of physicians employed by internal medicine departments (ie, internal medicine, pediatrics, psychiatry and radiology) among respondents with those among both nonrespondents and national physicians.

Bivariate analyses were performed to identify factors that might be associated with current participation in clinical research. We used chi-square tests for categorical variables and *t* test for continuous variables. The continuous variables in this dataset were age range (decade) and knowledge of Helsinki. Correlation analyses were performed to test for multicollinearity between 5 sets of factors we hypothesized might be highly correlated (age range and status, past participation in clinical research and past submission for publication of a manuscript on clinical research, past participation in clinical research and past writing of a research protocol, past participation in clinical research and prospective participation in clinical research, and past submission for publication of a manuscript on clinical research and past writing of a research protocol). Decisions to include factors in the multiple logistic regression analysis were based on the strength of correlated factors ($r < 0.75$) or a $P$ value < .05 on bivariate analyses. We performed multiple logistic regression analysis to identify factors that were correlated with participation in clinical research.

A $P$ value of less than 0.05 was considered to be statistically significant. Analysis was performed using STAT View (SAS Institute Inc, Cary, NC).

## Results

### *Characteristics of respondents*

Among 602 physicians from the 31 departments who received the questionnaire, a total of 51.5% (310 of 602) completed the questionnaire. A total of 175 faculty and 58 residents responded; 173 faculty and 243 residents did not respond ($P < 0.001$). As to age range, 47.8% of nonrespondents were aged 20 to 29, 16.8% of nonrespondents were aged 30 to 39, and 24.2% of nonrespondents were 40 to 49. Table 1 provided age range of respondents. There were statistically significant difference between respondents and nonrespondents on age range ($P < 0.001$). The survey respondents were not representative of all physicians at KUH: Faculty was more likely to complete survey than were residents, possibly because many junior residents did not receive the questionnaire. As junior residents rotate through various specialties, some of the person in charge of each department hesitated to distribute the questionnaire to junior residents. A total of 96 faculty and residents employed in internal medicine departments responded to the questionnaire, and 137 faculty and residents in surgical or other departments responded to the questionnaire. There were 164 nonrespondents in internal medicine departments and 252 nonrespondents in surgical or other departments ($P$ =

**Table 1: Characteristics of the 304 respondents**

| Characteristic | Percent* | | |
|---|---|---|---|
| | resident or doctoral student (n = 129) | faculty (n = 175) | total (n = 304) |
| Age range | | | |
| <= 29 | 15.5 | 0.6 | 6.9 |
| 30-39 | 82.2 | 31.4 | 53.0 |
| 40-49 | 2.3 | 53.1 | 31.6 |
| >= 50 | 0.0 | 14.9 | 8.6 |
| Internal medicine departments | 50.4 | 36.0 | 42.1 |
| Current participation in clinical research | 48.8 | 82.3 | 68.1 |
| Past participation in clinical research | 53.5 | 89.1 | 74.0 |
| Prospective participation in clinical research | 61.2 | 89.7 | 77.6 |
| Previous training course in clinical research | 11.6 | 18.9 | 15.8 |
| Do you consider it is necessary for physicians to conduct clinical research? | | | |
| yes | 96.1 | 97.1 | 96.7 |
| Have you ever written research protocol? | | | |
| yes | 14.0 | 51.4 | 35.5 |
| Have you submitted for publication of a manuscript on clinical research? | | | |
| yes | 24.8 | 50.9 | 39.8 |
| Do you know "World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects"? | | | |
| I know very well | 10.9 | 28.0 | 20.7 |
| I know to some degree | 81.4 | 68.0 | 73.7 |
| I don't know | 5.4 | 2.9 | 3.9 |

* Percent values were expressed as ratio in respondents of each age range. Percentage may not total 100% due to missing or blank data.

0.657 vs respondents). In comparison, there were 77358 physicians in internal medicine departments and 90969 physicians in surgical or other departments in hospitals in Japan in December 2006 [10] ($P = 0.146$ vs respondents). Respondents did not differ from nonrespondents and national physicians in the proportion of physicians who belonged to internal medicine departments.

Table 1 lists the respondents' characteristics by status: resident or doctoral student vs faculty. Six respondents with other status or with blank data for status were deleted. Among respondents, 68% of physicians reported current participation in clinical research; 74% reported past participation in clinical research. More faculty than resident or doctoral student reported past participation in, current participation in and prospective participation in clinical research. Most physicians (97%) believed that it is necessary for physicians to conduct clinical research. More than half of faculty had written a research protocol and reported submitting for publication of a manuscript on clinical research, whereas 14% of counterpart had written a research protocol and 25% of counterpart reported submitting for publication of a manuscript on clinical research. However, only 16% had taken a training course in clinical research offered by either the Japan Clinical Oncology Group (9), Kyoto University Graduate School of Medicine (9), other domestic universities and scientific societies (9), or foreign institutions (2). Most physicians (94%) were aware of the World Medical Association Declaration of Helsinki; 4% were not.

### *Attitudes*

Respondents were queried regarding the benefits of conducting clinical research. Obtaining a better understanding of disease was the most frequently cited benefit, and was mentioned by 255 physicians (47.3%). Enhanced standing in society or the hospital was the second most frequently cited benefit, and was mentioned by 150 physicians (27.8%), followed by obtaining research grants or awards. Eleven respondents (2.0%) felt that there was no benefit (Table 2).

**Table 2: Attitude towards clinical research**

| Question | Percent*(%) |
|---|---|
| What benefits do you think are brought to physicians of conducting clinical research? | |
| Physicians can obtain a better understanding of disease | 47.3 |
| Physicians will enhance standing in society or in hospital | 27.8 |
| Physicians will obtain research grants or awards | 12.8 |
| Physicians will obtain credits to be board certified doctor | 4.1 |
| There is no benefit to physicians | 2.0 |
| Which lecture topics related to clinical research are interesting or useful? | |
| Statistical analysis | 25.3 |
| How to write a protocol | 20.7 |
| Paperwork and procedures† | 13.2 |
| Cost management for clinical research | 12.7 |
| Informed consent form to patients | 10.5 |
| Compensation | 9.2 |
| Medical ethics | 8.0 |
| What were the criticisms of reviewers when you submitted for publication a manuscript on clinical research ? | |
| Statistical analysis | 36.9 |
| Selection of patients | 21.0 |
| Aim or meaning of research | 19.1 |
| Definition of the technical terms | 10.2 |
| Ethical problems | 5.7 |
| What difficulties did you meet of conducting clinical research? | |
| The paperwork was complicated and onerous | 26.2 |
| Eligible patients were very few | 18.9 |
| Lack time | 17.6 |
| Too many examinations were scheduled | 11.5 |
| There was no benefit to patients | 8.6 |
| I could not continue clinical research because of transfer of physicians | 6.3 |
| Patients missed appointments | 5.4 |
| Patients didn't consent to take placebo | 3.6 |
| Doctor-patient relationships were damaged by offering clinical research | 0.8 |

* Percent values were expressed as ratio in total answers. Percentage may not total 100% due to missing or blank data.
†Paperwork and procedures mean production and management of study documents regarding submission to institutional review board and completion of case report form.

Most physicians (93.2%) wanted to attend lectures or seminars on one or more topics related to clinical research. The most frequently cited desired lecture topics were statistical analysis, how to write a protocol, paperwork and procedures (production and management of study documents regarding submission to institutional review board and completion of case report form), and cost management in clinical research (Table 2).

Respondents who had submitted research papers for publication were asked to indicate the criticisms of reviewers. Statistical analysis was the most frequent reviewer criticism, followed by selection of patients, aim or meaning of research, and definition of technical terms (Table 2).

Regarding the difficulties of conducting clinical research, respondents indicated that the "paperwork was complicated and onerous", that there were "few eligible patients", and that the respondents "lack time" (Table 2).

### *Factors associated with current participation in clinical research*

Age range had moderate correlation with status (r = 0.635), as did past participation in clinical research with prospective participation in clinical research (r = 0.505). Past participation in clinical research had some correlation with past submission for publication of a manuscript on clinical research (r = 0.413), as did past submission for publication of a manuscript on clinical research with past writing of a research protocol (r = 0.311) and past participation in clinical research with past writing of a research protocol (r = 0.282).

In bivariate analyses, current participation had statistically significant correlation with status, age range, past participation in clinical research, prospective participation in clinical research, past submission for publication a manuscript on clinical research, training course in clinical research, past writing a research protocol and knowledge of the World medical Association Declaration of Helsinki. A multivariable logistic regression model was developed including all these correlated factors as variables. Current participation was positively associated with past participation in, prospective participation in clinical research and past writing of a research protocol (Table 3). Age range of 30-39 was negatively associated with current participation in clinical research: Respondents aged 30 to 39 were less than quarter (odds ratio, 0.24; 95% confidence interval,

**Table 3: Effect of status, age range, and attitudes to current participation in clinical research**

| Characteristic | Odds ratio (95% CI) | *P* value* |
|---|---|---|
| Status | | |
| resident or doctoral student | reference | |
| faculty | 1.416(0.568-3.531) | 0.4554 |
| Age range, y | | |
| <=29 | reference | |
| 30-39 | 0.240(0.064-0.907) | 0.0353 |
| 40-49 | 0.354(0.069-1.822) | 0.2142 |
| >=50 | 0.218(0.028-1.684) | 0.1442 |
| Past participation in clinical research | | |
| yes | 5.680(2.40-13.441) | < 0.0001 |
| no | reference | |
| Prospective participation in clinical research | | |
| yes | 5.756(2.508-13.212) | < 0.0001 |
| no | reference | |
| Previous training course in clinical research | | |
| yes | 2.081(0.678-6.389) | 0.2002 |
| no | reference | |
| Previous writing of a research protocol | | |
| yes | 2.631(1.130-6.125) | 0.0249 |
| no | reference | |
| Previous submission for publication of a manuscript on clinical research | | |
| yes | 1.798(0.815-3.967) | 0.1464 |
| no | reference | |
| Do you know "WORLD MEDICAL ASSOCIATION DECLARATION OF HELSINKI Ethical Principles for Medical Research Involving Human Subjects"? | | |
| I know very well | 4.219(0.561-31.728) | 0.1619 |
| I know to some degree | 2.457(0.413-14.623) | 0.3233 |
| I don't know | reference | |

* P < 0.05 is considered statistically significant.
The R2 value was 0.378. CI, confidence intervals

0.064-0.907) as likely to participate in clinical research currently as respondents aged 20 to 29. There was no association between current participation and either status or previous training course in clinical research.

## Discussion

In this questionnaire survey of physicians at KUH, most respondents were currently participating in clinical research and felt that clinical research was necessary. As compared to physicians participating in clinical research, smaller proportions of physicians had formal training in clinical research. The majority reported a need to acquire concepts and skills regarding clinical research, especially those related to statistics. Both previous participation in and prospective participation in clinical research were positively associated with current participation in clinical research, suggesting that physicians who were accustomed to clinical research were participating in and would participate in clinical research.

Our findings indicate that the contention that "doctors (in Japan) simply don't want to take part in clinical trials"[11] is a misunderstanding. Indeed, our results indicate that if an adequate trial infrastructure is present, Japanese physicians are eager to conduct clinical research.

KUH is an important research center in Japan, and this likely explains why the rates of participation in and acknowledgement of the importance of clinical research were high among respondents. Studies have reported a wide range in the percentage of physicians participating in clinical research, from 13% to 90% [12-14]. In a questionnaire survey at Tokushima University Hospital[8], 61% of faculty had contributed to IND application trials and 58% of those wanted to participate in IND application trials, whereas in our survey at KUH, 89% of faculty reported past participation in clinical research. The difference in participation rates could be the result of different criteria of clinical research in the questionnaire. As mentioned above, many non-notified trials are carried out at KUH and other hospitals. Perhaps the rate of participation was high because, with the exception of notified trials, physicians in Japan are able to initiate clinical research with only minimal ethical oversight.

In the present study, the difficulties that physicians faced in conducting clinical research are similar to those noted in previous studies [14-16]. Paperwork was cited as a major hurdle, even though the limited number of regulatory obstacles in Japan would be expected to lessen paperwork demands. Perhaps because physicians have a low opinion of the necessity for preparing and managing study documents, they perceive extra paperwork as onerous. Therefore, we suggest that a clinical support center should be available to provide initial advice and support regarding the production and design of documents, thereby establishing good practice. Lack of time was also reported as a major hurdle. Most physicians in university hospitals in Japan are involved in both patient care and research on molecular and cellular biology including experiments with animals. Because researchers could study molecular and cellular biology on a smaller budget than clinical research, which is the evaluation of new treatment involving human subjects, they studied it since it was introduced to Japan. As a result, there are few highly skilled clinical researchers in Japan and opportunities to learn the principles and methodology of clinical research are limited for young Japanese physicians.

Physicians who are familiar with clinical research are able to conduct clinical research more easily than those who are not, as they know the guidelines and laws necessary for conducting clinical research and can use their pre-existing network of experienced research collaborators [17]. In addition, physicians who have completed clinical trials can obtain funding more easily than those who have not; however, they gain no special treatment or financial incentives [11]. As the majority of physicians indicated that obtaining a better understanding of disease was the greatest benefit of conducting clinical research, the pleasure of discovery would appear to have more than repaid them for their efforts.

In our model with respect to current participation in clinical research, the previous training in clinical research was not found to be a significant factor. As various training providers were reported in this questionnaire, the programs and the length of these training courses should be variable. Universities or university hospitals should develop a standardized training program on clinical research that physicians can learn essential knowledge before they initiate such research.

The current study did have some limitations. The most significant of these is that the clinical research referred to in this survey comprised a variety of research types, ranging from epidemiological and observational studies to clinical trials, including IND application trials. Nevertheless, the research support section that serves the university hospital assists with a variety of clinical research designs, and a commonality of needs among physicians was demonstrated in our survey. Another limitation was that the response rate was much higher among faculty than among resident, which may influence the final logistic regression analysis. In addition, this survey took place at a single institution, so the possibility for generalization is limited. However, the difficulties indicated by respondents were quite consistent with those of prior reports. Moreover, an ongoing international collaboration project is attempting to compare the status and attitudes of physicians, and to

seek strategies to promote clinical research. The results of this study have contributed much to the refinement and modification of the questionnaire used for the international attitude study. We aim to identify unique and universal problems regarding academic clinical research, and to submit them to academic societies and governing bodies in order to improve the situation. In addition, after completion of our questionnaire survey, Ethical Guidelines for Clinical Studies were just revised and enacted in April 2009. Under the revised guidelines, investigators are now required to register their trials at a public trial registry, to obtain insurance for trial subjects, and to have adequate training in clinical research. Concern for the welfare of trial subjects may have increased, but this may create another barrier to perform clinical research by requesting more paperwork and more funding for insurance for trial subjects.

## Conclusions

Physicians in university hospitals need more administrative assistance and greater knowledge of the principles and techniques of clinical research, especially the concepts of biostatistics. Our results highlight the need for training in clinical research and biostatistics and the necessity for administrative assistance in the production of study documents requested by the institutional Independent Ethics Committee.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

ES conceived the study and participated in the design, management, data analysis, and preparation of the manuscript. TM participated in the study design and in the preparation of the manuscript. MY participated in the study design and participant recruitment. All authors read and approved the final manuscript.

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*Circ J* 2009; **73:** 1337–1341

# N-Acetylcysteine Reduces the Severity of Atherosclerosis in Apolipoprotein E-Deficient Mice by Reducing Superoxide Production

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***Background:*** Oxidative stress may play an important role in the development of atherosclerosis. Because N-acetylcysteine (NAC) is able to reduce oxidative stress, the present study assessed the hypothesis that NAC may reduce the severity of atherosclerosis in apolipoprotein (apo) E-deficient mice.

***Methods and Results:*** Atherosclerosis was induced in apoE-deficient mice fed a high-fat diet containing 0.3% cholesterol. Mice were injected intraperitoneally with NAC ($20\,mg \cdot kg^{-1} \cdot day^{-1}$) 3 times per week over 8 weeks. Fatty streak plaque developed in the apoE-deficient mice, but not in mice treated with NAC. In addition, NAC reduced superoxide production in the aortic walls, as detected by ethidium staining. NAC treatment did not significantly modify the serum lipid profiles.

***Conclusions:*** In this animal model NAC may suppress atherosclerosis via reducing superoxide production. (*Circ J* 2009; **73:** 1337–1341)

**Key Words:** Atherosclerosis; Free radicals; N-acetylcysteine; Oxidative stress

Many kind of stresses, especially oxidative stress and free radicals, may be key factors in the development of atherosclerosis[1,2] In addition, the significance of systemic inflammation in the development of atherosclerosis is now well known[1,2] For example, angiotensin II is a major mediator of oxidative stress by activating NADH/NAD(P)H oxidase via the type 1 receptor, which results in the production of the superoxide anion[3,4] Thus, angiotensin II has deleterious effects on vessel walls. Recent reports indicate that N-acetylcysteine (NAC), a potent antioxidant, inhibits inflammatory cardiovascular diseases[5–7] and it has also been reported that NAC inhibits not only inflammatory cytokines, but also free radical production. These results imply that NAC may be an effective agent against inflammatory reactions and oxidative stress in the vessel walls. However, the effects of NAC on atherosclerosis are still unknown, irrespective of the previous reports[8,9]

## Methods

### Experimental Atherosclerosis

ApoE-deficient 129ola×C57BL/6 hybrid mice were the generous gift of Dr Edward M. Rubin (University of California, Berkeley, CA, USA). They were mated with C57BL/6 mice to produce $F_1$ hybrids. The $F_1$ apo $E^{+/-}$ mice were then backcrossed to C57BL/6 mice for 10 generations. Mice homogeneous for the apoE-null allele on a C57BL/6 background were subsequently generated. Male mice were used in the subsequent experiments. They were kept in a temperature-controlled facility on a 14.10-h light–dark cycle with free access to food and water.

After being weaned at 4 weeks of age, mice were fed a normal chow diet (Oriental Yeast) until 6 weeks of age, when they were switched to a high-fat diet containing 20% fat and 0.3% cholesterol as previously described[10,11]

The experimental protocols were approved by the Ethics Committee for Animal Experiments of Kyoto University.

### Treatment Protocol

At 6 weeks of age, mice given a daily intraperitoneal injection of either saline (control group, n=9) or $20\,mg \cdot kg^{-1} \cdot day^{-1}$ of NAC (NAC group, n=9) 3 times per week on alternate days for 8 weeks. The dosage of the drug was determined from previous reports[5–7] At 14 weeks the mice were killed by puncture of the ventricle under ether anesthesia. The organs were weighed, and the ratio of heart weight (HW) to body weight (BW) was calculated.

### Tissue Processing

Mice were killed by exsanguination after puncturing the ventricle. The aortas were removed 36 h after the last injection of NAC. The vasculature was perfused with sterile phosphate-buffered saline and 6.8% sucrose. The root of the aorta was dissected under a microscope and frozen in OCT embedding medium for serial cryosectioning covering 1.0 mm of the root. The first section was harvested when the first cusp became visible in the lumen of the aorta. Four sections of 6-$\mu$m thickness were harvested per slide, and thus 8 slides per mouse were prepared. All sections

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were immersed for 15 s in 60% isopropanol, stained for 30 min in a saturated oil-red-O solution at room temperature, counterstained with hematoxylin, and then mounted under coverslips with glycerol gelatin[11,12]

### In Situ Detection of Superoxide Production

To evaluate in situ superoxide production by the aorta, unfixed frozen cross-sections of the specimens were stained with dihydroethidium (DHE; Molecular Probe, OR, USA) according to a previously validated method[13–15] In the presence of superoxide, DHE is converted to the fluorescent molecule, ethidium, which can then label nuclei by intercalating with DNA. Briefly, the unfixed frozen tissues were cut into 10-$\mu$m sections, and incubated with 10 $\mu$mol/L DHE at 37°C for 30 min in a light-protected humidified chamber. The images were obtained with a laser scanning confocal microscope. Superoxide production was demonstrated by red fluorescence labeling.

For quantification of ethidium fluorescence from the aortas, fluorescence (intensity×area) was measured using a high-power image.

### Immunohistochemistry

Anti-macrophage (anti-M$\phi$, M 3184, 1:400, PharMigen) and anti intercellular adhesion molecule-1 (ICAM-1) (M-19, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies were applied to acetone-fixed cryosections of the aortic roots. After being washed, the sections were then exposed to the second antibody (horseradish peroxidase-conjugated antibody), and antibody binding was visualized with diaminobenzidine. Sections were counterstained with methyl green or Mayer's hematoxylin.

The positive-staining macrophages were counted in several fields at ×400 magnification (within a 1-mm$^2$ grid), and the percentage of positive-staining cells/total infiltrating cells was calculated.

### Western Blotting

Protein expression of ICAM-1 was examined as described previously[8] Protein samples were probed with the anti-ICAM-1 antibody. The $\beta$-actin samples were probed in the blots as internal controls for loading. Resulting bands were quantified as optical density×band area by the image analysis system.

### Lipid Measurement

Serum was separated by centrifugation and stored at −80°C. Serum total cholesterol and triglyceride levels were measured.

### Statistical Analysis

Values are expressed as means±SD. Student's t-test was performed, and $P<0.05$ was considered statistically significant.

## Results

### Effects of NAC on Organ Weights

HW, BW and the HW/BW ratio were not significantly different between the NAC group and the control group (**Table 1**).

**Table 1. Organ Weights**

| | n | BW (g) | HW (g) | HW/BW (mg/g) |
|---|---|---|---|---|
| Control | 9 | 25.3±4.1 | 0.15±0.03 | 6.09±1.02 |
| NAC | 9 | 24.0±2.4 | 0.15±0.02 | 6.06±0.46 |

Mean±SD.
BW, body weight; HW, heart weight; NAC, N-acetylcysteine.

### Effects of NAC on Atherosclerotic Lesions

ApoE-deficient mice were kept on a cholesterol-rich diet for 8 weeks to induce fatty streak formation. The surface

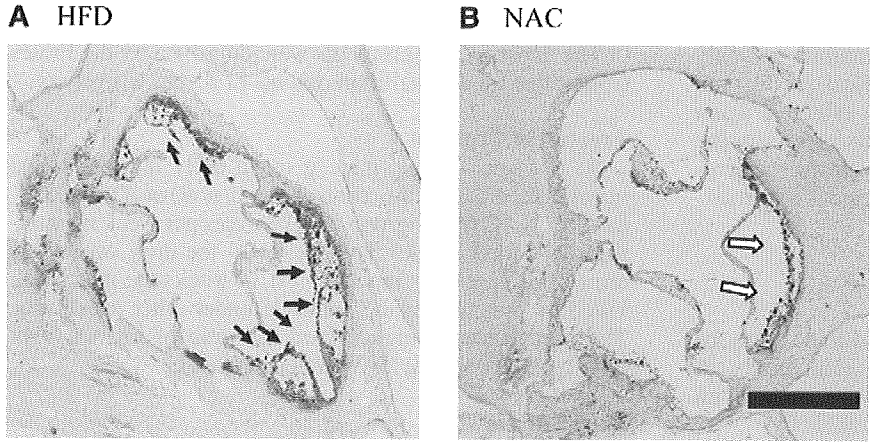


**Figure 1.** Effects of N-acetylcysteine (NAC) treatment on atherosclerotic lesions in the NAC-treated mouse (**B**, white arrows), which were smaller and covered less of the inner circumference of the aortic root than those (black arrows) in the control mouse (**A**). Bar=500 $\mu$m. Oil-red-O stain.

**Table 2. Lesion Area**

| | n | Lesion area, $\mu$m$^2$ (%) | M$\phi$-positive cells (%) | Ethidium fluorescence (units) |
|---|---|---|---|---|
| Control | 9 | 65.91±20.60×10$^3$ (8.45±2.64) | 14.3±4.2 (n=5) | 1.00±0.12 (n=5) |
| NAC | 9 | 35.49±19.97×10$^3$ (4.55±2.56)* | 4.5±4.0* (n=5) | 0.84±0.04* (n=5) |

Mean±SD.
*P<0.05 vs Control group.
M$\phi$, macrophage. Other abbreviation see in Table 1.

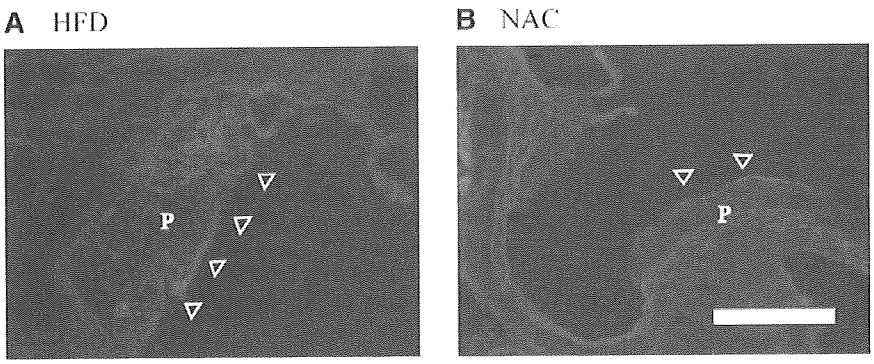


**Figure 2.** Effects of N-acetylcysteine (NAC) on superoxide production. The degree of ethidium fluorescence (arrowheads) in the NAC group is less than that in the control group, suggesting less superoxide production after NAC treatment. HFD, high-fat diet; P, plaque. Bar=50$\mu$m (dihydroethidium stain; ×100).

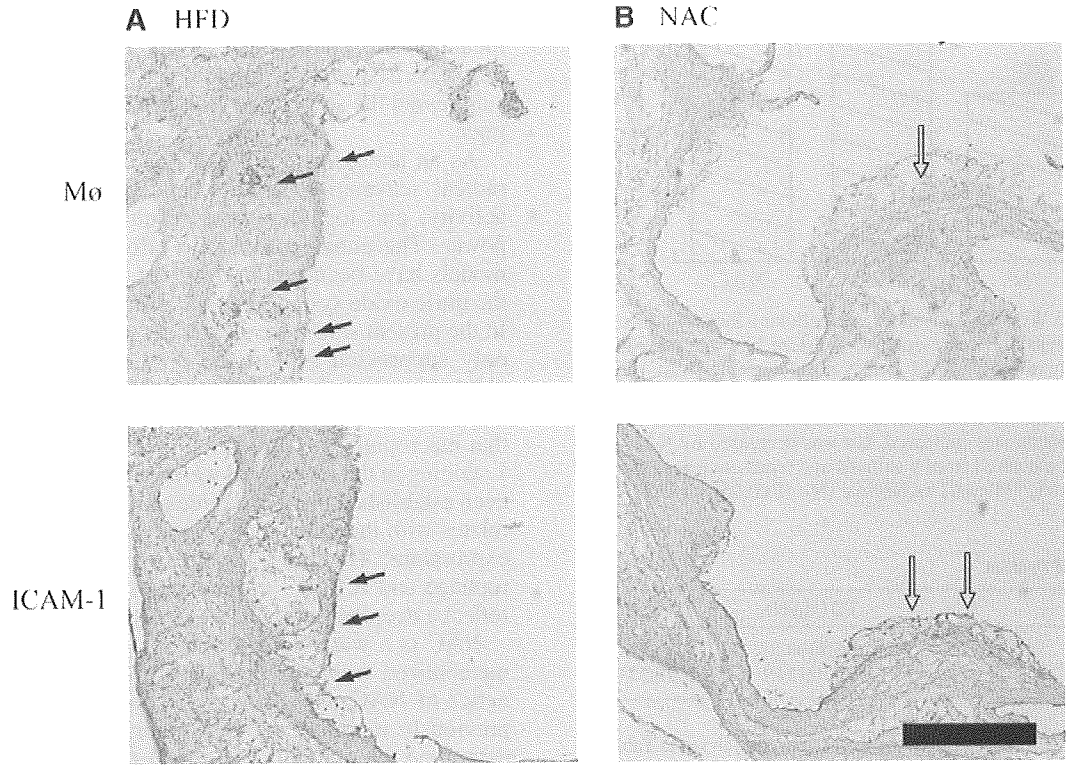


**Figure 3.** Effects of N-acetylcysteine (NAC) on macrophage and intercellular adhesion molecule-1 (ICAM-1) expressions. The expression of macrophages (M$\phi$) and ICAM-1 in the lesions (white arrows) of the NAC-treated mouse (**B**) is less compared with that (black arrows) of the control mouse (**A**). Brown staining shows the positive cells for M$\phi$ and ICAM-1 expressions in the atherosclerotic plaques. Bar=50$\mu$m (×100). HFD, high-fat diet.

area covered by fatty streak lesions was quantified in oil red-O-stained samples, and specimens from the control group were compared with those from the NAC group. Controls developed extensive lesions in the root of the aorta (**Figure 1**). In mice treated with NAC, the fractional area of the lesions was reduced compared with the controls (**Table 2**, **Figure 1**).

### Superoxide Production

Ethidium fluorescence in the NAC group was significantly weaker than that in the control group (**Table 2**, **Figure 2**). Namely, the brightness of the DHE-stained lesions from NAC treated mice was less than that from control mice. The origin of the superoxide might have been mainly from macrophages, but also partly from endothelial cells, considering the results of both a previous report[14] and the current study.

### Macrophage and ICAM-1 Expressions

Both the accumulation of macrophages and expression of ICAM-1 were decreased by NAC treatment compared with the controls (**Table 2**, **Figure 3**). The percentage of lesions was less in NAC-treated mice than that in the control mice.

### Western Blotting

Decreased ICAM-1 expression in NAC-treated mice was confirmed by Western blot analysis (**Figure 4**).

### Lipid Profiles

NAC treatment did not significantly modify the serum lipid profiles (**Table 3**).

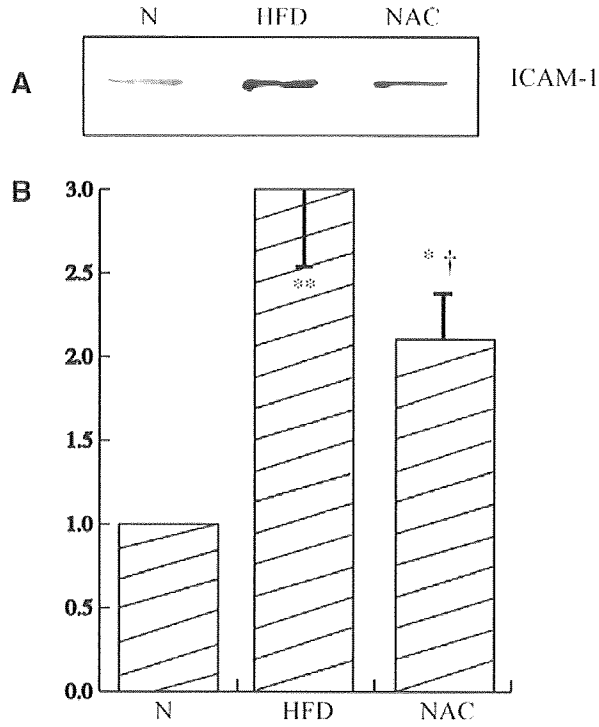


**Figure 4.** (**A**) Western analysis of intercellular adhesion molecule-1 (ICAM-1) expression. Representative Western blot analysis showed decreased expression of ICAM-1 in N-acetylcysteine (NAC)-treated mice compared with control mice. (**B**) Densitometric analysis of relative protein levels. In mice with the control (high-fat diet (HFD)), ICAM-1 expression was increased, but was decreased by NAC treatment. Values are derived from 5 animals and represent a percentage of controls. *P<0.05, **P<0.01 vs Normal (N); †P<0.05 vs HFD.

**Table 3. Lipid Profiles**

| | n | TC (mg/dl) | TG (mg/dl) |
|---|---|---|---|
| Control | 9 | 648.8±97.5 | 93.2±92.6 |
| NAC | 9 | 644.0±174.1 | 29.0±20.0 |

Mean±SD.
TC, total cholesterol; TG, triglycerides. Other abbreviation see in Table 1.

## Discussion

The results of the current study show that NAC treatment suppressed the development of experimental atherosclerosis in apoE-deficient mice by reducing both superoxide production and macrophage accumulation in the aortic walls.

Chronic inflammation is thought to be of central importance in atherosclerosis[1,16] It has been shown that regular and chronic exercise can suppress overt and subclinical inflammation[17,18] based on the fact that atherosclerosis can be considered as a generalized manifestation of an inflammatory disease[1] We and other investigators had already reported that experimental atherosclerosis in apoE-deficient mice was markedly suppressed by the Fcγ portion of immunoglobulin administration, possibly by an antiinflammatory action via the inhibitory Fcγ receptor IIB[19–21]

There is also increasing evidence to support the critical role of both free radicals and oxidative stress in the development of atherosclerosis[22–25] and in heart failure[26,27] We had already demonstrated that MCI-186, a free radical scavenger, and olmesartan, an angiotensin type 1 receptor antagonist, suppress the severity of experimental atherosclerosis[11] Indeed, angiotensin stimulation has been reported to produce free radicals from various cells[28] Free radicals from vessel walls are thought to play a critical role in atherogenesis. It is considered that free radicals induce the expression of adhesion molecules and chemokines, accelerate atherosclerotic plaque formation, increase matrix metalloprotease production, and cause vulnerable plaques[29] The superoxide anion is a free radical.

Psychological and behavioral stress are now recognized as important contributors to inflammatory and free radical mediated cardiovascular diseases. Both clinical and experimental evidence support the hypothesis that oxidative stress is linked to hypertension, heart failure, and atherosclerosis. Accordingly, the present study determined the protective effects of NAC, a potent, antioxidative agent[5–7] on experimental atherosclerosis, irrespective of the results of previous reports[8,9] and we found that NAC treatment clearly suppressed the severity of atherosclerosis in apoE-deficient mice.

As an antioxidant, NAC, has a chelating capacity, free radical scavenging activity, and peroxidation inhibiting activity, and together these result in a potent reducing power. The antiatherosclerotic effects of NAC in animal models have been reported in part, which were related to the nitric oxide system[8] as well as its anti-oxidative effects[9] In the present study, we clearly demonstrated that NAC not only suppressed superoxide production but also macrophage accumulation, assessed by ICAM-1 expression, in the aortic walls. Our immunohistochemical study showed that macrophage accumulation and the intensity of ICAM-1 staining in the aortic wall were very similar. It has already been established that the degree of instability of plaque correlates with the amount of macrophages, and thus ICAM-1 expression[29] Therefore, a decrease in the intensity of macrophage and ICAM-1 expressions in the aortic walls may reflect a decrease in oxidative stress by NAC treatment.

NAC is a water-soluble material and may be smoothly metabolized from the plasma. However, the pharmacokinetics of NAC may differ between mice and humans, as previously reported[5–9,30–32] Further studies are needed to determine the optimal dose–effect relationship in humans. In the current study, however, we chose an intraperitoneal injection of $20\,mg \cdot kg^{-1} \cdot day^{-1}$ of NAC, guided by previous reports[5–7]

## Conclusion

NAC treatment protected against experimental atherosclerosis in apoE-deficient mice by suppressing superoxide production in the atherosclerotic lesions. Our results suggest that anti-oxidative agents, food and antioxidant behavior may be beneficial for protecting against atherosclerosis in the clinical setting.

### Acknowledgments

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### Disclosure

There is no conflict of interest.

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# Mulberry leaf ameliorates the expression profile of adipocytokines by inhibiting oxidative stress in white adipose tissue in db/db mice

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ABSTRACT

Previous study showed that mulberry (*Morus Alba* L.) leaf (ML) ameliorates atherosclerosis in apoE$^{-/-}$ mice. Although the adipocytokine dysregulation is an important risk factor for atherosclerotic cardiovascular disease, the effect of ML on metabolic disorders related to adipocytokine dysregulation and inflammation has not been studied. Therefore, we studied the effects of ML in metabolic disorders and examined the mechanisms by which ML ameliorates metabolic disorders in db/db mice. We treated db/db mice with ML, pioglitazone, or both for 12 weeks and found that ML decreased blood glucose and plasma triglyceride. Co-treatment with ML and pioglitazone showed additive effects compared with pioglitazone. Moreover, their co-treatment attenuated the body weight increase observed under the pioglitazone treatment. ML treatment also increased the expression of adiponectin, and decreased the expression of TNF-α, MCP-1, and macrophage markers in white adipose tissue (WAT). Furthermore, ML decreased lipid peroxides and the expression of NADPH oxidase subunits in WAT and liver. Their co-treatment enhanced these effects. Thus, ML ameliorates adipocytokine dysregulation at least in part through inhibiting oxidative stress in WAT of db/db mice, and that ML may be a basis for a pharmaceutical for the treatment of the metabolic syndrome as well as reducing adverse effects of pioglitazone.



## 1. Introduction

Recent study showed that white adipose tissue (WAT) produces and secretes a variety of adipocytokines involved in metabolic syndrome [1,2]. Increased production of monocyte chemoattractant protein-1 (MCP-1) from WAT contributes to macrophage infiltration into WAT and causes inflammation [3], while tumor necrosis factor-α (TNF-α) causes insulin resistance [4]. In contrast, adiponectin, which is an adipocyte-specific endocrine protein, exhibits anti-atherogenic and anti-diabetic properties, and its plasma level is decreased in visceral obesity [5,6].

In addition to inflammation, oxidative stress also plays critical roles in the metabolic syndrome [7]. Oxidative stress is shown to be increased in obesity via NADPH oxidase activation [8]. NADPH oxidase is a major source of reactive oxygen species (ROS) in various organs, especially in WAT [8]. NADPH oxidase consists of membrane-associated flavocytochrome b558 family of proteins, which include gp91$^{phox}$ and p22$^{phox}$ as well as cytosolic components p47$^{phox}$, p67$^{phox}$, and p40$^{phox}$ [9]. Because macrophages are also known to produce ROS in addition to inflammatory adipocytokines, such as TNF-α [10], infiltrated macrophages might be involved in augmented NADPH oxidase and elevate ROS production in WAT. Furthermore, in adipocytes ROS themselves have been shown to augment expression of NADPH oxidase subunits as well as PU.1, a member of the ETS family of transcription factors required for the development of multiple hematopoietic lineages [8]. Thus, increased oxidative stress in WAT might cause dysregulated production of adipocytokines, which induces macrophage infiltration into WAT, causing more inflammation, and induction of oxidative stress. Furthermore, previous study showed that anti-oxidants such

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as vitamin C, E, and α-lipoic acid ameliorate insulin resistance [11]. Thus anti-oxidant may be a potential agent for the metabolic syndrome.

We have studied the role of mulberry leaf (ML), because it contains various nutritional components, such as flavonoids. Dietary ML also shows hypoglycemic [12] and anti-atherogenic effects [13,14] in certain animal models. Recently, we demonstrated that ML treatment reduced atherosclerotic lesions in apoE$^{-/-}$ mice by inhibiting lipoprotein oxidation [13]. We also showed that ML-derived aqueous fractions (MLAF) inhibit TNF-α-induced nuclear factor κB activation and lectin-like oxidized low-density lipoprotein receptor-1 expression in vascular endothelial cells [15]. However, roles and mechanisms of ML in metabolic disorders and inflammation in WAT have not been investigated.

Therefore, in this study, we examined the effects of ML on the expression profile of adipocytokines and related metabolic disorders in obese diabetic db/db mice, and compared its effect with that of a PPAR-γ agonist, pioglitazone. We also investigated the mechanisms by which ML improves development of metabolic disorders.

## 2. Materials and methods

### 2.1. Mulberry leaves

Mulberry trees were cultured in mulberry plantation of Center for Bioresource Field Science, Kyoto Institute of Technology by a standard method in Japan. Mulberry (*Morus Alba* L.) race used was "Shin-Ichinose". Mulberry leaves were harvested in July 2006 and immediately dried by air flush at 180 °C for 7 s. The average diameter of the dried powder used in this experiment was 20 μm.

### 2.2. Animals and experimental protocol

All animals were obtained from Oriental Bio-Service (Kyoto, Japan) and housed in a temperature-, humidity-, and light-controlled room (14-h light and 10-h dark cycle) and had free access to water and chow. In db/db mice studies, male mice at 9 weeks of age were treated with each diet for 12 weeks ($n$ = 5–6 in each group). Briefly, mice in the ML group were fed with regular chow containing 3% (w/w) ML powder, mice in the Pio group were fed with regular chow containing 0.01% (w/w) pioglitazone (Takeda Pharmaceutical, Osaka, Japan) and mice in the ML + Pio group were fed with regular chow containing both 3% ML powder and 0.01% pioglitazone. Mice at 21 weeks of age were euthanized, blood was collected, and epididymal WAT and liver tissue were dissected out and frozen in liquid nitrogen. Samples were stored at −80 °C until use. All animal experiments were performed according to the guidelines of Kyoto University Animal Research Committee.

### 2.3. Body fat composition analysis

For computed tomography (CT) analysis of body fat composition, mice were anesthetized and then scanned using a LaTheta (LCT-100 M) experimental animal CT system (Aloka, Tokyo, Japan). Body fat mass was analyzed quantitatively using LaTheta software (version 1.00), as previously described [16].

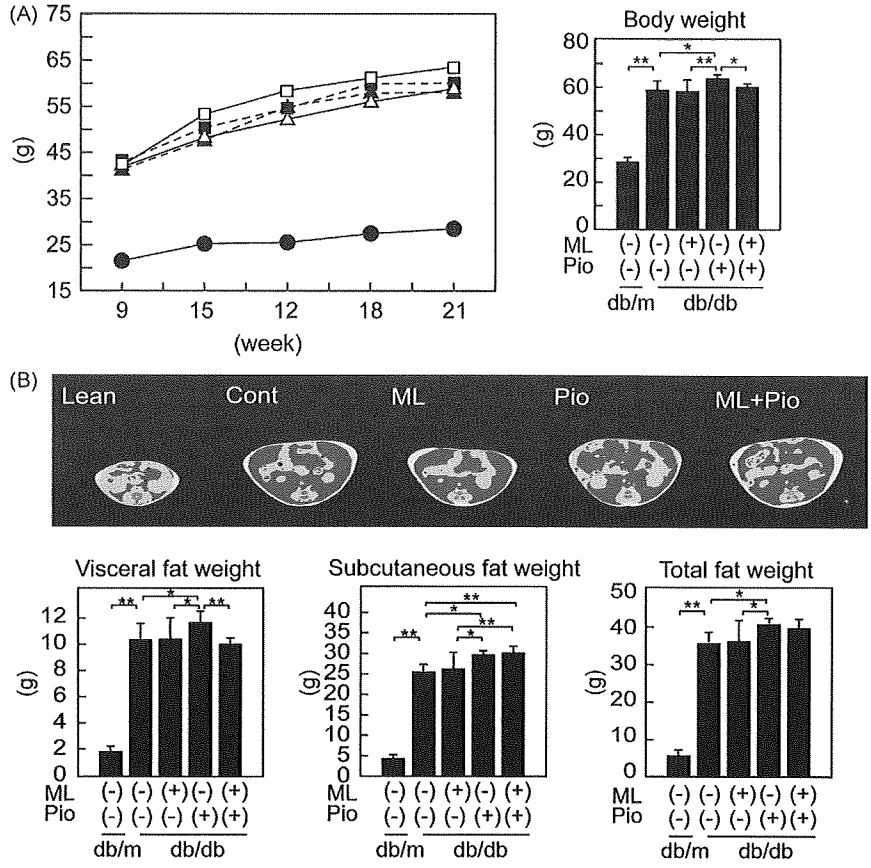


**Fig. 1.** Effect of mulberry leaf, pioglitazone, or both treatment for 12 weeks on body and fat weight. (A) Growth curve during experiment and body weight at the end of experiment of db/m (Lean) and db/db mice on control (Cont), 3% ML-supplemented (ML), 0.01% pioglitazone-supplemented (Pio), or co-supplemented (ML + Pio) diet for 12 weeks, respectively. Closed circle, Lean ($n$ = 6); open triangle, Cont ($n$ = 5); closed triangle, ML ($n$ = 5); open square, Pio ($n$ = 6); closed square, ML + Pio ($n$ = 6). (B) Representative CT sections of abdominal regions and weight of visceral, subcutaneous, and total fat in db/m and db/db mice on each treatment calculated from CT scan data. Pink areas show visceral fat, while yellow areas show subcutaneous fat. Data are expressed as means ± SD. $n$ = 5 or 6. $^{*}P < 0.05$; $^{**}P < 0.01$.

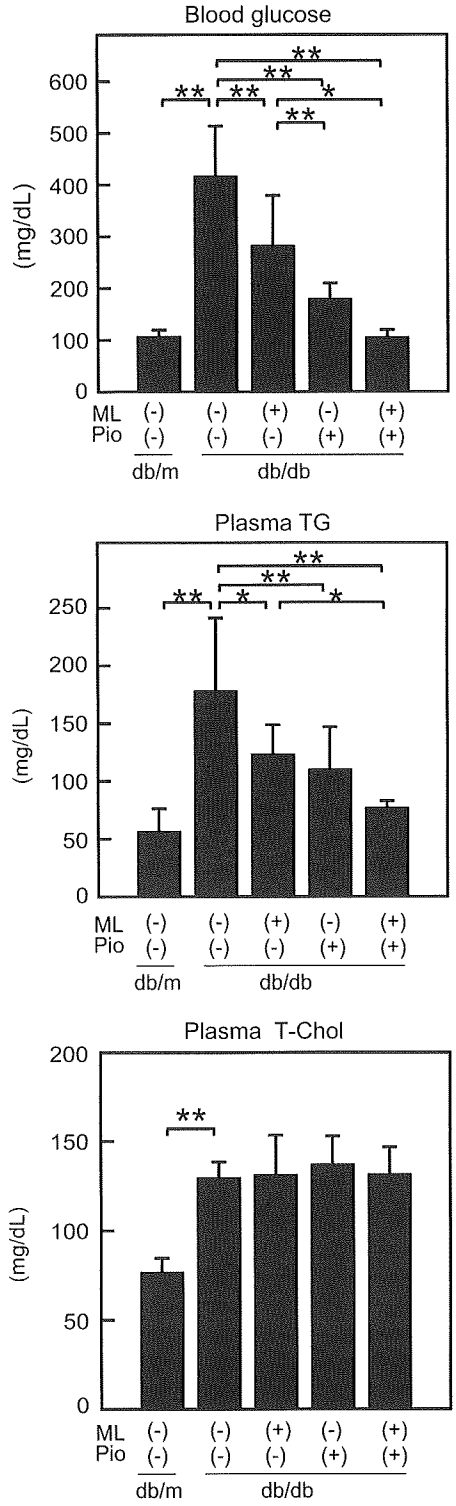


**Fig. 2.** Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on glucose and lipid metabolism. Blood glucose levels and plasma concentrations of triglyceride and total cholesterol in each group of mice are shown. Data are expressed as means ± SD. $n = 5$ or 6. $^{*}P < 0.05$; $^{**}P < 0.01$.

### 2.4. Analysis of metabolic parameters

All blood samples were collected after overnight fasting. Blood glucose level and plasma concentrations of triglyceride (TG), total cholesterol (T-Chol) and adiponectin were measured by automatic glucometer (Glutest Ace, Sanwa Chemical, Hiratsuka, Japan),

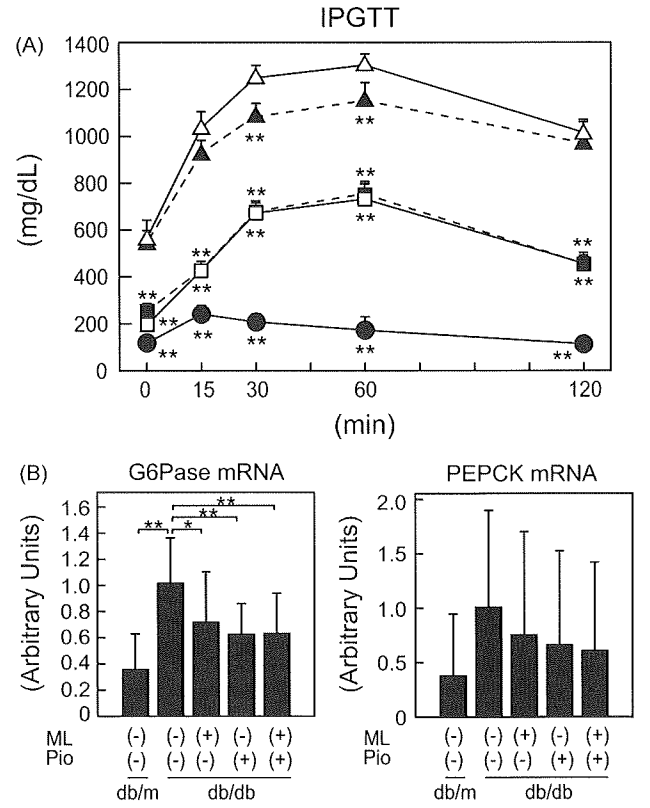


**Fig. 3.** Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on abnormal glucose tolerance and the expression of genes related to gluconeogenesis. (A) Plasma glucose levels were determined in each group of mice are shown. $n = 5$ or 6. $^{**}P < 0.01$ versus db/db mice on control diet. (B) Expression of G6Pase and PEPCK in the liver of db/m and db/db mice on each diet after 12 weeks is shown. Data are expressed as means ± SD. $n = 5$ or 6. $^{**}P < 0.01$.

triglyceride E-test Wako, cholesterol E-test Wako (Wako Pure Chemical), and adiponectin ELISA kit (Otsuka Pharmaceutical, Tokyo, Japan), respectively. For intraperitoneal glucose tolerance tests (IPGTT), mice were starved for 16 h and then injected with 1.5 mg/kg body weight of glucose. Blood samples were collected before and after injection, and plasma glucose concentration was measured with an automatic glucometer.

### 2.5. Quantitative real time polymerase chain reaction (PCR)

Total RNA was extracted from WAT and liver tissue using RNeasy lipid tissue kit (Qiagen, Valencia, CA). Real time PCR was performed on an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) using the SYBR GREEN PCR Master Mix (Applied Biosystems). Primer sets used for quantitative real time PCR are shown in Supplementary Table. mRNA levels were normalized relative to the amount of 18S rRNA and expressed in arbitrary units.

### 2.6. Lipid peroxide concentration

WAT and liver tissue were homogenized in lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Nondient-P40, 0.25% SDS). Tissue suspension was centrifuged at 1600 × *g* for 10 min at 4 °C, and the supernatants were collected and used for assay. The levels of lipid peroxide in tissue homogenate were measured as thiobarbituric acid reactive substance (TBARS) using the TBARS Assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacture's recommendation.

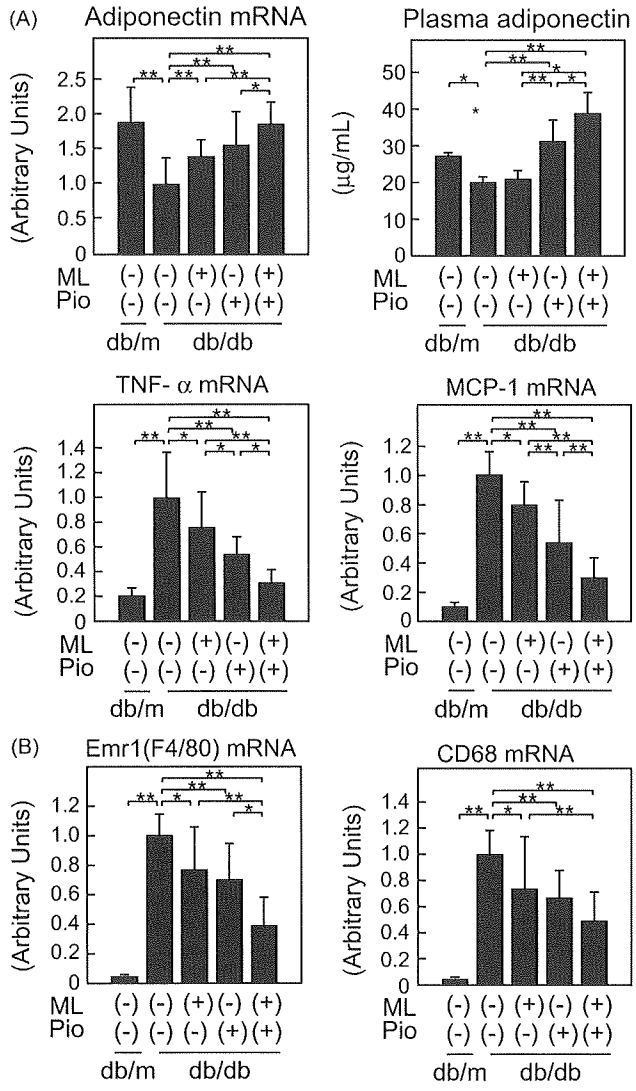


**Fig. 4.** Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on the expression of adipocytokines and macrophage infiltration in white adipose tissue. (A) Levels of adiponectin in WAT and in plasma in each group of mice are shown. (B) Expression of TNF-α and MCP-1 mRNAs and Emr1 (F4/80) and CD68 mRNAs in WAT in each group of mice are shown. Data are expressed as means ± SD. $n = 5$ or 6. $^{*}P < 0.05$; $^{**}P < 0.01$.

### 2.7. Hepatic TG content

Hepatic TG content was measured as previously described [17].

### 2.8. NADPH oxidase activity

Liver tissue was homogenized on ice in ice-cold Tris–sucrose buffer (10 mM Tris (pH 7.6), 340 mM sucrose, 1 mM EDTA, 1 mM PMSF, 0.5% Protease inhibitor cocktail (Sigma–Aldrich)). The tissue suspension was centrifuged at 15,000 × *g* for 20 min at 4 °C, and the supernatant was collected and used for assay. NADPH oxidase activity was measured as previously described [18]. NADPH oxidase activity was expressed as relative NADPH oxidase activity versus the rate of NADPH consumption of non-treated db/db mice.

### 2.9. Statistical analysis

The results are expressed as means ± SD. The statistical significances of differences among multiple groups were evaluated using ANOVA and post hoc Fischer's PLSD tests. Values of $p < 0.05$ were considered significant.

## 3. Results

### 3.1. Effect of ML, pioglitazone, and their co-treatment on body weight and body fat mass

Db/db mice (9 weeks of age) were treated with ML, pioglitazone, or both for 12 weeks and the changes of body weight were examined. ML did not affect the body weight gain of db/db mice, whereas pioglitazone slightly but significantly increased it by 7% compared with non-treated db/db mice. Their co-treatment significantly attenuated the body weight gain induced by pioglitazone (Fig. 1A). Next, we analyzed the body fat composition by CT scan. As previously shown, pioglitazone significantly increases visceral, subcutaneous, and total fat mass. Interestingly, the addition of ML to pioglitazone inhibited the increase of visceral fat mass induced by pioglitazone, while ML did not affect the visceral, subcutaneous, or total fat mass (Fig. 1B).

### 3.2. Effect of ML, pioglitazone, and their co-treatment on energy homeostasis and lipolysis

To investigate the effect of ML on energy homeostasis and lipolysis, we next measured the expression of uncoupling protein (UCP)-1, 2, and β3-adorenoceptor (β3AR), which regulate energy expenditure and lipolysis [19] in WAT and liver. However, ML had no effect on the expression of UCP-1, 2 or β3AR in WAT, or UCP-2 in the liver (Supplementary Fig. 1). In addition, co-treatment of ML and pioglitazone did not affect total fat mass.

### 3.3. Effect of ML, pioglitazone, and their co-treatment on blood glucose, plasma TG and T-Chol

Next, we measured the changes in blood glucose, plasma TG and T-Chol levels. Although all these blood parameters were higher in db/db mice than in db/m mice, ML decreased blood glucose level by 32% and plasma TG level by 30% compared with non-treated db/db mice. Furthermore, co-treatment of ML and pioglitazone showed further 40% reduction in glucose level, and 30% reduction in TG level compared with pioglitazone alone. On the other hand, any treatment did not affect plasma T-Chol levels (Fig. 2).

### 3.4. Effect of ML, pioglitazone, and their co-treatment on glucose homeostasis

To investigate the effect of ML on glucose homeostasis, we performed IPGTT. ML significantly improved abnormal glucose tolerance, while pioglitazone, or their co-treatment markedly improved it (Fig. 3A). We also measured the expression of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), both of which regulate gluconeogenesis in the liver. Although the expression of G6Pase was significantly higher in db/db mice than in db/m mice, ML, pioglitazone, or their co-treatment significantly decreased the expression of G6Pase by 24, 37, and 31%, respectively. However, any treatment did not affect the expression of PEPCK (Fig. 3B).

### 3.5. Effect of ML, pioglitazone, and their co-treatment on adipocytokine expression

We next measured the levels of adipocytokines in epididymal WAT and plasma. Adiponectin levels in WAT and in plasma were

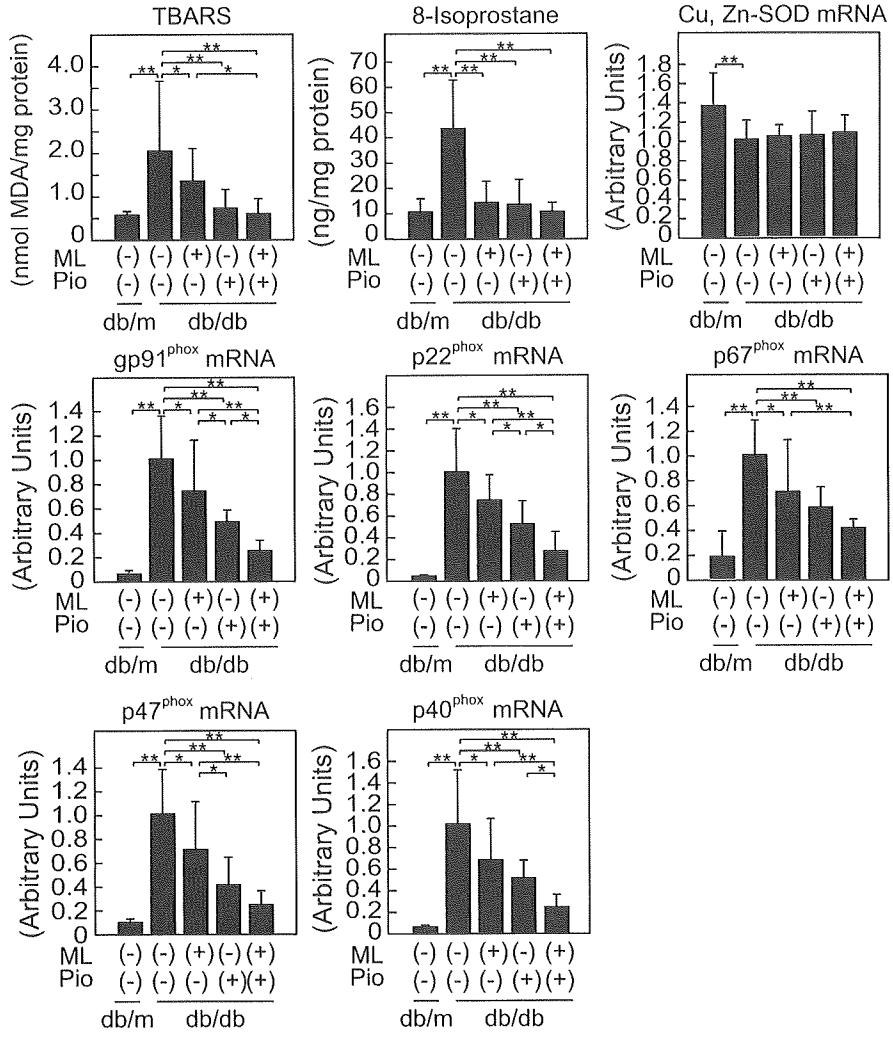


**Fig. 5.** Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on oxidative stress in white adipose tissue. Levels of TBARS, Cu, Zn-SOD mRNA, and NADPH oxidase subunits, gp91$^{phox}$, p22$^{phox}$, p67$^{phox}$, p47$^{phox}$ and p40$^{phox}$ and PU.1 mRNAs in WAT from each group of mice are shown. Data are expressed as means ± SD. $n = 5$ or 6. $^{*}P < 0.05$; $^{**}P < 0.01$.

significantly lower in db/db mice than in db/m mice. ML significantly increased adiponectin levels in WAT by 40% compared with non-treated db/db mice, but not in plasma. Co-treatment further increased adiponectin levels by 17% in WAT and by 25% in plasma compared with pioglitazone alone. In contrast, the expression of inflammatory adipocytokines, such as TNF-α and MCP-1 in WAT was markedly increased in db/db mice than in db/m mice. However, ML decreased the expression of TNF-α and MCP-1 by 25 and 20% in db/db mice, respectively. In addition, co-treatment resulted in a further decrease by approximately 45% compared with pioglitazone alone (Fig. 4A).

We also measured the expression of two macrophage markers, F4/80 antigen, Emr1, and CD68 in WAT. Expression of Emr1 and CD68 was markedly increased in db/db mice than in db/m mice. However, ML significantly decreased the expression of Emr1 and CD68 by 13 and 16% in WAT, respectively. Co-treatment further decreased the expression of Emr1 and CD68 by 46 and 26%, respectively, compared with pioglitazone alone (Fig. 4B).

### 3.6. *Effect of ML, pioglitazone, and their co-treatment on oxidative stress in WAT and liver*

We next measured adipose TBARS concentrations to investigate the effect of ML on oxidative stress. Although adipose TBARS concentrations were markedly higher in db/db mice than in db/m mice, treatment with ML, pioglitazone, or both significantly decreased them in db/db mice by 43, 62, and 72%, respectively.

We also investigated the effects of ML, pioglitazone, or their co-treatment on gene expression related to the production and removal of ROS in WAT. Expression of genes related to the production of ROS, including all NADPH oxidase subunits and PU.1 was markedly increased in epididymal WAT of db/db mice, but ML significantly decreased them. Further, co-treatment consistently decreased the expression of these genes compared with pioglitazone alone. On the other hand, expression of Cu, Zn-SOD, the ROS-elimination system, was decreased in db/db mice compared with that in db/m mice. However, any treatment did not affect the expression of Cu, Zn-SOD (Fig. 5).

In the liver TG accumulation was higher in db/db mice than in db/m mice. ML significantly decreased hepatic TG content by 44% in db/db mice. TBARS concentrations and NADPH oxidase activity were also higher in db/db mice than in db/m mice. Treatment with ML, pioglitazone, or both markedly decreased hepatic TBARS concentrations by 35, 33, and 59%, respectively, and NADPH oxidase activity by 37, 65, and 74%, respectively in db/db mice. Furthermore, although we could not show a significant effect of each treatment on the expression of NADPH oxidase subunits, gp91$^{phox}$ and p47$^{phox}$, and Cu, Zn-SOD, tendencies were

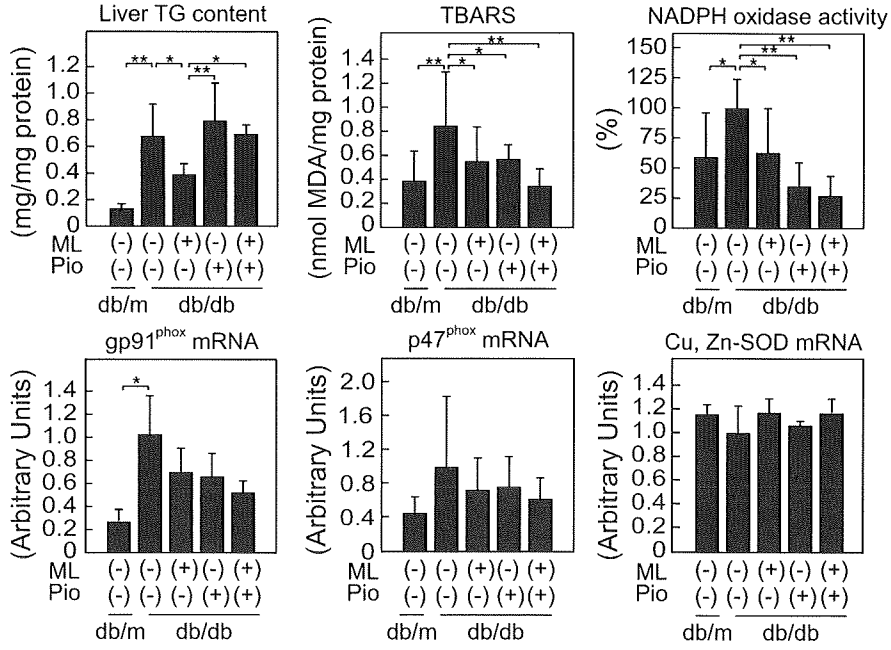


**Fig. 6.** Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on oxidative stress in the liver. Levels of TBARS, Cu, Zn-SOD mRNA, and NADPH oxidase from each group of mice are shown. Data are expressed as means ± SD. $n = 5$ or 6. $^{*}P < 0.05$; $^{**}P < 0.01$.

observed to show the effect of ML, Pio, and their co-treatment (Fig. 6).

### 3.7. *Effect of MLAF on glucose metabolism, oxidative stress and macrophage infiltration*

We previously showed that MLAF shows the strongest anti-oxidant effect [13,15]. Therefore, we treated db/db mice with MLAF for 5 weeks. We found that MLAF ameliorated result of IPGTT (Supplementary Fig. 2A) and IP insulin tolerance test (ITT) (Supplementary Fig. 2B). Furthermore, MLAF decreased plasma and urine 8-isoprostane levels (Supplementary Fig. 3A), and TBARS levels in skeletal muscle (Supplementary Fig. 3B), which are markers of lipid peroxides. In addition, MLAF also significantly decreased the ration of F4/80 positive cells in WAT (Supplementary Fig. 4).

## 4. Discussion

In the present study, we have shown that ML ameliorates metabolic disorders and adipocytokine dysregulation in db/db mice. Intriguingly, these effects of ML are additive to the effects of pioglitazone. We also proposed that these effects are mediated, at least in part through inhibiting oxidative stress in WAT and liver of obese mice.

Our results indicate that ML ameliorates adipocytokine dysregulation and suppresses macrophage infiltration, which are involved in the development of obesity [8]. We also demonstrated that ML, pioglitazone, and their co-treatment attenuated TBARS concentrations and the expression of NADPH oxidase subunits in WAT. The oxidation of fatty acids is an important source of ROS in fatty liver [20]. ML also decreased hepatic TG and TBARS concentrations by inhibiting NADPH oxidase activity through decreased expression of NADPH oxidase subunits and induction of the expression of Cu, Zn-SOD in the liver. These results could indicate that the inhibition of ROS generation via NADPH oxidase in WAT and liver may be one of the mechanisms by which ML can ameliorate metabolic disorders.

In accordance with the effects of ML on adipocytokine dysregulation, ML decreased blood glucose and plasma TG levels as previously described [12]. Previous study demonstrated that ML contains α-glucosidase inhibitor, 1-deoxynojirimycin (1-DNJ) [21]. Therefore, we expected that the effect of ML on glucose metabolism can be partly attributed to the inhibition of glucose absorption from intestine by 1-DNJ. However, as a novel mechanism, we propose that ML ameliorates adipocytokine dysregulation and ROS production through inhibiting oxidative stress in WAT, because we previously showed that MLAF shows the strongest anti-oxidant effect. Furthermore, we also found that MLAF ameliorated result of IPGTT and IPITT. MLAF also decreased plasma and urine 8-isoprostane levels, and TBARS levels in skeletal muscle and macrophage infiltration into WAT. These data may strengthen that ML ameliorates metabolic disorders and inflammation through its anti-oxidative effect in addition to the inhibition of glucose absorption from the gut by 1-DNJ.

We showed that administration of ML in addition to pioglitazone attenuated the body weight gain observed under pioglitazone treatment. Clinical study shows that treatment with thiazolidinediones such as pioglitazone is associated with edema and weight gain [22]. Previous study showed that pioglitazone induces fat mass by increasing the number of small adipocytes in Zucker (*fa/fa*) rats by an activation of PPAR-γ [23]. Another study showed that mice treated with pioglitazone experience weight gain from epithelial $Na^+$ channel (ENaC)-mediated renal salt absorption [24]. In this study, ML attenuated pioglitazone-induced visceral fat mass gain. Therefore, we speculated that ML might attenuate visceral fat gain through promotion of energy consumption by increasing adiponectin. However, ML had no effect on the expression of UCP-1, 2 or β3AR in WAT, or UCP-2 in the liver. In addition, co-treatment of ML and pioglitazone did not affect total fat mass. Thus, the ameliorative effect of ML on body weight gain might depend on another mechanism. Although we did not study the effect of ML on ENaC or urine volume, previous study shows diuretic effects of γ-aminobutyric acid [25], which is abundantly contained in ML [26]. Therefore, although we did not study the brown adipose tissue, which mainly regulates thermogenesis, this effect of ML might be

caused by amelioration of edema through its diuretic action more than promotion of energy expenditure.

The present study clearly demonstrated that ML ameliorates metabolic disorders including diabetes and dyslipidemia, and shows additive effects with pioglitazone. As an expected mechanism, we propose that ML could ameliorate adipocytokine dysregulation at least in part through inhibiting oxidative stress in WAT. In addition, we showed that ML attenuated the body weight gain caused by pioglitazone treatment. Thus, our study implicates that ML may be a basis for a pharmaceutical for the treatment of the metabolic syndrome as well as inducing effects of pioglitazone while reducing its adverse effects.

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We thank Ms. Ayumi Hosotani (Kyoto University) for excellent technical assistance, Dr. Masaru Iwai (Ehime University) for advice for our experiment. This study was supported by Grants-in Aid from the Ministry of Education, Science, Sports, Culture, and Technology of Japan and a research grant for health sciences from the Japanese Ministry of Health and Welfare.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2008.10.021.

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# Distribution of Receptor Glycolipids for *Lactobacilli* in Murine Digestive Tract and Production of Antibodies Cross-reactive with them by Immunization of Rabbits with *Lactobacilli**

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**In the digestive tract of mice (HR-1 strain), glycolipids belonging to the ganglio-series were revealed to be expressed in region-specific manners, *i.e.* FGA1 and FGM1 in the stomach, GA1 in the small intestine, and FGA1 and sulphatides in the cecum. The amount of GA1 as a receptor glycolipid for *Lactobacilli* was especially higher in the small intestine than in the other regions, it comprising 1.6–2.8 μg/mg dry weight. On immunization of rabbits with *Lactobacillus johnsonii* and *Lactobacillus intestinalis*, both of which are murine intestinal bacteria, antibodies toward bacterial glycolipids, *i.e.* Galα1–2Glcα1–3DG, and tri- and tetrahexaosyl DGs, were effectively generated and, in addition, they were found to cross-react with GA1 and GalCer, but not with structurally related glycolipids such as $Lc_4Cer$, $nLc_4Cer$ and $IV^3Gal\alpha$-$nLc_4Cer$, indicating that GA1 is a preferable antigen for anti-lactobacillus antisera and suggesting the presence of epitopes common to both *Lactobacilli* and the host. In fact, molecules reacting with anti-GA1 antibodies were detected among bacterial proteins on Western blotting, indicating a possible occurrence of the carbohydrate structure mimicking GA1 in bacterial proteins.**

**Key words: asialo GM1, bacterial receptor, digestive tract, glycoglycerolipids, TLC-immunostaining.**

Abbreviations: CL, cardiolipin; CMH, ceramide monohexoside; CS, cholesterol sulphate; DG, diacyl glycerol; Hep, L-glycero-D-mannoheptose; FGA1, fucosyl asialo GM1; FGM1, fucosyl GM1; GA1, asialo GM1; PG, phosphatidyl glycerol; Sul, sulphatide.

Glycolipids are ubiquitous membrane components of mammalian tissues and cells, and their carbohydrate moieties are included in antigens concerning blood group, species specificity and cellular differentiation and transformation, and receptors for bacteria, bacterial toxins and viruses (*1*). In the murine digestive tract, glycolipids belonging to the globo- and ganglio-series are separately distributed in the mesenchymal and epithelial tissues, respectively, and GA1, as the backbone structure of ganglio-series glycolipids, is expressed in association with cellular differentiation from the crypt to the villus in the intestinal microvilli (*2*), and provides receptors for several bacteria, such as *Lactobacillus casei*, *Lactobacillus reuteri*, *Lactobacillus johnsonii* (*L. acidophilus*), *Bifidobacterium bifidum*, *Pseudomonas aeruginosa*, *Actinomyces maeslundii* and *Neisseria gonorrhoeae* (*1*). However, the carbohydrate moiety of GA1 has been revealed to be modified through transcriptional regulation of the sugar transferase gene on bacterial infection (*3–6*). Under conventional breeding conditions, expression of FGA1 from the fucosyltransferase gene in the small intestine occurs during the postnatal period from early suckling to weaning (*7–9*), and is enhanced by food containing dietary fibre at the time of weaning (*10*). Fucosylation does not occur under germ-free conditions, but infection by indigenous filamentous bacteria and wild-type *Bacteroides thetaiotaomicron* in germ-free mice triggers expression of the FUT2 gene, a counterpart of the human secretor (Se) gene, for synthesis of FGA1 only in the small intestine, *i.e.* not in other regions of the digestive tract (*5*, *6*). In fact, differences in the regulation of gene expression among different regions have been revealed by targeted deletion of the FUT1 and FUT2 genes (*6*, *11*). Although fucosylated glycolipids completely disappear from the antrum, cecum and colon of FUT2-null mice, those in the small intestine of FUT2-null mice are maintained at similar levels to those in the wild-type (*6*). In contrast, the amounts of fucosylated glycolipids in the stomach, cecum and colon are not affected by targeted deletion of the FUT1 gene, but those in the small intestine of FUT1-null mice are rather increased compared to in wild-type and

*The nomenclature for glycolipids and gangliosides is based on the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [IUPAC-IUB Commission on Biochemical Nomenclature. (1977) The nomenclature of lipids. *Eur. J. Biochem.* **179**, 11–21] and Svennerholm [Svennerholm, L. (1963) Chromatographic separation of human brain gangliosides. *J. Neurochem.* **10**, 613–623], respectively.

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FUT2-null mice (*6*). These findings indicate that fucosylation in the antrum, cecum and colon is preferentially due to the FUT2 gene, but that in the small intestine to either the FUT1 or FUT2 gene. Accordingly, fucosylation of glycolipids in the stomach, cecum and colon due to the FUT2 gene occurs in a development-dependent manner, but that in the small intestine is regulated by the surrounding circumstances in mice, *i.e.* suppression of FUT2-gene expression under germ-free conditions, and activation of FUT2-gene expression under conventional breeding conditions and targeted deletion of the FUT1 gene. Thus, expression of GA1 in the epithelial cells of the digestive tract is actively regulated through modification of the carbohydrate structures, and is thought to influence the colonization of bacteria having the ability to bind with GA1 as a receptor, particularly of symbiotic bacteria, *i.e. Lactobacillus* and *Bifidobacterium* (*12–16*), which play a role in protection from infection by harmful bacteria passing into the digestive tract together with food.

However, the profile of glycolipid expression under region-specific regulation in the digestive tract is not clearly understood yet, and therefore we determined the glycolipid compositions in different regions of the digestive tract of conventional breeding mice, with special reference to the receptor distribution for intestinal *Lactobacilli*, the receptor glycolipids for *L. johnsonii* having been characterized as GlcCer, $Gb_3Cer$, GA1, $nLc_4Cer$ and $Lc_4Cer$ (*13*). In addition, we prepared antisera by immunization of rabbits with *L. johnsonii* and *L. intestinalis*, both of which are murine intestinal bacteria, and found that the antisera cross-reacted with GalCer and GA1, indicating the occurrence of an intestinal glycolipid-like structure in *Lactobacilli*, which might be related with a mechanism for evading immunological surveillance by the host for symbiosis with *Lactobacilli* in the digestive tract of mice.

## MATERIALS AND METHODS

*Lactobacilli—Lactobacillus johnsonii* (*L. acidophilus*) (JCM No. 1022), *Lactobacillus casei* (JCM No. 1134) and *Lactobacillus intestinalis* (JCM No. 7548) were purchased from the Japan Collection of Microorganisms (JCM), RIKEN BioResource Center (Wako, Saitama, Japan), and cultured in *Lactobacilli* MRS broth (Difco, Beckton-Dickinson, Sparks, MD, USA).

*Glycolipids and phospholipids*—The glycolipids used in this experiment were purified from various sources in our laboratory: Galα1–2Glcα1–3DG from *L. casei* (*17*), GlcCer, LacCer, $Gb_3Cer$, $Gb_4Cer$, GM3 and $IV^3$NeuAc-$nLc_4Cer$ from human erythrocytes, $IV^3$GalNAcα-$Gb_4Cer$ (Forssman antigen) from equine kidney, $Gg_3Cer$ from guinea pig erythrocytes, GalCer containing non-hydroxy fatty acids (GalCer NFA) and 2-hydroxy fatty acids (GalCer HFA), sulphatides and GM1 from bovine brain, FGM1 from bovine thyroid, $Lc_4Cer$ from human meconium and $IV^3$Galα-$nLc_4Cer$ from rabbit erythrocytes (*18*). GA1, FGA1 and $nLc_4Cer$ were prepared from GM1, FGM1 and $IV^3$NeuAc-$nLc_4Cer$, respectively, by treatment with *Arthrobacter ureafaciens* sialidase (*19*). *N*-Stearoyl glycolipids, as standards, for TLC-densitometry were prepared by deacylation with sphingolipid ceramide *N*-deacylase (*Pseudomonas sp.* TK4), followed by reacylation with stearoyl chloride. Dioleoyl derivatives of phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), phosphatidyl serine (PS), phosphatidyl glycerol (PG), phosphatidic acid (PA) and sphingomyelin (SM) from human erythrocytes were kindly provided by Alfresa Pharma Co. (Osaka), and cardiolipin (CL) and phosphatidyl inositol (PI) were purchased from Sigma (St Louis, MO, USA). The concentrations of standard phospholipids in chloroform/methanol (1:1, v/v) were determined by the phosphomolybdate procedure after decomposition of the lipids with 70% $HClO_4$ and $H_2O_2$ (*20*).

*Antisera*—Rabbit polyclonal antibodies toward *L. johnsonii* and *L. intestinalis* were generated by immunizing rabbits (Japanese White; Japanese Biological Materials, Tokyo) intradermally with a water-in-oil emulsion prepared by mixing 15 mg of each bacterium in 1 ml of phosphate-buffered saline (PBS) with 1 ml of Freund's complete adjuvant (Sigma), and the antibody titres were subsequently monitored by enzyme-linked immunosorbent assaying (ELISA) with the respective bacterium (2 μg/well) as the antigen. In a similar way, polyclonal antibodies toward Forssman antigen and GA1 were generated by immunizing rabbits with 1 mg of glycolipids together with Freund's complete adjuvant, and also monoclonal antibodies toward FGM1 and FGA1 were prepared by immunizing mice with 20 μg of glycolipids together with *Salmonella minnesota* as the adjuvant, followed by hybridization of lymphocytes with murine myeloma P3X63Ag8. Anti-Forssman, anti-GA1, anti-FGM1 and anti-FGA1 antibodies generated characteristically reacted with the respective glycolipid antigens and no cross-reaction to structurally related glycolipids were observed (*5*, *21*).

*Quantitation of lipids in murine tissues and bacteria*—Mice (HR-1, 10 weeks of age) were kept under conventional breeding conditions at a room temperature of $24 \pm 1°C$ and a humidity level of $55 \pm 10\%$ with food and water *ad lib.* Animal treatment followed the animal care guidelines of Kinki University.

Tissues, *i.e.* stomach, duodenum, jejunum, ileum, cecum and colon, were rinsed with PBS and then lyophilized. Total lipids were extracted from the lyophilized tissues with chloroform/methanol/water (20:10:1, 10:20:1 and 1:1:0, v/v/v), and then fractionated into neutral and acidic lipids on a DEAE-Sephadex column (A-25, acetate form; GE Healthcare Bioscience, Piscatway, NJ, USA). Then, the neutral glycolipids were separated from the unabsorbed neutral lipid fraction by acetylation, separation of the acetylated derivatives, deacetylation and desalting, whereas the absorbed acidic lipid fraction containing gangliosides and cholesterol sulphate (CS) was saponified with 0.5 M NaOH in methanol to remove the ester-containing lipids, followed by dialysis (*22*, *23*).

The total lipids, and acidic and neutral lipids thus obtained were examined by TLC. The following solvent mixtures were used as the developing solvents for TLC, chloroform/methanol/water (65:35:8, v/v/v) for phospholipids and neutral glycolipids, chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, v/v/v) for cholesterol sulphate and sulphatides, and chloroform/methanol/0.5%

$CaCl_2$ (55:45:10, v/v/v) for gangliosides, and the detection reagents were as follows, cupric acetate–phosphoric acid reagent for neutral lipids, phospholipids and CS, Dittmer's reagent for phospholipids, orcinol–sulphuric acid reagent for glycolipids and resorcinol–hydrochloric acid reagent for gangliosides. The density of spots on TLC plates was determined by image analysis (NIH image). Standard lipids, *i.e. N*-stearoyl derivatives of GalCer, LacCer, $Gb_3Cer$, GM3, $Gb_4Cer$, Forssman glycolipid, GA1, GM1, FGM1 and GD1a, and dioleoyl derivatives of PE, PS, PC, PG and PA, and CS (0.1–1.5 μg), were developed on the same TLC plates for the preparation of standard curves (*24*). Analysis of lipids from *Lactobacilli* was carried out by the same procedure, but quantitation of mono- to tetrahexaosyl DGs in *Lactobacilli* was performed using the standard curves for mono- to tetrahexaosyl ceramides as described above.

*TLC-immunostaining*—Lipids were developed on plastic-coated TLC plates (Macherey-Nagel, Düren, Germany), which were then blocked with blocking buffer (PBS containing 1% polyvinylpyrrolidone and 1% ovalbumin), and the spots were visualized by immunostaining with the above anti-glycolipid and anti-lactobacilli antibodies (1:500) diluted with dilution buffer (PBS containing 3% polyvinylpyrrolidone), followed by immunostaining with peroxidase-conjugated anti-rabbit IgG and IgM (1:1,000; Jackson Immunoresearch Lab., PA, USA), and anti-murine IgG and IgM antibodies (1:1,000; Sigma), and peroxidase substrates, 4-chloro-1-naphthol and $H_2O_2$, according to the procedure reported previously (*25*). Control staining with normal rabbit serum was performed simultaneously under the same conditions.

*SDS–PAGE and Western blotting*—*Lactobacilli* were suspended in PBS by ultrasonication, and the protein concentration of the resulting solution was measured by Bradford's procedure with bovine serum albumin as the standard protein (*26*). Then the solution, corresponding to 8 μg of protein, was denatured with 15 μl of sample buffer [1% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.125% bromophenol blue in 0.06 M Tris–HCl (pH 6.8)] at 95°C for 4 min, and then electrophoresed on an acrylamide gel (12%), which was stained with Coomassie brilliant blue (CBB) for proteins (*27*). Also, proteins on a SDS–PAGE gel were electrically transferred to a nitrocellulose membrane according to the manufacturer's instructions (Bio-Rad Lab., Hercules, CA, USA), and the membrane was immunostained with anti-lactobacilli and anti-GA1 antibodies according to the procedure for TLC-immunostaining described above.

## RESULTS AND DISCUSSION

*Glycolipid receptors for Lactobacilli in the murine digestive tract*—As shown in Figs 1 and 2, the glycolipids, particularly ganglio-series ones, were revealed to be distinct in different regions of the murine digestive tract. Although Forssman glycolipid was present in all regions of the tract, amounting to 0.12–1.11 μg/mg dry weight, GA1 as a receptor for intestinal *Lactobacilli* was predominant in the duodenum, jejunum and ileum, in comparison to in the stomach, cecum and colon, where it was not detectable or was only present in a trace amount, as judged on TLC-immunostaining with anti-GA1 antibodies (Fig. 2). The amounts of GA1 in the duodenum, jejunum and ileum were 1.6, 2.8 and 2.2 μg/mg dry weight, respectively, corresponding to ~36–50% of the total neutral glycolipids. In contrast, FGA1 was abundant in the stomach and cecum, amounting to 0.9 and 1.6 μg/mg dry weight, respectively, corresponding to ~20% of the total neutral glycolipids. On comparison of the ratio of FGA1 to GA1 plus FGA1 as the rate of fucosylation of GA1, GA1 in the stomach, cecum and colon was revealed to be almost completely converted to FGA1, but the fucosylation rates in the duodenum, jejunum and ileum were only 8, 0.3 and 10%, respectively (Table 1). On the other

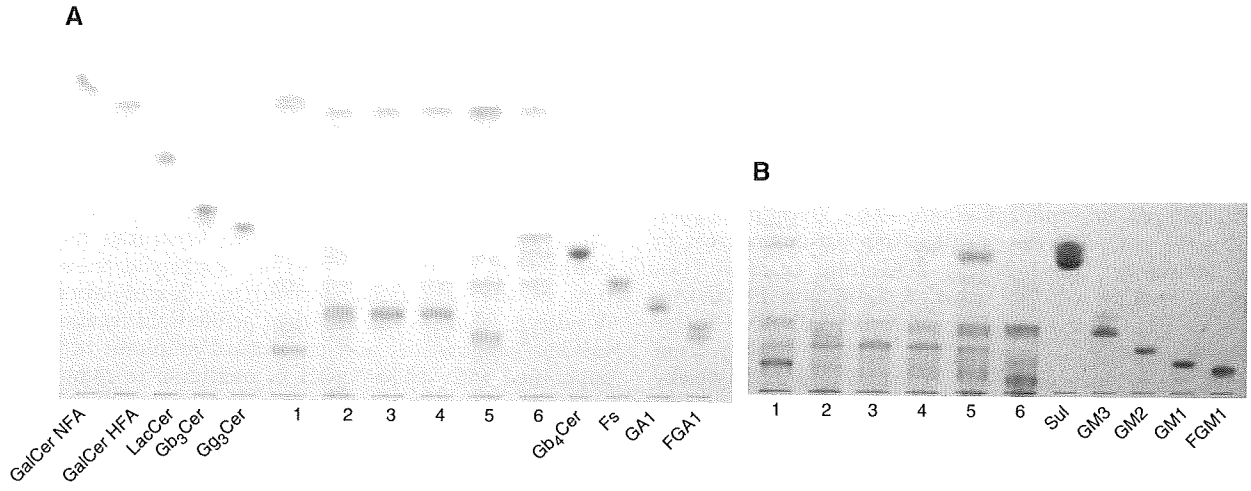


Fig. 1. **TLC of neutral (A) and acidic (B) glycolipids from different regions of the murine digestive tract.** Neutral glycolipids and gangliosides, corresponding to 0.5 mg dry weight, were developed on TLC plates with chloroform/methanol/water (65:35:8, v/v/v) for A and chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, v/v/v) for B, and were detected with orcinol–sulphuric acid reagent. 1, Stomach; 2, duodenum; 3, jejunum; 4, ileum; 5, cecum; 6, colon.