disease, osteoporosis, and geriatric syndrome. Many studies have been done by doctors, nurses, physical or occupational therapists, care workers, and the government to assess and improve the LTCI system (Ikegami et al., 2003; Ozawa and Nakayama, 2005; Kikuchi et al., 2006). We should try our best to improve this system in our country by supporting caregivers with professional training in improvement of long-term care for elderly patients, evaluation of long-term care services, and further assistance for care workers.

### 3. The health insurance system for elderly 75 and over and comprehensive geriatric assessment

The second problem tackled was the health insurance system specifically for elderly people aged 75 and over, which began on April 1, 2008. This has been unpopular due to several problems in the insurance system. However, the notion that people aged 75 and over should have their own primary-care physicians is useful for the transition to the home medical care. Some people are concerned about this system, because the "free-access" maintained in the Japanese medical system is no longer guaranteed for the very elderly. In spite of this concern, the idea should be generally implemented to avoid adverse drug effects related to polypharmacy, because elderly patients tend to see many doctors. This is also a good chance for our society to appreciate the role of geriatricians. Along with the enforcement of this insurance system, the Japan Geriatrics Society started a new education program for geriatricians and general practitioners to teach care of elderly patients. In the program, the Japan Geriatrics Society is trying to educate in the practical aspects of geriatric medicine required for the care of elderly patients and how to deal with clinical problems, especially geriatric syndrome. In this program the role of comprehensive geriatric assessment (CGA) has been stressed to maintain activities of daily living and quality of life of elderly patients. CGA is an integral part of geriatric care throughout the world, where the assessment of physiological, psychosocial, and cognitive aspects in elderly is essential (Rubenstein and Wieland, 1989). The frail elderly often have multiple chronic illnesses, functional disabilities, and psychosocial problems. Therefore, their needs obviously extend far beyond the treatment of any single medical condition. CGA also requires a multidisciplinary team approach and the use of guidelines and procedures to identify and address potentially reversible problems. The ultimate goal is to systematically restore and maintain the functions essential to preserving quality of life. Assessing how we offer medical examinations, treatment plans, rehabilitation, and the care services on the basis of a result of CGA is important. Among Japanese geriatricians, CGA is becoming more and more popular and many physicians are becoming aware of the importance of CGA. The applicability and effect of CGA on in-patient care, as well as community health care, have been reported (Onishi et al., 2004; Iizaka et al., 2008). Thus, the medical care system for the very elderly is a touchstone of our society.

### 4. Role of geriatricians in the aged society

To ensure that every older person receives high-quality, patient-centered health care, the role of geriatricians and the Japan Geriatrics Society is enormous. The first department of geriatric medicine in Japan was founded in the University of Tokyo in 1962. Our department of geriatric medicine at the Kyoto University Graduate School of Medicine is the second oldest and was established in 1967. Since then, many medical schools have established departments of geriatric medicine. However, at the moment less than 30% of Japan's 79 medical schools have departments of geriatric medicine. Therefore, not all the medical students receive the education in geriatric medicine and many graduate without even learning what the geriatric medicine is. Another sad fact is that some departments of geriatric medicine are managed without full professors. Generally speaking, undergraduate education in geriatrics in Japan emphasizes the theoretical aspects of the aging process and the features of disease in the elderly, but tends to omit attention to the practical aspects of care. In contrast, education in the United Kingdom and other countries places emphasis on practical aspects of elderly care. Thus, the education of geriatric medicine in our country is still remarkably poor in spite of the demand from society. I personally think that training in geriatric medicine should be required in all medical schools, and be taken into the medical-training system of young residents. Therefore, to expand the geriatrics knowledge base, increase the number of health-care professionals who employ the principles of geriatric medicine in caring for older persons, and recruit physicians and other health-care professionals into careers in geriatric medicine, the Japan Geriatrics Society is a key leader of change to achieve the goals of geriatric medicine and optimize the health of our aging population. At the moment the Japan Geriatrics Society has 6,134 members, and has approved 1,487 specialists of geriatrics. I hope that more and more young physicians are interested in geriatric medicine and are involved in the care of elderly people along with other health-care professionals. I do hope that our efforts will bring a brighter future for the Asian countries that follow Japan in terms of an aging society.

### 5. Conclusions

We have made significant progress in geriatrics and gerontology in the last two decades in Japan, as well as establishing our own health-care systems. Because Japan is the most aged country in the world – the second and third also being Asian countries – collaboration among Asian geriatric societies is important for the improvement of elderly health care in Asia.

### Conflict of interest statement

The author has no conflicts of interest to report.

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Atherosclerosis 204 (2009) 388–394

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

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

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ARTICLE INFO

*Article history:*
Received 26 May 2008
Received in revised form 6 October 2008
Accepted 12 October 2008
Available online 30 October 2008

*Keywords:*
Macrophage
Adipocytokine
Oxidative stress
Insulin resistance
Obesity

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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doi:10.1016/j.atherosclerosis.2008.10.021

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].

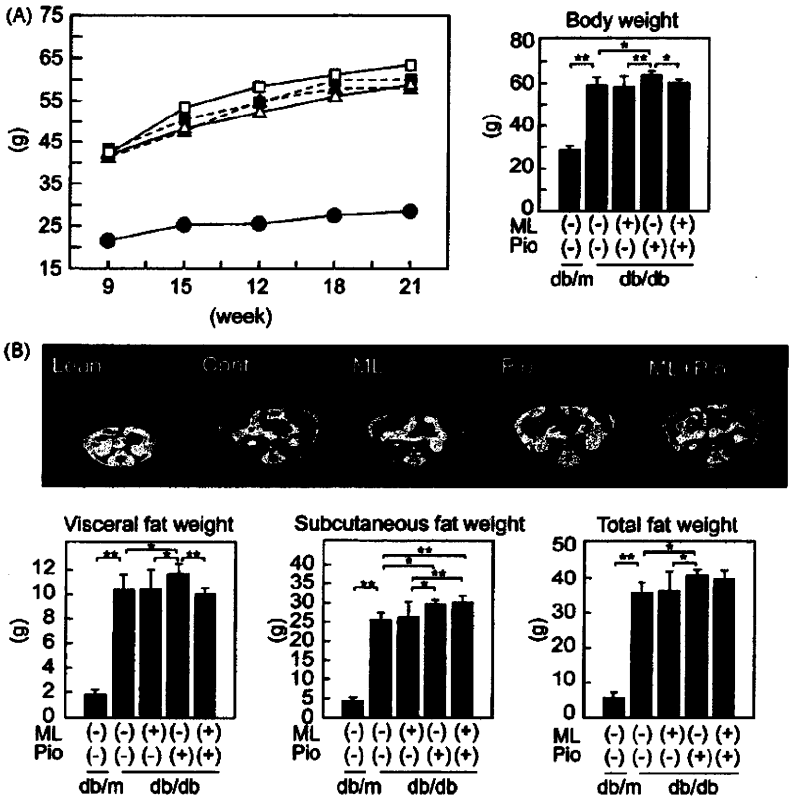


**Fig. 1.** Effect of mulberry leaf, pioglitazone, or both treatment for 12 weeks on body and fat weight. (A) Growth curve during experiment and body weight at the end of experiment of db/m (Lean) and db/db mice on control (Cont), 3% ML-supplemented (ML), 0.01% pioglitazone-supplemented (Pio), or co-supplemented (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. $^{*}P < 0.05$; $^{**}P < 0.01$.

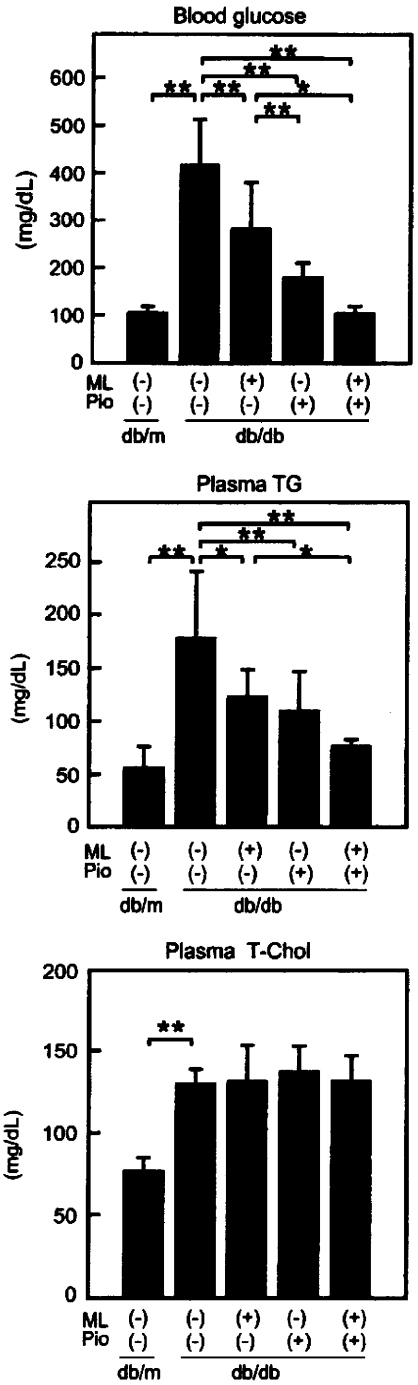


**Fig. 2.** Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on glucose and lipid metabolism. Blood glucose levels and plasma concentrations of triglyceride and total cholesterol in each group of mice are shown. Data are expressed as means ± SD. $n = 5$ or 6. $^{*}P < 0.05$;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

### 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),

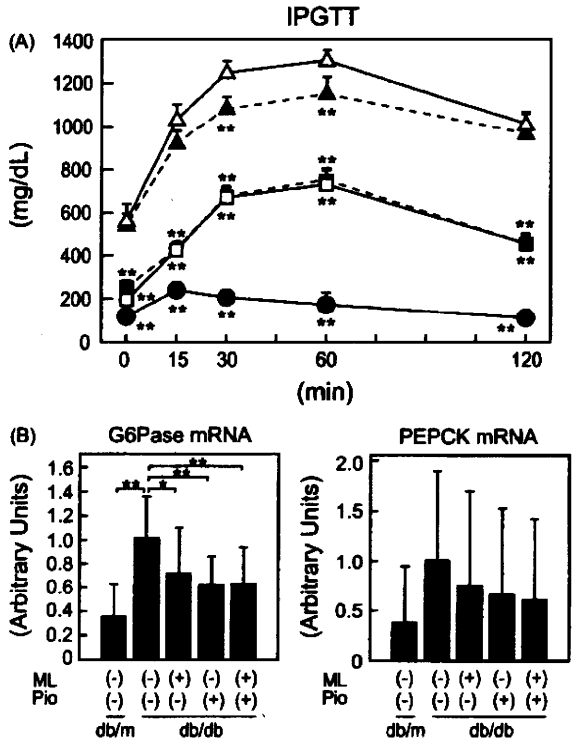


**Fig. 3.** Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on abnormal glucose tolerance and the expression of genes related to gluconeogenesis. (A) Plasma glucose levels were determined in each group of mice are shown. $n = 5$ or 6. $^{**}P < 0.01$ versus db/db mice on control 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.

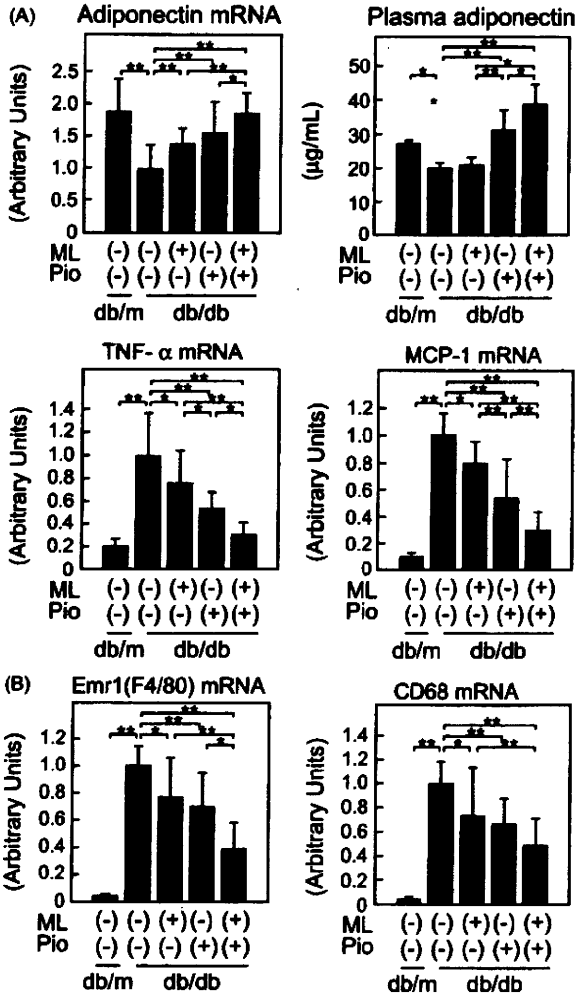


**Fig. 4.** Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on the expression of adipocytokines and macrophage infiltration in white adipose tissue. (A) Levels of adiponectin in WAT and in plasma in each group of mice are shown. (B) Expression of TNF-α and MCP-1 mRNAs and Emr1 (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

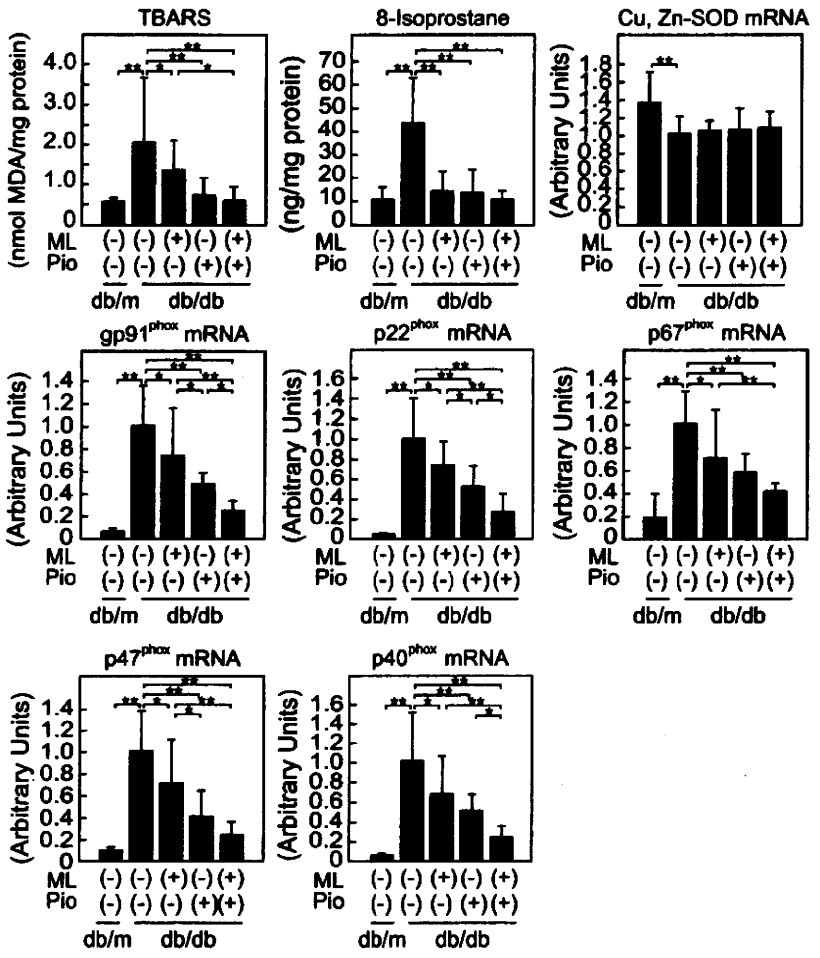


**Fig. 5.** Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on oxidative stress in white adipose tissue. Levels of TBARS, Cu, Zn-SOD mRNA, and NADPH oxidase subunits, $gp91^{phox}$, $p22^{phox}$, $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$ and PU.1 mRNAs in WAT from each group 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

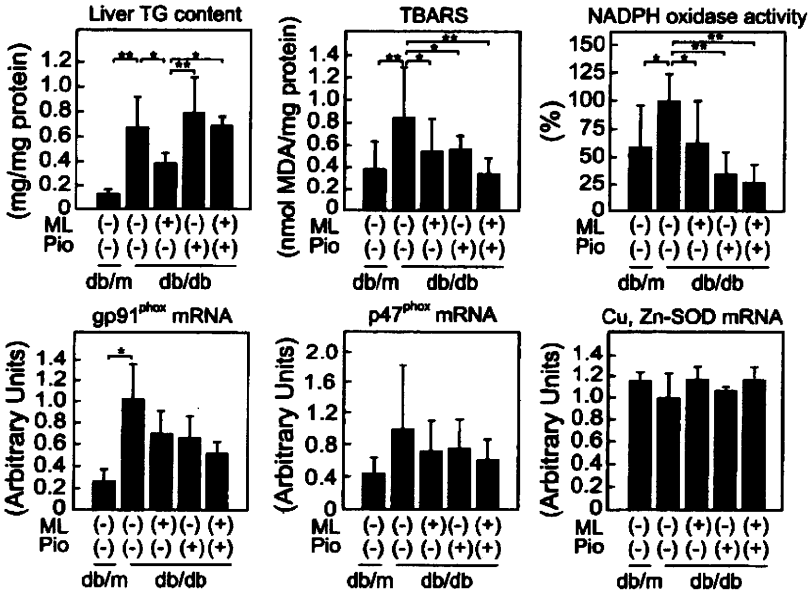


**Fig. 6.** Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on oxidative stress in the liver. Levels of TBARS, Cu, Zn-SOD mRNA, and NADPH oxidase from each group of mice are shown. Data are expressed as means ± SD. $n = 5$ or 6. $^{*}P < 0.05$; $^{**}P < 0.01$.

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.

## Acknowledgements

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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一般演題 3)

# メタボリックシンドロームに対する食事・運動療法による効果の検討

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京都大学医学部附属病院に通院中の患者でメタボリックシンドロームと診断された患者に対し、食事指導・運動指導を行うことにより、メタボリックシンドロームの各パラメータがどのように変化するかを検討した。介入を行った26名の中で、食事・運動目標達成群は10名にとどまり、10名は食事目標のみ達成した。食事・運動目標達成群においては体重、ウエスト周囲径、アポB及び高感度CRPは有意に低下したが、他のパラメータは改善傾向を示すものの、有意差を示すに至らなかった。今回の介入研究により、メタボリックシンドローム患者に対する標準指導法として毎日10,000歩のウォーキングと理想体重×25カロリーの食事療法が有効であることが示された。

キーワード：メタボリックシンドローム、食事療法、運動療法、アディポネクチン

## 目 的

近年生活習慣の欧米化により肥満、脂質異常症、糖尿病が増加しており、動脈硬化性疾患の増加が懸念されている。なかでも内臓肥満、脂質異常症、耐糖能異常、高血圧などの危険因子が合併した病態であるメタボリックシンドロームは、動脈硬化性疾患のハイリスクな病態として注目され、2005年4月に8学会合同の新しい診断基準が作成された。このメタボリックシンドロームに対する治療方針としては運動と食事療法による減量が有効であると考えられているが、どの程度の運動を行い、どの程度の食事制限をすれば減量ができ、それぞれの危険因子が改善するかについては明らかになっていない。今回の研究においては新しい診断基準で診断を行ったメタボリックシンドローム患者において運動療法と食事療法を行うことにより、どの程度体重、ウエスト周囲径が減少し、さらには血清脂質値や血糖値、血圧が改善するかについて検討する。

## 方 法

### 1．対象

京都大学医学部附属病院に通院中で、平成17年4月に作成された日本におけるメタボリックシンドロームの診断基準を用いてメタボリックシンドロームと診断された35～75歳の男女。急性心筋梗塞発症後6ヶ月以内の患者、不安定狭心症の患者、重篤な心疾患の既往・合併のある患者、心血管再建術施行後6ヶ月以内の患者、

重篤な肝疾患または腎疾患（血清クレアチニン2.5mg/dl以上）を合併している患者、悪性腫瘍を合併している患者、コントロール不良の糖尿病患者あるいは高血圧患者、全身麻酔での手術を予定している患者、関節疾患などのために運動療法ができない患者、その他主治医が不適当と判断した患者は除いた。

**2．調査・観察・検査項目**

運動／食事療法施行前の検査項目

年齢、性別、身長、体重、BMI、立位でのウエスト周囲径、血圧、脈拍数、喫煙歴、アルコール摂取量、既往歴、家族歴、使用薬剤

採血項目：12時間以上の絶食空腹時、血清脂質：総コレステロール、中性脂肪、HDLコレステロール、LDLコレステロール、AST、ALT、γ-GTP、アルブミン、クレアチニン、尿酸、BUN、空腹時血糖、CPK、$HbA_{1c}$を必須項目とした。アディポネクチン、可溶型LOX-1、高感度CRP、small dense LDLは介入開始前と6ヶ月後に測定した。

**3．介入方法**

メタボリックシンドロームの診断基準を満たし、文書による本研究への参加の同意を得たものに対し、以下の介入を行った。介入を行う期間は6ヶ月とした。

(A) 運動処方：原則として30分／日以上、3回／週以上

1．歩数計で10,000歩／日以上（週5日以上）を推奨した。

(B) 食事療法

食事：原則として推奨摂取エネルギーを男女とも理想体重（身長(m)$^2$×22）×25カロリー／日とした。

栄養士が栄養指導を行い、自己申告で月3日間の栄養摂取量を食事記録法により栄養士が評価した（3，6ヶ月後）。これにより平均のエネルギー摂取量を計算した。

原則として投与中の薬剤の変更は研究期間中行わないこととしたが、臨床的に必要な場合は変更を認めた。なお、運動療法および食事療法を継続するにあたって、なんらかの問題が生じた場合には研究を中止した。本プロトコルに関しては京都大学医の倫理委員会において承認された（C-30）。

## 結　果

37歳から75歳までの男女33名から研究参加の同意を得たが、7名が途中で脱落し、6ヶ月間の介入を終了したのは26名であった。Tab.1に参加者の臨床データを示す。平均年齢は61.5歳で、男性がやや多かった。26名中10名の患者が食事・運動目標を達成した。食事目標のみの達成者は10名であった。いずれの目標を達成しなかったのは5名であった。Tab.2に示すように食事・運動目標達成群において有意に体重、ウエスト周囲径、アポB、高感度CRPが減少し

Tab.1　対象患者のベースラインデータ

| | 平　均 | SD or Median |
|---|---|---|
| 年齢（歳） | 61.5 | 63 |
| 男女比 | 男性57.7% | |
| 体重（kg） | 73.6 | 11.3 |
| 腹囲（cm） | 97.6 | 7.1 |
| 収縮期血圧（mmHg） | 142 | 20.7 |
| 拡張期血圧（mmHg） | 82.4 | 12.5 |
| 総コレステロール（mg/dl） | 197 | 33.6 |
| HDLコレステロール（mg/dl） | 50.2 | 10 |
| LDLコレステロール（mg/dl） | 121 | 31 |
| トリグリセリド（mg/dl） | 130 | 122 |
| 空腹時血糖（mg/dl） | 124 | 28 |
| AST（IU/L） | 26 | 9.5 |
| ALT（IU/L） | 34 | 19.9 |
| γ-GTP（IU/L） | 52 | 38.6 |
| Alb（g/dl） | 4.3 | 0.3 |
| Cre（mg/dl） | 0.8 | 0.2 |
| 尿酸（mg/dl） | 6.0 | 1.2 |
| BUN（mg/dl） | 15 | 3.9 |
| アポB（mg/dl） | 98 | 22.3 |
| アポA1（mg/dl） | 135 | 21.7 |
| アディポネクチン（μg/ml） | 6.8 | 2.6 |
| 高感度CRP（mg/L） | 3.1 | 0.9 |

Tab.2　各群におけるパラメータの変化

| | 食事・運動達成群 | | 食事達成群 | | 未達成群 | |
|---|---|---|---|---|---|---|
| | 平均値 | 減少率（%） | 平均値 | 減少率（%） | 平均値 | 減少率（%） |
| 体重 | 73.1 | −6.1 | 75.0 | −2.2 | 74.2 | 1.9 |
| ウエスト周囲径 | 96.1 | −5.7 | 97.4 | −0.4 | 102.2 | 0.7 |
| 収縮時血圧 | 143.2 | −5.2 | 144.1 | −3.7 | 132.4 | −3.6 |
| 拡張時血圧 | 84.0 | −3.7 | 83.5 | 0.7 | 76.2 | 1.6 |
| 総コレステロール | 209.5 | −7.3 | 201.3 | 1.3 | 170.0 | −1.6 |
| HDL コレステロール | 46.2 | 5.5 | 53.0 | 2.1 | 49.2 | 6.1 |
| LDL 直接 | 124.9 | −9.9 | 118.6 | −3.6 | 95.6 | −8.4 |
| LDL 計算 | 128.7 | −8.2 | 126.4 | 1.5 | 99.0 | −10.3 |
| トリグリセリド | 166.0 | −26.6 | 112.1 | 20.2 | 109.0 | 20.0 |
| AST | 26.2 | −11.1 | 26.3 | −9.5 | 26.4 | 19.7 |
| ALT | 32.4 | −20.7 | 38.1 | −22.8 | 30.2 | 31.8 |
| γ - GTP | 45.5 | −13.2 | 67.8 | −2.5 | 31.4 | 31.2 |
| 空腹時血糖 | 115.9 | −1.6 | 128.3 | −3.2 | 135.2 | 1.2 |
| $HbA_{1c}$ | 6.3 | −2.2 | 6.6 | 1.0 | 7.2 | 1.9 |
| アポリポ蛋白 B | 107.8 | −14.1 | 98.3 | −4.1 | 84.0 | −7.4 |
| アポリポ蛋白 A1 | 128.6 | −1.4 | 142.3 | 1.9 | 131.3 | 7.4 |
| アディポネクチン | 5.7 | 8.0 | 6.9 | 3.3 | 6.9 | −4.4 |
| 高感度 CRP（対数） | 6.7 | −10.9 | 7.4 | −11.3 | 7.9 | −8.8 |
| UA | 6.0 | 0 | 5.9 | 0.3 | 6.4 | −0.1 |

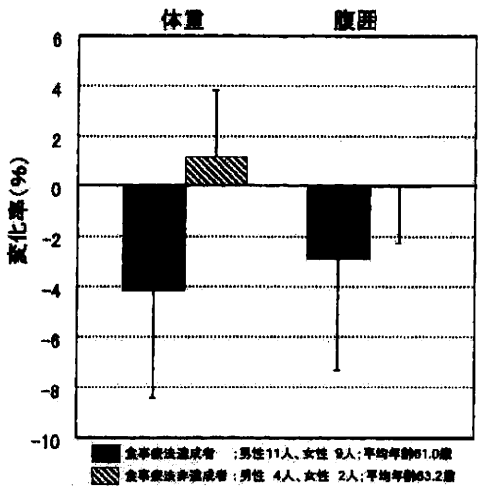


Fig.1　食事療法達成群、非達成群における体重と腹囲の変化率

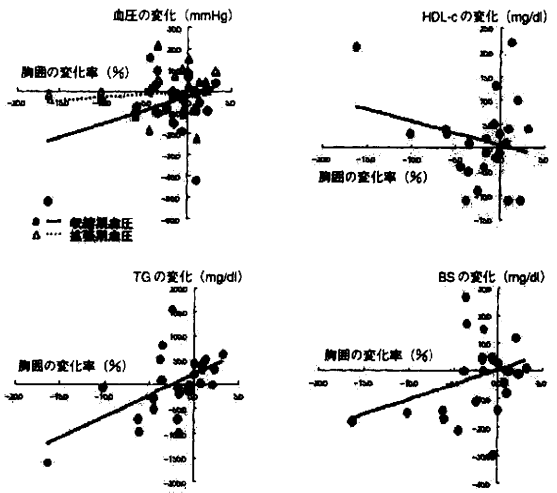


Fig.2　ウエスト周囲径の変化と各パラメータの変化との相関

た。LDL コレステロール、中性脂肪、収縮期・拡張期血圧、血糖、$HbA_{1c}$ も低下傾向を示したが、有意差は示さなかった。HDL コレステロール、アディポネクチンは増加傾向を示した。また、目標達成群と非達成群を比較するとウエスト周囲径、$HbA_{1c}$ が高い傾向が認められたが、統計学的に有意差はなかった。

食事療法の達成と体重変化率、腹囲変化率を Fig.1に示す。食事療法の達成群では非達成群に比して有意差をもって体重減少と腹囲の減少が得られた。また、腹囲の変化率と血圧、血清 HDL コレステロール値、中性脂肪値、空腹時血糖値

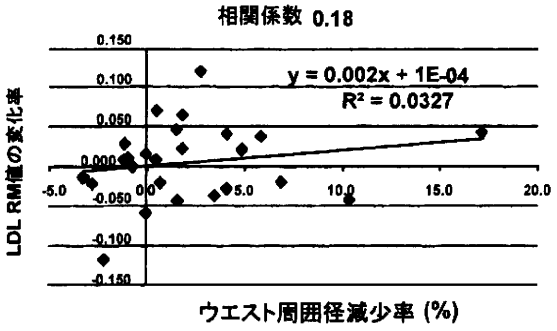


Fig.3　ウエスト周囲径と LDL RM 値の変化

それぞれの変化との相関を Fig.2に示す。腹囲の減少は高血圧、脂質異常症、耐糖能異常いずれの改善を伴うことが明らかとなった。すなわち、腹囲の減少は収縮期血圧、拡張期血圧の低下、血清 HDL コレステロール値の増加、中性脂肪値の減少、空腹時血糖値の低下を伴った。

リポ蛋白分画精密測定（ポリアクリルアミドゲル・ディスク電気泳動）により、LDL の peak の相対移動度（RM; VLDL から LDL peak までの移動度／ VLDL から HDL までの移動度）を計算し、0.4以上を small dense LDL とすると、2名の患者のみがこの基準を満たした。両名とも運動・食事目標を達成し体重とウエスト周囲径はそれぞれ 2 ～4.2%、0.5～5.9%減少し、6 ヶ月後には0.441→0.405、0.405→0.336と、ともに LDL の RM 値が改善した。一方、介入により、0.293→0.411と RM 値が悪化した患者が 1 名いたが、この患者は運動目標のみの達成で、体重は0.4%、ウエスト周囲径も2.2%増加していた。

リポ蛋白分画精密測定を行った25名について、介入によるウエスト周囲径の減少 (%) と、LDL の RM 値の変化についての相関分析を行うと、Fig.3に示すように、弱い正の相関（相関係数 0.18）が見られた。体重減少率と RM 値の間には相関はなかった（相関係数－0.068)。

## 考　察

今回の介入では n が十分ではなく、体重、ウエスト周囲径、アポ B、高感度 CRP 以外に有意差は出なかったが、n を増やせば有意差が出るものと期待される。大学病院外来において栄養士による栄養指導と医師による運動指導を行ったにもかかわらず、両者を達成したのは約25%にとどまり、肥満者における生活習慣の改善の困難さが確認された。

## 結　論

メタボリックシンドローム患者に対する標準指導法として毎日10,000歩のウォーキングと理想体重×25 カロリーの食事療法が有効であることが示された。しかしながら、その達成率から食事指導、運動指導により行動変容を計ることの困難さが明らかとなり、行動変容を計るアプローチにより工夫が必要であると考えられた。

# Effects of Walking and Dietary Management of Metabolic Syndrome

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Key words : metabolic syndrome, dietary management, walking, adiponectin

In this study we examined whether diet and exercise intervention can ameliorate each metabolic parameter as well as high sensitivity CRP and adiponectin in patients with metabolic syndrome in Kyoto University Hospital. We recruited 26 out-patients with the metabolic syndrome and asked them to comply with the lifestyle change: walking 10,000 steps a day and calorie restriction to 25 kcal × ideal weight, and followed them for 6 months. Among them 10 patients attained the goal of diet and exercise, and 10 patients only attained the goal of diet. Only patients who attained the goals of diet and exercise showed a significant reduction in body weight, waist circumference, apolipoprotein B, and high sensitivity CRP. Other parameters also ameliorated, but the difference was not statistically significant. No significant improvement was found in other patients. Thus walking 10,000 steps a day and calorie restriction are effective for the treatment of metabolic syndrome in Japanese.

# Ⅱ. アポ蛋白機能異常症
# 1. 家族性Ⅲ型高脂血症（アポリポ蛋白E遺伝子変異）

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附属病院内科 教授
**衛藤　雅昭**

同　薬学部疾患薬理学
**服部　由香，寺澤　理恵，
斉藤美恵子**

**[Summary]**

家族性Ⅲ型高脂血症はアポリポ蛋白（アポ）E2/2遺伝型（まれにアポE欠損）を基盤として発症する．レムナントリポ蛋白（レムナント）が血中に増加する典型的な疾患である．レムナントはLDLと同様に動脈硬化惹起性リポ蛋白である．アポE2/2遺伝型に加えて，糖尿病やメタボリックシンドロームを合併して発症することが多い．レムナントの増加のために，早期に冠動脈硬化症を発症する．血中トリグリセリド，総コレステロール両方高値（トリグリセリド高値が優位），総コレステロールが高値にかかわらずLDLコレステロールが低値，かつレムナント（RLP）コレステロールが異常高値であることを確認することが診断の要点である．Ⅲ型高脂血症は治療によく反応することから，早期診断と早期治療がきわめて重要である．薬物療法としては，レムナント低下作用が最も強力なフィブラート系薬剤が第一選択薬となる．

**Key Words:**

*家族性Ⅲ型高脂血症□レムナントリポ蛋白□*
*アポリポ蛋白E遺伝子変異□アポリポ蛋白E2/2遺伝型□*
*RLPコレステロール*

## はじめに

家族性Ⅲ型高脂血症はアポリポ蛋白（アポ）E2/2遺伝型（まれにアポE欠損）を基盤として発症し[1]，トリグリセリド（TG）richリポ蛋白のひとつであるレムナントリポ蛋白（レムナント）が血中に増加する典型的な疾患である．高レムナント血症・高TG血症を呈する．TG/レムナントはLDLと同様に動脈硬化惹起性リポ蛋白であり[2]，Ⅲ型高脂血症の存在はTG/レムナントの催動脈硬化性を証明したといっても過言ではない．レムナントコレステロールが増加しており，血中総コレステロールは高値であるがLDLコレステロールは低値であることも特徴である．レムナントの増加のために，早期に冠動脈硬化症を起こしやすい[3]．Ⅲ型高脂血症は治療によく反応することから，早期診断と早期治療がきわめて重要である．薬物療法としては，レムナント低下作用が最も強力なフィブラート系薬剤が第一選択薬となる．

## 家族性Ⅲ型高脂血症

### 1．病因・病態

Ⅲ型高脂血症の特徴は血中レムナントの増加である．レムナントはTGとコレステロールをほぼ半々含有するので，

血中ではTGとコレステロールが増加する．レムナントはカイロミクロンレムナントとVLDLレムナント(intermediate density lipoprotein; IDL)に由来する．

カイロミクロンとVLDLはリポ蛋白リパーゼによって水解され，相対的にコレステロールとアポEに富んだレムナント(それぞれカイロミクロンレムナントとVLDLレムナント)になる．レムナントの代謝はアポEをリガンドとする肝臓レムナントレセプター(アポEレセプター)によって行われる．LDLレセプター(アポB，Eレセプター)もアポEに親和性を示し，レムナント代謝に関与している．カイロミクロンレムナントはレムナントレセプターを介して肝臓に取り込まれるが，VLDLレムナントの一部はレムナントレセプターとLDLレセプターを介して肝臓に取り込まれ，残りは肝TGリパーゼ(hepatic triglyceride lipase; HTGL)によって代謝されLDLへと変換される．Ⅲ型高脂血症はこのレムナントの代謝が障害され，血中にレムナントが蓄積する高脂血症である[4]．

レムナントの表面に存在するアポEにはE2，E3，E4の3種類のイソ蛋白が存在するが，これらはアポE対立遺伝子すなわちε2，ε3，ε4によってそれぞれコードされており，六つのアポE遺伝型/表現型(E2/2，E3/2，E3/3，E4/2，E4/3，E4/4)が存在する．このうち，アポE3/3遺伝型が標準型であり，日本人の70%はアポE3/3遺伝型である．Ⅲ型高脂血症はアポE2のホモ接合体すなわちアポE2/2(ε2/ε2)遺伝型から発症する[1]．レムナントは通常そのアポEがレムナントレセプターあるいはLDLレセプターに認識されることにより血中から速やかに消失するが，アポE2のレセプターへの結合能がきわめて低いため(レセプター結合活性はアポE3を100とすると，アポE2は2以下である)，アポE2のホモ接合体では血中にレムナントが蓄積し，Ⅲ型高脂血症を発症する[4]．この結果，肝臓でのコレステロールプールが減少し，LDLレセプターのup-regulationが誘導され，血中LDLが減少する[5]．また，アポEを介したVLDLレムナントからLDLへの変換がアポE2のホモ接合体では低下しているともいわれている[5]．

表　日本人家族性Ⅲ型高脂血症患者における平均血中脂質値と背景

| | | (正常値) |
|---|---|---|
| トリグリセリド | 381 mg/dL | (<150) |
| 総コレステロール | 253 mg/dL | (<220) |
| LDLコレステロール(直接法，超遠心法) | 74 mg/dL | (<140) |
| IDLコレステロール(超遠心法) | 39 mg/dL | (<10) |
| アポE | 16.6 mg/dL | (<4.6) |
| RLPコレステロール(超遠心法) | 48.3 mg/dL | (<5.2) |
| RLPコレステロール/TG比 | 0.13 | (<0.1) |
| 糖尿病 | 43.8% | |
| 肥満 | 75.0% | |
| 冠動脈硬化 | 37.5% | |

n = 16

(文献6より引用)

アポE2/2遺伝型保有者がすべて臨床的にⅢ型高脂血症を発症するわけではない．発症には他の因子すなわち糖尿病，肥満(メタボリックシンドローム)，甲状腺機能低下，妊娠などが関与している[3,6]．前二者におけるインスリン不足あるいはインスリン抵抗性によるLPL活性の低下は，アポE遺伝子変異に加えてⅢ型高脂血症の発症に大きく関与しているものと考えられる．われわれの調査では，日本人Ⅲ型高脂血症における糖尿病の合併は43.8%，肥満の合併は75.0%と高頻度であった(表)[6]．

## 2．臨床症状

動脈硬化性疾患の合併頻度が高く，冠動脈硬化症だけでなく下肢動脈硬化症も起こしやすい．黄色腫，特に手掌線条黄色腫が認められることがある[3]．欧米では約半数に黄色腫が認められると報告されているが[3]，日本人のⅢ型高脂血症ではその頻度は低い[6,7]．

欧米では冠動脈硬化症の合併率は25%と報告されている[3]．われわれの調査研究では，日本人Ⅲ型高脂血症における冠動脈硬化症の合併率は37.5%と欧米並みに高頻度であり(表)[6]，冠動脈硬化症の頻度が低い国民であることを考慮するとⅢ型高脂血症，特にレムナントの高値が日本人の冠動脈硬化症の発症に強く影響しているといえる[6]．

発症年齢は通常成人以降である．欧米では16~95歳と報告されているが[3]，われわれの調査では9~81歳であった[6]．

## 3．検査・診断

診断は厚労省特定疾患「原発性高脂血症」調査研究班の基準により行われる．すなわち，①血漿TG，総コレステロール（TC）が共に高値を示す，②アポE2/2遺伝型（きわめてまれであるがアポEの欠損）を証明する，③血漿リポ蛋白のポリアクリルアミドゲル電気泳動ではレムナント（IDL）の増加を示す．

血中アポEは増加し10mg/dL以上であればⅢ型高脂血症を疑う（正常値＜4.6mg/dL）．アポE/血中コレステロール比は0.05以上となる．またLDLコレステロールは減少し，アポBも減少する．Ⅲ型高脂血症ではLDLコレステロールをFriedewaldの式により求めることができないことに留意する．脂質を専門とする研究室などでは，血漿を超遠心法により分離しLDLコレステロールを測定することができるが，一般的には直接法によりLDLコレステロールを測定する．

Ⅲ型高脂血症の診断において最も重要なことは，アポE2/2遺伝型と血中レムナントの増加を証明することである．アポE2/2遺伝型の測定は保険適応されていないが，われわれのような大学の研究室で行われている．

血中レムナントは，現在保険適応されているRLP（remnant lipoprotein like particles；レムナント様粒子）コレステロールとして測定するのが最適である．空腹時で中嶋らの免疫吸着・酵素法により測定し，30mg/dL以上（正常値5.2mg/dL未満）であれば，Ⅲ型高脂血症の可能性が高い．さらに，RLPコレステロール/TG比0.1以上がⅢ型高脂血症の診断に有用である[8]．最近，RLPコレステロールを直接法で測定することも可能になった．

日本人Ⅲ型高脂血症16名における分析結果を表に示した[6]．血中TG値381mg/dL，総コレステロール値253mg/dLと両方高値であった．TG値＞総コレステロール値がⅢ型高脂血症の特徴である．LDLコレステロール値は74mg/dLと低値であった．アポE値は16.6mg/dLと高値であった．レムナントは，超遠心法によるIDLコレステロール値は39mg/dL，RLPコレステロール値は48.3mg/dLと異常高値であった．RLPコレステロール/TG比は0.13と0.1以上の症例が多く，Ⅲ型高脂血症の診断に有用と考えられた．

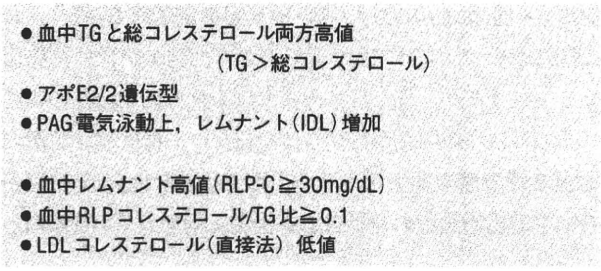


**図　家族性Ⅲ型高脂血症診断の要点**

TG：トリグリセリド，PAG：ポリアクリルアミドゲル

図にⅢ型高脂血症診断の要点を示した．血中TG，総コレステロール両方高値（TG高値が優位），総コレステロールが高値にかかわらずLDLコレステロールが低値で，かつレムナント（RLP）コレステロールが異常高値であるということである．必ずしもアポE2/2遺伝型を証明できなくても，上記により疑うことができるが，確定診断はIEF法[9]，PCR-RFLP法あるいはIEF-immunoblotting法によりアポE2/2遺伝型（表現型）を証明することにより行う．Ⅲ型高脂血症は治療によく反応することから，早期診断と早期治療がきわめて重要である．

## 4．頻　度

われわれの一般人口（n=576）における調査では，日本人におけるアポE遺伝子頻度はε2：3.7%，ε3：84.6%，ε4：11.7%であり，ε2およびε4遺伝子の頻度が欧米人よりも有意に少ないのが特徴であった[10]．これよりアポE2/2の頻度は0.14%（10,000人中14人）と計算される．このうち約10%が発症すると仮定すると，家族性Ⅲ型高脂血症の頻度は10,000人中1~2人と推定される．欧米ではε2遺伝子の頻度が高いためアポE2/2の頻度は約1%と高く，Ⅲ型高

脂血症の頻度は10,000人中2~10人と推定されている[3]．わが国において，アポE2/2遺伝型のうち何％がⅢ型高脂血症を発症するか，また日本人におけるⅢ型高脂血症の頻度については今後明らかにせねばならない．糖尿病や肥満の増加，欧米型の食生活パターンによりアポE2/2遺伝型からのⅢ型高脂血症の発症はわが国でも増加しているものと予想される．

## 5．治　療

Ⅲ型高脂血症は治療によく反応するといわれている．すなわち食事療法を含めた生活習慣改善が有効である．肥満があれば体重是正，糖尿病があれば血糖コントロールする．しかし難治性のものも存在することは確かである．

薬物療法としては血中TG/レムナント低下作用が最強であるフィブラート系薬剤が第一選択であることはいうまでもない[3]．Ⅲ型高脂血症の本態はレムナントの増加であり，そのレムナントを低下させるのはフィブラート系薬剤である．したがって，LDL低下作用が主たるスタチン系薬剤はⅢ型高脂血症の第一選択薬にはならない．

日本人Ⅲ型高脂血症14名における治療結果を報告した[6]．4名は食事療法を中心とした生活習慣改善のみ，8名はベザフィブラート(5名)あるいはフェノフィブラート(3名)，1名は甲状腺ホルモン＋フィブラート，1名は分娩後改善例である．平均血中TG値は412mg/dLから187mg/dLへ，総コレステロール値は266mg/dLから175mg/dLへ低下した．Ⅲ型高脂血症における血中脂質の目標値に関して定説はないが，動脈硬化を防ぐためには血中レムナント(RLP)コレステロールを5.2mg/dL未満にする必要があると考えられる[11]．

## おわりに

家族性Ⅲ型高脂血症はアポE遺伝子変異すなわちアポE2/2遺伝型を基盤として発症し，その本態は血中レムナントの増加である．家族性高コレステロール血症がLDLの動脈硬化惹起性を証明したように，家族性Ⅲ型高脂血症はレムナントの動脈硬化惹起性を証明したといえる．Ⅲ型高脂血症に限らず，一般的な高TG症の基盤をなすのもレムナントであり，日常診療におけるTG/レムナントの管理がいかに重要であるかを，本疾患は教えている．

家族性Ⅲ型高脂血症は診断が難しく(アポE2/2遺伝型の証明が必要)，また比較的まれな疾患であるため，わが国におけるその実態解明は遅れている．現在，厚労省難治性疾患克服研究事業「原発性高脂血症に関する調査研究」(筑波大学 山田信博班長)が進行中であり，筆者らもその一員として家族性Ⅲ型高脂血症の調査研究を行っており，わが国における本疾患の実態を今後明らかにしたい．

最近，われわれはⅢ型高脂血症合併糖尿病患者が腎症を早期にかつ高頻度に発症することを発見した[12]．すなわち，レムナントコレステロールは腎症の危険因子でもあった[13]．Ⅲ型高脂血症において冠動脈硬化症予防だけではなく，腎症予防をも念頭にいれたTG/レムナント低下療法を積極的に行うことが重要であろうと考えられる．その最も有望な薬剤がフィブラート系薬剤であろう[14]．

**謝　辞**

厚労省難治性疾患克服研究事業「原発性高脂血症に関する調査研究」(平成21年度)の支援を受けた．

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Atherosclerosis 205 (2009) 413–419

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

# On the mechanism for PPAR agonists to enhance ABCA1 gene expression

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ARTICLE INFO

*Article history:*
Received 20 October 2008
Received in revised form 7 January 2009
Accepted 8 January 2009
Available online 19 January 2009

*Keywords:*
ABCA1
PPAR
LXR
ABCA1
HDL
Cholesterol

ABSTRACT

Expression of ATP binding cassette transporter A1 (ABCA1), a major regulator of high density lipoprotein (HDL) biogenesis, is known to be up-regulated by the transcription factor liver X receptor (LXR) α, and expression is further enhanced by activation of the peroxisome proliferator activated receptors (PPARs). We investigated this complex regulatory network using specific PPAR agonists: four fibrates (fenofibrate, bezafibrate, gemfibrozil and LY518674), a PPAR δ agonist (GW501516) and a PPAR γ agonist (pioglitazone). All of these compounds increased the expression of LXRs, PPARs and ABCA1 mRNAs, and associated apoA-I-mediated lipid release in THP-1 macrophage, WI38 fibroblast and mouse fibroblast. When mouse fibroblasts lacking expression of PPAR α were examined, the effects of fenofibrate and LY518674 were markedly diminished while induction by other ligands were retained. The PPAR α promoter was activated by all of these compounds in an LXR α-dependent manner, and partially in a PPAR α-dependent manner, in mouse fibroblast. The LXR responsive element (LXRE)-luciferase activity was enhanced by all the compounds in an LXR α-dependent manner in mouse fibroblast. This activation was exclusively PPAR α-dependent by fenofibrate and LY518674, but nonexclusively by the others. We conclude that PPARs and LXRs are involved in the regulation of ABCA1 expression and HDL biogenesis in a cooperative signal transduction pathway.



## 1. Introduction

High density lipoprotein (HDL) plays a central role in transporting cholesterol from extrahepatic tissues to the liver for its catabolism to bile acids, and thus it is thought to contribute to removing cholesterol from peripheral tissues and possibly lowering its deposits in atherosclerotic lesions. This idea is based on epidemiological evidence that plasma HDL concentrations are inversely related to cardiovascular risk, and on experimental results showing that HDL may remove cholesterol from vascular cells in culture. HDL removes cholesterol from cells via two independent mechanisms [1]. One is by non-specific exchange of cholesterol, in which the driving forces for its net release may be cholesterol esterificaiton on HDL and the presence of ATP binding cassette transporter (ABC) G1 in the cell membrane. The other mechanism is through HDL biogenesis by helical apolipoproteins and cellular lipids in the presence of ABCA1 [1]. The latter is an almost exclusive source of HDL biogenesis and one of the important rate limiting factors for plasma HDL concentration [2]. Many drug reagents are known to influence this reaction by modulating ABCA1 activity. ABCA1 is therefore an important target for development of drugs that impact atherogenesis.

Peroxisome proliferator activated receptor (PPAR) agonists are known to increase expression of ABCA1 and enhance biogenesis of HDL in vitro and in vivo. Fibrates act mainly as PPARα agonists [3] to increase ABCA1 gene transcription [4,5]. Fenofibrate and LY518674 exclusively activate PPAR α while bezafibrate and gemfibrozil activate PPARδ and PPARγ as well [6–8]. More specific activation of PPARδ and PPARγ also results in increased ABCA1 transcription [4,9]. All of these events seem to involve the liver X receptors (LXRs), especially LXRα [4,5,8], one of the main regulators of ABCA1 gene transcription by sensing oxysterol [10], although specific pathways for these cascades are yet to be clarified.

Fibrates are drugs widely used to decrease plasma triglyceride (TG). This effect is expected to reduce atherosclerotic disease through a decrease in TG-rich atherogenic lipoproteins as well as by reducing other risk factors secondarily caused by the increase of TG-rich lipoprotein, such as low HDL and "small and dense" LDL.

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doi:10.1016/j.atherosclerosis.2009.01.008

Fibrates also increase HDL independently of TG reduction by direct up-regulation of the genes related to HDL biogenesis as indicated above [4,5]. Eventually, fibrates were shown to decrease secondary or primary coronary heart disease events in several large-scale prevention trials [11]. Statistical analysis of these data suggested independent contribution of HDL increase to the risk reduction [12].

Chronic inflammation is also thought to be involved in atherogenesis, such that accumulation of cholesterol-rich lipoproteins results in the recruitment of circulating monocytes, their adhesion to the endothelium and differentiation into macrophages. Lipid-loaded macrophages may produce chemokines, cytokines, and reactive oxygen species as early atherogenic process. Activation of PPARs was shown to suppress such processes.

PPARs act as ligand-activated transcription factors mainly to regulate target genes related to energy metabolism. The PPAR family consists of PPARα, δ and γ, which display distinct expression patterns, different ligand specificities and different biological functions with some degree of overlap between PPARα and PPARδ. PPARα is mainly expressed in liver, kidney, heart, and muscle, tissues with a high rate of fatty acid catabolism. PPARα up-regulates the expression of genes involved in fatty acids oxidation, lipolysis, HDL metabolism, down-regulates very low density lipoprotein synthesis and cholesterol esterification [13], and inhibits inflammatory mediators [14]. PPARγ is mainly expressed in adipose tissue, skeletal and cardiac muscle, and also in human monocytes [15]. It plays a role in adipocyte differentiation and fat storage; up-regulation of PPARγ increases insulin sensitivity [16]. PPARγ also up-regulates the expression of genes involved in HDL metabolism, down-regulates cholesterol esterification, and inhibits inflammatory mediators [4–17]. PPAR pathways are thus integrated in atherogenesis and the use of the fibrates (PPARα agonists) and thiazolidinediones (PPARγ agonists) may extend beyond the treatment of hyperlipidemia or insulin resistance [18]. PPARδ is ubiquitously expressed and its role in atherogenesis is controversial. Disruption of the PPARδ gene suggested its important roles in skin biology, lipid metabolism, and energy homeostasis [19]. PPARδ agonist (GW501516), however, reportedly enhanced ABCA1 expression and increased apoAI-mediated lipid release to maintain macrophage cholesterol homeostasis [20] and suppressed inflammation to reduce atherosclerosis [21]. On the other hand, a different PPARδ agonist promoted lipid accumulation in macrophages [22].

As various PPAR agonists are clinically used, it is important to provide information about their detailed reaction mechanism with respect to signaling and cross-talk among the transcriptional factors relating the energy metabolism induced by these drugs. We therefore investigated profiles of the network of transcriptional factors by which PPAR agonists modulate ABCA1 gene expression. The results suggest that PPARs and LXRs are involved in the regulation of ABCA1 expression and HDL biogenesis in a complicated signal transduction network.

## 2. Methods

### 2.1. Cell culture

THP-1 (human monocytic leukemia) cells ($4.0 \times 10^6$ cells per well) in six well plates were differentiated with $3.2 \times 10^{-7}$ M phorbol 12-myristate 13-acetate (PMA) (Wako) in 10% FBS (PAA Laboratories)-RPMI 1640 medium (IWAKI Glass) for 72 h at 37 °C in a humidified atmosphere of 5% $CO_2$ (THP-1 macrophages). WI38 human fibroblasts (RIKEN Cell Bank) were incubated in Eagle's minimum essential medium (MEM) with 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% $CO_2$. Fibroblasts were prepared from C57BL6 mice and PPAR-null/C57BL6 (PPAR(−/−)) mice [23] and bred in the Nagoya City University Animal Experiment Facility. Briefly, 13–14th day fetuses were harvested and suspended in Hank's EGTA solution containing 100 units/ml of penicillin and 0.1 mg/ml of streptomycin (PCSM). Associated membranes and placentas were dissected and rinsed thoroughly. The fetal trunks were transferred to fresh solution and finely minced. The suspension was centrifuged at 1000 rpm for 3 min and the cells were re-suspended into MEM medium containing 10% FCS and PCSM. The cells were cultured in 37 °C with 5% $CO_2$ incubator over five passages and used for the experiments. The experimental protocol was pre-approved by the institutional Animal Welfare Committee. Cells were seeded into a 100-mm dish at a density of $1.5 \times 10^6$ cells/ml. When the WI38 cells and mouse primary fibroblasts were grown to 80% of a confluent stage, cells were washed with phosphate-buffered saline (PBS) twice and cultured an additional 20 h in the presence of apoA-I (10 μg/ml). PPARα activators, fenofibric acid (Tyger Scientific, referred as fenofibrate hereafter), bezafibrate (Sigma), gemfibrozil (Sigma), and LY518674 [24] (synthesized in house), PPARδ activator GW501516 [20] (kindly provided by Aska Pharmaceutical Co. Ltd.), PPARγ activator pioglitazone (kindly provided Takeda Pharmaceutical Co. Ltd.), and an LXR agonist TO901317 (Sigma) were dissolved in dimethyl sulfoxide and added to the culture medium containing 0.02% bovine serum albumin (BSA) (Sigma). The experimental procedure had been approved by the animal welfare committee of the institution.

### 2.2. Cellular lipid release

WI38 cells and mouse primary fibroblasts were grown to 80% confluence, and twice washed with PBS. The cells were cultured for additional 20 h in the presence of apoA-I (10 μg/ml) and PPAR activators described above. THP-1 macrophages were also treated with PPAR activators similarly for apoA-I-mediated lipid release in 0.02% BSA-RPMI 1640 medium (serum-free). Cholesterol and choline-phospholipid released into the medium by apoA-I were determined enzymatically and the apoA-I-dependent release was evaluated by subtracting the background with BSA, as described in detail previously [5].

### 2.3. SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting

Cells incubated with and without apoA-I and PPAR activators for 20 h were harvested in cold PBS and collected by centrifugation. Membrane fractions were prepared for detection of ABCA1. The cell pellet was suspended in 5 mmol/l Tris–HCl, pH 7.5, containing 0.3% protease inhibitor cocktails (Sigma) and 1 mM phenylmethane sulfonyl fluoride and 1 mM benzamidine for 30 min in ice with vortexing at every 10 min. The cell debris and nuclei were removed by centrifugation at $800 \times g$ for 5 min at 4 °C, and the supernatant was centrifuged at $99{,}000 \times g$ for 60 min to prepare the membrane fraction as a pellet. The pellet was resuspended in 50 mM Tris–HCl, pH 7.5, containing 5 mM EDTA, 10 mM EGTA, 1 mM phenylmethane sulfonyl fluoride, 10 mM benzamidine, 1% Triton X-100, and 1% protease inhibitor cocktails. Membrane fraction protein (20–60 μg) was dissolved in 9 M urea, 2% triton X-100, 1% dithiothreitol and analyzed by 6% polyacrylamide electrophoresis for immunoblotting by using specific antibodies against ABCA1 and BIP/GRP78.

### 2.4. RNA extraction and real time quantitative polymerase chain reaction (PCR)

Cellular RNA was extracted by using RNA extraction reagent (Isogen, Nippon Gene). Single strand cDNA was synthesized by High capacity cDNA archive kit (applied biosystem) from 5 μg of the total RNA. PCR was carried out for the cDNA by using primers (sense and antisense) of human ABCA1 (5′-GAA CTG GCT GTG TTC CAT GAT-3′ and 5′-GAT GAG CCA GAC TTC TGT TGC-3′), human LXRα (5′-TCT