large waist circumference. *Lancet* 351: 853–856, 1998
7. Nakamura T, Tokunaga K, Shimomura I, Nishida M, Yoshida S, Kotani K, Islam AHMW, Keno Y, Kobatake T, Nagai Y, Fujioka S, Tarui S, Matsuzawa Y: Contribution of visceral fat accumulation to the development of coronary artery disease in non-obese men. *Atherosclerosis* 107:239–246, 1994
8. Lemieux I, Pascot A, Couillard C, Lamarche B, Tchernof A, Almeras N, Bergeron J, Gaudet D, Tremblay G, Prud'homme D, Nadeau A, Despres JP: Hypertriglyceridemic waist: a marker of the atherogenic metabolic triad (hyperinsulinemia; hyperapolipoprotein B; small, dense LDL) in men. *Circulation* 102:179–184, 2000
9. Ryo M, Maeda K, Onda T, Katashima M, Okumiya A, Nishida M, Yamaguchi T, Funahashi T, Matsuzawa Y, Nakamura T, Shimomura I: A new simple method for the measurement of visceral fat accumulation by bioelectrical impedance. *Diabetes Care* 28:451–453, 2005
10. Examination Committee of Criteria for 'Obesity Disease' in Japan; Japan Society for the Study of Obesity: New criteria for 'obesity disease' in Japan. *Circ J* 66:987–992, 2002
11. Eberly LE, Stamler J, Neaton JD, Multiple Risk Factor Intervention Trial Research Group: Relation of triglyceride levels, fasting and nonfasting, to fatal and nonfatal coronary heart disease. *Arch Intern Med* 163:1077–1083, 2003
12. Ahmad J, Hameed B, Das G, Siddiqui M, Ahmad I: Postprandial hypertriglyceridemia and carotid intima-media thickness in north Indian type 2 diabetic subjects. *Diabetes Res Clin Pract* 69:142–150, 2005
13. American Diabetes Association: Clinical practice recommendations 2000: screening for type 2 diabetes. *Diabetes Care* 23 (Suppl. 1):S20–S23, 2000
14. Despres JP, Lamarche B: Effects of diet and physical activity on adiposity and body fat distribution: implications for the prevention of cardiovascular disease. *Nutr Res Rev* 6:137–159, 1993
15. Janssen I, Katzmarzyk PT, Ross R: Waist circumference and not body mass index explains obesity-related health risk. *Am J Clin Nutr* 79:379–384, 2004
16. Fujioka S, Matsuzawa Y, Tokunaga K, Kawamoto T, Kobatake T, Keno Y, Kotani K, Yoshida S, Tarui S: Improvement of glucose and lipid metabolism associated with selective reduction of intra-abdominal visceral fat in premenopausal women with visceral fat obesity. *Int J Obes* 15: 853–859, 1991
17. Ross R, Dagnone D, Jones PJ, Smith H, Paddags A, Hudson R, Janssen I: Reduction in obesity and related comorbid conditions after diet-induced weight loss or exercise-induced weight loss in men: a randomized, controlled trial. *Ann Intern Med* 133:92–103, 2000
18. Miyatake N, Nishikawa H, Morishita A, Kunitomi M, Wada J, Suzuki H, Takahashi K, Makino H, Kira S, Fujii M: Daily walking reduces visceral adipose tissue areas and improves insulin resistance in Japanese obese subjects. *Diabetes Res Clin Pract* 58:101–107, 2002
19. Slentz CA, Aiken LB, Houmard JA, Bales CW, Johnson JL, Tanner CJ, Duscha BD, Kraus WE: Inactivity, exercise, and visceral fat: STRRIDE: a randomized, controlled study of exercise intensity and amount. *J Appl Physiol* 99:1613–1618, 2005

ELSEVIER

Atherosclerosis 191 (2007) 281–289

ATHEROSCLEROSIS

www.elsevier.com/locate/atherosclerosis

Potent free radical scavenger, edaravone, suppresses oxidative stress-induced endothelial damage and early atherosclerosis

Hang Xi [a], Masahiro Akishita [b,*], Kumiko Nagai [a], Wei Yu [a], Hiroshi Hasegawa [a], Masato Eto [b], Koichi Kozaki [a], Kenji Toba [a]

[a] *Department of Geriatric Medicine, Kyorin University School of Medicine, Tokyo, Japan*
[b] *Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*

Received 28 November 2005; received in revised form 9 May 2006; accepted 19 May 2006
Available online 27 June 2006

Abstract

Objective: Effects of potent free radical scavenger, edaravone, on oxidative stress-induced endothelial damage and early atherosclerosis were investigated using animal models and cultured cells.
Methods and results: Endothelial apoptosis was induced by 5-min intra-arterial exposure of a rat carotid artery with 0.01 mmol/L H_2O_2. Edaravone treatment (10 mg/kg i.p.) for 3 days suppressed endothelial apoptosis, as evaluated by chromatin staining of *en face* specimens at 24 h, by approximately 40%. Similarly, edaravone dose-dependently inhibited H_2O_2-induce apoptosis of cultured endothelial cells in parallel with the inhibition of 8-isoprostane formation, 4-hydroxy-2-nonenal (4-HNE) accumulation and VCAM-1 expression. Next, apolipoprotein-E knockout mice were fed a high-cholesterol diet for 4 weeks with edaravone (10 mg/kg i.p.) or vehicle treatment. Edaravone treatment decreased atherosclerotic lesions in the aortic sinus (0.18 ± 0.01 to 0.09 ± 0.01 mm^2, $P < 0.001$) and descending aorta (5.09 ± 0.86 to 1.75 ± 0.41 mm^2, $P < 0.05$), as evaluated by oil red O staining without influence on plasma lipid concentrations or blood pressure. Dihydroethidium labeling and cytochrome *c* reduction assay showed that superoxide anions in the aorta were suppressed by edaravone. Also, plasma 8-isoprostane concentrations and aortic nitrotyrosine, 4-HNE and VCAM-1 contents were decreased by edaravone treatment.
Conclusions: These results suggest that edaravone may be a useful therapeutic tool for early atherosclerosis, pending the clinical efficacy.

Keywords: Atherosclerosis; Reactive oxygen species; Free radical scavenger; Edaravone; 4-HNE; Apolipoprotein E knockout mouse

1. Introduction

Accumulating evidence has shown that stress-induced injury of vascular endothelial cells (ECs) is an initial event in the development of atherosclerosis [1]. In particular, oxidative stress has been implicated in endothelial injury caused by oxidized LDL and smoking as well as hypertension, diabetes and ischemia-reperfusion [1–3]. This notion is supported by the findings that the production of reactive oxygen species (ROS) is upregulated in vascular lesions [4,5], and that lesion formations such as endothelial dysfunction [6] and atherosclerosis [7] are accelerated by superoxide anion ($O_2^{\bullet -}$).

Experimental studies have shown the protective effects of antioxidants on atherosclerosis and endothelial injury. Dietary antioxidants were reported to preserve endothelial function [8,9] and inhibit atherosclerosis [10] in cholesterol-fed rabbits. In a well employed animal model of atherosclerosis, apolipoprotein E knockout (ApoE-KO) mouse fed a high fat diet, it has been shown that there was a significant increase in basal superoxide products [11,12], and that both $O_2^{\bullet -}$ levels and aortic lesion areas were attenuated by treatment with Vitamin E [11] or superoxide dismutase [13]. By contrast, it has been reported that elimination of NAD(P)H oxidase [14] or disruption of its subunit p47phox [15] had no effect on lesion size in ApoE-KO mice. Clinical experiments have

* Corresponding author. Tel.: +81 3 5800 8832; fax: +81 3 5800 8831.
E-mail address: akishita-tky@umin.ac.jp (M. Akishita).

0021-9150/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2006.05.040

also shown that antioxidants such as Vitamins C and E can ameliorate endothelial dysfunction in patients with hypercholesterolemia or atherosclerosis [16,17], although recent clinical trials have failed to prove the protective effects of Vitamin E on cardiovascular events in patients with risk factors [18] and in healthy subjects [19].

Edaravone is a potent free radical scavenger that has been clinically used to reduce the neuronal damage following ischemic stroke [20]. Edaravone has promising property to quench hydroxyl radical ($^{\bullet}OH$) and show inhibitory effects on peroxynitrite ($ONOO^-$) and both water-soluble and lipid-soluble peroxyl radical ($LOO^{\bullet}$) [21,22]. Accordingly, this compound exerts a wide range of antioxidant activity on ROS beyond the effects of water-soluble or lipid-soluble antioxidant vitamins. Based on this idea, we hypothesized that edaravone would inhibit the process of atherosclerosis.

To test this hypothesis, we investigated the effects of edaravone in two experimental models. First, we examined whether edaravone could inhibit hydrogen peroxide (H_2O_2)-induced EC apoptosis in a rat model [23] and cultured ECs. Second, we examined whether edaravone could suppress the atherosclerotic lesion formation in ApoE-KO mice.

2. Methods

2.1. Animals

Male Wistar rats aged 10–12 weeks (Japan Clea), and male C57BL/6 mice and ApoE-KO mice on C57BL/6 background aged 4–6 weeks (Jackson Laboratory) were used in this study. All of the experimental protocols were approved by the Animal Research Committee of the Kyorin University School of Medicine.

2.2. H_2O_2-induced EC apoptosis in rats and in culture

EC apoptosis was induced by 5-min intra-arterial treatment of a rat carotid artery with 0.01 mmol/L H_2O_2 as previously described [23]. Briefly, edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one; 3 or 10 mg/kg; donated by Mitsubishi Pharma Corporation, Japan) or its vehicle was intraperitoneally injected daily for 3 days before H_2O_2 treatment. A catheter was placed in the common carotid artery via the external carotid artery. The lumen was flushed with saline, replaced with 0.01 mmol/L H_2O_2 diluted with saline for 5 min and recovered. At 24 h after H_2O_2 treatment, EC apoptosis was evaluated by chromatin staining of *en face* specimens of the carotid artery using Hoechst 33342 dye. Apoptotic cells were identified by their typical morphological appearance; chromatin condensation, nuclear fragmentation, or apoptotic bodies. The numbers of apoptotic cells and intact cells were counted in 10 high-power fields for each specimen by an observer blinded to the treatment group.

Apoptosis of ECs isolated from a bovine carotid artery was induced as previously described [24]. Briefly, subconfluent ECs were pretreated for 24 h with culture medium containing edaravone or vehicle. After washing twice with Hank's balanced salt solution, the cells were exposed to H_2O_2 (0.2 mmol/L) diluted in Hank's balanced salt solution for 1.5 h at 37 °C to induce apoptosis. Then ECs were cultured in culture medium containing edaravone or vehicle until assay. Apoptosis was evaluated at 24 h after H_2O_2 treatment as histone-associated DNA fragments using a photometric enzyme immunoassay (Cell Death Detection ELISA, Roche), according to the manufacturer's instructions.

2.3. Atherosclerosis in ApoE-KO mice

ApoE-KO mice received a high-cholesterol diet (1% cholesterol, 10% fat in CE-2 standard diet; Japan Clea) for 4 weeks. Simultaneously, edaravone (10 mg/kg) or its vehicle was intra-peritoneally injected daily throughout the experiments. Body weight and systolic blood pressure were recorded every week in a conscious state by the tail cuff method (BP-98A; Softron, Tokyo).

At 4 weeks of treatment, mice were sacrificed with an overdose of diethyl ether and perfusion-fixed. Atherosclerotic lesions in the aortic sinus were quantified according to the method described previously [25]. We also measured the surface area of atherosclerotic lesions in the whole descending aorta including the abdominal aorta just proximal to the iliac bifurcation. *En face* specimens of the descending aorta were stained with oil red O, photographed and analyzed using the NIH image software. Total cholesterol, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol in mice plasma were determined by a commercial laboratory (SRL, Japan).

2.4. Measurement of ROS

Aortic samples for ROS measurements were prepared separately from those for atherosclerosis evaluation. At 4 weeks of treatment, ApoE-KO mice were sacrificed with CO_2 inhalation. Descending aortas were rapidly removed and placed into chilled modified Krebs/HEPES buffer. C57BL/6 mice fed a standard diet were also used as the control. To determine superoxide production *in situ*, frozen cross-sections of the aorta were stained with 10 μmol/L dihydroethidium (DHE; Molecular Probes), followed by fluorescent microscopy [26]. Also, superoxide production in aortic rings was quantified using the superoxide dismutase-inhibitable cytochrome *c* reduction assay as previously described [27]. Immunohistochemical detection of 3-nitrotyrosine in the aorta was visualized by diaminobenzidine as reported previously [28].

Intracellular production of superoxide anions was measured using DHE as described previously [29], and the intensity values were calculated using the Metamorph software [24]. Concentrations of 8-isoprostane (8-iso prostaglandin

$F_{2\alpha}$) in the culture supernatants and mouse plasma were measured using a commercially available EIA kit (Cayman Chemical). Culture supernatants were directly applied to EIA, while plasma was applied to EIA after solid phase extraction purification according to the manufacturer's instructions.

2.5. Western blotting

Western blotting was performed as previously described [30], to detect the expression of VCAM-1 and 4-HNE in cultured ECs and mouse aortas. Descending aortas were prepared as described in ROS measurements. The antibodies used in this study were anti-4-HNE monoclonal antibody (JaICA, Shizuoka, Japan), anti-VCAM-1 polyclonal antibody (Santa Cruz Biotechnology) and anti-3-nitrotyrosine monoclonal antibody (Upstate). Densitometric analysis was performed using an image scanner and the NIH software.

2.6. Data analysis

All values are express as mean ± S.E.M. Data were analyzed using one-factor ANOVA. If a statistically significant effect was found, Newman–Keuls' test was performed to isolate the difference between the groups. Differences with a value of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of edaravone on H_2O_2-induced EC apoptosis and ROS

As shown in Fig. 1A, edaravone dose-dependently inhibited EC apoptosis in culture, which was induced 24 h after H_2O_2 treatment. Edaravone was then employed in a rat model of H_2O_2-induced EC apoptosis. Consistent with the *in vitro* experiment, edaravone of 10 mg/kg/day decreased EC apoptosis of the rat carotid artery by approximately 40% (Fig. 1B).

We next examined whether edaravone decreased ROS production in the process of H_2O_2-induced EC apoptosis. For this purpose, DHE fluorescent, a marker of intracellular production of superoxide anions, release of 8-isoprostane into the culture supernatants and accumulation of 4-HNE, a pivotal end-product of lipid peroxidation [31], were measured using cultured ECs. We also examined the expression of VCAM-1 as a marker of endothelial injury or activation [32]. Edaravone decreased DHE fluorescent, 8-isoprostane formation and VCAM-1 expression at 3 h after H_2O_2 treatment in a dose-dependent manner (Fig. 2A–C). As shown in Fig. 2D, multiple bands showing 4-HNE-Michael protein adducts [33,34] were accumulated after H_2O_2 treatment in a time-dependent manner. Consequently, the effect of edaravone on 4-HNE expression was examined at 3 h after H_2O_2 treatment (4.5 h after H_2O_2 was initially added). Edaravone decreased 4-HNE expression in a dose dependent manner.

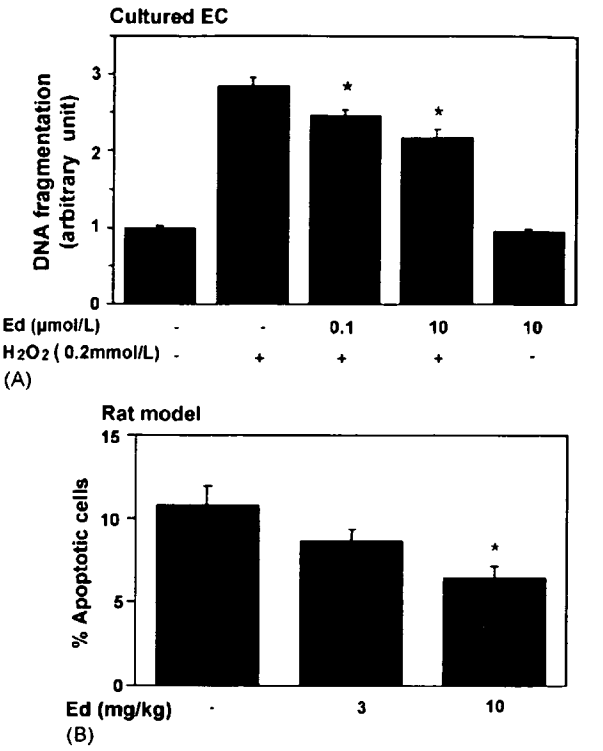

Fig. 1. Effects of edaravone (Ed) on H_2O_2-induced EC apoptosis in culture (A) and in a rat model (B). (A) Ed or its vehicle was added to the culture medium 24 h before H_2O_2 treatment until assay. EC apoptosis was evaluated 24 h after H_2O_2 treatment (0.2 mmol/L) by means of DNA fragmentation. Values are expressed as mean ± S.E.M. ($n = 3$). $^*P < 0.05$ vs. H_2O_2 (+) + Ed (−). (B) Ed or its vehicle was intraperitoneally injected once a day for 3 days before H_2O_2 treatment. At 24 h after H_2O_2 treatment, apoptotic ECs were counted per high power field and the ratio of the apoptotic cell number to the intact cells was calculated using *en face* specimens of the carotid artery stained with Hoechst 33342. Values are expressed as mean ± S.E.M. ($n = 7$). $^*P < 0.05$ vs. vehicle.

3.2. Effects of edaravone on atherosclerotic lesions and ROS in ApoE-KO mice

In the next set of experiments, we examined whether edaravone could suppress the atherosclerotic lesions in ApoE-KO mice fed a high cholesterol diet for 4 weeks. As shown in Fig. 3A and B, atheromatous lesions both in the aortic sinus and the descending aorta were smaller in mice treated with 10 mg/kg/day edaravone than in those with vehicle. This dose of edaravone did not influence body weight, blood pressure or plasma LDL and HDL cholesterol levels (Table 1).

Then, we examined whether the anti-atherogenic effects of edaravone were associated with the decrease in ROS production. Peroxynitrite formation was assessed as 3-nitrotyrosine accumulation in the aorta [28]. Both immunohistochemistry and Western blotting showed that edaravone inhibited nitrotyrosine accumulation in the aorta of ApoE-KO mice (Fig. 4A(a) and A(b)). Superoxide production *in situ* was examined using DHE staining of the descend-

Table 1
Body weight, blood pressure and plasma lipid levels in ApoE-KO mice treated with edaravone or vehicle

	Vehicle	Edaravone
Body weight (g)	21.4 ± 0.5	21.0 ± 0.5
Systolic blood pressure (mmHg)	106 ± 2	103 ± 3
Total cholesterol (mg/dL)	1967 ± 38	1872 ± 66
HDL cholesterol (mg/dL)	66 ± 6	82 ± 9
LDL cholesterol (mg/dL)	602 ± 24	602 ± 12

The values are shown as mean ± S.E. ($n = 14$). There were no significant differences in the values between the two groups.

ing aorta. As shown in Fig. 4B, ethidium fluorescence, which was amplified in ApoE-KO mice, was decreased by edaravone treatment. A quantitative analysis by the superoxide dismutase-inhibitable cytochrome *c* reduction assay revealed that $O_2^{\bullet -}$ levels in aortic rings of ApoE-KO mice were decreased by 43% in edaravone-treated ApoE-KO mice compared to those in vehicle-treated mice (Fig. 4C). Consistent with these results, plasma 8-isoprostane levels and 4-HNE expression in the descending aorta, both of which were elevated in ApoE-KO mice compared to

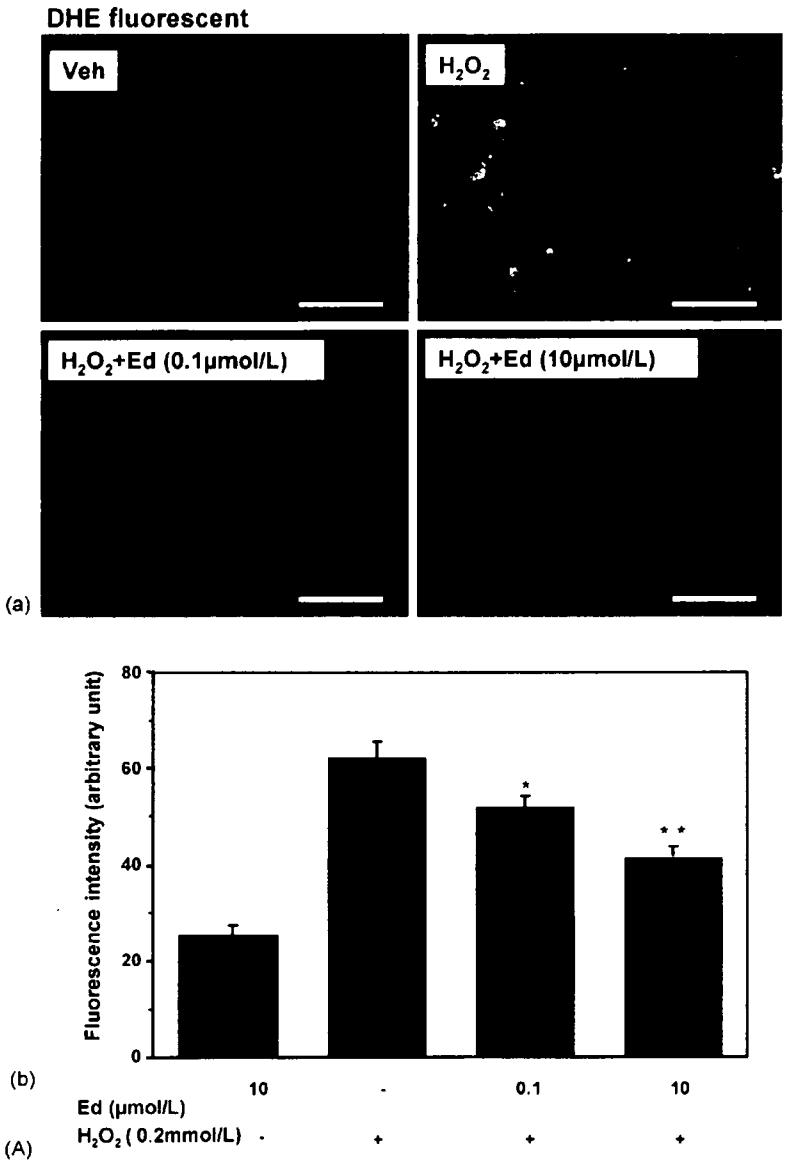

Fig. 2. Effects of edaravone (Ed) on DHE fluorescent (A) and 8-isoprostane formation (B), VCAM-1 expression (C) and 4-HNE expression (D) in cultured EC. Ed or its vehicle was added to the culture medium 24 h before H_2O_2 treatment until assay. DHE fluorescent ($n = 6$), 8-isoprostane concentration ($n = 3$) and VCAM-1 expression ($n = 3$) in the cell lysate were measured 3 h after H_2O_2 treatment. Values are expressed as mean ± S.E.M. Time dependent changes of 4-HNE expression after H_2O_2 treatment was detected by Western blotting. Representative image showed that 4-HNE-Michael protein adducts were accumulated after treatment (D(a)). The major 97 kDa band was measured 4.5 h after H_2O_2 treatment in the presence or absence of edaravone (D(b)). Values are expressed as mean ± S.E.M. ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$ vs. H_2O_2 (+) + Ed (−).

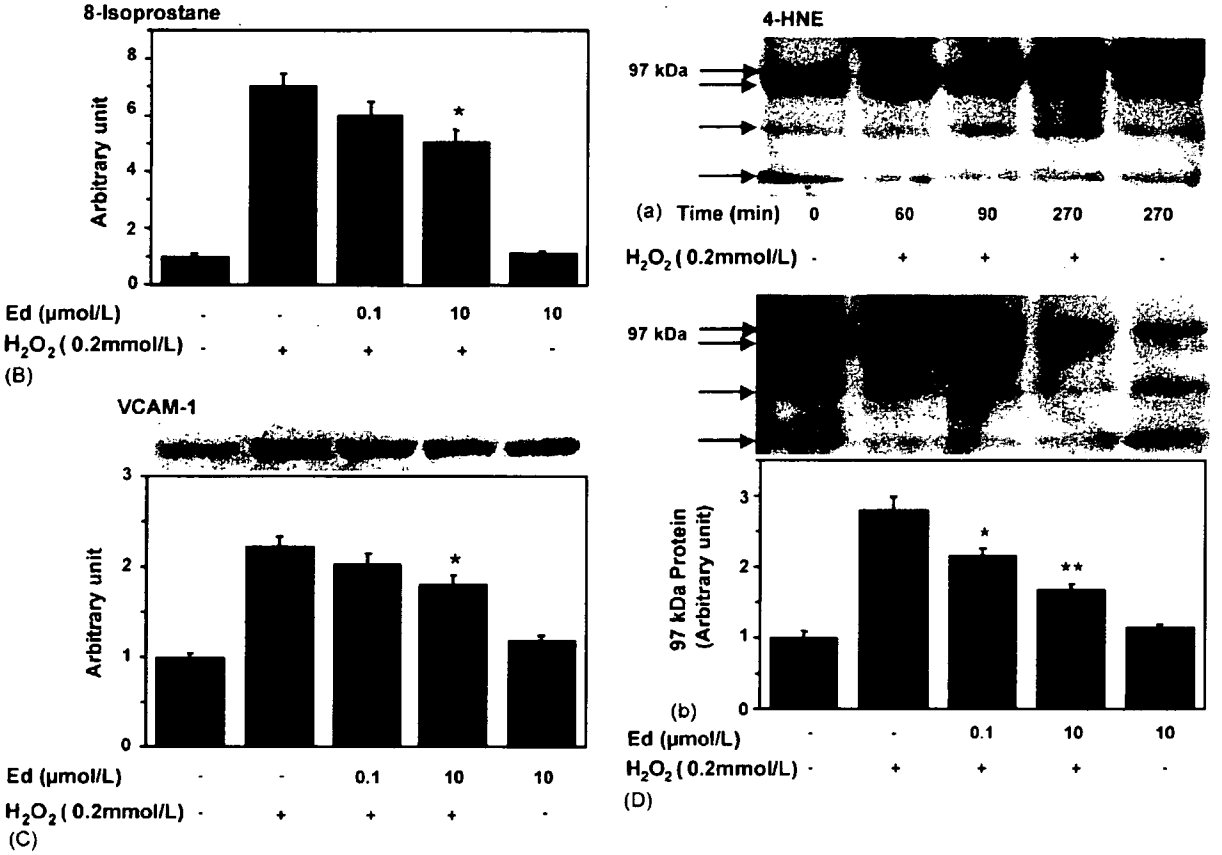

Fig. 2. (*Continued*).

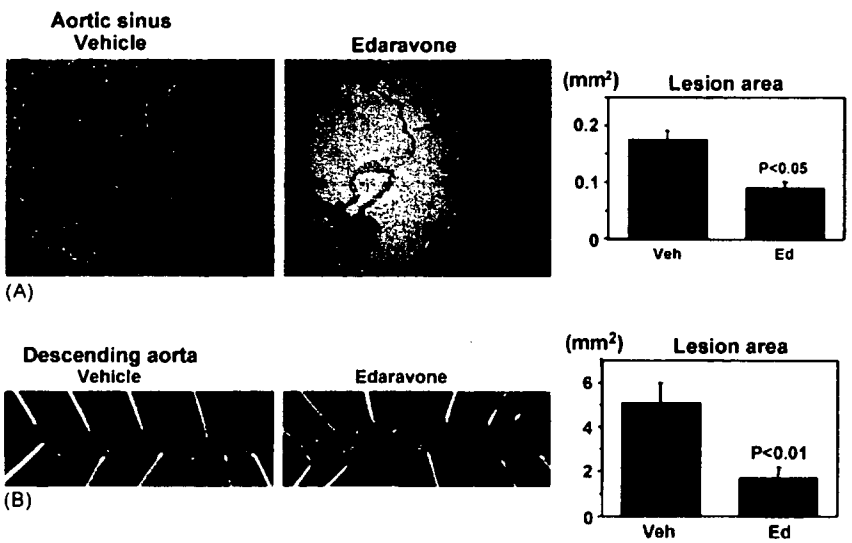

Fig. 3. Effects of edaravone on atherosclerotic lesion in ApoE-KO mice. ApoE-KO mice were fed a high-cholesterol diet for 4 weeks with the administration of edaravone (10 mg/kg daily) or its vehicle by i.p. injection. (A) Oil red O-stained cross-sections of the aortic sinus (bar = 100 μm) and morphometric analysis of the lesions are shown. (B) Oil red O-stained *en face* specimens of the descending aorta (bar = 5 mm) and morphometric analysis of the lesions are shown. Values are expressed as mean ± S.E.M. ($n = 14$).

those in wild-type C57BL/6 mice fed a normal chow, were decreased by edaravone treatment (Fig. 4D and E). Finally, the increase in VCAM-1 expression in the aorta of ApoE-KO mice was attenuated by edaravone as well (Fig. 4F).

4. Discussion

A number of studies have shown that ROS contribute to the pathogenesis of endothelial dysfunction and atherosclerosis formation. In addition to $O_2^{\bullet -}$ that is predominantly pro-

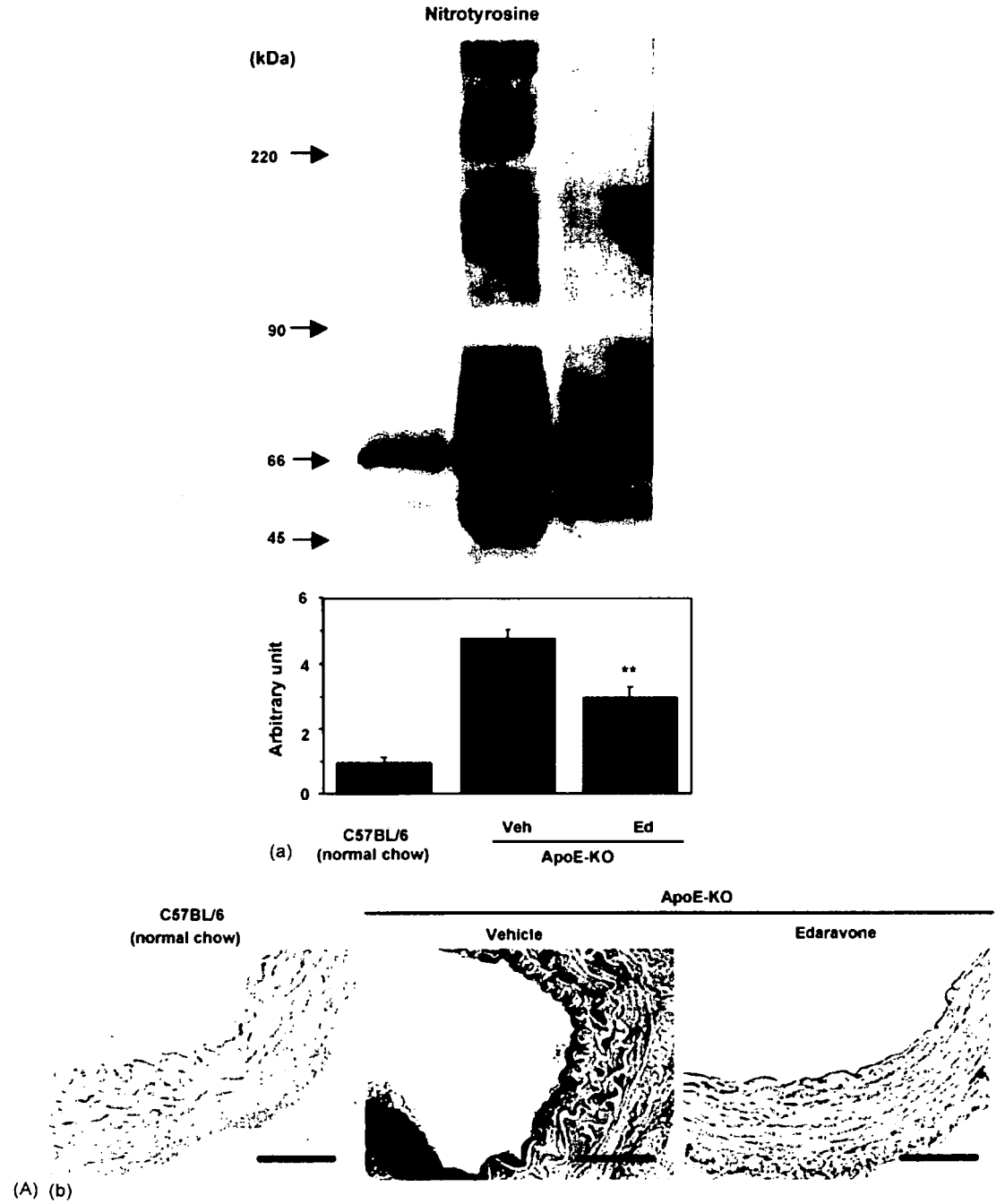

Fig. 4. Effects of edaravone (Ed) on ROS production (A–E) and VCAM-1 expression (F) in ApoE-KO mice. (A) Nitrotyrosine contents in the aorta was examined by Western blot analysis (A(a), $n = 6$) and immunohistochemistry (A(b)). Bar = 50 μm. (B) Fresh-frozen cross-sections of the aorta were stained with DHE, and representative fluorescent micrographs are shown (bar = 100 μm). (C) Superoxide anion in aortic rings was determined using SOD inhibitable-cytochrome *c* reduction assay ($n = 6$). (D) 8-Isoprostane level in mouse plasma was measured with EIA ($n = 6$). (E and F) Representative Western blotting for 4-HNE (97 kDa band) and VCAM-1 expression in the aorta and densitometric analysis are shown ($n = 3$). Values are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle (Veh). C57/BL6 mice fed a normal chow serve as the control.

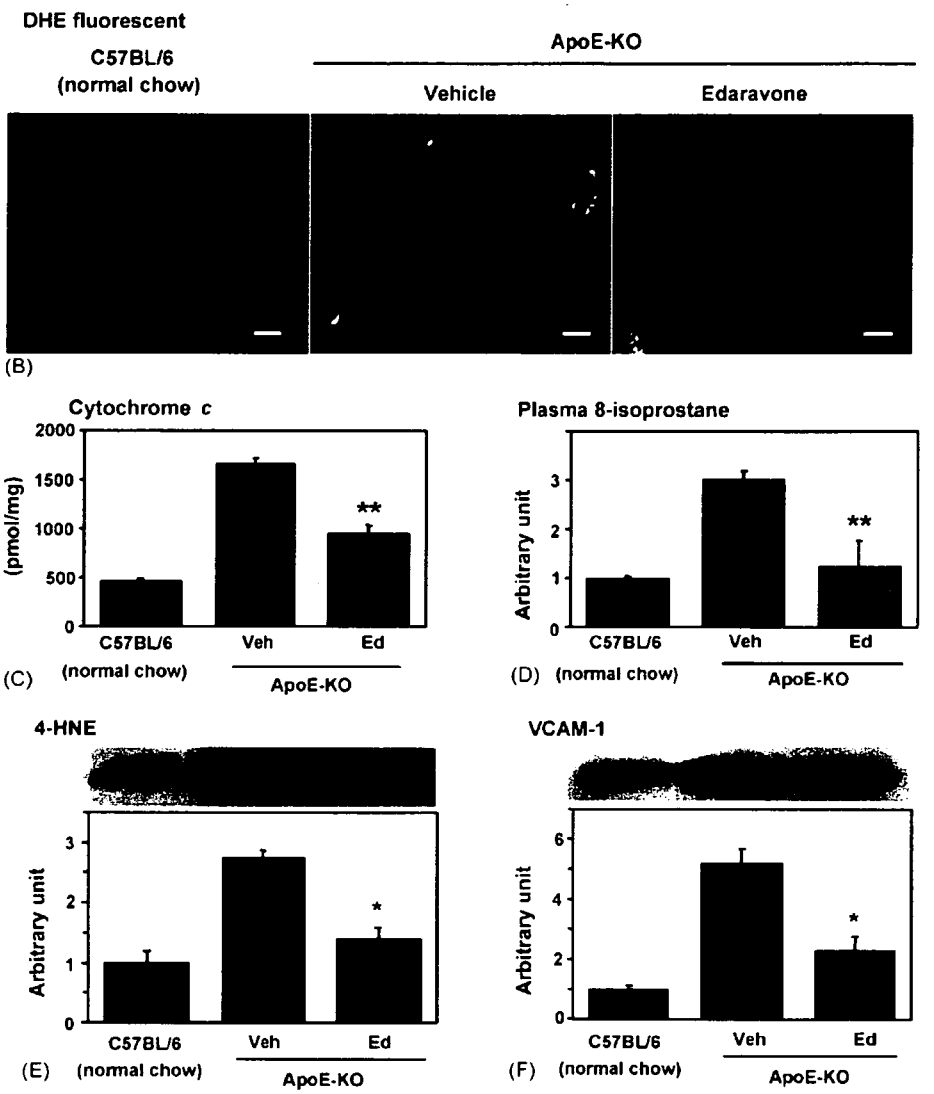

Fig. 4. (*Continued*).

duced via NAD(P)H oxidase [35], •OH as well as LOO• [36] and $ONOO^-$ [37] play a role in atherogenesis. In particular, •OH is extremely strong in terms of oxidative activity and cellular damage [38]. Therefore, it might be essential to scavenge the wide range of ROS for the prevention of atherosclerosis. As a matter of fact, recent clinical trials have denied the protective effects of Vitamin E, which predominantly reacts with LOO• [39], on cardiovascular events [18,19].

Edaravone, a potent free radical scavenger with unique properties, works by donating an electron from edaravone anion to free radicals [22]. Edaravone quenches •OH and inhibits both •OH-dependent and •OH-independent lipid peroxidation [22]. Edaravone shows inhibitory effects on both water-soluble and lipid-soluble LOO-induced peroxidation systems [22]. Edaravone also inhibits $ONOO^-$-induced tyrosine nitration [22]. These properties are different from those of water-soluble Vitamin C and lipid-soluble Vitamin E.

In the present study, we demonstrated that edaravone suppressed endothelial apoptosis and fatty streak formation. Reduced expression of VCAM-1, a marker of vascular injury and activation [32], were corroborated with these results. In cultured ECs, protein expression of VCAM-1 was induced as early as 3 h after H_2O_2 treatment (actually 4.5 h after addition of H_2O_2, Fig. 2C). This is reasonable based on our time course experiments (data not shown), and is consistent with the previous reports that VCAM-1 protein has been induced 4–6 h after cytokine stimulation through an antioxidant-sensitive mechanism [40,41]. Although the experimental conditions were different between the cell culture and animal studies, edaravone inhibited both the rapid induction of VCAM-1 in cultured ECs and the chronic upregulation of VCAM-1 in the aorta of ApoE-KO mice, further supporting the vasoprotective effects of edaravone.

Edaravone has been clinically used as a neuroprotectant in the treatment of ischemic stroke in Japan from 2001. The dose of edaravone used in this study (intraperitoneal injection of 10 mg/kg) has been reported to be comparable to that of intravenous injection in clinical use in terms of plasma concentration [42]. This compound has been reported to preserve endothelial function in ischemic brain [43] and ameliorate ischemia-reperfusion injury in various organs such as kidney [44] and heart [45]. Also, edaravone has been shown to inhibit pressure overload-induced cardiac hypertrophy [42]. To our knowledge, however, the effect of edaravone on atherosclerosis has never been reported till now.

The effects of edaravone on endothelial injury and atherosclerosis were associated with the decrease in ROS production including peroxynitrite, superoxide anion and 8-isoprostane, suggesting the mechanistic role of antioxidation in vascular protection. Edaravone also inhibited the expression of 4-HNE in vascular tissues, further indicating the antioxidant activity and suggesting the signaling cascade leading to endothelial injury, because 4-HNE triggers cellular damages through the MAP kinase pathway as an end-product of ROS [34]. Antioxidant effects of edaravone on lipoproteins were not determined in the present study because of the methodological limitation in mice. It has been reported, however, that edaravone can inhibit oxidative modification of low-density lipoprotein *in vitro* and in rats [46]. Consequently, it is likely that reduced lipoprotein oxidation would have played a role in the anti-atherosclerotic effects of edaravone in ApoE-KO mice. Furthermore, edaravone has been reported to stimulate the expression of endothelial nitric oxide synthase in cultured ECs [46] and the artery [47], leading to the increased production of nitric oxide. Taken together with the effects on peroxynitrite formation, edaravone might synergistically increase the availability of nitric oxide, which exerts vasoprotective and anti-atherosclerotic action.

The effects of edaravone on advanced and complicated lesions of atherosclerosis were not investigated in this study. Neither, the effects on plaque ruptures nor consequent cardiovascular events are known. This study demonstrated that edaravone might be a potential new therapeutic agent for the prevention and treatment of early atherosclerosis. For the purpose of chronic use, however, the innovation of drug preparation for oral administration is necessary. Another application of edaravone might be the prevention of restenosis after percutaneous coronary interventions, since ROS plays an important role in neointimal formation after angioplasty [48]. Intravenous injection of edaravone for several days might inhibit neointimal formation in addition to ischemia reperfusion injury of cardiomyocytes [45]. Taken together, edaravone is expected to show protective effect on ROS-related vascular diseases beyond cerebral infarction.

In summary, edaravone, a free radical scavenger with unique properties, attenuated oxidative stress-induced endothelial damage in rats and early atherosclerosis in ApoE-KO mice in association with the inhibition of ROS formation. These findings provide new information on the role of ROS in atherogenesis and the therapeutic strategy for atherosclerosis.

Acknowledgements

We thank Ms. Mariko Sawano for her excellent technical assistance. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan (13670741), and by Health and Labour Sciences Research Grants (H15-Choju-013, H15-Choju-015 and H17-Choju-046) from the Ministry of Health, Labour and Welfare of Japan.

References

[1] Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med 1999;340:115–26.
[2] Griendling KK, Sorescu D, Lassegue B, Ushio-Fukai M. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler Thromb Vasc Biol 2000;20:2175–83.
[3] Zalba G, San Jose G, Moreno MU, et al. Oxidative stress in arterial hypertension: role of NAD(P)H oxidase. Hypertension 2001;38:1395–9.
[4] Sorescu D, Weiss D, Lassegue B, et al. Superoxide production and expression of nox family proteins in human atherosclerosis. Circulation 2002;105:1429–35.
[5] Spiekermann S, Landmesser U, Dikalov S, et al. Electron spin resonance characterization of vascular xanthine and NAD(P)H oxidase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. Circulation 2003;107:1383–9.
[6] Rey FE, Li XC, Carretero OA, Garvin JL, Pagano PJ. Perivascular superoxide anion contributes to impairment of endothelium-dependent relaxation: role of gp91(phox). Circulation 2002;106:2497–502.
[7] Barry-Lane PA, Patterson C, van der Merwe M, et al. p47phox is required for atherosclerotic lesion progression in ApoE(−/−) mice. J Clin Invest 2001;108:1513–22.
[8] Keaney Jr JF, Gaziano JM, Xu A, et al. Dietary antioxidants preserve endothelium-dependent vessel relaxation in cholesterol-fed rabbits. Proc Natl Acad Sci (USA) 1993;90:11880–4.
[9] Keaney Jr JF, Xu A, Cunningham D, Jackson T, Frei B, Vita JA. Dietary probucol preserves endothelial function in cholesterol-fed rabbits by limiting vascular oxidative stress and superoxide generation. J Clin Invest 1995;95:2520–9.
[10] Lamb DJ, Reeves GL, Taylor A, Fems GA. Dietary copper supplementation reduces atherosclerosis in the cholesterol-fed rabbit. Atherosclerosis 1999;146:33–43.
[11] Pratico D, Tangirala RK, Rader DJ, Rokach J, FitzGerald GA. Vitamin E suppresses isoprostane generation in vivo and reduces atherosclerosis in ApoE-deficient mice. Nat Med 1998;4:1189–92.
[12] Li Z, Iwai M, Wu L, et al. Fluvastatin enhances the inhibitory effects of a selective AT1 receptor blocker, valsartan, on atherosclerosis. Hypertension 2004;44:758–63.
[13] Fennell JP, Brosnan MJ, Frater AJ, et al. Adenovirus-mediated overexpression of extracellular superoxide dismutase improves endothelial dysfunction in a rat model of hypertension. Gene Ther 2002;9:110–7.
[14] Kirk EA, Dinauer MC, Rosen H, Chait A, Heinecke JW, LeBoeuf RC. Impaired superoxide production due to a deficiency in phagocyte NADPH oxidase fails to inhibit atherosclerosis in mice. Arterioscler Thromb Vasc Biol 2000;20:1529–35.

[15] Hsich E, Segal BH, Pagano PJ, et al. Vascular effects following homozygous disruption of p47(phox): an essential component of NADPH oxidase. Circulation 2000;101:1234–6.
[16] Fang JC, Kinlay S, Beltrame J, et al. Effect of Vitamins C and E on progression of transplant-associated arteriosclerosis: a randomised trial. Lancet 2002;359:1108–13.
[17] Engler MM, Engler MB, Malloy MJ, et al. Antioxidant Vitamins C and E improve endothelial function in children with hyperlipidemia: endothelial assessment of risk from lipids in youth (EARLY) trial. Circulation 2003;108:1059–63.
[18] Lonn E, Bosch J, Yusuf S, et al. Effects of long-term Vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. JAMA 2005;293:1338–47.
[19] Lee IM, Cook NR, Gaziano JM, et al. Vitamin E in the primary prevention of cardiovascular disease and cancer: the Women's Health Study: a randomized controlled trial. JAMA 2005;294:56–65.
[20] Study-Group E. Effect of a novel free radical scavenger, edaravone (MCI-186), on acute brain infarction. Randomized, placebo-controlled, double-blind study at multicenters. Cerebrovasc Dis 2003;15:222–9.
[21] Abe S, Kirima K, Tsuchiya K, et al. The reaction rate of edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186)) with hydroxyl radical. Chem Pharm Bull (Tokyo) 2004;52:186–91.
[22] Watanabe T, Yuki S, Egawa M, Nishi H. Protective effects of MCI-186 on cerebral ischemia: possible involvement of free radical scavenging and antioxidant actions. J Pharmacol Exp Ther 1994;268:1597–604.
[23] Sudoh N, Toba K, Akishita M, et al. Estrogen prevents oxidative stress-induced endothelial cell apoptosis in rats. Circulation 2001;103:724–9.
[24] Akishita M, Nagai K, Xi H, et al. Renin–angiotensin system modulates oxidative stress-induced endothelial cell apoptosis in rats. Hypertension 2005;45:1188–93.
[25] Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis 1987;68:231–40.
[26] Miller Jr FJ, Gutterman DD, Rios CD, Heistad DD, Davidson BL. Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. Circ Res 1998;82:1298–305.
[27] Landmesser U, Dikalov S, Price SR, et al. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J Clin Invest 2003;111:1201–9.
[28] Francia P, Delli Gatti C, Bachschmid M, et al. Deletion of p66shc gene protects against age-related endothelial dysfunction. Circulation 2004;110:2889–95.
[29] Carter WO, Narayanan PK, Robinson JP. Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. J Leuk Biol 1994;55:253–8.
[30] Xi H, Shin WS, Suzuki J, et al. Dystrophin disruption might be related to myocardial cell apoptosis caused by isoproterenol. J Cardiovasc Pharmacol 2000;36(Suppl 2):S25–9.
[31] Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 1991;11:81–128.
[32] Meigs JB, Hu FB, Rifai N, Manson JE. Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. JAMA 2004;291:1978–86.
[33] Uchida K, Toyokuni S, Nishikawa K, et al. Michael addition-type 4-hydroxy-2-nonenal adducts in modified low-density lipoproteins: markers for atherosclerosis. Biochemistry 1994;33:12487–94.
[34] Usatyuk PV, Natarajan V. Role of mitogen-activated protein kinases in 4-hydroxy-2-nonenal-induced actin remodeling and barrier function in endothelial cells. J Biol Chem 2004;279:11789–97.
[35] Jiang F, Guo Y, Salvemini D, Dusting GJ. Superoxide dismutase mimetic M40403 improves endothelial function in apolipoprotein (E)-deficient mice. Br J Pharmacol 2003;139:1127–34.
[36] O'Donnell VB, Chumley PH, Hogg N, Bloodsworth A, Darley-Usmar VM, Freeman BA. Nitric oxide inhibition of lipid peroxidation: kinetics of reaction with lipid peroxyl radicals and comparison with alpha-tocopherol. Biochemistry 1997;36:15216–23.
[37] White CR, Brock TA, Chang LY, et al. Superoxide and peroxynitrite in atherosclerosis. Proc Natl Acad Sci USA 1994;91:1044–8.
[38] Zheng H, Dimayuga C, Hudaihed A, Katz SD. Effect of dexrazoxane on homocysteine-induced endothelial dysfunction in normal subjects. Arterioscler Thromb Vasc Biol 2002;22:E15–8.
[39] Terentis AC, Thomas SR, Burr JA, Liebler DC, Stocker R. Vitamin E oxidation in human atherosclerotic lesions. Circ Res 2002;90:333–9.
[40] Marui N, Offermann MK, Swerlick R, et al. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. J Clin Invest 1993;92:1866–74.
[41] Park SH, Park JH, Kang JS, Kang YH. Involvement of transcription factors in plasma HDL protection against TNF-alpha-induced vascular cell adhesion molecule-1 expression. Int J Biochem Cell Biol 2003;35:168–82.
[42] Tsujimoto I, Hikoso S, Yamaguchi O, et al. The antioxidant edaravone attenuates pressure overload-induced left ventricular hypertrophy. Hypertension 2005;45:921–6.
[43] Amemiya S, Kamiya T, Nito C, et al. Anti-apoptotic and neuroprotective effects of edaravone following transient focal ischemia in rats. Eur J Pharmacol 2005;516:125–30.
[44] Doi K, Suzuki Y, Nakao A, Fujita T, Noiri E. Radical scavenger edaravone developed for clinical use ameliorates ischemia/reperfusion injury in rat kidney. Kidney Int 2004;65:1714–23.
[45] Tsujita K, Shimomura H, Kawano H, et al. Effects of edaravone on reperfusion injury in patients with acute myocardial infarction. Am J Cardiol 2004;94:481–4.
[46] Yoshida H, Sasaki K, Namiki Y, Sato N, Edaravone TN, a novel radical scavenger, inhibits oxidative modification of low-density lipoprotein (LDL) and reverses oxidized LDL-mediated reduction in the expression of endothelial nitric oxide synthase. Atherosclerosis 2005;179:97–102.
[47] Zhang XH, Matsuda N, Jesmin S, et al. Normalization by edaravone, a free radical scavenger, of irradiation-reduced endothelial nitric oxide synthase expression. Eur J Pharmacol 2003;476:131–7.
[48] Cipollone F, Fazia M, Iezzi A, et al. High preprocedural non-HDL cholesterol is associated with enhanced oxidative stress and monocyte activation after coronary angioplasty: possible implications in restenosis. Heart 2003;89:773–9.

Effects of Aerobic Exercise on Metabolic Syndrome Improvement in Response to Weight Reduction

Tomohiro Okura,† Yoshio Nakata,*† Kazunori Ohkawara,‡ Shigeharu Numao,‡ Yasutomi Katayama,‡ Tomoaki Matsuo,‡ and Kiyoji Tanaka*†*

Abstract

OKURA, TOMOHIRO, YOSHIO NAKATA, KAZUNORI OHKAWARA, SHIGEHARU NUMAO, YASUTOMI KATAYAMA, TOMOAKI MATSUO, AND KIYOJI TANAKA. Effects of aerobic exercise on metabolic syndrome improvement in response to weight reduction. *Obesity*. 2007;15:2478–2484.

Objective: The objective was to test effects of aerobic exercise training on metabolic syndrome (MetSyn) improvement in response to weight reduction.

Research Methods and Procedures: A total of 459 overweight and obese women (age, 49 ± 9 years; BMI, 28 ± 3 kg/m^2) were recruited for a baseline examination to test the relationship between cardiorespiratory fitness and metabolic syndrome prevalence; among these, 67 subjects with MetSyn were treated with 14-week weight-loss programs, which included low-calorie diet and aerobic exercise. The MetSyn was defined according to the Examination Committee of Criteria for "Metabolic Syndrome" in Japan. Maximal oxygen uptake ($\dot{V}O_{2max}$) during a maximal cycling test was measured as an index of cardiorespiratory fitness at baseline and after the intervention.

Results: In the baseline examination, age- and BMI-adjusted odds ratios for MetSyn prevalence in the low, middle, and upper thirds of $\dot{V}O_{2max}$ were 1.0 (referent), 0.50 (95% confidence interval, 0.26 to 0.95), and 0.39 (95% confidence interval, 0.14 to 0.96), respectively (linear trend, p = 0.02). The adjusted odds ratios for MetSyn improvement in the two interventions with diet alone and diet plus exercise were 1.0 and 3.68 (95% confidence interval, 1.02 to 17.6; p = 0.04), respectively.

Discussion: These results suggest that adding aerobic exercise training to a dietary weight-reduction program further improves MetSyn (adjusted odds ratio, 3.68) in obese women, compared with diet alone. Further studies on an association between $\dot{V}O_{2max}$ change and MetSyn improvement are needed.

Key words: exercise intervention, diet, aerobic exercise, metabolic syndrome

Introduction

Metabolic syndrome is a cluster of interrelated risk factors (visceral obesity, dyslipidemia, hyperglycemia, and hypertension) (1) that increase susceptibility to cardiovascular disease (2,3) and type 2 diabetes (4,5). The National Cholesterol Education Program's Adult Treatment Panel III report (6) stated that the increasing prevalence of obesity has been accompanied by a parallel increase in the prevalence of metabolic syndrome, which together constitutes the "obesity epidemic."

Cross-sectional data indicate that high levels of cardiorespiratory fitness are associated with low prevalence of metabolic syndrome (7–9). Several prospective studies have found that cardiorespiratory fitness is a significant predictor for metabolic syndrome incidence (10,11). Another study found that 20 weeks of aerobic exercise training reduced metabolic syndrome prevalence (12). Clinical intervention studies in obese people have also revealed that regular aerobic exercise training clearly improves risk factors for metabolic syndrome (13,14).

Detecting metabolic syndrome in asymptomatic obese individuals is useful in identifying high-risk individuals for intensive primary preventive therapy (15), and lifestyle

Received for review June 5, 2006
Accepted in final form February 26, 2007.
The costs of publication of this article were defrayed, in part, by the payment of page charges. This article must, therefore, be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
*Graduate School of Comprehensive Human Sciences, †Center for Tsukuba Advanced Research Alliance, and ‡Doctoral Program in Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan.
Address correspondence to Tomohiro Okura, 1-1-1 Ten-nodai, Tsukuba, Ibaraki 305-8574, Japan.
E-mail: okura@taiiku.tsukuba.ac.jp

therapy is recognized as an important approach in the various clinical and educational settings of obesity treatment. However, little is known of the effects of diet and/or aerobic exercise training on metabolic syndrome improvement in obese individuals.

We have investigated these issues in overweight and obese Japanese women. On the basis of the studies cited above, we hypothesized that change in cardiorespiratory fitness, defined as maximal oxygen uptake ($\dot{V}O_{2max}$)[1], would be a predictor for improvement in metabolic syndrome in obese subjects during weight reduction. We first determined whether cardiorespiratory fitness was associated with metabolic syndrome prevalence at baseline. Next, we assigned subjects with metabolic syndrome to two treatment groups, which received diet therapy alone or with aerobic exercise training, and we investigated the effects of cardiorespiratory fitness change and these two treatments on metabolic syndrome improvement during weight reduction.

Research Methods and Procedures

Subjects

Participants were sedentary overweight and obese Japanese women who were recruited through advertisements in local newspapers in Ibaraki prefecture, Japan, and participated in a 14-week weight-reduction program between 2000 and 2004. Before the program, we excluded subjects who smoked, had concomitant renal, hepatic, or cardiac disease, or were being treated with hormone replacement or drugs, which could affect the variables of the study. Consequently, 459 women, 34 to 66 years of age, were chosen as subjects (Table 1) after they met the following criteria: 1) sedentariness, defined as exercise-induced energy expenditure of <60 minutes/wk, and 2) overweight or obesity, defined as a BMI of, respectively, >25 kg/m^2 and >30 kg/m^2 (16). Of these women, 185 were postmenopausal and 274 were premenopausal. Menopause was defined as the absence of menses for at least 12 months, as reported by questionnaire. This study conformed to the principles outlined in the Helsinki Declaration and was approved by the Review Board of the University of Tsukuba. The aim and design of the study were explained to each subject before she gave her written, informed consent.

Research Procedures

First, we cross-sectionally examined the relationship between cardiorespiratory fitness and metabolic syndrome prevalence in all subjects. Next, 67 subjects were diagnosed as having the metabolic syndrome according to the criteria for the Japanese population, which are described below (17). To increase subjects' adherence to the weight loss programs, the subjects' personal lifestyles (occupations, daily schedules, etc.) and preferences were taken into account, and the 67 subjects were assigned to two 14-week weight-reduction programs consisting of a low-calorie diet (n = 24; target energy intake, 1200 kcal/d) or the diet-plus-aerobic exercise (n = 43). Three subjects in the diet alone group and 5 in the diet plus exercise group were unable to complete the study successfully for personal reasons. As a consequence, 21 subjects in the diet alone group and 38 subjects in the diet plus exercise group completed the study requirements. Assays and measurements were carried out before and after the 14-week intervention period. We prospectively examined the relationship between cardiorespiratory fitness change and metabolic syndrome improvement in response to weight reduction.

Table 1. Baseline characteristics of subjects (n = 459)

Characteristic	Value
Age (yrs)	49 ± 9
BMI (kg/m^2)	27.5 ± 3.4
Waist (cm)	99.4 ± 9.5
Visceral fat area (cm^2)	96 ± 47
Systolic BP (mm Hg)	132 ± 18
Diastolic BP (mm Hg)	83 ± 11
Triglycerides (mM)	1.21 ± 0.86
HDL-C (mM)	1.64 ± 0.39
Glucose (mM)	5.49 ± 1.16
$\dot{V}O_{2max}$ (mL/kg per min)	25.2 ± 4.0
$\dot{V}O_{2max}$ (mL/min)	1714 ± 280
Visceral fat obesity (%)	42
High BP (%)	54
High triglycerides (%)	16
Low HDL-C (%)	2
High glucose (%)	13
No. of subjects with metabolic syndrome (%)	67 (15)

BP, blood pressure; HDL-C, high-density lipoprotein cholesterol; $\dot{V}O_{2max}$, maximal oxygen uptake. Values are mean ± SD unless specified otherwise.

Anthropometric Variables

Body mass was measured to the nearest 0.1 kg using a digital scale, height was measured to the nearest 0.1 cm using a wall-mounted stadiometer, and BMI was calculated as mass (kg) divided by height squared (m^2). Waist girth was measured to the nearest 0.1 cm at the level of the umbilicus with subjects in the standing position.

[1] Nonstandard abbreviations: $\dot{V}O_{2max}$, maximal oxygen uptake; CT, computed tomography; CI, confidence interval.

Visceral Fat Area by CT Scans

Visceral fat area (cm^2) was measured at the level of the umbilicus (L4–L5) using computed tomography (CT) scans (SCT-6800TX, Shimadzu, Tokyo, Japan) performed on subjects in the supine position and was calculated using a computer software program (FatScan, N2system, Osaka, Japan) (18). The intra-class correlation for repeated determinations of visceral fat area in our laboratory was 0.99.

Definition of Metabolic Syndrome

For the Japanese population, the Examination Committee of Criteria for "Metabolic Syndrome" in Japan (17) defined metabolic syndrome as the presence of visceral fat obesity (visceral fat area $\geq$100 cm^2) and two or more of the following criteria: 1) triglycerides $\geq$1.70 mM (150 mg/dL) and/or high-density lipoprotein cholesterol $<$1.04 mM (40 mg/dL), 2) systolic blood pressure $\geq$140 mm Hg and/or diastolic blood pressure $\geq$90 mm Hg, and 3) fasting plasma glucose $\geq$6.1 mM (110 mg/dL). Systolic and diastolic blood pressures were taken from the left arm using a sphygmomanometer after the subjects rested at least 20 minutes in a sitting position. Cuff sizes were selected based on upper arm girth and length. A blood sample of ~10 mL was drawn from each subject after an overnight fast. Triglycerides were determined enzymatically, and fasting plasma glucose was assayed by a glucose oxidase method. Serum high-density lipoprotein-cholesterol was measured by the heparin-manganese precipitation method.

Maximal Oxygen Uptake

Maximal oxygen uptake ($\dot{V}O_{2max}$, mL/kg per min and mL/min) was determined during a graded exercise test using a cycle ergometer (818E, Monark, Stockholm, Sweden). After a 2-minute warm-up, the subject started with a workload of 15 W, which was increased by 15 W each minute until volitional exhaustion occurred. Pulmonary ventilation and gas exchange were measured breath-by-breath with an online data acquisition system (Oxycon α System, Mijnhardt, Breda, Netherlands).

Diet and Exercise Regimens

Dietary Protocol. Subjects were instructed to take a well-balanced supplemental food product (MicroDiet, Sunny-Health, Nagano, Japan) every day. It was developed for very low-energy diets (170 kcal per pack) and is comprised of protein, carbohydrates, fat, various amino acids, vitamins, and minerals. Two other meals per day were allowed, consisting, on average, of 240 kcal of protein, 480 kcal of carbohydrate, and 240 kcal of fat. Subjects also kept daily food diaries during the 14-week intervention period and learned about proper daily nutrition through weekly lectures and counseling by skilled dieticians.

Exercise Protocol. In addition to restricting energy intake, the subjects from the diet plus exercise training group performed a bench-stepping exercise 3 days/wk for 45 minutes per session, supervised in the hospital by two or three physical trainers. The bench-stepping exercise is a combination exercise of low impact aerobic dance and stepping with a step bench (10 to 20 cm high) (19). The exercise started with basic steps for the first 4 weeks and then progressed to combination of basic steps and lunge steps for the next 6 weeks, and finally progressed to more advanced lunge steps for the last 4 weeks. Subjects were instructed to perform the aerobic dance at a level that raised their heart rate to 70% to 85% of the corresponding heart rate at their $\dot{V}O_{2max}$. The target Borg's scale (ratings of perceived exertion) (20) ranged from 13 (fairly hard) to 17 (very hard).

Statistical Analysis

Values are mean $\pm$ standard deviation. Paired t tests were used to assess differences between variables before and after the weight-reduction intervention period. Unpaired t tests were used to test difference in variables between the two treatment groups. Qualitative data were analyzed by a χ^2 test. We used logistic regression to estimate odds ratios and 95% confidence intervals (CIs) as an index of the strength of associations between cardiorespiratory fitness and metabolic syndrome prevalence or improvement, and between treatment (diet alone vs. diet plus exercise) and metabolic syndrome improvement. Multivariate analyses were adjusted for age (years), menopause (yes/no), BMI (kg/m^2), and body weight change (kg). General linear model analyses [repeated-measure two-by-two way (baseline vs. after treatment) ANOVA with post hoc tests] were used to test for difference in measurement variables between groups with diet alone and diet plus exercise, and between baseline and after treatment. In each statistical analysis, probability values below 0.05 were regarded as significant. The data were analyzed with the Statistical Analysis System, version 9.01 for Microsoft Windows (SAS Institute, Inc., Cary, NC).

Results

At baseline, we observed an inverse gradient (linear trend, $p < 0.05$) of age- and BMI-adjusted odds ratios for metabolic syndrome prevalence in the low (average $\dot{V}O_{2max}$, 20.8 mL/kg per min), middle (average $\dot{V}O_{2max}$, 25.2 mL/kg per min), and upper (average $\dot{V}O_{2max}$, 29.5 mL/kg per min) thirds of $\dot{V}O_{2max}$. They were 1.0 (referent), 0.50 (95% CI, 0.26 to 0.95), and 0.39 (95% CI, 0.14 to 0.96), respectively (linear trend, $p = 0.02$) (Table 2). The significant trend (linear trend, $p = 0.03$) remained after adjustment for age, BMI, and menopausal status. The adjusted risks of metabolic syndrome were 48% (−6% to 75%) and 63% (−4% to 87%) lower in the middle and upper thirds of fitness, respectively, compared with the lower third. On average, each 1 mL/kg per min increment in $\dot{V}O_{2max}$ was associated with 7% lower risk of metabolic syndrome.

Table 2. Odds ratios and 95% CIs for metabolic syndrome according to $\dot{V}O_{2max}$ (mL/kg per min)

Covariates	$\dot{V}O_{2max}$ tertile			Linear trend (p)
	Low	Middle	High	
All (n = 459)				
No adjustment	1.0 (referent)	0.38 (0.21 to 0.68)	0.13 (0.05 to 0.28)	<0.001
Age, baseline BMI	1.0 (referent)	0.50 (0.26 to 0.95)	0.39 (0.14 to 0.96)	0.02
Age, menopause, baseline BMI	1.0 (referent)	0.52 (0.25 to 1.06)	0.37 (0.13 to 1.04)	0.03
Postmenopausal (n = 143)				
No adjustment	1.0 (referent)	0.39 (0.15 to 0.96)	0.24 (0.08 to 0.66)	<0.01
Age, baseline BMI	1.0 (referent)	0.45 (0.16 to 1.21)	0.45 (0.13 to 1.47)	0.14
Premenopausal (n = 212)				
No adjustment	1.0 (referent)	0.35 (0.09 to 1.12)	0.18 (0.03 to 0.70)	0.02
Age, baseline BMI	1.0 (referent)	0.53 (0.13 to 1.83)	0.34 (0.06 to 2.14)	0.26

CI, confidence interval; $\dot{V}O_{2max}$, maximal oxygen uptake.

Sixty-seven women (15% of all subjects) were diagnosed as having metabolic syndrome. The subjects were assigned to two groups, treated with a low-calorie diet (n = 24) or the diet-plus-aerobic exercise training (n = 43) (Table 3). Three subjects in the diet alone group and five in the diet plus exercise group were unable to complete the weight-reduction program successfully, for personal reasons. Consequently, 21 subjects in the diet alone group and 38 subjects in the diet plus exercise group were included in the final analysis. The average weight reductions in the diet group and diet plus exercise group were 6.0 kg and 8.8 kg, respectively. The prevalence of metabolic syndrome and metabolic syndrome risk factors was significantly decreased and improved in both groups. For the group treated with diet alone, of the 21 subjects with the metabolic syndrome at baseline, 15 (71%) were no longer diagnosed with the metabolic syndrome after the weight-loss treatment. For the group treated with diet plus exercise, of the 38 subjects with the metabolic syndrome at baseline, 36 (95%) were no longer diagnosed as having the metabolic syndrome after the weight-loss treatment.

We next examined whether treatment (diet alone vs. diet plus exercise) affected metabolic syndrome improvement in response to weight reduction (Table 4). The adjusted odds ratios in the groups with diet alone and diet plus exercise for metabolic syndrome improvement were 1.0 (referent) and 3.68 (95% CI, 1.02 to 17.6; linear trend, p = 0.04).

Discussion

Several organizations have recommended clinical criteria for the diagnosis of the metabolic syndrome (1,21). There are some slight differences in the criteria for diagnosis of the metabolic syndrome used by these organizations. According to the definition of the World Health Organization (22), insulin resistance is a required component and two other risk factors are sufficient for a diagnosis of metabolic syndrome. The National Cholesterol Education Program's Adult Treatment Panel III has stated that when three of five listed characteristics are present, a diagnosis of metabolic syndrome can be made (6). The criteria of the International Diabetes Federation include "central obesity" as an essential component and ethnic-specific values for waist girth (23). In the present study, we used Japanese-specific criteria recommended by the Examination Committee of Criteria for Metabolic Syndrome in Japan (17). This is in accordance with the International Diabetes Federation definition, whereas a slight difference was found in the criteria of low high-density lipoprotein cholesterol and high fasting plasma glucose between the two organizations. It is well known that Japanese individuals are likely to develop obesity-related disorders with even mild obesity (24). Inter-relations among anthropometric variables, body composition, fat distribution, and lipid/glucose metabolism, which may be affected by genetic factors, are quite different in the Japanese, U.S., and European populations. Therefore, we decided to use the Japanese-specific definition of metabolic syndrome.

A few prospective studies have revealed that physical activity and cardiorespiratory fitness are predictors of metabolic syndrome incidence (10,11). One study reported that a 20-week supervised aerobic exercise training reduced metabolic syndrome prevalence by 31% (12). The subjects in the above studies, however, were not all obese. Clinical intervention studies have shown that regular aerobic exercise training clearly improved risk factors for metabolic syndrome in obese people (13,14), but no study, to our

Table 3. Descriptive characteristics of subjects with metabolic syndrome at baseline (n = 67) and after treatment (n = 59)

	Diet alone			Diet plus exercise		
	Baseline (n = 24)	After treatment (n = 21)	p	Baseline (n = 43)	After treatment (n = 38)	p
No. of subjects						
With metabolic syndrome	24	6		43	2	
Without metabolic syndrome	0	15	<0.001	0	36	<0.001
Age (yrs)	52 ± 8			55 ± 6		
BMI (kg/m^2)	30.4 ± 4.9	27.1 ± 4.3	<0.001	29.2 ± 2.3	25.8 ± 2.2	<0.001
Visceral fat area (cm^2)	161 ± 52	119 ± 45	<0.001	136 ± 24	92 ± 26	<0.001
Systolic BP (mm Hg)	150 ± 15	133 ± 11	<0.001	147 ± 19	133 ± 16	<0.001
Diastolic BP (mm Hg)	91 ± 10	87 ± 9	<0.05	89 ± 10	81 ± 10	<0.01
Triglycerides (mM)	1.77 ± 0.75	1.23 ± 0.55	<0.001	2.19 ± 0.94	1.09 ± 0.29	<0.001
HDL-C (mM)	1.43 ± 0.34	1.46 ± 0.29	NS	1.43 ± 0.34	1.51 ± 0.29	<0.05
Glucose (mM)	6.82 ± 2.27	5.77 ± 2.27	<0.01	6.99 ± 2.11	5.60 ± 1.11	<0.001
$\dot{V}O_{2max}$ (mL/kg per min)	22.6 ± 3.4	24.0 ± 3.5	<0.05	22.9 ± 3.2	27.0 ± 3.8	<0.001
$\dot{V}O_{2max}$ (mL/min)	1617 ± 320	1584 ± 291	NS	1596 ± 258	1657 ± 284	NS
Visceral fat obesity (%)	100	76	<0.001	100	47	<0.001
High BP (%)	96	76	<0.001	84	74	<0.01
High triglycerides (%)	58	48	<0.05	67	13	<0.001
Low HDL-C (%)	8	5	NS	10	5	NS
High glucose (%)	67	48	<0.01	58	34	<0.01

BP, blood pressure; HDL-C, high-density lipoprotein cholesterol; $\dot{V}O_{2max}$, maximal oxygen uptake; NS, not significant. Values are mean ± standard deviation unless otherwise specified. Qualitative data were analyzed by a χ^2 test.

knowledge, has confirmed the improvement of metabolic syndrome with increased cardiorespiratory fitness. To our knowledge, the present study is, therefore, the first to investigate the association between cardiorespiratory fitness and metabolic syndrome in overweight and obese populations.

Table 2 suggests that, even with overweight and obesity, high $\dot{V}O_{2max}$ is associated with low prevalence of metabolic syndrome. This finding is in accordance with previous cross-sectional studies on the association of physical activity and cardiorespiratory fitness with the prevalence of metabolic syndrome (7–9). In obese patients, high cardiorespiratory fitness may prevent metabolic syndrome. Hence, clinicians should counsel their sedentary patients with obesity to become more physically active.

It is well known that an excess visceral fat accumulation is strongly associated with a high prevalence of risk factors

Table 4. Odds ratios and 95% confidence intervals for improvement of metabolic syndrome according to treatment

Treatment		
Diet alone	Diet plus exercise	Linear trend (p)
No adjustment		
1.0 (referent)	7.20 (1.47–53.1)	0.02
Adjusted for age and body weight change		
1.0 (referent)	3.68 (1.02–17.6)	0.04

for coronary heart disease, such as lipid metabolic disorders, hypertension, and type 2 diabetes. Moreover, several studies have shown that the visceral fat area above which metabolic disturbances increase is 100 or 110 cm^2 (25,26). Japan Society for the Study of Obesity has adopted visceral fat area of 100 cm^2 as the cut-off point for diagnosing high-risk obesity (16). Our data showed that average visceral fat area became <100 cm^2 in the group treated with diet plus exercise after weight loss, but that still remained >100 cm^2 in the group with diet alone. Table 3 shows that, although the prevalence of metabolic syndrome and metabolic syndrome risk factors was significantly decreased in both groups, the decreases tended to be larger in the group with diet plus exercise compared with the diet alone group. These data were accordance with previous studies on the association of visceral fat accumulation with the prevalence of risk factors for coronary heart disease.

In the present study, relative values of $\dot{V}O_{2max}$ (unit, mL/kg per min) increased significantly between baseline and after weight reduction, whereas a significant increase was not found in absolute values of $\dot{V}O_{2max}$ (unit, mL/min), even in the group treated with diet plus exercise. A study design that includes exercise of higher intensity and greater frequency and a longer intervention period, which could increase cardiorespiratory fitness, might be needed.

Aerobic exercise training may, however, be essential in treating obese patients with metabolic syndrome, even if the exercise does not increase their $\dot{V}O_{2max}$. The adjusted odds ratio for metabolic syndrome improvement in our study was 3.68 in the group with diet plus exercise training compared with diet alone, when adjusted for age and body weight change. That is to say, adding aerobic exercise training to dietary weight reduction may have a synergistic effect on the improvement of risk factors for metabolic syndrome. This is partly supported by our previous studies (27,28). In one (27), we found that the addition of exercise training contributes to the maintenance of fat-free mass and might be more effective for improving physical fitness and risk factors for coronary heart disease during weight reduction in obese women, compared with diet alone. Another study revealed that a 14-week weight-loss program with diet plus aerobic exercise training reduced visceral adipose tissue by a factor of 1.3 (diet plus exercise, 49.3 cm^2; diet alone, 37.8 cm^2 by CT scans) compared with diet alone, after adjustment for age, menopausal status, and body weight reduction (28). These studies suggest that adding aerobic exercise training to a dietary weight-reduction program further reduces visceral adipose tissue and further improves coronary heart disease risk factors compared with diet alone, even if weight reduction is the same with either treatment.

This study has some limitations. Our findings apply primarily to overweight and obese Japanese women. Although the external validity of our data is limited, the homogeneity of our subjects reduces confounding by sociodemographic factors, thus enhancing its internal validity. Second, subjects were not randomized to the treatments. Because our goal, in particular, was to increase subjects' adherence to the weight loss programs, the subjects' personal lifestyles and preferences were preferentially taken into account. Consequently, the numbers of subjects were imbalanced in two treatment groups. The study design without randomization may be concomitant with a type II error because of some confounding variables. This factor might partly preclude our definitive conclusion. However, at baseline, no differences were found in any variables between the groups treated with diet alone and with diet plus exercise. This suggests that assigning rather than randomizing subjects had little, if any, influence on the measurement variables.

In summary, our cross-sectional data suggest that, for overweight and obese women, a physically active lifestyle and maintenance of high cardiorespiratory fitness can be useful in primary prevention of metabolic syndrome. Our interventional study revealed that, for overweight and obese women with metabolic syndrome, adding aerobic exercise training to dietary weight reduction is a more effective (adjusted odds ratio = 3.68) treatment for improving metabolic syndrome than diet alone. However, weight-loss intervention trials of longer duration, with more frequent, higher-intensity exercise training and larger samples of obese patients with metabolic syndrome are needed to confirm the association between cardiorespiratory fitness change and metabolic syndrome improvement.

Acknowledgments

This work was supported in part by Grants-in-Aid from the Japanese Society of Physical Fitness and Sports Medicine (1998–2000), by Grants-in-Aid from the Uehara Memorial Foundation (2005), by the Tanaka Project (2004–2006) of TARA (Tsukuba Advanced Research Alliance) at University of Tsukuba, and by the twenty-first century COE (Center of Excellence) program, Ministry of Education, Culture, Sports, Science and Technology (2002–2006 Nishihira Project: Promotion of Health and Sport Scientific Research).

References

1. **Grundy SM, Brewer HB Jr, Cleeman JI, Smith SC Jr, Lenfant C.** Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association Conference on scientific issues related to definition. *Circulation.* 2004;109:433–8.
2. **Lakka HM, Laaksonen DE, Lakka TA, et al.** The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. *JAMA.* 2002;288:2709–16.
3. **Hu G, Qiao Q, Tuomilehto J, Balkau B, Borch-Johnsen K, Pyorala K.** Prevalence of the metabolic syndrome and its relation to all-cause and cardiovascular mortality in nondiabetic European men and women. *Arch Intern Med.* 2004;164:1066–76.

4. **Lorenzo C, Okoloise M, Williams K, Stern MP, Haffner SM.** The metabolic syndrome as predictor of type 2 diabetes: the San Antonio Heart Study. *Diabetes Care.* 2003;26: 3153–9.
5. **Laaksonen DE, Lakka HM, Niskanen LK, Kaplan GA, Salonen JT, Lakka TA.** Metabolic syndrome and development of diabetes mellitus: application and validation of recently suggested definitions of the metabolic syndrome in a prospective cohort study. *Am J Epidemiol.* 2002;156:1070–7.
6. **National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III).** Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation.* 2002;106:3143–21.
7. **Lakka TA, Laaksonen DE, Lakka HM, et al.** Sedentary lifestyle, poor cardiorespiratory fitness, and the metabolic syndrome. *Med Sci Sports Exerc.* 2003;35:1279–86.
8. **Jurca R, Lamonte MJ, Church TS, et al.** Associations of muscle strength and fitness with metabolic syndrome in men. *Med Sci Sports Exerc.* 2004;36:1301–7.
9. **Farrell SW, Cheng YJ, Blair SN.** Prevalence of the metabolic syndrome across cardiorespiratory fitness levels in women. *Obes Res.* 2004;12:824–30.
10. **Carnethon MR, Gidding SS, Nehgme R, Sidney S, Jacobs DR Jr, Liu K.** Cardiorespiratory fitness in young adulthood and the development of cardiovascular disease risk factors. *JAMA.* 2003;290:3092–100.
11. **LaMonte MJ, Barlow CE, Jurca R, Kampert JB, Church TS, Blair SN.** Cardiorespiratory fitness is inversely associated with the incidence of metabolic syndrome: a prospective study of men and women. *Circulation.* 2005;112:505–12.
12. **Katzmarzyk PT, Leon AS, Wilmore JH, et al.** Targeting the metabolic syndrome with exercise: evidence from the HERITAGE family study. *Med Sci Sports Exerc.* 2003;35:1703–9.
13. **Watkins LL, Sherwood A, Feinglos M, et al.** Effects of exercise and weight loss on cardiac risk factors associated with syndrome X. *Arch Intern Med.* 2003;163:1889–95.
14. **Frank LL, Sorensen BE, Yasui Y, et al.** Effects of exercise on metabolic risk variables in overweight postmenopausal women: a randomized clinical trial. *Obes Res.* 2005;13:615–25.
15. **Grundy SM, Hansen B, Smith SC Jr, Cleeman JI, Kahn RA.** Clinical management of metabolic syndrome: report of the American Heart Association/National Heart, Lung, and Blood Institute/American Diabetes Association Conference on scientific issues related to management. *Circulation.* 2004; 109:551–6.
16. **Japan Society for the Study of Obesity.** Examination Committee of Criteria for 'Obesity Disease' in Japan: new criteria for 'obesity disease' in Japan. *Circ J.* 2002;66:987–92.
17. **Examination Committee of Criteria for 'Metabolic Syndrome' in Japan.** Criteria for 'metabolic syndrome' in Japan [in Japanese]. *J Jpn Soc Intern Med.* 2005;94:188–203.
18. **Yoshizumi T, Nakamura T, Yamane M, et al.** Abdominal fat: standardized technique for measurement at CT. *Radiology.* 1999;211:283–6.
19. **Olson MS, Williford HN, Blessing DL, Greathouse R.** The cardiovascular and metabolic effects of bench stepping exercise in females. *Med Sci Sports Exerc.* 1991;23:1311–7.
20. **Borg G.** Perceived exertion: a note on 'history' and methods. *Med Sci Sports.* 1973;5:90–3.
21. **Alberti KGM, Zimmet P, Shaw J.** The metabolic syndrome: a new worldwide definition. *Lancet.* 2005;366:1059–62.
22. **Word Health Organization.** *Definition, diagnosis and classification of diabetes mellitus and its complications: report of a WHO consultation. Part 1: diagnosis and classification of diabetes mellitus.* http://whqlibdoc.who.int/hq/1999/WHO_NCD_NCS_99.2.pdf (Accessed March 30, 2006).
23. **International Diabetes Federation.** *The IDF consensus worldwide definition of the metabolic syndrome.* http://www.idf.org/webdata/docs/Metac syndrome def.pdf (Accessed March 30, 2006).
24. **Nakamura T, Tsubono Y, Kameda-Takemura K, et al.** Magnitude of sustained multiple risk factors for ischemic heart disease in Japanese employees: a case-control study. *Jpn Circ J.* 2001;65:11–7.
25. **Despres JP, Lamarche B.** Effects of diet and physical activity on adiposity and body fat distribution: implications for the prevention of cardiovascular disease. *Nutr Res Rev.* 1993;6: 137–59.
26. **Williams MJ, Hunter GR, Kekes-Szabo T, et al.** Intraabdominal adipose tissue cut-points related to elevated cardiovascular risk in women. *Int J Obes Relat Metab Disord.* 1996;20:613–7.
27. **Okura T, Nakata Y, Tanaka K.** Effects of exercise intensity on physical fitness and risk factors for coronary heart disease. *Obes Res.* 2003;11:1131–9.
28. **Okura T, Nakata Y, Lee DJ, Ohkawara K, Tanaka K.** Effects of aerobic exercise and obesity phenotype on abdominal fat reduction in response to weight loss. *Int J Obes.* 2005;29:1259–66.

Med Mol Morphol (2007) 40:55–67
DOI 10.1007/s00795-007-0366-7

REVIEW

Makoto Nishida · Tohru Funahashi · Iichiro Shimomura

Pathophysiological significance of adiponectin

Received: March 13, 2007 / Accepted: March 16, 2007

Abstract Adipose tissue, which classically has been considered as an energy-storing organ, is now viewed as a massive source of bioactive substances such as leptin, tumor necrosis factor (TNF)-α, and adiponectin. Adiponectin was discovered to be the most abundant adipose-specific transcript. Its function had been unclear, but epidemiological and clinical studies have demonstrated that serum levels of adiponectin are inversely associated with body weight, especially abdominal visceral fat accumulation. In addition, adiponectin was inversely related to cardiovascular risk factors, such as insulin resistance, blood pressure, and low-density lipoprotein (LDL) cholesterol and triglyceride levels, and was positively related to high-density lipoprotein (HDL) cholesterol levels. Moreover, low adiponectin concentration is associated with a high incidence of cardiovascular disease (CVD), diabetes, some kinds of cancer, and other various diseases. These associations suggest the clinical significance of adiponectin, and a number of investigations are now being conducted to clarify the biological functions of adiponectin. Recent studies have revealed that adiponectin exhibits antiinflammatory, antiatherogenic, and antidiabetic properties. In addition, adiponectin has been thought to be a key molecule in "metabolic syndrome," which is an epidemiological target for preventing cardiovascular disease. Various functions of adiponectin may possibly serve to prevent and treat obesity-related diseases and CVD. Furthermore, enhancement of adiponectin secretion or action may become a promising therapeutic target.

Key words Adiponectin · Visceral fat · Adipocytokine · Cardiovascular disease · Metabolic syndrome

M. Nishida (✉)
Health Care Center, Osaka University 1-17 Machikaneyama, Toyonaka, Osaka 560-0043, Japan
Tel. +81-6-6850-6015; Fax +81-6-6850-6040
e-mail: makoton@imed2.med.osaka-u.ac.jp

T. Funahashi · I. Shimomura
Department of Metabolic Medicine, Graduate School of Medicine, Osaka University, Osaka, Japan

Introduction: the discovery of adiponectin

Obesity, in particular, abdominal visceral fat accumulation, is an important risk factor for hyperlipidemia, diabetes mellitus, hypertension, cardiovascular disease (CVD), and some kinds of cancer. However, the molecular mechanism underlying these linkages had not been previously elucidated. We investigated the characteristics of adipose tissue by analyzing the gene expression profile in visceral and subcutaneous fat in the human complementary DNA project.[1] Of approximately 1000 independent clones, 60% of the whole genes were already identified as known human genes. The remaining 40% of genes were novel genes.[2] Although adipose tissue was considered as an energy-storing organ, we found unexpectedly high frequencies of the genes encoding secretory proteins.[3] In subcutaneous adipose tissue, approximately 20% of all known genes were the genes encoding secretory protein (Fig. 1).[2,3] Furthermore, its frequency reached approximately 30% in visceral adipose tissue. In addition, leptin and tumor necrosis factor (TNF)-α had been well recognized as bioactive substances from adipose tissues that regulate the functions of other organs. We named these adipose tissue-derived bioactive substances adipocytokines,[3] although some of them are not cytokines according to the classical category.

We identified the gene that expressed most abundantly and specifically in adipose tissue in 1996.[1] The molecule encoded by this gene, adipose most abundant gene transcript-1 (apM-1), possesses a signal peptide and collagen-like motif (Fig. 2).[1] We termed this matrix-like protein adiponectin. Adiponectin was independently isolated from human plasma as gelatin-binding protein-28.[4] The mouse homologue of adiponectin was cloned as ACRP30 and AdipoQ at the same time.[5,6] However, the significance of this novel molecule was unclear. Then, we developed a method for the measurement of plasma adiponectin levels using an enzyme-linked immunosorbent assay.[7] Measurement of plasma adiponectin revealed the clinical significance of adiponectin.

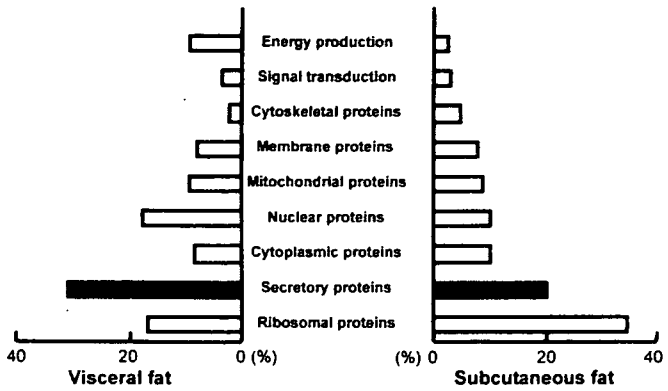

Fig. 1. The high frequency of genes for secretory proteins in adipose tissue. Although adipose tissue has been considered to be an energy-storing organ, an unexpectedly high frequency of the genes encoding secretory proteins is demonstrated. In subcutaneous adipose tissue, approximately 20% of all known genes are the genes encoding secretory protein. Furthermore, its frequency reaches approximately 30% in visceral adipose tissue. [Reproduced with permission from Maeda et al.[2] (1997) Gene 190:227–235; Funahashi et al.[3] (1999) Intern Med 38:202–206]

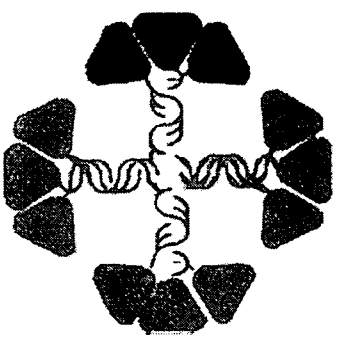

Fig. 2. Structure of adiponectin. This 244-amino-acid protein contains a signal sequence, and a collagen-like domain at the N-terminus and a C1q-like globular domain at the C-terminus. Some units of the trimer of adiponectin are bound in a bouquet-like formation in plasma. [Reproduced with permission from Matsuzawa et al.[10c] (2004) Arterioscler Thromb Vasc Biol 24:29–33]

Molecular characteristics of adiponectin

Structure

The gene encoding adiponectin is located on chromosome 3q27, and recent genome-wide scans have mapped a diabetes susceptibility locus to this chromosome.[8–10] This 244-amino-acid protein contains a signal sequence, and a collagen-like domain at the N-terminus and a C1q-like globular domain at the C-terminus (see Fig. 2).[1] The globular domain has sequence homology to collagens VIII and X and complement factor C1q. The crystal structure is similar to that of the TNF family, which has identical folding topologies and similar trimer interfaces.[11] Some units of the trimer of adiponectin are bound up in a bouquet-like formation.[12] Two major oligomeric forms of adiponectin, a hexamer and a 400-kDa high molecular weight (HMW) complex, exist in plasma. The HMW form of adiponectin has been shown to be more active than low molecular weight forms.[13] Hydroxylation and glycosylation of the lysine residues within the collagenous domain of adiponectin are critically involved in regulating the formation of its HMW oligomeric complex.[14]

Full-length adiponectin protein is proteolytically cleaved, with a smaller form, including the globular domain, although in very small amounts.[15] It is reported that the globular domain of adiponectin exhibits more extensive biological activity than the full-length form. However, further studies are needed to clarify the biologically active form of adiponectin and the relative abundance of the different cleavage products in plasma under physiological conditions.

Localization

Adiponectin messenger RNA is exclusively expressed in adipose tissue of humans[1,4] and experimental animals.[5,16] Recent evidence indicated that adiponectin can be produced by organs other than adipose tissue, such as bone marrow,[17] bone-forming cells,[18] fetal tissue,[19] myocytes, cardiomyocytes,[20] and salivary gland epithelial cells,[21] but the major source of plasma adiponectin in adults is the adipocyte. Plasma levels of adiponectin usually range from 3 to 30 μg/ml in adults, whereas adiponectin levels in umbilical venous blood from human fetuses was about 30 μg/ml.[19] Adiponectin was detected in several fetal tissues at mid- and late gestation (from 14 to 36 weeks) but not in the placenta. Adiponectin was detected in human fetal tissues of mesodermic origin, such as brown and white adipocytes, skeletal muscle fibers of diaphragm and iliopsoas, smooth muscle cells of small intestine and arterial walls, perineurium and renal capsule, and tissues of ectodermal origin, such as epidermis and ocular lens. The distribution of adiponectin detection in nonadipose tissues showed a general decline during the progression of gestation.[19] It should be noted that these results do not necessarily demonstrate the production of adiponectin in nonadipose tissues but represent the existence of adiponectin that may adhere to the tissue. In mouse embryos, production of adiponectin was demonstrated in brown adipose tissues (BAT) and surrounding immature tissues using immunohistochemical staining and in situ hybridization.[22] This interspecies difference of fetal localization of adiponectin may be attributed to the small amount of BAT in humans. These studies suggest the adiponectin may have a role during fetal development.

Secretion

Recent studies established the concept that adipose tissue is not only a fuel storage depot but also a critical endocrine tissue secreting a variety of bioactive adipocytokines into the circulation. Despite the importance of adipocytokines in metabolism, the mechanism of secretion from adipocytes remains poorly elucidated. Cellular localization of adiponectin in the steady state is predominantly in the Golgi apparatus or trans-Golgi network (TGN).[23–25] Treatment of 3T3-L1

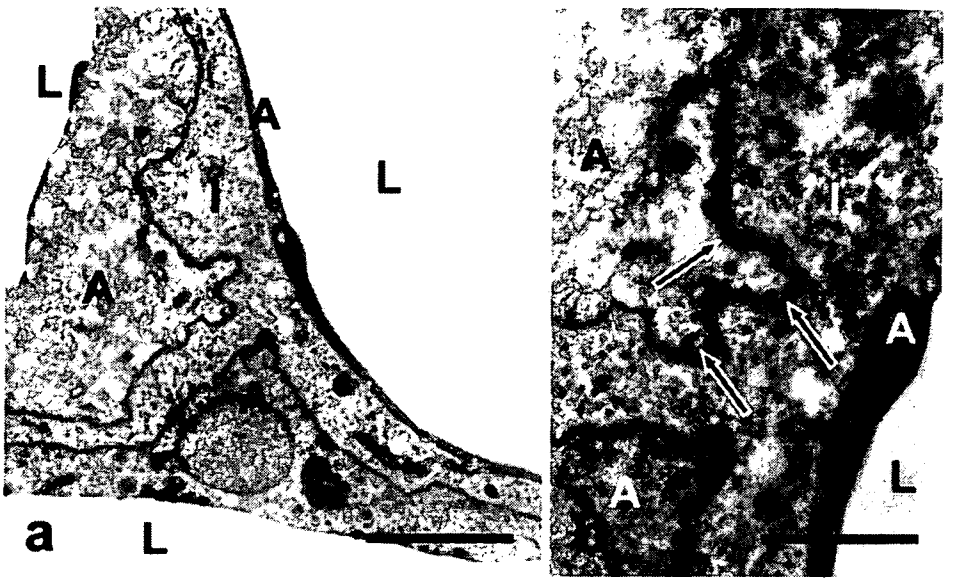

Fig. 3. Caveolae of adipocytes. Three human adipocytes are demonstrated in the *left panel* (a). Many caveolae on the cell membrane (*arrows*) are found at a higher magnification (b). Most studies of function of caveolae have focused on endocytosis and signal transduction. Further morphological studies in adipocytes as secretory cells may reveal a new secretory mechanism of adipocytokines including adiponectin. *A*, adipocytes; *I*, interstitial tissue; *L*, lipid droplet. *Bars* a 2 μm; b 1 μm

adipocytes with brefeldinA (BFA), an inhibitor of the post-Golgi trafficking pathway, severely inhibited the secretion of adiponectin.[24,25] There should be secretory pathways of adiponectin from Golgi/TGN to the cell surface. Moreover, fractional analyses demonstrated that the adiponectin fraction overlapped with transferrin receptor-positive membranes, indicating that secretory pathways of adiponectin involve the transferrin receptor-positive endosomal system.[25] Generally, some secretory cargo can traffic to the cell surface via the endosomal system.[26,27] The traffic in the endosomal system is controlled by a variety of small molecular weight GTPases of both the Rab and ADP ribosylation factor (Arf) classes.[28–31] Rab11 has been involved in insulin-dependent trafficking in adipocytes.[32] Arf6 controls cell phosphatidylinositol-(4,5)-bisphosphate levels in the plasma membrane and may be involved in regulated exocytosis.[33] In addition, Arf6 may play a role in the recycling of endosomal components with the plasma membrane.[34,35] In 3T3-L1 adipocytes, it has been demonstrated that Rab11 and Arf6 are important mediators of constitutive and insulin-stimulated secretion of adiponectin.[25]

Regulated exocytosis in adipocytes has been investigated by insulin-stimulated secretion of peptides and recycling of vesicles containing GLUT4 to the cell surface.[24] A confocal microscopic study demonstrated that the subcellular distributions of adiponectin and GLUT4 are distinct and non-overlapping,[23] although some molecules have been shown to regulate both adiponectin and GLUT4, implying the existence of common trafficking pathways between adiponectin and GLUT4. One molecule is the GGA1 (for Golgi localizing γ-adaptin ear homology domain ARF-binding protein) protein, which is a monomeric clathrin adaptor that mediates sorting at the TGN of specific cargo in an Arf-dependent manner. Inhibition of GGA1 blocks both traffic of the GLUT4 to its insulin-sensitive intracellular compartment and secretion of adiponectin (but not leptin).[24] It is also speculated that GGA proteins regulate selective cargo formation at the TGN and that insulin may act via a distal (post-TGN) compartment.[24] Another molecule is a kind of v-SNARE. It is suggested that the v-SNARE Vti1a is likely regulating a common and early step in the trafficking of both adiponectin and GLUT4 in 3T3-L1 adipocytes.[36]

There are many caveolae in the cell membrane of adipocytes[37] (Fig. 3). Most studies on the functions of caveolae have focused on endocytosis and signal transduction. Regarding secretion, cholesterol efflux mediated by caveolae or rafts has been investigated. Further morphological studies in adipocytes as secretory cells may reveal a new secretory mechanism of adipocytokines, including adiponectin.

Receptor

Two adiponectin receptors were identified in 2003.[38] AdipoR1 is a receptor for globular adiponectin that is abundantly expressed in skeletal muscle. AdipoR2 is a receptor for full-length adiponectin that is mainly expressed in the liver. Expression of AdipoR1 and -R2 was also detected in the hypothalamus, and increased AdipoR2 expression was found in the paraventricular nucleus (PVN), which may be involved in energy regulation.[39] These molecules are distantly related to the family of seven-transmembrane-spanning G protein-coupled receptors. They have an inverted topology with the N-terminus intracellular and the extracellular portion being small, as distinct from members of this class of receptors that bind peptide hormones.[38,40] Further studies are needed to elucidate the physiological role and the signal transduction pathways of these receptors.

T-cadherin has been demonstrated as a receptor for hexameric and HMW adiponectin but not for trimeric or globular adiponectin.[40] T-cadherin is a glycosylphosphatidylinositol (GPI)-anchored extracellular protein. Tissue distribution of T-cadherin is widespread in cardiovascular system, nervous system, and muscle. T-cadherin is involved in signal transduction in addition to cell–cell ad