSS 14.0J for Windows (SPSS Japan Inc., Tokyo, Japan) was used for the statistical analyses. All probability values were two-tailed and the significance level was set at $P < 0.05$.

## Results

The baseline risk characteristics of the 4502 participants grouped by BMI are summarized in Table 1. For both sexes, approximately 20% of all participants had a BMI of 25.0 or over, and approximately 1% had a BMI of 30.0 or over. For both sexes, the $25.0 \leq$ BMI category had the highest prevalence of hypertension, hypercholesterolaemia and diabetes mellitus in the three BMI categories. The BMI $< 18.5$ category had the highest mean age and the highest prevalence of current smokers and drinkers.

**Table 1** Baseline risk characteristics of 4502 National Health Insurance beneficiaries in Shiga, Japan from 1989–1991, grouped by sex and body mass index

| | Body mass index (BMI) (kg/m$^2$) category | | |
|---|---|---|---|
| | BMI ≤ 18.5 | 18.5 ≤ BMI < 25.0 | 25.0 ≤ BMI |
| Men | | | |
| Number of participants (percentage) | 95 (4.9%) | 1492 (77.1%) | 349 (18.0%) |
| Age (year)[a,c] | 58.3 ± 8.0 | 54.0 ± 8.3 | 52.4 ± 7.6 |
| Body mass index (kg/m$^2$)[a,c] | 17.6 ± 0.7 | 22.0 ± 1.7 | 26.7 ± 1.6 |
| Current smoker (%)[b,c] | 74.7 | 60.5 | 54.4 |
| Current drinker[b,c] | | | |
| Occasional drinker (%) | 18.9 | 20.8 | 24.1 |
| Daily drinker (%) | 46.3 | 60.1 | 52.6 |
| Hypertension (%)[b,c] | 27.4 | 33.6 | 57.6 |
| Hypercholesterolaemia (%)[b,c] | 11.6 | 16.2 | 25.8 |
| Diabetes mellitus (%)[b] | 5.3 | 4.1 | 5.7 |
| Women | | | |
| Number of participants (percentage) | 125 (4.9%) | 1842 (71.8%) | 599 (23.3%) |
| Age (year)[a,c] | 56.3 ± 7.9 | 54.3 ± 8.2 | 54.4 ± 7.2 |
| Body mass index (kg/m$^2$)[a,c] | 17.5 ± 0.9 | 22.1 ± 1.7 | 27.1 ± 1.9 |
| Current smoker (%)[b,c] | 11.2 | 3.0 | 2.8 |
| Current drinker[b] | | | |
| Occasional drinker (%) | 13.6 | 16.1 | 17.3 |
| Daily drinker (%) | 7.2 | 4.2 | 2.2 |
| Hypertension (%)[b,c] | 23.2 | 29.7 | 53.9 |
| Hypercholesterolaemia (%)[b,c] | 21.6 | 28.6 | 37.4 |
| Diabetes mellitus (%)[b,c] | 2.4 | 1.4 | 3.8 |

a: One way analysis of variance.
b: Chi-square test.
c: Significant difference among the three BMI categories, $P < 0.05$.

**Table 2** Medical costs per person and all-cause mortality grouped by body mass index, from a 10-year follow-up from 1990 to 2001, in National Health Insurance in Shiga, Japan

| Body mass index (BMI) (kg/m$^2$) category | Number of participants | Medical costs per person | | | | | All-cause mortality | |
|---|---|---|---|---|---|---|---|---|
| | | Total | | Outpatient | | Inpatient | | |
| | | Arithmetic mean | Adjusted geometric mean[a] | Arithmetic mean | Adjusted geometric mean[a] | Arithmetic mean[c] | Number | Adjusted hazard ratio (95% CI)[d] |
| BMI < 18.5 | 220 | 189 Euros | 65 Euros | 83 Euros | 43 Euros | 105 Euros | 22 | 1.76(1.12–2.77) |
| 18.5 ≤ BMI < 25.0 | 3334 | 134 Euros | 56 Euros | 74 Euros | 42 Euros | 60 Euros | 150 | 1.00 |
| 25.0 ≤ BMI | 948 | 155 Euros | 73 Euros[b] | 86 Euros | 53 Euros[b] | 68 Euros | 40 | 1.21(0.85–1.73) |

1 Euros = 143 Japanese Yen or 1.21 US Dollars, at the foreign exchange rate on 1 April 2006
a: Analysis of covariance adjusted for age, sex, smoking habit and drinking habit.
b: Significant difference, vs. 18.5 ≤ BMI < 25.0, for multiple post-hoc comparisons with Bonferroni correction, $P < 0.05$.
c: Wilcoxon's rank sum test.
d: Analysis of a Cox proportional hazards regression model adjusted for age, sex, smoking habit and drinking habit.

The total person-years were 40 565 and mean follow-up period was 9.0 years. There was no interaction for total medical costs and all-cause mortality between BMI and sex. Furthermore, when we performed sex-specific analyses of the relationships between BMI and total medical costs or all-cause mortality, the pattern of results was similar for both men and women. Therefore, we reported the relationships for both sexes combined. The relationship between BMI and total medical costs per person was J-shaped, with the nadir of the curve occurring at a BMI of 18.5–24.9, as shown in Table 2. For the multivariate-adjusted geometric means of total medical costs, the differences among the three BMI categories were statistically significant ($P < 0.01$). The 25.0 ≤ BMI category showed a statistically significant 1.3-fold increase compared to the 18.5 ≤ BMI < 25.0 category. The BMI < 18.5 category also showed a 1.2-fold increase compared to the 18.5 ≤ BMI < 25.0 category, although the increase was not statistically significant. Similar statistically significant differences were observed in outpatient medical costs as well ($P < 0.01$). Inpatient medical costs showed statistically significantly differences among the three BMI categories ($P < 0.01$). When we performed the analyses with the obese participants classified into the two sub-categories, the arithmetic means for total medical costs were 139 Euros (per month) (25.0 ≤ BMI < 30.0; $n = 888$) and 386 Euros (30.0 ≤ BMI; $n = 60$). The adjusted geometric means of the total medical costs were 70 Euros (per month) (25.0 ≤ BMI < 30.0) and 125 Euros (30.0 ≤ BMI) (data not shown in the table). On the other hand, the relationship between BMI and all-cause mortality was inversely J-shaped, as shown in Table 2.

The pattern of personal medical costs was J-shaped among the non-smoking participants as well as the current smokers (data not shown in the table). The adjusted geometric means

for the total medical costs were 66 Euros (per month) (BMI < 18.5 non-current smokers; $n = 135$), 55 Euros ($18.5 \leq$ BMI < 25.0 non-current smokers; $n = 2376$), 72 Euros ($25.0 \leq$ BMI non-current smokers; $n = 741$), 63 Euros (BMI < 18.5 current smokers; $n = 85$), 59 Euros ($18.5 \leq$ BMI < 25.0 current smokers; $n = 958$) and 75 Euros ($25.0 \leq$ BMI current smokers; $n = 207$).

The pattern of personal medical costs was J-shaped after excluding the early deceased participants (data not shown in the table). The adjusted geometric means for the total medical costs were 61 Euros (per month) (BMI < 18.5; $n = 212$), 54 Euros ($18.5 \leq$ BMI < 25.0; $n = 3264$) and 70 Euros ($25.0 \leq$ BMI; $n = 931$).

The excess medical costs attributable to the $25.0 \leq$ BMI category as contrasted with the $18.5 \leq$ BMI < 25.0 category were estimated to be 19 908 Euros (per month), and were calculated as follows: (155 Euros − 134 Euros) × 948 participants with a BMI of 25.0 or over. Accordingly, the excess medical costs attributable to obesity, which was defined as a BMI of 25.0 or over, represented 3.1% of entire total medical costs for the 4502 participants (634 105 Euros), and was calculated as follows: 19 908 Euros/634 105 Euros.

## Discussion

To our knowledge, few studies on medical costs for obesity have been conducted for Asian populations[12], and no long-term investigations have been conducted. The strength of the present study is that we conducted a much longer follow-up period (10-year) compared to previous studies.[12] We demonstrated that the relationship between BMI and medical costs in a general Japanese population was J-shaped, with the nadir of the curve occurring at a BMI between 18.5 and 24.9, after adjusting for confounding factors. In particular, personal total medical costs for groups with a BMI of 30.0 or over were much higher than those in groups with a BMI between 25.0 and 29.9. Smoking habit did not modify the J-shaped pattern of total medical costs. A similar J-shaped pattern was observed even after excluding participants who had died in the first 5 years of follow-up. After a 10-year follow-up, the excess medical costs attributable to participants with a BMI of 25.0 or over represented 3.1% of the total medical costs for all groups.

Obesity has been identified as a significant risk factor for hypertension[23,24], diabetes mellitus[23,25] and dyslipidaemia.[23,25] A combination of these syndromes is known as metabolic syndrome[26], which is a major risk factor for cardiovascular disease.[27,28] Obesity has also been identified as a significant risk factor for colorectal, prostate, endometrial, ovary and breast cancer.[29] Furthermore, obesity is a risk for knee osteoarthritis.[23,30,31] Some obese patients with knee osteoarthritis may require symptomatic relief or joint replacement surgery.[30] Obesity can lead to increased mortality and medical costs as a result of the associated diseases previously mentioned. In fact, the present study showed that the obese participants had a higher prevalence of hypertension, hypercholesterolaemia and diabetes mellitus at baseline. The prevalence of hypertension in the obese participants was substantially high. Accordingly, some obese participants in the present study may have incurred medical costs due to these disorders. Furthermore, serious diseases caused by these disorders (e.g. cardiovascular disease)[27,28] may also have led to increased medical costs of the obese participants. The latter possible explanation is supported by the higher hazard ratio for all-cause mortality in the obese participants.

Kuriyama *et al.*[12] reported a J-shaped relationship between BMI and medical costs after a 4-year follow-up in Miyagi Prefecture, East Japan. In their study[12], the estimated excess medical costs attributable to obesity from a BMI of 25.0 or over represented 3.2% of the entire costs for their study population. Our results are consistent with these results in spite of the different regions and follow-up periods. Accordingly, our results may be applicable to the Japanese population in general, despite some regional differences in lifestyle.[32] Wolf *et al.*[10] reported that medical costs associated with obesity defined as a BMI of 30.0 or over represented 5.7% of National Health Expenditure in the United States in 1995. The prevalence of people with a BMI of 30.0 or over in Western populations is 20–30%[33,34] which is almost equal to the prevalence of people with a BMI of 25.0 or over in the Japanese population.[13,15] These results suggest that the impact of people with a BMI of 25.0 or over on medical economics for the entire Japanese population is almost two-thirds that of people with a BMI of 30.0 or over in the United States.

Being underweight, which is usually defined as a BMI below 18.5[20,21], also represents a high risk of death when there has been unintentional weight loss.[22] Unintentional weight loss is significantly associated with older age, a lower BMI, a smoking habit or poor health status.[22] Furthermore, Wannamethee *et al.*[35] reported that increased mortality of underweight people was likely to be a direct result of a pre-existing disease which led to the underweight condition.[35] Smokers are especially likely to have a lower BMI than non-smokers due to serious diseases associated with smoking.[36–38] Therefore, some of the underweight participants in the present study, especially those with smoking habit, may also have had a serious disease which caused unintentional weight loss, thus leading to increased mortality and medical costs. Meanwhile, it is also possible that some of the normal weight participants may have experienced weight loss prior to baseline due to pre-existing diseases, which may have influenced medical costs during follow-up. The differences in medical costs between the obese participants and the normal weight participants, as well as the underweight participants, may have been underestimated in the present study. In order to examine the effects of smoking habit or pre-existing diseases, we performed analyses taking into account smoking habit and excluded premature death. We still found a J-shaped pattern of personal medical costs in these further analyses. Medical costs of underweight people are likely to be higher than those of normal weight people, irrespective of smoking habit or premature death. As for smoking habit, Hayashi *et al.*[5] reported increased all-cause mortality in the lower BMI groups regardless of smoking status (never smokers, ex-smokers and current smokers) for Japanese men. This result supports our result demonstrating increased medical costs of underweight participants with or without current smoking habit.

The present study has several limitations. First, medical cost data from the official medical insurance records in Japan do not include costs for any services used to prevent disease or to promote health status (e.g. special diet for weight control). If the obese participants took advantage of such services more frequently than the normal weight participants, the obese participants would have incurred medical costs more in excess of what we observed. Therefore, there may be a possibility that we underestimated obesity cost in the present study. However, all beneficiaries can take advantage of therapeutic services without paying an extra insurance fee, even if he or she suffers from a serious disease. Therefore, the results in the present study may be sufficient to reveal long-term medical costs of obese people. Second, participation was limited to NHI beneficiaries belonging to self-employed occupational groups in one area of Shiga prefecture in Japan.[15,16] The socio-economic status and lifestyle of these NHI beneficiaries may have had an effect on their health status, and may be a confounding factor among the three BMI categories. However,

socioeconomic status and lifestyle were not available in the present study. Third, we had no serial data for obesity-associated factors such as blood pressure after the baseline survey. Furthermore, medical diagnosis, medical treatment status and causes of mortality during the follow-up period were also not available in the present study. Therefore, we could not identify the events which directly increased medical costs among the obese participants. Finally, it is better to classify obesity into the two subcategories, $25.0 \leq \text{BMI} < 30.0$ and $30.0 \leq \text{BMI}$. However, we did not classify obesity further in the analysis, because the number of obese participants with a BMI of 30.0 or over was very small ($n = 60$) in our study population.

In spite of the limitations previously mentioned, we believe that medical costs of the obese or underweight participants increased due to diseases associated with a higher or lower BMI, and that the J-shaped relationship between BMI and medical costs in the present study are reasonable, and are supported by similar relationships between BMI and mortality in previous studies on Japanese populations.[3–5]

In conclusion, approximately 20% of the Japanese NHI beneficiaries in the present study had a BMI of 25.0 or over, and this BMI level was associated with a burden on medical economics in Japan.

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## Key points

- The relationship between BMI and medical costs in a general Japanese population was J-shaped, with the nadir of the curve occurring at a BMI between 18.5 and 24.9, after a 10-year follow-up.
- Smoking habit did not modify the J-shaped pattern of total medical costs.
- The excess medical costs attributable to obese individuals having a BMI of 25.0 or over represented 3.1% of the total medical costs for all groups.
- Our results are consistent with finding reported after a 4-year follow-up, in spite of differing regions and follow-up periods, and may be applicable to the Japanese population in general.
- A BMI level of 25.0 or over may be associated with a burden on medical economics in Japan.

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## Appendix

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Chairman: Hirotsugu Ueshima (Department of Health Science, Shiga University of Medical Science).

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Secretary Members: Yukio Tobita, Kanehiro Okamura, Kiminobu Hatta, Takao Okada, Michiko Hatanaka (the Shiga NHI Organizations).

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厚生労働科学研究費補助金（循環器疾患等生活習慣病対策総合研究事業）
分担研究報告書

# 大阪府八尾市南高安地区地域コホート研究

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研究要旨
八尾市南高安地区住民の循環器疾患発生者の危険因子をretrospectiveに検討した結果、発生前5年以内に住民健診を受けていた者の割合は28%と低率であった。発生前の危険因子の保有状況をみると脳卒中では発生前に高血圧を有する者の割合が83%、虚血性心疾患では喫煙者の割合が88%と最も高かった。

## A．研究目的

われわれは、大阪府八尾市南高安地区住民を対象として、長期間、循環器疾患の発生調査を行っている。本研究では、最近5年間の発生者の発生前の健診所見を分析し、当地区における脳卒中と虚血性心疾患の危険因子をretrospectiveに検討した。

## B．研究方法

当地区の全住民（2005年人口23276人）を対象として、既定の方法により、脳卒中（脳出血、脳梗塞、くも膜下出血）と虚血性心疾患（心筋梗塞、労作性狭心症）の発生者を把握した。今回、2001～2005年の発生者について、発生前の健診受診歴や健診所見を検討した。

## C．研究結果

脳卒中93人（男54人・女39人・発生時平均年齢71歳）、虚血性心疾患55人（男46人・女9人・発生時平均年齢63歳）の計148人の発生者が把握された。この中で発生以前5年以内に住民健診を受診していた者は41人（受診割合28%）であった。

発生前5年以内の直近の健診における主な危険因子の保有者割合は、脳卒中24人中、割合の大きい順に、高血圧20人（83%）、高コレステロール血症13人（54%）、飲酒11人（46%）であった。メタボリックシンドローム（MetS）（日本の基準に準じる、腹部肥満をBMI≧25kg/m$^2$で代用）を有する者は5人（21%）であった。逆に、肥満していないリスク集積者（2個以上）は、9人（38%）であった。同様に、虚血性心疾患17名中、有所見者の割合が大きかったのは喫煙15名（88%）、高血圧8名（47%）、高コレステロール血症8名（47%）であり、MetSは2名（12%）であった。

## D．考察

発生前5年以内に健診を受診していた者の割合が3割未満であったため、断言はできないが、今回の検討結果より、循環器疾患の発生前には高血圧や喫煙等の危険因子の保有割合が比較的高率であった。今後、健診の受診率を高め、ウエスト高値を指標としたMetSの保有状況の検討が必要である。

## E．結論

地域における循環器疾患の発生予防の観点からみると、MetS対策のみならず、高血圧や喫煙などの危険因子についても重点的に対策を行う事が重要であることが示唆された。

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## G．研究発表

1．論文発表
無し

2．学会発表
山野賢子、北村明彦、他．八尾市南高安地区における脳卒中・虚血性心疾患発生調査の現状と課題．日本公衛誌 54（特別附録）；418:2007

## H．知的財産権の出願・登録状況
無し

厚生労働科学研究費補助金（循環器疾患等生活習慣病対策総合研究事業）
分担研究報告書

# 保健指導への活用を前提としたメタボリックシンドロームの診断・管理のエビデンス創出のための横断・縦断研究

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研究要旨
沖縄県の人間ドック受診者において、生活習慣病関連の危険因子を調査した。内臓肥満症、高血圧、耐糖能異常、脂質異常症のいずれも全国平均より高いことがわかった。また内臓肥満を基盤とするメタボリックシンドロームを有することが心臓血管病の発症リスクとなることが示された。

A．研究目的

沖縄県における動脈硬化リスクファクターの陽性率とその要因を検討する。特にメタボリックシンドロームのコンポーネントの陽性率と心血管イベントの発生率との関係を明らかにする。

B．研究方法

研究1： 2003年5月から2004年3月まで豊見城中央病院健康管理センターを人間ドックのため受診した者6985名（年齢30～69才、男性3839名、女性3146名）。メタボリックシンドロームの各コンポーネントの陽性率と他の動脈硬化危険因子の陽性率を調査する。研究2：研究1で対象となった症例につき、毎年の受診歴をカルテ上で確認し、受診歴の不明なものに対して、往復はがきで健康状態、死別の有無に関する調査を行う。メタボリックシンドローム診断基準は注1に示す通り。メタボリックシンドロームに対して、食事療法、運動療法、薬物療法の有無を調査し、注2に示すプライマリーエンドポイントおよびセカンダリーエンドポイントを判定する。

注1：メタボリックシンドロームの診断

メタボリック症候群診断基準検討会が公式発表した下記の基準（2005年4月） を満たすもの。

① 内臓肥満（ウエスト周囲径で男性 ≧ 85ｃｍ、女性 ≧ 90ｃｍ）

②高血圧（ ≧ 130/85ｍｍＨｇ）

③高中性脂肪血症（ ≧ 150ｍｇ/ｄｌ または低ＨＤＬ－Ｃ血症 （＜ 40ｍｇ/ｄｌ）

④空腹時血糖 ＞ 110ｍｇ/ｄｌ

・①は必須条件で、②から④の中から２項目以上を満たす。

・入院・外来は不問。

・試験に際し、インフォームドコンセントを行い、文面にて患者同意の得られた症例。

・投薬に関する安全性について主治医が本調査に適応と判断した症例。

注2：プライマリーエンドポイント（前、3ヶ月、その後一年毎5年後まで）

OGTT、HOMA-IR、FFA、ｱﾃﾞｨﾎﾟﾈｸﾁﾝ、h-CRPへの影響

セカンダリーエンドポイント（エントリーから5年間経過を追う）

心血管イベント（急性冠症候群、脳血管障害、末梢血管障害、心不全、不整脈）発症および全死亡

（倫理面への配慮）

ヘルシンキ宣言（http://www.wma.net/e/policy/b3.htm）を遵守する。特に、予後の有無について問い合わせるはがきでは、調査に

関する同意を得た上で情報保護シールを貼ること並びに個人情報の保護管理を徹底する。

C．研究結果

人間ドック受診者で、メタボリックシンドロームの頻度並びにリスクファクターの頻度について調べた。腹部肥満陽性率は、男性57%、女性17%であった。空腹時高血糖の陽性率は、男性20%、女性7%であった。高中性脂肪血症の陽性率は、男性38%、女性13%であった。低HDL血症の陽性率は、男性15%、女性4%であった。高血圧症の陽性率は、男性53%、女性35%であった。メタボリックシンドロームの陽性率は男性は、30才代で20%、40才代で28%、50—70才代は30%を超えた。女性は、各年代とも10%以下であった。

前向きコホートで、メタボリックシンドロームを有することは男女とも、心血管イベントの発症率を増加させた（ロジスティック解析）。心血管イベント発症のオッズ比は男性で男性2.5倍、女性1.8倍であった。

D．考察

沖縄県人間ドック受診者（全国平均との比較のため40才以上のみを示す）の腹部肥満陽性率は、男性59.8%、女性18.5%であった。肥満指数（BMI）をもとに報告された統計で、沖縄県の肥満陽性率は、男性46.9%、女性26.1%（2004年度社会保険庁）であったが、腹部肥満の基準による陽性率は男性は全国平均（男性55.8%）を上回っていたが、女性では全国平均（女性22.0%）以下であった。

一方、高血圧（140/90mmHg以上）は男性30.6%、女性19.6%と、全国平均（男性18.0%、女性11.2%）に比べ男女とも高く、耐糖能異常は男性20.3%、女性7.0%と、全国平均（男性15.6%、女性7.2%）に比べ男性のみで高いことがわかった（日本人間ドック学会、2005年）。

高中性脂肪血症は、男性38.6%、女性14.5%と、全国平均（男性19.1%、女性8.1%）に比べ男女とも多いことがわかった。高コレステロール血症は、男性30.3%、女性34.3%と、全国平均（男性25.6%、女性25.4%）に比べ男女とも高いことがわかった。

人間ドック受診者にエントリーした前向きコホートで、メタボリックシンドロームを有することは、男女とも心血管イベントの発症率を増加させることがわかった。中間解析では、男性でのイベント内訳は、心筋梗塞5例、大動脈疾患3例、脳梗塞3例、狭心症2例、心不全2例、不整脈1例であり、女性は、脳梗塞1例、狭心症2例であった。男性のイベント発症が16例、女性が3例と男性が著明に多く、観察期間をさらに延長して男女別の解析を実施する予定である。

E．結論

沖縄県の人間ドック受診者における生活習慣病関連の危険因子は全国平均よりそれぞれ高いことがわかった。その要因として内臓肥満を増加させる生活習慣との関連が疑われた。また内臓肥満を基盤とするメタボリックシンドロームを有することが心臓血管病の発症リスクとなることが示唆された。

G．研究発表

1．論文発表

別紙４参照

2．学会発表

別紙４参照

H．知的財産権の出願・登録状況（予定を含む。）

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

別紙4

研究成果の刊行に関する一覧表

雑誌

| 発表者氏名 | 論文タイトル名 | 発表誌名 | 巻 | ページ | 出版年 |
|---|---|---|---|---|---|
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| Shimabukuro M, Tanaka H, Shimabukuro T. | Effects of telmisartan on fat distribution in subjects with the metabolic syndrome. | J Hypertens | 25 | 841-848 | 2007 |
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| 島袋充生 | サロゲートマーカーを用いた臨床試験：糖尿病・メタボリックシンドローム | 臨床薬理 | 第38巻 | | 2007 |
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# Vascular Lipotoxicity: Endothelial Dysfunction via Fatty-Acid-Induced Reactive Oxygen Species Overproduction in Obese Zucker Diabetic Fatty Rats

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**Vascular endothelial dysfunction has been demonstrated in obesity, but the molecular basis for this link has not been clarified. We examined the role of free fatty acids (FFA) on vascular reactivity in the obese *fa/fa* Zucker diabetic fatty (ZDF) rat. Addition of acetylcholine produced a dose-dependent relaxation in aortic rings of ZDF and lean +/+ rats, but the $ED_{50}$ value was higher in ZDF ($-6.80 \pm 0.05$ *vs.* $-7.11 \pm 0.05$ $\log_{10}$ mol/liter, $P = 0.033$). A 2-wk treatment with a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, pitavastatin (3 mg/kg/d) or a reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, apocynin (5 mmol/liter in drinking water), improved the response in ZDF ($ED_{50}$, $-7.16 \pm 0.03$ and $-7.14 \pm 0.05$ $\log_{10}$ mol/liter, $P = 0.008$ and $P = 0.015$ *vs.* vehicle, respectively). Vasodilator response to sodium nitroprusside was identical between ZDF and +/+ rats. Vascular reactive oxygen species (ROS) levels and NADPH oxidase activity in aorta were increased in ZDF rats but were decreased by pitavastatin. In *in vitro* cell culture, intracellular ROS signal and NADPH oxidase subunit mRNA were increased by palmitate, but this palmitate-induced ROS production was inhibited by NADPH oxidase inhibitor or pitavastatin. In conclusion, FFA-induced NADPH oxidase subunit overexpression and ROS production could be involved in the endothelial dysfunction seen in obese ZDF rats, and this could be protected by pitavastatin or NADPH oxidase inhibitors. (*Endocrinology* 148: 160–165, 2007)**

THE PRESENCE OF vascular endothelial dysfunction has been demonstrated in subjects with insulin resistance/visceral fat obesity (1–3). Namely, blood flow response to an endothelium-dependent vasodilator such as methacholine chloride but not to an endothelium-independent vasodilator such as sodium nitroprusside was impaired in obese insulin-resistant subjects (1). One common metabolic feature of insulin resistance/obesity is a deficit in insulin-mediated glucose disposal. Recent evidence raised the possibility that tissue accumulation of free fatty acids (FFA) mainly causes abnormalities of insulin secretion and actions and consecutive metabolic derangements, named lipotoxicity (4–6). Because FFA are supplied excessively and persistently to the bloodstream from visceral fat tissues, we and others assumed that an elevation of circulating FFA might be causally related to the onset and progression of endothelial dysfunction in patients with insulin resistance/visceral fat obesity (2, 3).

Although direct inhibitory effects of FFA on endothelial function have already been shown in humans (2, 3, 7), the mechanism by which FFA cause such inhibition has not been clear. Several *in vitro* studies reported that FFA can enhance production of reactive oxygen species (ROS) (8, 9), but a functional link of circulating FFA to endothelial function through ROS production has not been evaluated. It has been reported that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statin) improve endothelial function and also reduce vascular superoxide production via inhibition of vascular NADPH oxidase activation (10, 11). Thus, statin might attenuate FFA-induced endothelial dysfunction via inhibition of vascular superoxide production.

In the present study, we examined 1) the role of FFA and the oxidases responsible for ROS production in vascular reactivity and 2) effects of statin on vascular ROS production in a rodent model of visceral fat obesity, Zucker diabetic fatty (ZDF) rat, which shows hyperphagia and obesity-related diabetes, dyslipidemia, and hypertension resulting from a loss-of-function mutation in the leptin receptor (12).

## Materials and Methods

### *Animals*

Studies were carried out in male obese homozygous (*fa/fa*) ZDF rats and lean wild-type (+/+) littermates (Charles River Laboratories, Wilmington, MA). All rats were fed standard laboratory chow and given tap water *ad libitum*. Their genotype was determined as described (12). ZDF *fa/fa* and +/+ rats received either pitavastatin (3 mg/kg/d) or vehicle from 7–9 wk of age via an orogastric tube. A group of *fa/fa* ZDF rats received apocynin (5 mmol/liter in drinking water). Rats were housed individually in metabolic cages for monitoring food intake, urine volume, and body weight. All procedures were performed in accordance with the guidelines of the University of the Ryukyus Committee on Animal Care and Handling.

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Abbreviations: DPI, Diphenyleneiodonium; eNOS, endothelial nitric oxide synthase; 8-epi-$PGF_2\alpha$, 8-epi-prostaglandin-$F_2\alpha$; FFA, free fatty acids; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high-density lipoprotein; HUVEC, human umbilical vein endothelial cells; NAC, *N*-acetyl-L-cysteine; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substance; ZDF, Zucker diabetic fatty.

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### *Biochemical measurements*

Plasma glucose levels were measured by the glucose oxidase method with the Glucose Analyzer II (Beckman Coulter Inc., Fullerton, CA). Plasma insulin levels were assessed using an insulin ELISA kit. Serum levels of cholesterol and triglyceride, and FFA were measured using routine enzymatic assays. Plasma levels of lipid peroxidation were measured as thiobarbituric acid reactive substance (TBARS) using the LPO test (Wako Pure Chemical Industries, Osaka, Japan) (13). Plasma and urinary 8-epi-prostaglandin-$F_2\alpha$ (8-epi-$PGF_2\alpha$) was extracted on C-18 SPE cartridges (Waters Corp., Milford, MA) and assayed by competitive immunoassay using a Cayman Chemical 8-epi-$PGF_2\alpha$ EIA kit (Ann Arbor, MI) (13). Plasma levels of adiponectin (Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan) and TNF-$\alpha$ (JIMRO Co., Ltd., Takasaki, Gunma, Japan) were measured by sandwich ELISA as previously described (3).

### *Vascular reactivity*

After a midlaparotomy under pentobarbital sodium anesthesia, the aorta was rapidly excised for vascular reactivity measurements (14), and nonfasting blood samples were obtained from the inferior vena cava. A portion of aorta was frozen in liquid nitrogen and stored at −70 C. Fresh aorta were cleared of periadventitial tissue and cut transversely into rings 1.5–2.0 mm in diameter. Vascular rings, handled carefully to avoid damage to the inner surface, were mounted on wires in the chambers of a multivessel myograph (J.P. Trading, Tokyo, Japan) and bathed in Krebs' buffer. The medium was gassed with 95% $O_2$ and 5% $CO_2$ and maintained at 37 C (pH 7.4). After equilibration (30 min), the rings were set to an isometric force-displacement transducer (TB-611T; Nihon Kohden, Tokyo, Japan) for measurement of changes in tension and allowed to stabilize for another 30 min. The rings were then depolarized with potassium chloride (60 mmol/liter) to evaluate maximal contraction. After washing with a Krebs' buffer, the vascular preparations were contracted with phenylephrine ($10^{-6}$ mol/liter), and when the contractile response was stabilized (steady-state phase, 15 min), vasorelaxing responses to cumulative increments in the concentration of acetylcholine or sodium nitroprusside were examined. The resting tension of the rings was adjusted to 1.0 g. Changes in vascular tension were recorded on a pen-writing recorder (WT-645G; Nihon Kohden).

### *Human umbilical vein endothelial cells (HUVEC) study*

HUVEC are plated in a 100-mm culture dish at the density of $2.0 \times 10^6$ cells per dish. After 16–24 h, the cells were incubated with 0.1–1 mM palmitate, a major fraction of saturated FFA in plasma, with a 1-h prior incubation each of vehicle, 1 mmol/liter pitavastatin, 10 $\mu$mol/liter diphenyleneiodium (DPI), 20 mmol/liter *N*-acetyl-L-cysteine (NAC). After treatment of indicated conditions, cells were harvested from the dish with 0.5× Trypsin-EDTA and then immediately subjected to cytoplasmic mRNA extraction by RNA-Easy kit (QIAGEN GmbH, Hilden, Germany).

### *ROS signals*

The intracellular ROS formation in HUVEC was detected using the fluorescent probe 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's protocol. Preliminarily, we confirmed the satisfactory efficacy of this probe to detect intracellular ROS signal induced by $H_2O_2$ in several cell lines including HUVEC.

### *Immunoprecipitations and Western blotting*

Western blot analysis was performed as described previously (15). Protein samples (50 $\mu$g) were prepared from thoracic aortas of four groups of mice and denatured and run on polyacrylamide gels. After transfer onto polyvinylidene difluoride transfer membranes, the membranes were blocked for 90 min in 5% nonfat milk solution. For immunoprecipitation, the primary antibodies [endothelial nitric oxide synthase (eNOS) or p47phox] were used at a 1:1000 dilution in 5% nonfat milk solution for 12 h at 4 C (16, 17). Bound antibodies were detected with horseradish-peroxidase-conjugated antimouse IgG and visualized with an enhanced chemiluminescence detection system (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL). For anti-phosphotyrosine or anti-phosphoserine blots, nitrocellulose membranes were blocked by incubation in Tris-buffered saline/Tween 20 containing 1% BSA for 2 h, followed by a 20-min incubation in anti-phosphotyrosine (eNOS) or anti-phosphoserine (p47phox) antibody diluted in blocking buffer. The membranes were washed extensively in Tris-buffered saline/Tween 20 and developed by using the above system. Band intensity was quantified by NIH ImageJ 1.32j, and the ratio of anti-phosphotyrosine or anti-phosphoserine blot intensity to those of eNOS or p47phox blot intensity was used to represent the enzyme catalytic activities. Lipid peroxidation level of aorta was determined using the TBARS assay kit (ZeptoMetrix Corp., Buffalo, NY).

### *Real-time RT-PCR*

RT-PCR was done with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Japan K.K., Tokyo, Japan) and SYBR green on an ABI PRISM 7000 real-time PCR system (Applied Biosystems Japan Ltd., Tokyo, Japan) (13). Primers used were as follows: p22phox (GenBank NM_000101) forward, ATTACTATGTTCGGGCCGTCCT, and reverse, GGTAGATGCCGCTCGCAAT; p40phox (NM_000631) forward, ATGCGGATACCTGCCCTCAA, and reverse, CTCTGAGTCATAGGGCGACTGGTAA; p47phox (NM_000265) forward, GATGCCCAAAGATGGCAAGAGTA, and reverse, GCTTTCATCTGACAGAACCACCAA; p67phox (NM_000433) forward, AGCTCCGGCTGGAACACACTA, and reverse, GGCACCAGCTCATTGCTGTC; gp91phox (NM_000397) forward, AAATGGATCGCATCTGTGTGAC, and reverse, TGGCCACACTAACAGTGATTTAGAG; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM_002046) forward, GGCCTCCAAGGAGTAAGACC, and reverse, AGGGGTCTACATGGCAACTG. Results are expressed as fold change in gene expression by determining the ratio of copy number of the gene of interest corrected for expression of GAPDH in the samples.

### *Statistical analysis*

Values are expressed as the mean ± SE. Two-tailed unpaired Student's *t* test or one-way factorial ANOVA, followed by Bonferroni's *post hoc* comparisons, was used to compare group means. Comparisons of dose-response curves were made by two-factor repeated-measures ANOVA. A $P$ value $< 0.05$ was considered statistically significant. Analyses were processed using StatView J-5.0 software package (SAS Institute Inc., Cary, NC) or InStat 3 for Macintosh version 3.0b (GraphPad Software, Inc., San Diego, CA).

## Results

### *Metabolic features in obese ZDF (fa/fa) rats*

The mean body weight, plasma glucose, insulin, FFA, and triglyceride of obese male ZDF (*fa/fa*) rats at 9 wk of age were all higher than age-matched (+/+) controls (Table 1). Pitavastatin treatment did not significantly change plasma levels of total and high-density lipoprotein (HDL)-cholesterol, triglyceride, and FFA in ZDF rats.

### *Effects of pitavastatin or apocynin on vascular reactivity*

In aortic rings, vasoconstrictive response to phenylephrine was almost identical between +/+ and ZDF rats (Fig. 1, *left*). Addition of acetylcholine produced a dose-dependent relaxation ($10^{-9}$ to $10^{-4}$ mol/liter) in both +/+ and ZDF rats (Fig. 1, *center*), but the $ED_{50}$ value was significantly higher in ZDF rats (Table 2). A 2-wk treatment of pitavastatin or apocynin improved the dose-relaxation response in ZDF rats (Table 2). The vasodilator response to sodium nitroprusside was almost identical between +/+ and ZDF rats and was not affected by pitavastatin or apocynin treatment (Fig. 1, *right*).

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**TABLE 1.** General characteristics of animals after a 2-wk treatment with vehicle or pitavastatin

| | Lean +/+ | | ZDF *fa/fa* | | |
|---|---|---|---|---|---|
| | Vehicle | Pitavastatin | Vehicle | Pitavastatin | Apocynin |
| Body weight (g) | 153 ± 28 | 150 ± 6 | 220 ± 19[b] | 203 ± 21[b] | 227 ± 12 |
| Food intake (g/d) | 18 ± 1 | 17 ± 1 | 24 ± 2[a] | 23 ± 1[a] | 28 ± 2[a] |
| Glucose (mmol/liter) | 7.9 ± 0.5 | 7.8 ± 0.4 | 10.3 ± 0.8 | 7.9 ± 0.8 | 10.6 ± 1.3 |
| Insulin (pmol/liter) | 9.6 ± 1.8 | 9.7 ± 1.6 | 83.3 ± 13.6[a] | 79.2 ± 8.0[a] | 34.5 ± 8.6[a] |
| Total cholesterol (mmol/liter) | 2.85 ± 0.11 | 2.72 ± 0.07 | 3.02 ± 0.35 | 3.43 ± 0.67 | 2.59 ± 0.09 |
| Triglyceride (mmol/liter) | 0.41 ± 0.09 | 0.32 ± 0.08 | 1.44 ± 0.40[b] | 1.13 ± 0.59[b] | — |
| HDL-cholesterol (mmol/liter) | 0.91 ± 0.03 | 0.82 ± 0.09 | 1.08 ± 0.14 | 1.02 ± 0.11 | 1.28 ± 0.08 |
| FFA (mmol/liter) | 0.33 ± 0.03 | 0.30 ± 0.03 | 0.92 ± 0.16[a] | 0.50 ± 0.18[a] | 0.53 ± 0.11[a] |
| Plasma TBARS (nmol MDA/ml) | 1.0 ± 0.1 | 1.3 ± 0.3 | 8.08 ± 1.533[b] | 7.29 ± 1.64[b] | 7.00 ± 0.35[b] |
| Urinary 8-epi-$PGF_2\alpha$ (pg/mg creatinine) | 533 ± 132 | 545 ± 147 | 890 ± 75[b] | 795 ± 157[b] | 892 ± 24[b] |

Values are the mean ± SEM of three to six rats. MDA, Malondialdehyde.
[a] $p < 0.017$ *vs.* lean +/+ vehicle (*post hoc* Bonferroni test).
[b] $p < 0.05$ *vs.* lean +/+ vehicle (*t* test).

*Circulating ROS and vascular activities of NADPH oxidase and eNOS*

Levels of plasma TBARS and urinary 8-epi-$PGF_2\alpha$ were increased in ZDF rats (Table 1). A 2-wk treatment with pitavastatin did not change plasma TBARS and urinary 8-epi-$PGF_2\alpha$ in ZDF rats. The level of eNOS phosphorylation was not different between +/+ and ZDF rats (Fig. 2, *left*), but the level of p47phox serine phosphorylation, a marker of NADPH oxidase activity, was increased in aorta homogenates of ZDF rats, and it was decreased by pitavastatin treatment (Fig. 2, *center*). Vascular ROS production was also increased in ZDF rats, and the increase was inhibited by pitavastatin to the comparable level of +/+ (Fig. 2, *right*).

*HUVEC study*

Using *in vitro* cell culture, palmitate increased ROS signals dose dependently (0.1–2 mmol/liter) and time dependently (up to 24 h) (data not shown). The palmitate-induced ROS formation was inhibited completely by pitavastatin and a general antioxidant, NAC, and partially by DPI, a NADPH oxidase inhibitor (Fig. 3). Palmitate increased expression levels of p22phox, p40phox, p47phox, p67phox, and gp91phox subunit gene (Fig. 3). A prior treatment with pitavastatin inhibited palmitate-induced up-regulation in p22phox, p40phox, and p47phox mRNA, but did not change p67phox and gp91phox.

## Discussion

The major findings of the present study were that 1) the vasodilator response to acetylcholine, but not to sodium nitroprusside, was impaired in prediabetic obese ZDF rats, 2) elevations in circulating FFA and ROS and an enhancement of NADPH oxidase activation and vascular ROS production were also observed in ZDF rats, 3) pitavastatin recovered vasodilator responses in ZDF rats with a reduction of vascular NADPH oxidase activity and ROS production, and 4) FFA enhanced production of ROS and expression of NADPH oxidase subunit mRNA, and those were inhibited by pitavastatin.

*Vascular reactivity and vascular ROS*

Using a rodent model of visceral fat obesity, the ZDF (*fa/fa*) rat (4, 5), we measured the vascular response to vasodilatory and vasoconstrictive agents. The vasodilator response to acetylcholine, but not to sodium nitroprusside, was impaired in ZDF rats. This indicates that endothelium-dependent vasodilatation, frequently represented by the response to acetylcholine, was impaired, but endothelium-

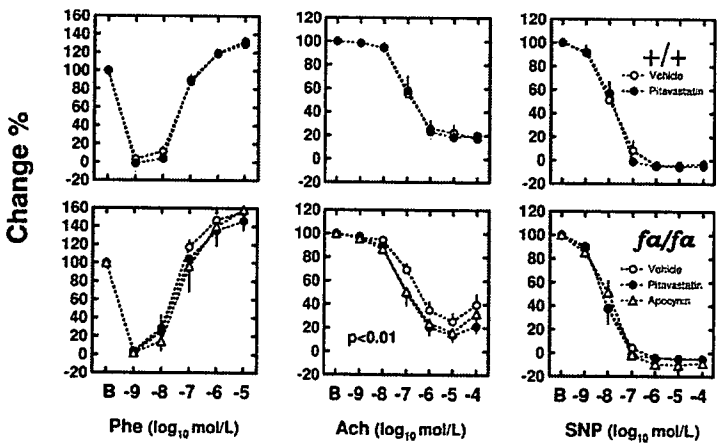


FIG. 1. Vascular reactivity in aorta isolated from +/+ and *fa/fa* ZDF rats. Rats were treated with vehicle (○), pitavastatin (●), or apocynin (△) from 7–9 wk of age. Vascular reactivity to phenylephrine (Phe), acetylcholine (Ach), or sodium nitroprusside (SNP) was determined in +/+ (*upper panels*) and ZDF rats (*lower panels*). Data represent the mean ± SEM (n = 3–6). The *P* values for curve difference by two-factor repeated-measures ANOVA are shown.

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**TABLE 2.** $ED_{50}$ value of vascular reactivity

| | Lean +/+ | | ZDF *fa/fa* | | |
|---|---|---|---|---|---|
| | Vehicle | Pitavastatin | Vehicle | Pitavastatin | Apocynin |
| Phenylephrine ($log_{10}$ mol/liter) | −8.247 ± 0.126 | −8.255 ± 0.122 | −7.431 ± 0.074 | −7.391 ± 0.080 | −7.194 ± 0.128 |
| 95% confidential interval | −9.842 to −6.651 | −9.806 to −6.705 | −8.374 to −6.489 | −8.411 to −6.371 | −8.825 to −5.563 |
| Acetylcholine ($log_{10}$ mol/liter) | −7.111 ± 0.053 | −7.021 ± 0.017 | −6.803 ± 0.050[a] | −7.156 ± 0.030[b] | −7.143 ± 0.048[b] |
| 95% confidential interval | −7.278 to −6.943 | −7.075 to −6.967 | −7.016 to −6.590 | −7.286 to −7.027 | −7.348 to −6.938 |
| Sodium nitroprusside ($log_{10}$ mol/liter) | −7.942 ± 0.025 | −7.870 ± 0.038 | −8.155 ± 0.035 | −8.153 ± 0.016 | −7.904 ± 0.074 |
| 95% confidential interval | −8.022 to −7.863 | −7.989 to −7.750 | −8.268 to −8.042 | −8.205 to −8.102 | −8.138 to −7.670 |

Values are the mean ± SEM of three to six rats.
[a] $P < 0.05$ *vs.* lean +/+ vehicle.
[b] $P < 0.05$ *vs.* ZDF *fa/fa* vehicle.

independent vasodilatation, represented usually by the response to sodium nitroprusside, was preserved in ZDF rats. Levels of plasma TBARS and urinary 8-epi-$PGF_2\alpha$ were increased in ZDF rats, indicating an increase in circulating ROS. Because accumulated fat is possibly a principal source of circulating ROS in obesity (13), the circulating ROS might be coming mainly from accumulated fat. However, the level of vascular ROS production was also increased in ZDF rats. Serine phosphorylation of p47phox, which is a critical step for cytoplasmic complex formation of NADPH oxidase and serves as NADPH oxidase activation (17), was enhanced in ZDF aorta, indicating that ROS production was also locally amplified. Increased ROS, regardless of whether it was locally produced or fat-derived remote ROS (13), may be associated with endothelial dysfunction (18).

Under normal conditions, NO released by eNOS stimulates soluble guanylyl cyclase, increasing cGMP, activating cGMP-dependent protein kinase 1, and finally eliciting vasodilation (18). When vascular ROS is in excess, it can react with NO, thereby generating peroxynitrite, the most stable and potent oxidant. Peroxynitrite uncouples eNOS, switching the NO-producing process to a ROS reproduction process (18). In ZDF rats, excess vascular ROS can come from increased activity of NADPH oxidase and normal activity of eNOS. A 2-wk treatment with a NADPH oxidase inhibitor, apocynin (13), almost completely recovered the vascular response to acetylcholine in ZDF rats, supporting the notion that vascular ROS is the major cause of endothelial dysfunction.

Hyperglycemia is the other possible mechanism of vascular endothelial dysfunction in visceral obesity. Our ZDF rats at 9 wk of age were at the phase of glucose intolerance, showing mild hyperglycemia (nonfasting was 7.9 mmol/liter *vs.* age-matched control was 10.3 mmol/liter), hyperinsulinemia (9.6 *vs.* 83.3 pmol/liter) and hyperlipacidemia (0.33 *vs.* 0.92 mmol/liter) (4–6). We confirmed that this level of mild hyperglycemia did not impair vascular reactivity to acetylcholine and did not increase ROS production (data not shown). Although we cannot completely exclude the role of hyperglycemia in impairing vascular reactivity, it is likely that mild hyperglycemia is not the primary cause of ROS-associated endothelial dysfunction in prediabetic ZDF rats.

It had been shown that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statin) reduce ROS production (10, 11). In our study, pitavastatin did not change levels of plasma TBARS and urinary 8-epi-$PGF_2\alpha$ in ZDF rats but improved the vasodilator response to acetylcholine. The level of vascular ROS was decreased by pitavastatin simultaneously with a decrease of vascular p47phox serine phosphorylation, indicating that pitavastatin somehow inhibited the activation process of NADPH oxidase (10, 11). Pitavastatin did not change the plasma levels of adipocyte-derived cytokines such as TNF-$\alpha$ (vehicle 4.00 ± 0.75 *vs.* pitavastatin 4.77 ± 1.69 pg/ml) and adiponectin (8.75 ± 0.95 *vs.* 7.27 ± 0.48 $\mu$g/ml) in ZDF rats (19, 20) (see also IDF Worldwide Definition of the Metabolic Syndrome at http://www.idf.org/home/). Collectively, pitavastatin did not affect circulating ROS level but decreased vascular ROS production, suggesting direct vascular effects.

### *FFA and vascular NADPH oxidase activity*

A common feature of visceral fat obesity is an oversupply of FFA to the bloodstream from adipose tissues, and FFA can enhance vascular production of ROS (8, 9). We thus tested whether FFA do directly activate vascular ROS production

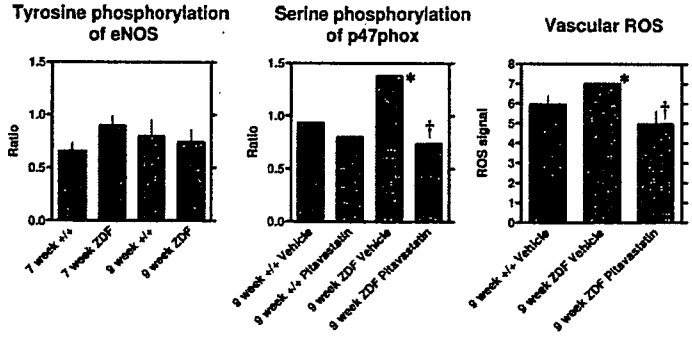


FIG. 2. Activities of eNOS and p47phox and ROS signal in aorta isolated from +/+ and *fa/fa* ZDF rats. Rats were treated either with vehicle or pitavastatin from 7–9 wk of age. RDS signal was expressed in pmol malondialdehyde/$\mu$g protein. Data represent the mean ± SEM (n = 3–6). *, $P < 0.05$ *vs.* 9-wk +/+ vehicle; †, $P < 0.05$ *vs.* 9-wk *fa/fa* ZDF rats.

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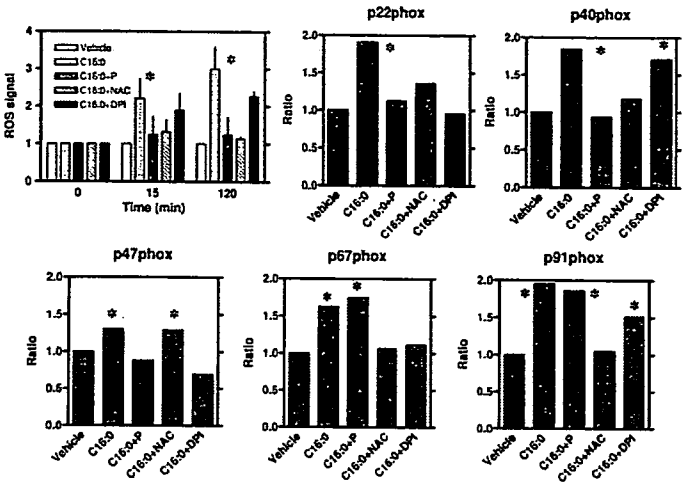


FIG. 3. Effects of palmitate on ROS production and levels of vascular NADPH oxidase subunit gene in HUVEC. HUVEC were incubated for 0, 15, and 120 min with vehicle or 1 mM palmitate (C16:0) during a 1-h previous incubation each of 1 mmol/liter pitavastatin (P), 20 mmol/liter *N*-acetyl-L-cysteine (NAC), or 10 μmol/liter diphenyleneiodium (DPI). The intracellular ROS formation was detected using the fluorescent probe, 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester. Expression levels of p22phox, p40phox, p47phox, p67phox, and gp91phox subunit gene were quantified by real-time PCR and corrected by GAPDH level. Data represent the mean ± SEM (n = 3–6). *, $P < 0.0125$ *vs.* vehicle.

and, if so, whether it can be through NADPH oxidase activation.

First we confirmed that palmitate, a major fraction of saturated FFA in plasma, directly increased intracellular ROS signals dose dependently and time dependently (data not shown) (8). The palmitate-induced increases in vascular ROS signals were inhibited completely by pitavastatin and a general antioxidant, NAC, and partially by DPI, a NADPH oxidase inhibitor.

The major source of superoxide anion in the vasculature is the NADPH oxidase family of enzymes (21, 22). Vascular NADPH oxidase is a multisubunit enzyme complex that includes the membrane-bound flavocytochrome b558 formed by gp91phox and p22phox and the cytosolic proteins p47phox, p67phox, and Rac. We thus determined the effects of palmitate on the expression levels of the vascular NADPH oxidase subunit gene. Palmitate increased expression levels of p22phox, p40phox, p47phox, p67phox, and gp91phox. A prior treatment with pitavastatin inhibited the palmitate-induced increases in p22phox, p40phox, and p47phox mRNA but did not change p67phox and gp91phox.

Two general mechanisms underlying activation of NADPH oxidase are either an acute increase in oxidase complex formation secondary to posttranslational modification of regulatory subunits (p47phox and Rac) or a chronic increase in the expression and abundance of component subunits (18, 21, 22). As we and others reported previously (5, 8), palmitate directly increases diacylglycerol levels and protein kinase C activation, which is the well-known signal for activation of NADPH oxidase. A key mechanism of acute activation by palmitate can be that protein kinase C-dependent phosphorylation of the p47phox regulatory subunit and its translocation to the Nox2/p22phox heterodimer to form fully assembled complexes. Increased expression of NADPH oxidase subunits might be the mechanism of chronic NADPH activation by palmitate.

As recently reported, mitochondrial uncoupling could be another source of ROS production in FFA-treated endothelial cells (23). Adenoviral overexpression of uncoupling protein 1 (UCP-1) or inhibition of mitochondrial FFA oxidation by carnitine palmitoyltransferase I (CPT-I) inhibitor (etomoxir) could inhibit such FFA-induced ROS production. NADPH oxidase and mitochondrial uncoupling could independently contribute to FFA-induced ROS production in vascular system.

Activated Rac in its GTP-bound state binds to the cytosolic p67phox subunit and activates the oxidase. Pitavastatin may inhibit palmitate-induced activation of NADPH oxidase through Rac inactivation, because Rac activation requires its posttranslational modification by isoprenylation, a process that is inhibited by statin (10, 11). Inhibition of palmitate-induced up-regulation of NADPH oxidase subunits may be another mechanism of NADPH inactivation by pitavastatin.

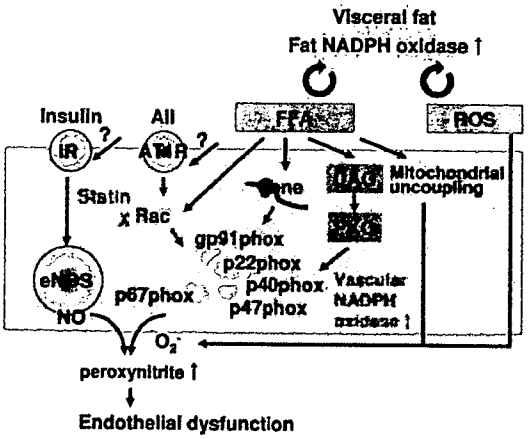


FIG. 4. A working hypothesis of vascular lipotoxicity. Visceral fat is the major source of circulating FFA and ROS. Circulating FFA induces vascular ROS production via up-regulation of vascular NADPH oxidase. The locally produced ROS and fat-derived ROS concomitantly react with NO, generate peroxynitrite, and finally impair cGMP-dependent vasodilatation. Statin may block FFA-induced ROS production via inhibition of NADPH oxidase expression and Rac inactivation. There is controversy about effects of FFA on insulin-mediated eNOS activation and angiotensin type 1 receptor (AT1R) signaling. AII, Angiotensin II; DAG, diacylglycerol.

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*Conclusion*

Endothelial dysfunction and NADPH oxidase activation were concomitantly observed in obese ZDF rats, but those were improved by pitavastatin and apocynin treatment. It is suggested that pitavastatin might inhibit FFA-induced NADPH oxidase subunit gene expression and ROS production in endothelial cells and then protect the endothelial dysfunction seen in obese ZDF rats. Visceral fat obesity is the essential component of the metabolic syndrome including hypertension, dyslipidemia, and glucose intolerance (Ref. 19 and http://www.idf.org/home/). Endothelial dysfunction, which is a systemic disorder and a key variable in the initiation and progression of atherosclerosis and its complications (20), occurs frequently in subjects with visceral fat obesity (1–3). As shown in Fig. 4, we suggest that FFA-induced ROS overproduction might be a possible underlying mechanism(s) for the impaired endothelial function in visceral fat obesity, vascular lipotoxicity (Fig. 4).

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# Effects of dietary composition on postprandial endothelial function and adiponectin concentrations in healthy humans: a crossover controlled study[1,2]

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## ABSTRACT

**Background:** Abnormalities during the postprandial state contribute to the development of atherosclerosis. Reportedly, postprandial hyperglycemia, hypertriglyceridemia, and hyperlipacidemia independently cause postprandial cytokine activation. However, it is not clear which dietary composition preferentially affects postprandial endothelial function in healthy subjects.

**Objective:** We aimed to examine the associations of dietary composition and postprandial endothelial function in healthy subjects.

**Design:** The effects of a single ingestion of a high-carbohydrate meal (300 kcal, 100% carbohydrate), a high-fat meal (30 g fat/m$^2$, 35% fat), or a standard test meal (478 kcal; 16.4% protein, 32.7% fat, 50.4% carbohydrate) on postprandial plasma concentrations of adiponectin and forearm blood flow (FBF) during reactive hyperemia were studied in healthy subjects.

**Results:** The peak FBF response and the total reactive hyperemic flow (flow debt repayment; FDR), indexes of resistance artery endothelial function, were unchanged after ingestion of a high-carbohydrate and standard test meal but decreased 120 and 240 min after a high-fat meal. After a high-fat meal, decreases in peak FBF and FDR were well correlated with an increase in plasma free fatty acid (FFA) concentrations but not with the other biochemical variables, including triacylglycerol, insulin, glucose, total cholesterol, HDL cholesterol, and adiponectin.

**Conclusions:** Postprandial endothelial function was impaired only after the high-fat diet and not after the high-carbohydrate or standard test meal in healthy subjects. Because such endothelial dysfunction after a high-fat meal was closely correlated with FFA concentrations, postprandial state could be hazardous, mostly through acute hyperlipacidemia in healthy subjects. *Am J Clin Nutr* 2007;86:923–8.


**KEY WORDS** Free fatty acids, endothelial function, adiponectin, postprandial state, hyperlipidemia, hyperglycemia

## INTRODUCTION

The abnormalities of the postprandial state are important contributing factors to the development of atherosclerosis. There is evidence that postprandial hypertriglyceridemia is a risk factor for cardiovascular diseases (CVD) (1), whereas in diabetic subjects, postprandial hyperglycemia has been proposed as an independent risk factor for CVD (2).

The risk of coronary artery disease is increased by consumption of a diet rich in saturated fatty acids (3). In both healthy subjects and in diabetic patients, a single high-fat meal induces endothelial activation, which is associated with increased inflammatory cytokine production (4). Meanwhile, postprandial hypertriglyceridemia (4–6) and hyperglycemia (7) can elicit endothelial dysfunction via an independent and a cumulative increase in oxidative stress. Free fatty acids (FFAs) elevated by intravenous lipid or heparin infusion directly impair the vasodilatory response to acetylcholine in healthy humans, which may be pathophysiologically relevant to the development of postprandial endothelial dysfunction in patients with obesity and insulin resistance (8–10).

Adiponectin is an adipocyte-derived plasma protein (adipocytokine) that accumulates in injured arteries and has potential antiatherogenic properties. Maintenance of adiponectin concentrations is closely associated with normal endothelial function in humans (11, 12); therefore, postprandial changes in plasma adiponectin concentrations (13–15) could be related to postprandial endothelial dysfunction. However, such a relation has not been evaluated. We investigated effects of diet composition on postprandial plasma concentrations of adiponectin and endothelial function in healthy subjects.

## SUBJECTS AND METHODS

### Subjects

Twelve healthy subjects (6 men and 6 women) aged 30–42 y ($\bar{x}$ ± SD: 36 ± 1 y) participated in this study. The subjects had a mean (±SD) body weight of 62 ± 4 kg, body mass index (in kg/m$^2$) of 23.3 ± 0.9, systolic blood pressure of 121 ± 5 mm Hg, diastolic blood pressure of 72 ± 3 mm Hg, and heart rate of 63 ± 2 beats/min. All subjects gave their written informed consent before the study began. The study protocol was approved by the Ethical Committee of the University of Ryukyus and was carried

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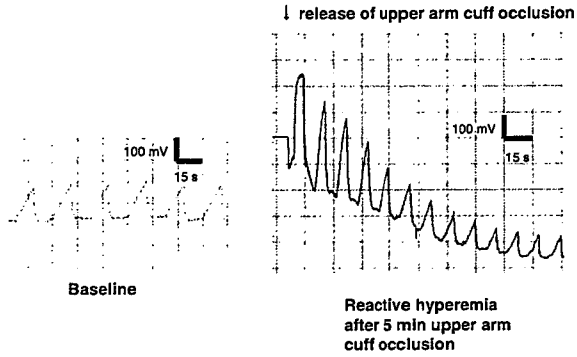


**FIGURE 1.** Representative tracing of forearm blood flow (FBF). FBF was calculated from gradient of tracing (ΔmV/s) with a mercury-filled strain-gauge plethysmograph at baseline and after 5 min of upper arm cuff occlusion.

out in accordance with the principles of the Declaration of Helsinki as revised in 2000. None of the subjects had an acute or chronic illness, had experienced recent body weight changes, or were taking medication regularly. All subjects abstained from alcohol, tobacco, and strenuous physical activity for 24 h and caffeine-containing drinks overnight.

### Endothelial function

On 3 separate mornings ≥7 d apart, participants ingested a high-carbohydrate meal (300 kcal, 100% carbohydrate) (16), a high-fat meal (30 g fat/$m^2$, 35% fat, 342 kcal/100 g), or a standard test meal (478 kcal; 16.4% protein, 32.7%fat, 50.4% carbohydrate; proposed by a working group of the Japan Diabetes Society) after fasting overnight (17). The order for meal ingestion was randomized in a crossover design. Endothelial function was measured by using forearm blood flow (FBF) before and 120 and 240 min after the ingestion of each meal. Blood samples were obtained at 0, 30, 60, 120, and 240 min. FBF was measured with the use of a mercury-filled silastic strain-gauge plethysmograph (EC-5R; DE Hokanson Inc, Issaquah, WA) as described (**Figure 1**) (11, 16).

The strain gauge was attached to the upper arm, held above the right atrium, and connected to a plethysmographic device. At baseline and during 180 s after release of 5-min occlusion of the upper arm at 200 mm Hg (reactive hyperemia), FBF was measured as follows. First, the wrist cuff was inflated to a pressure of 200 mm Hg to exclude hand circulation, and then the upper arm cuff was repetitively inflated to 40 mm Hg to occlude venous outflow from the arm (7 s) and deflated (8 s) in each 15-s cycle with a rapid cuff inflator (EC-20; DE Hokanson Inc). The FBF output signal was transmitted to a recorder (U-228, Advance Co, Nagoya, Japan). FBF was calculated from gradient of tracing (ΔmV/s) with a mercury-filled strain-gauge plethysmograph at baseline and after 5 min of upper arm cuff occlusion and was expressed as milliliters per minute per 100 mL of forearm tissue. A representative tracing of an FBF curve is shown in Figure 1.

Calculations of blood flow debt incurred during arterial occlusion, reactive hyperemic flow, and blood flow debt repayments (FDR) were made as described (11) below:

$$\text{Blood flow debt (mL)} = \text{control flow rate (mL/s)} \times \text{duration of occlusion (s)} \quad (1)$$

$$\text{Reactive hyperemic flow (mL)} = [\text{total flow during reactive hyperemia (mL)}] - [\text{control flow rate (mL/s)} \times \text{duration of reactive hyperemia (s)}] \quad (2)$$

$$\text{Blood flow debt repayment (\%)} = (\text{reactive hyperemic flow/blood flow debt}) \times 100 \quad (3)$$

Before and after release of a 5-min upper arm cuff occlusion at 200 mm Hg (reactive hyperemia) and after a single sublingual administration of 0.3 mg nitroglycerin (Nihonkayaku Co, Tokyo, Japan). In the preliminary study, we confirmed the reproducibility of reactive hyperemia and sublingual nitroglycerin-induced vasodilatation on 2 separate occasions in healthy male subjects (11).

### Biochemical measurements

Before and after meal ingestion, venous blood was collected from subjects by using a vacuum tube with serum separation reagents or heparin sodium and then stored frozen until used. Preliminary effects of blood sampling conditions, including serum separation reagents, heparin sodium, and EDTA-$Na_2$, on adiponectin measurements were compared. There was a good correlation between adiponectin concentration and serum and heparin-treated plasma ($r = 0.997$; regression line $y = 0.96x + 0.05$) and also between serum and EDTA-treated plasma ($r = 0.999$; regression line $y = 0.93x + 0.08$).

Plasma glucose concentration was measured with a glucose oxidase method, and insulin was measured by enzyme-linked immunosorbent assay (ELISA). Serum concentrations of total cholesterol, HDL cholesterol, and triacylglycerols were measured by routine enzymatic methods. Plasma and serum adiponectin concentrations were determined with a latex-particle-enhanced turbidimetric immunoassay (LTIA) (Human Adiponectin Assay Kit; Mitsubishi Kagaku Iatron Inc, Chiba, Japan) with an automated analyzer (Hitachi H7170) (18). The LTIA results were well correlated with the commercially available ELISA assay (18).

### Statistical analysis

Values are expressed as means ± SEMs. Multigroup comparisons were made by one-factor repeated-measures analysis of variance (ANOVA). Multigroup comparisons of time course curves were first analyzed by two-factor repeated-measures ANOVA. If the multigroup difference was significant, intragroup comparisons were made by one-factor repeated-measures ANOVA and followed by Tukey's post hoc test. For comparisons between metabolic variables and endothelial function during meal loading, the analysis was adjusted by analysis of covariance (ANCOVA). Probabilities were considered to be significant if $<0.05$. The data were processed by using the GRAPHPAD INSTAT 3 for Macintosh (GraphPad Software Inc, San Diego, CA) software package.

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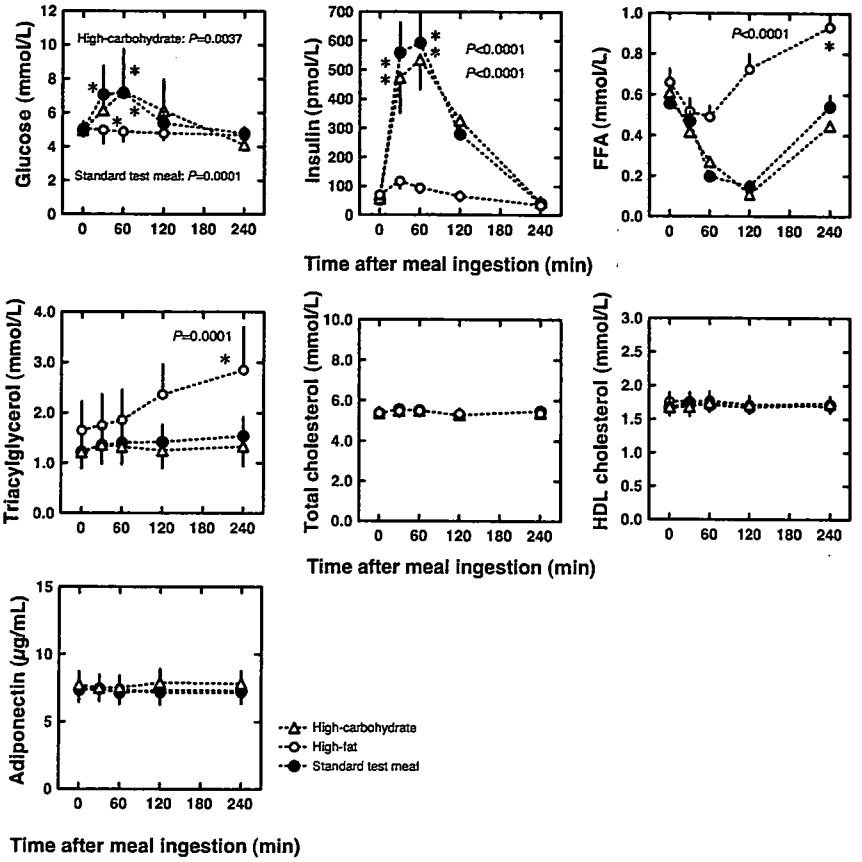


**FIGURE 2.** Mean (±SEM) blood concentrations of glucose, insulin, free fatty acid (FFA), triacylglycerols, total and HDL cholesterol, and adiponectin after loading of a high-carbohydrate, high-fat, and standard test meal in 12 healthy subjects (6 men and 6 women). Multigroup comparisons of time course curves were analyzed by 2-factor repeated-measures ANOVA. If the multigroup difference was significant, intragroup comparisons were made by one-factor repeated-measures ANOVA followed by Tukey's post hoc test. *Significantly different from 0 min, $P < 0.01$ (Tukey's post hoc test).

## RESULTS

The meals were well tolerated by all patients, and no adverse events were observed during the study. Systemic hemodynamic and metabolic variables before test meal ingestion were comparable on the 3 study mornings.

The effects of the high-carbohydrate, high-fat, and standard test meals on plasma glucose, plasma insulin, serum lipid, and serum adiponectin concentrations are shown in **Figure 2**. After the high-carbohydrate and standard test meals, plasma glucose concentrations increased from baseline by ≈5 mmol/L to a peak of ≈7.2 mmol/L at 60 min and returned to baseline at 240 min (Figure 2). Plasma glucose concentrations did not change after the high-fat meal. After the high-carbohydrate and standard test meals, plasma insulin concentrations increased from baseline by ≈50 pmol/L to a peak of 540–590 pmol/L at 60 min. The plasma insulin concentration did not change after the high-fat meal. Serum FFA concentrations decreased during the 120 min after the high-carbohydrate and standard test meals and returned to baseline at 240 min. Serum FFA concentrations increased to 0.87 ± 0.06 mmol/L at 240 min after the high-fat meal. Serum triacylglycerol concentrations increased to 2.77 ± 0.83 mmol/L at 240 min after the high-fat meal, but did not change after the high-carbohydrate and standard test meals. Serum concentrations of total and HDL cholesterol and adiponectin did not change during 240 min after either test meal.

The effects of the high-carbohydrate, high-fat, and standard test meals on FBF are shown in **Figure 3**, A, B, and C. The peak FBF was unchanged before and after the high-carbohydrate and standard test meals, but decreased significantly 120 and 240 min after the high-fat meal (Figure 3, A and B). The total reactive hyperemic flows (FDR), indexes of resistance artery endothelial function, also decreased 240 min after the high-fat meal (Figure 3, C).

After the high-fat meal, changes from baseline in peak FBF (Δpeak FBF) and FDR (ΔFDR) were inversely well correlated with the change in plasma FFA concentration (ΔFFA) (**Figure 4**) but not with the other biochemical variables, including triacylglycerol, insulin, glucose, total cholesterol, HDL cholesterol, and adiponectin (data not shown).

## DISCUSSION

In this study, we investigated the effects of diet composition on postprandial plasma concentrations of adiponectin and endothelial function in healthy subjects. Effects of the high-carbohydrate, high-fat, and standard test meals on plasma

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