Diabetes and functional disability

One of the most serious geriatric symptoms is functional disability. Cross-sectional studies in the USA showed that diabetes was associated with a twofold increased risk of being unable to perform daily physical tasks.[5,6] Gregg *et al.* showed that, among 6588 community-dwelling individuals aged 60 years or more, 32% of women and 15% of men reported an inability to walk one-quarter of a mile, do housework or climb stairs, compared with 14% of women and 8% of men without diabetes, respectively.[5] The Women's Health and Aging Study reported that women with diabetes had a 1.6-fold greater risk of basic activities of daily living (bADL) disability (bathing, transferring from bed to chair, using the toilet, dressing and eating) and a 2.3-fold greater risk of severe walking limitation.[6] We assessed the disability of 1135 elderly diabetic outpatients using the Tokyo Metropolitan Institute of Gerontology Index of Competence (TMIG-IC; 13 items), which included instrumental activities of daily living (IADL) (tasks of using public transportation, shopping for daily necessities, preparing meals, paying bill and handling one's own banking), intellectual activity (ability to complete the pension form; to read newspapers, books or magazines; and to be interested in news stories or programs dealing with health) and social role.[7] The prevalence of disability on at least one item of the TMIG-IC was approximately 45%. When we divided the subjects into three age groups, the oldest group (aged ≥80 years) reported a higher prevalence of disabilities for IADL, such as using public transportation, shopping, preparing meals and paying bills, compared with the youngest group (aged 65–69 years) (Fig. 1). A prospective study of women aged 65 years or more in the Study of Osteoporosis Fractures also demonstrated that diabetes was associated with a 2- to 2.5-fold increased incidence of functional disability for doing housework or walking two or three blocks.[8] Coronary heart disease (CHD), obesity (women), stroke (men), peripheral artery disease (PVD), neuropathy, arthritis and depression were important contributors to diabetes-related functional disabilities in US studies.[5,8] In contrast, in a Japanese study, a low sense of well-being, insulin treatment, cognitive impairment and visual impairment were associated with functional disabilities after adjustment for age, sex, body mass index (BMI), duration of diabetes, HbA1c, microangiopathy and macroangiopathy.[7] In addition to diabetic complications, aging per se contributed to diabetes-related disability, which may lead to difficulty in performing self-care activities such as exercise and diet therapy.

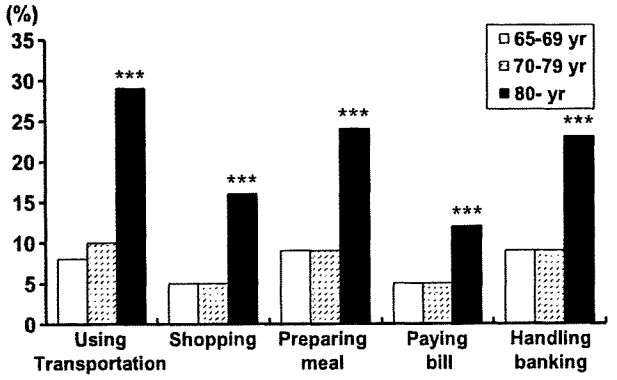

Figure 1 Age-related changes in functional disabilities of elderly patients with diabetes mellitus. The oldest group (aged ≥80 years) reported a higher prevalence of disabilities on tasks for instrumental activities of daily living: public transportation, shopping, preparing meals, paying bills and handling banking) than the youngest group (aged 65–69 years). ***$P < 0.001$.

Diabetes and depression or low sense of well-being

The prevalence and incidence of depressive symptoms are greater in diabetic than in non-diabetic people. Approximately 30% of people with diabetes have depressive symptoms, while 5–10% have major depression.[9] The Health, Aging, and Body Composition Study, a cohort study of subjects aged 70–79 years, showed that diabetic people had an increased incidence of depressed mood compared with those without diabetes (23.5% vs 19.0%; hazard ratio [HR], 1.31; 95% confidence interval [CI], 1.07–1.61).[10] A community-based study in Spanish elderly subjects demonstrated that diabetes was associated with an increased risk of prevalent depression (odds ratio [OR], 1.47; CI, 1.16–1.83) and incident depression (HR, 1.40; CI, 1.03–1.90).[11] Because the presence of comorbid diseases increased the risk of incident depression, diabetes may play a role in the development of depression in the elderly.

The presence of high levels of depressive symptoms based on the Center for Epidemiologic Studies Depression Scale (CES-D) score of 16 or more was associated with diabetic complications, any activities of daily living (ADL) or IADL disabilities, urinary incontinence, visual impairment, poor perceived health status and increased number of hospitalizations.[9]

The impact of depression on disability is much greater in diabetic patients than in non-diabetic subjects. With no diabetes and no major depression as references, the adjusted OR of functional disability was 7.2 for people with diabetes and comorbid major depression, which was high compared with the OR of 2.4 for people with diabetes alone and 3.0 for those who had major depression alone.[12]

Reduced well-being or quality of life should be considered as part of the geriatric syndrome. When well-being, which included the concept of positive feeling of

happiness, life satisfaction and acceptance of aging as well as negative affects, was assessed using the Philadelphia Geriatric Center (PGC) morale scale in elderly people with diabetes, approximately 19% of them had a low sense of well-being (PGC morale score, ≤7). People with a low sense of well-being had 2- to 3-fold increased odds for disability on the TMIG-IC, except for shopping and use of transportation.[7] Multivariate analysis revealed that sex (female), macroangiopathy, high frequency of hypoglycemia, negative social support and reduced positive social support were independently associated with a low sense of well-being.[13]

Interestingly, a low sense of well-being was an independent predictor of stroke after adjustment for conventional risk factors in our 3-year follow-up study.[14] Similarly, depression also predicted stroke and cardiovascular disease independent of the other risk factors.[15,16] The association between depression or a low sense of well-being and atherosclerotic disease may be explained by the activation of platelets,[17] an activated sympathetic nervous system and the increased expression of inflammatory markers. The use of specific antidepressant agents, such as selective serotonin reuptake inhibitors (SSRI), inhibited the activation of platelets in patients with depression[17] and prevented the development of myocardial infarction.[18]

Depression predicted increased mortality, greater incidences of macro- and microvascular complications, and disability in ADL in older people with type 2 diabetes mellitus.[19] The interaction between depression and diabetes in old people (aged ≥65 years) was found to have a synergistic effect on adverse outcomes. Therefore, aggravation of psychological function in diabetic people may lead to adverse outcomes (micro- and macrovascular complications, disabilities and increased mortality) through multifactorial mechanisms.

Diabetes and falling

Falls may lead to fractures, aggravation of glycemic control and reduction of quality of life in diabetic people. Even non-injurious falls can result in a post-fall syndrome characterized by anxiety and reduced physical and social activities.

A growing amount of evidence suggests that diabetes mellitus is one of the major predictors of the risk of falling.[20,21] The Study of Osteoporotic Fractures, which involved 9247 women aged 67 years or more, showed that 18% of older women fell more than once a year. They showed that both non-insulin-treated and insulin-treated people with diabetes had an increased risk of falling compared with non-diabetic people (OR, 1.68; CI, 1.37–2.07; and OR, 2.78; CI, 1.82–4.25, respectively).[20] The Women's Health and Aging Study demonstrated that diabetes was associated with an increased risk of falling among disabled old women.[21] These studies show that poor lower extremity function, poor balance, a history of CHD, a history of arthritis, musculoskeletal pain, depression, poor vision, medication and peripheral neuropathy, overweight and insulin therapy are important predictors of falling among diabetic women.[20,21]

The increased risk of falling may be partially explained by the impairment of gait balance and gait in diabetic people.[22–24] A study reported that diabetic patients had poorer balance during standing in diminished light, and increased sway during standing.[22] Diabetes was associated with an increased risk of gait disturbance due to Parkinson's disease-like symptoms in a 9-year follow up of people aged 75 years and over.[23] Diabetic individuals with peripheral neuropathy had impaired peripheral sensation and reaction time, and had impaired ability to stabilize their body when walking on irregular surfaces.[24] They also had reduced walking speed and step length, and less rhythmic acceleration patterns at the head and pelvis compared with controls.

Diabetes and urinary incontinence

Diabetes is associated with increased risk of both stress incontinence and urge incontinence.[25–27] Furthermore, overflow incontinence can occur as a complication of autonomic neuropathy in diabetic people. Diabetic women have a threefold increased prevalence of urge incontinence and twice the prevalence of stress incontinence.[25] The association between diabetes and urinary incontinence was confirmed with a prospective study of 81 854 women for any incontinence (HR, 1.21; CI, 1.02–1.43) and for severe incontinence (HR, 1.40; CI, 1.15–1.71).[26] A cross-sectional study of postmenopausal women aged 55–75 years showed that 52% of diabetic women with diabetes had some form of incontinence in the past month and 15% had severe incontinence, and that diabetic women were more likely to have severe incontinence or mixed incontinence.[27] The study also showed that diabetes duration, neuropathy, retinopathy and a history of urinary tract infection were independently associated with severe incontinence in multivariate analysis, and that the association decreased after adjustment for BMI. In contrast, a lifestyle intervention that included weight reduction decreased the frequency of stress incontinence in the Diabetes Prevention Program Trial.[28]

Diabetes mellitus and malnutrition

Elderly people with diabetes may be at risk of malnutrition as compared with non-diabetic people. A case-control study showed that community-dwelling diabetic people had significantly lower scores of Mini-Nutritional Assessment (MNA) than non-diabetic subjects.[29] Anorexia due to morbidity (infectious disease,

end-stage renal failure or malignancy), drug adverse effects and excessive dietary restriction may be responsible for the malnutrition in older diabetic people. Because subclinical deficiencies in vitamin B groups (B1, B2, B12, B6 and folate) in the elderly are associated with cognitive impairment or decline,[30] sufficient intake of vitamin and micronutrients may be necessary in elderly patients with diabetes.

Even diabetic patients who are obese or have metabolic syndrome can be modified and overshadowed by malnutrition, which is associated with low BMI and low levels of leptin and insulin.[31] Sarcopenic obesity, which is defined as excess fat with loss of lean body mass, may be a major problem in the diabetic patients. Sarcopenic obesity preceded the onset of IADL disability in a community-dwelling elderly population.[32] The increased production of inflammatory adipokines may alter insulin sensitivity and muscle mass and strength in sarcopenic obesity in diabetic patients.

Malnutrition in the elderly leads to increased mortality, disability and life-threatening complications.[33,34] Because we found that people who had serum albumin levels of less than 3.5 mg/dL showed increased mortality in a 6-year follow-up study of 422 elderly diabetic patients (HR, 4.2; CI, 1.3–13.9) (A Araki *et al.* unpublished data) malnutrition should be included as important geriatric symptoms.

Diabetes and cognitive impairment

Diabetes mellitus is associated with moderate deficits in specific cognitive function domains, such as complex psychomotor skills, speed of information processing, and memory and learning.[35–37] Epidemiological studies have shown that the diabetic population has a 1.6- to 3.0-fold increased risk for Alzheimer's-type dementia and vascular dementia.[38,39] Hyperglycemia,[35–37] advanced glycation end products,[40] recurrent severe hypoglycemia,[41] symptomatic and asymptomatic cerebrovascular disease,[37] polyneuropathy,[42] insulin treatment,[38] hyperinsulinemia or insulin resistance,[43] depression,[44] and low serum albumin[44] were associated with cognitive impairment in diabetes mellitus. Cognitive impairment was predicted by brain structural changes, subcortical atrophy and subcortical white matter hyperintensity, cortical atrophy in the parietal lobe and thalamus, as well as cortical atrophy.[45] Probably decreased cerebral blood flow and hyperglycemia-induced metabolic derangement are involved in the pathogenesis of the diabetic complications in the central nervous system, which refers to diabetic encephalopathy. Cognitive function may be one of the important factors related to poor adherence to diabetic self-care activities, increased frequency of hospitalization, and increased need for assistance in personal care in older adults with diabetes.[46]

Glycemic control in the short term has some favorable effects on cognitive function in diabetic people. In particular, moderate impairment of learning, memory, and complex psychomotor skill was partially improved by glycemic control with oral drugs or insulin therapy for 3 weeks.[40,47]

Although insulin-treated diabetic patients had an increased risk of cognitive impairment or dementia,[38,39] the association may be explained by increased cerebral complications rather than effects of insulin in insulin-treated patients. In contrast, a defect in insulin signaling or insulin resistance in the brain has been proposed as one cause of Alzheimer's disease.[48] Treatment with intranasal insulin, which selectively acted on the central nervous system, improved the impairment of memory saving, attention and functional status in patients in the early stage of Alzheimer's disease.[49] Therefore, insulin may have some beneficial effects on cognition.

Glucose control and geriatric syndrome

Remarkable hyperglycemia may directly cause several forms of geriatric syndrome: functional disability due to general malaise, urinary incontinence due to polyuria, malnutrition due to increased protein catabolism and cognitive impairment. Poor glucose control in the long term also potentially affects forms of geriatric syndrome such as cognitive function and susceptibility to infection.

There is limited data as to what level should be an appropriate treatment goal of HbA1c for elderly people with diabetes. Gao *et al.* demonstrated that, in a longitudinal study of 1139 people aged 65 years and over in England and Wales, diabetic individuals who had HbA1c levels of 7.0% or had a significantly higher risk of all-cause and cardiovascular mortality, and dementia compared with the three tertiles of the subjects (HbA1c: 3.7%–5.2%, 5.3%–5.7%, and 5.8%–6.9%), suggesting a HbA1c goal of 7.0% or less.[50]

Interestingly, glucose control may affect the incidence of falling in people with diabetes. The Health, Aging and Body Composition Study involving a cohort of well-functioning older adults showed that, among those using insulin, HbA1c of 6% or less increased the risk of falls, although no association between HbA1c level and oral hypoglycemic medications was observed.[51] Nelson *et al.* pointed out that the risk of falling in community-dwelling diabetic people aged 75 years or more markedly increased when HbA1c was 7% or less, regardless of frailty status.[52] Although the frequency of hypoglycemia was not assessed in these studies, atypical hypoglycemic symptoms (e.g. unsteadiness, poor coordination, double vision and dizziness) have been considered to cause falling in elderly diabetic individuals.[53] Severe hypoglycemia may lead to transient depression as well as cognitive impairment.[54] Therefore, we consider that, for

well-functioning diabetic people free of geriatric syndrome, a HbA1c level between 6.5% and 7.0% would be an ideal goal in order to prevent severe hypoglycemia, diabetic complications, dementia and death.

In contrast, for elderly people with multiple symptoms of geriatric syndrome (i.e. geriatric syndromes) and multiple morbidities, the glucose control should be individualized. The "Guidelines for improving the care of older persons with diabetes mellitus" proposed that treatment goals for blood glucose in older people with diabetes may be individually determined based on age, life expectancy, patient preference and the presence of geriatric syndrome: depression, pain, falls, incontinence, polypharmacy and cognitive impairment.[55,56] Huang *et al.* calculated expected benefits of intensive glucose control (HbA1c level of 7.0%) versus moderate glucose control (HbA1c level of 7.9%) in a diabetic population 60–80 years of age as a quality-adjusted life expectancy from a decision analysis.[57] They showed that the expected quality of life benefit of intensive control was 106 days at 60–64 years of age and decreased to 52 days at 75–79 years of age with no comorbid illness or functional impairment. They also demonstrated that for people at 60–64 years of age who had had diabetes for 10–15 years the expected benefits decreased from 116 days for those who were at baseline good health (life expectancy, 13.5 years) to 36 days for those with 4 additional mortality index points, which was calculated from comorbid illnesses and functional impairment (life expectancy, 8.0 years) and to 8 days for those with 8 additional mortality index points (life expectancy, 3.9 years). Thus, in some diabetic patients with multiple morbidity and multiple functional impairments (such as dementia, disability and the other three serious diseases), the goal of a HbA1c level of less than 8.0% might be acceptable.

Geriatric syndromes and their risk factors

The prevalence, odds ratio and risk factors of the typical geriatric syndromes are shown in Table 1. Old diabetic people consistently have an increased risk of geriatric syndrome: functional disability, depression, falling, urinary incontinence, malnutrition and cognitive impairment. As shown in Figure 2, the aging per se, diabetic micro- and macrovascular complications (in particular autonomic neuropathy), hyperglycemia and hypoglycemia are risk factors for the geriatric syndromes. Multiple morbidity and lack of social support also may lead to the aggravation of geriatric syndrome in diabetic people. Some forms of geriatric syndrome such as depression and cognitive impairment adversely affect the risk factors: hyperglycemia and micro- and macrovascular complications to form a vicious cycle, leading to the increased mortality. The geriatric syndromes are multifactorial and interrelated, and share risk factors. For example, depression or reduced well-being is thought to be one of risk factors for disability, fall, cognitive impairment and malnutrition.

Assessment of geriatric syndrome

Based on the results discussed above, it is necessary to assess geriatric syndromes when treating diabetic patients, in particular old-old patients. Comprehensive geriatric assessment of geriatric syndromes including bADL, IADL, gait and balance, visual acuity, Mini-Mental State Examination, geriatric depression scores, history and risk of falling and urination status should be performed, as shown in Table 2. The assessment of family or social support, living accommodation and surroundings is also important. The measurement of both supine and standing blood pressures, residual urine volume, and electrocardiogram coefficient of variation of R-R variations may be helpful in the geriatric assessment of diabetic people because autonomic neuropathy may be involved in some geriatric syndromes.

Intervention in geriatric syndrome

The treatment of diabetic patients with geriatric syndrome should focus on a strategy for preventing the aggravation of geriatric syndrome.

The importance of exercise therapy, compared with diet therapy, may be greater in elderly than in younger patients. Muscle-strengthening training, as well as aerobic exercise, led by supervisors or exercise professionals is necessary in order to prevent the worsening of disability and to maintain good glycemic control.

Supervised resistance training for 16 weeks improved the muscle strength of the lower extremities, ADL and glycemic control in elderly patients with diabetes.[58] Supervised exercise may be one of the common strategies for the prevention of forms of geriatric syndromes (disability, depression, fall and cognitive impairment).

A fall prevention program should be implemented for diabetic individuals with geriatric syndrome. Multidisciplinary, multifactorial, health or environmental risk factor screening intervention programs; programs of muscle strengthening and balance training, home hazard assessment and withdrawal of psychotropic medication; and Tai Chi group exercise intervention may be effective in preventing falls in diabetic individuals.[59]

Psychological intervention,[60] such as counseling, group therapy, cognitive behavioral therapy, social support and exercise training,[61] may be necessary for the treatment of old diabetic patients with depressive symptoms or low sense of well-being. Anti-depressive medication, including SSRI drugs, may be indicated in

Table 1 Prevalence, odds ratio, and risk factors for geriatric syndromes in diabetic people

Geriatric syndrome	Ref	Prevalence or incidence	Odds ratio (95% CI)	Risk factors
Disability (inability on 1 or more tasks)	5	Men, 15.2% vs 7.8%; women, 32% vs 14.3%	Men, 2.71 (1.74–4.23); women, 3.27 (2.01–5.38)	CHD, stroke, arthritis, PVD, poor vision, CHD, high BMI, poor vision, stroke, arthritis
Disability (mobility)	6	Mobility disability, 62.2%; ADL disability, 41.2%	Mobility disability, 1.78 (1.06–2.97); ADL disability, 1.65 (1.08–2.52)	PVD, peripheral neuropathy, depression
Disability (any item)	7	45%		Old age, vascular complications, low well-being, low MMSE, low visual acuity, insulin treatment
Disability (any task)	8	Yearly incidence, 9.8% vs 4.8%	Incidence, 2.05 (1.77–2.37)	Old age, high BMI, CHD, arthritis, physical inactivity, severe visual impairment
Depression (CES-D ≥16)	9	31.1% vs 24.1%		CHD, kidney and eye problem, disability, hypertension, incontinence, visual impairment, poorer perceived health status, hospitalization
Depression (CES-D ≥10 or antidepressants)	10	23.5% vs 19.0% for 5.9 years	Incidence, 1.31 (1.07–1.61)	DM-related comorbidities
Depression (psychiatric diagnostic interview)	11	Prevalence, 15.4%, incidence, 16.5%	Prevalence, 1.47 (1.16–1.83; incidence, 1.40 (1.03–1.90)	
Fall (more than once a year)	20	Insulin-treated, 35.4% vs 17.0%; non-insulin-treated, 25.7% vs 17.0%	Insulin-treated, 3.98 (2.25–7.05); non-insulin-treated, 1.53 (1.14–2.04)	Balance, CHD, arthritis, peripheral neuropathy
Fall (any fall)	21	64.9% for 3 years	Incidence, 1.38 (1.04–1.81)	Insulin therapy, high BMI, lower extremity pain, poorer lower extremity performance
Incontinence (stress incontinence) (urge incontinence)	29	Stress incontinence, 30.2% vs 14.4%; urge incontinence, 7.7% vs 26.4%		Neuropathic pain, hysterectomy
Incontinence (very severe)	30	15% vs 7%	Prevalence, 1.78 (1.49–2.12); incidence, 1.97 (1.24–3.12)	Diabetes duration, treatment type, peripheral neuropathy, retinopathy
Dementia, Alzheimer's type, vascular type	38	4.2%	Alzheimer's type, 1.3 (0.9–1.9); vascular type, 2.1 (1.1–4.0)	Insulin treatment
Dementia, Alzheimer's type, vascular type (meta-analysis)	39		Alzheimer's type, OR = 1.4–2.4; vascular type, OR = 2.2–4.2	Stroke, hyperglycemia, insulin treatment, hypertension

ADL, activities of daily living; BMI, body mass index; CHD, coronary heart disease; CI, confidence interval; DM, diabetes mellitus; MMSE, Mini-Mental State Examination; OR, odds ratio; PVD, peripheral artery disease.

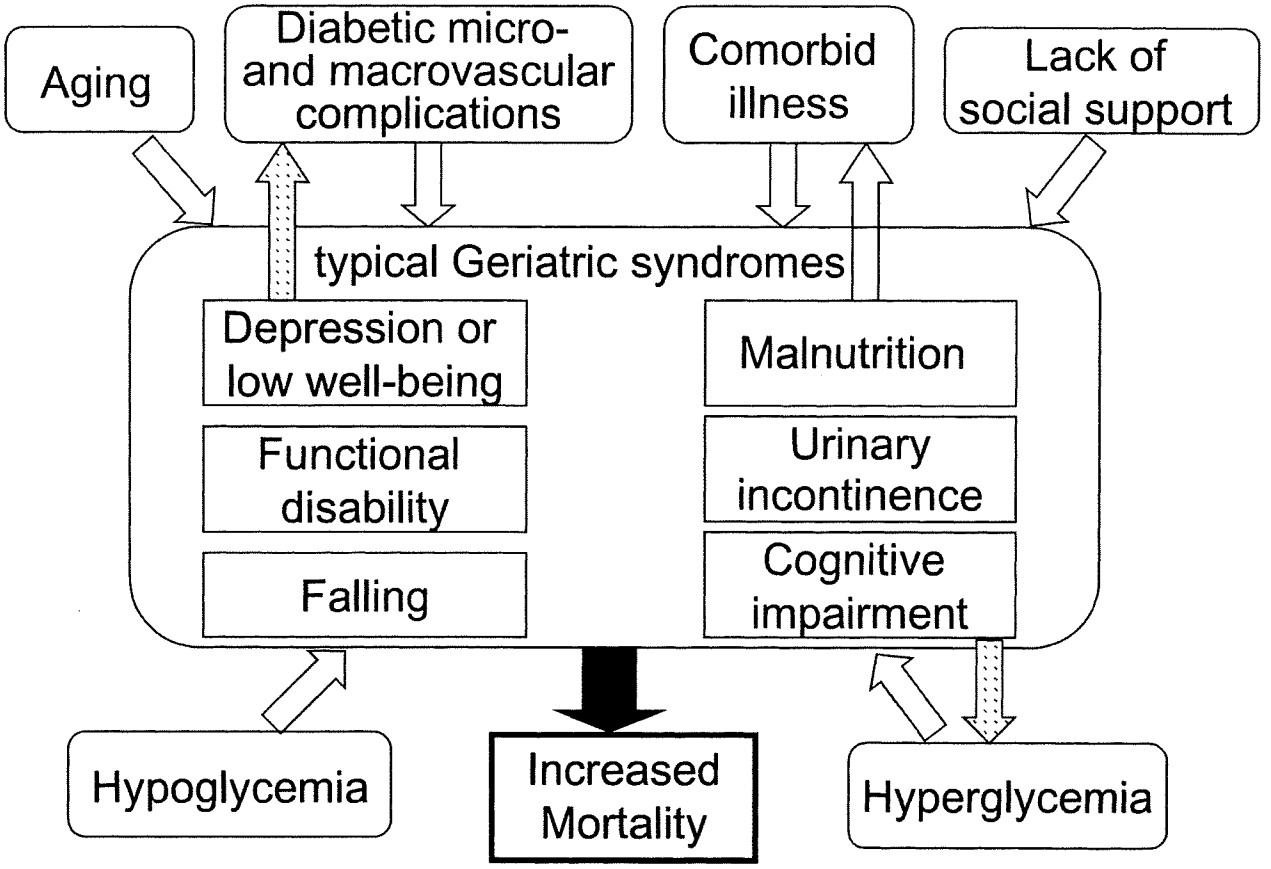

Figure 2 Relation between geriatric syndromes and their risk factors in elderly people with diabetes. Aging, diabetic micro- and macrovascular complications, hyperglycemia, hypoglycemia, multiple morbidity and lack of social support are risk factors for the geriatric syndromes. Some elements of geriatric syndrome such as depression adversely affect the risk factors: micro- and macrovascular complications a hyperglycemia to form a vicious cycle, leading to the increased mortality.

patients with diabetes and comorbid depression for the prevention of diabetic complications and increased mortality.

Diabetic women with urinary incontinence should be given exercise treatment, including pelvic floor muscle training,[62,63] weight reduction,[28] training mobility and toileting skills[64] for stress incontinence. Biofeedback therapy and behavioral training may be also effective for urge incontinence.[65]

Intensive care and social support are necessary for elderly people with diabetes and cognitive impairment. The management of cardiovascular risk factors, community-based supervised exercise, day-service activities and support for adherence to medication or insulin injection regimens may be helpful in the management of patients with both diabetes and cognitive impairment.

The avoidance of hypoglycemia and the ability to cope well with hypoglycemia may be also important for the prevention of falling and maintenance of well-being in elderly diabetic people. Prevention of hypoglycemia requires monitoring of HbA1c and self-monitoring of blood glucose, meticulous adjustment of sulfonylureas and insulin dosage, and educating patients, their families and care staff in coping skills for hypoglycemia and sick days.

Common strategy for geriatric syndrome

Because geriatric syndromes are multifactorial and share risk factors, a concentric approach, focusing on pathways associated with risk factor synergism, may be effective in the care of those who have geriatric syndromes.[4] Therefore, diabetic people with any geriatric symptoms could be treated with a multidisciplinary common concentric strategy: supervised exercise therapy including muscle-strengthening training, psychological support, social support for adherence to anti-diabetic medications or insulin, and good glycemic control with avoidance of hypoglycemia. Further studies are necessary including randomized trials of the efficacy of

Table 2 Assessment of typical geriatric syndromes in diabetes mellitus

Geriatric syndrome	Tools and risk assessment
Disability	Basic activities of daily living (ADL), instrumental ADL
Depression or low quality of life	15-Item Geriatric Depression Scale (GDS-15, GDS-5), PGC morale scale
Fall	Frequency of fall, gait, balance, blood pressures (supine and standing)
Urinary incontinence	Frequency and severity of incontinence, postvoid residual urine volume, nocturia
Dementia	Mini-Mental State Examination (MMSE)
Malnutrition	Subjective global assessment (SGA), Mini-Nutritional Assessment (MNA), objective data assessment (e.g. serum albumin, BMI, lymphocyte number)
Visual disturbance	Visual acuity

multidisciplinary common strategies based on geriatric syndrome in diabetic individuals.

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Cilostazol inhibits cytokine-induced nuclear factor-κB activation via AMP-activated protein kinase activation in vascular endothelial cells

Yoshiyuki Hattori*, Kunihiro Suzuki, Atsuko Tomizawa, Noriko Hirama, Toshie Okayasu, Sachiko Hattori, Hiroko Satoh, Kazumi Akimoto, and Kikuo Kasai

Department of Endocrinology and Metabolism, Dokkyo University School of Medicine, Mibu, Tochigi 321-0293, Japan

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Aims Cilostazol is a selective inhibitor of phosphodiesterase 3 that increases intracellular cyclic AMP (cAMP) levels and activates protein kinase A, thereby inhibiting platelet aggregation and inducing peripheral vasodilation. We hypothesized that cilostazol may prevent inflammatory cytokine induced-nuclear factor (NF)-κB activation by activating AMP-activated protein kinase (AMPK) in vascular endothelial cells.

Methods and results Cilostazol was observed to activate AMPK and its downstream target, acetyl-CoA carboxylase, in human umbilical vein endothelial cells (HUVEC). Phosphorylation of AMPK with cilostazol was not affected by co-treatment with an adenylate cyclase inhibitor, SQ 22536, and a cell-permeable cAMP analogue, pCTP-cAMP, did not induce AMPK phosphorylation and had no effect on cilostazol-induced AMPK phosphorylation, suggesting that cilostazol-induced AMPK activation occurs through a signalling pathway independent of cyclic AMP. Cilostazol also dose-dependently inhibited tumour necrosis factor alpha (TNFα)-induced NF-κB activation and TNFα-induced IκB kinase activity. Furthermore, cilostazol attenuated the TNFα-induced gene expression of various pro-inflammatory and cell adhesion molecules, such as vascular cell adhesion molecule-1, E-selectin, intercellular adhesion molecule-1, monocyte chemoattractant protein-1 (MCP-1), and PECAM-1 in HUVEC. RNA interference of AMPKα1 or the AMPK inhibitor compound C attenuated cilostazol-induced inhibition of NF-κB activation by TNFα.

Conclusion In the light of these findings, we suggest that cilostazol might attenuate the cytokine-induced expression of adhesion molecule genes by inhibiting NF-κB following AMPK activation.

1. Introduction

Treatment of thrombotic disease requires a delicate balance between the prevention of new thrombotic events and management of bleeding complications. Many antiplatelet and anticoagulant agents have been used to this end, with varying degrees of success. Among the many antiplatelet agents tested to date, cilostazol, which selectively targets phosphodiesterase 3 (PDE3), has several unique features. Cilostazol is classified as an antiplatelet agent because it inhibits the platelet aggregation induced by collagen, 5′-adenosine diphosphate (ADP), epinephrine, and arachidonic acid.[1] Unlike other antiplatelet agents, cilostazol not only inhibits platelet function, but also appears to have beneficial effects on endothelial cell function.[1] Since receiving approval in the USA for the treatment of intermittent claudication in 1999, cilostazol continues to demonstrate promise in the treatment of cardiovascular disorders. New data regarding the role of cilostazol in the prevention of recurrent cerebral infarction, the prevention of re-stenosis, and the treatment of periperal artery diseases, have increased recent interest in the drug.[2–5] However, because of the action mechanism of a PDE inhibitor, there have been concerns about the cardiovascular safety of cilostazol in patients with left ventricular dysfunction.[6,7] Furthermore, little is known about the clinical experience with cilostazol in acute myocardial infarction patients.[8]

Vascular endothelial cells play an important role in maintaining the antithrombotic properties of blood vessels, and functional damage to endothelial cells results in thrombus formation along the vessel wall.[9] The interaction that occurs between platelets and endothelium within the micro- and macro-vascular circulation has thrombotic effects by altering the status of platelet activation. Platelets, which are not activated by normal endothelium, are activated when they encounter activated endothelial cells following exposure to oxidative stress, for example, in patients with hypertension, diabetes mellitus, or hyperlipidaemia.[10]

* Corresponding author. Tel: +81 282 87 2150; fax: +81 282 86 4632.
E-mail address: yhattori@dokkyomed.ac.jp

The activated nuclear factor (NF)-κB has been identified in human atherosclerotic plaques, but is absent, or present only in very small amounts in vessels devoid of atherosclerosis.[11] A number of genes whose products have been implicated in the development of atherosclerosis are regulated by NF-κB. Various leukocyte adhesion molecules, such as the vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin, as well as various chemokines (chemoattractant cytokines), monocyte chemoattractant protein-1 (MCP-1), and IL-8, recruit circulating mononuclear leukocytes to the arterial intima.[12-14] The induction of other NF-κB-dependent genes, including tissue factors, might tip the pro/anti-coagulant balance of the endothelium towards coagulation. Still other products of target genes, including cyclin D1, may induce cell proliferation or stimulate cell survival at atherosclerotic deposits. Therefore, the coordinated induction of NF-κB-dependent genes might promote atherosclerosis.[15]

Cilostazol is unique in that it targets the endothelium, thereby inhibiting thrombosis and improving endothelial cell function, and reducing the number of partially activated platelets by interacting with activated endothelial cells. However, the exact mechanism by which cilostazol preserves endothelial function remains to be elucidated. In the present study, we hypothesized that cilostazol may prevent NF-κB activation in endothelial cells exposed to inflammatory cytokines. We examined the effects of cilostazol on NF-κB activation, as well as the expression of NF-κB-mediated genes, such as VCAM-1, ICAM-1, E-selectin, and MCP-1, in vascular endothelial cells. We found that cilostazol inhibits the cytokine-induced expression of pro-inflammatory and adhesion molecule genes by suppressing NF-κB activity via AMP-activated protein kinase (AMPK) activation and not via the cyclic AMP (cAMP)/protein kinase A (PKA) pathway.

2. Methods

2.1 Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA, USA) and cultured in EGM2 medium supplemented with 2% FCS in the standard fashion. The cells in this experiment were used within 3-4 passages and were examined to ensure that they demonstrated the specific characteristics of endothelial cells. SVEC4 cells (murine endothelial cell line; ATCC, Rockville, MD, USA) were also cultured in DMEM containing 10% FCS and observed to demonstrate the typical cobblestone morphological appearance of endothelial cells.[16] THP-1 cells, a human monocytic cell line (ATCC), were grown in RPMI-1640 medium containing 10% FCS.

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health and with the Declaration of Helsinki.

2.2 Western blot analysis

HUVEC treated with tumour necrosis factor alpha (TNFα) in the presence or absence of metformin for various intervals were lysed using cell lysis buffer (Cell Signalling, Beverly, MA, USA) with 1 mM PMSF. The protein concentration of each sample was measured using a Bio-Rad detergent-compatible protein assay. Subsequently, β-mercaptoethanol was added to a final concentration of 1%, after which each sample was denatured by boiling for 3 min. Samples containing 10 μg of protein were resolved by electrophoresis on 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinyldienefluoride (PVDF) membrane (Bio-Rad, Tokyo, Japan), after which they were incubated with anti-phospho-Thr-172 AMPK polyclonal antibody and anti-phospho-Ser-79 acetyl-CoA carboxylase (ACC) polyclonal antibody (1:1000, Cell Signalling). For the IκB experiments, the membranes were incubated with IκBα antibody or phospho-IκBα antibody (1:1000, Cell Signalling). The binding of each of these antibodies was detected using sheep anti-rabbit IgG horseradish peroxidase (1:20 000) and an ECL Plus system (Amersham, Buckinghamshire, UK).

2.3 Nuclear factor-κB activation

To study NF-κB activation, SVEC4 cells were stably transfected with a *cis*-reporter plasmid containing the luciferase reporter gene linked to five repeats of NF-κB binding sites (pNF-κB-Luc: Stratagene, La Jolla, CA, USA), as previously described.[17] For this, the pNF-κB-Luc plasmid was transfected, together with a pSV2neo helper plasmid, (Clontech, Palo Alto, CA, USA) into SVEC4 cells using a FuGEN 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany). The cells were then cultured in the presence of G418 (Clontech) at a concentration of 500 μg/mL, and the medium was replaced every 2-3 days. Approximately 3 weeks after transfection, G418-resistant clones were isolated using a cloning cylinder and analysed individually for the expression of luciferase activity. Several clones were also selected for the analysis of NF-κB activation. Luciferase activity was measured using a luciferase assay kit (Stratagene).

We also measured changes in the levels of NF-κB p50 and p65 in nuclear extracts from HUVEC using a transcription factor assay kit (Active Motif Japan, Tokyo, Japan). Nuclear extracts were prepared with a NE-PER nuclear extraction reagent (Pierce, Rockford, IL, USA), after which p50 and p65 were quantified using recombinant NF-κ B p50 and p65 protein (Active Motif) as the standard.

2.4 IκB kinase assay

IκB kinase (IKK) activity was examined using an immune complex kinase assay with GST-IκBα (1-55) as the substrate, as previously described.[18] Briefly, the cells were solubilized in ice-cold buffer, and then centrifuged at 15 000 × *g* for 20 min. IKKα and IKKβ were recovered from the cell lysate by immunoprecipitation, after which the immune complexes were incubated with 20 μL of reaction buffer containing 20 mM HEPES/NaOH (pH 7.4), 10 mM $MgCl_2$, 50 mM NaCl, 100 mM Na3VO4, 20 mM β-glycerophosphate, 1 mM DTT, 100 μM ATP, 0.1 μCi [γ-^{32}P]ATP, and 10 μg GST-IκBα (1-55), at 30°C for 20 min. Following SDS-PAGE, GST- IκBα phosphorylation was estimated using an Imaging plate (Fuji Film, Tokyo, Japan).

2.5 siRNA transfection

The day before transfection, the plates were inoculated with an appropriate number of HUVEC in serum-containing medium to ensure 50-70% confluence the following day. Control siRNA, and LKB1 siRNA (siL1 or siL2) or Ca^{2+}/calmodulin-dependent protein kinase β (CaMKKβ) siRNA (siC1 or siC2) (Santa Cruz Biotechnology, Santa Cruz, CA, USA and Dharmacon, Lafayette, CO, USA) mixed with siLentFect (Bio-Rad) were added to each plate of the cells at a concentration of 10 nM. Forty-eight hours after transfection, AMPK activation induced by cilostazol was assessed.

AMPK siRNA (Santa Cruz Biotechnology) mixed with siLentFect was added to SVEC4 cells at a concentration of 10 nM. Forty-eight hours after transfection, TNFα-induced NF-kB activity was compared with that of control SVEC4 cells.

2.6 Real-time PCR of human umbilical vein endothelial cells mRNA

For quantitative measurement of mRNA, 2 μg of total RNA was treated with DNase I for 15 min and subsequently used for cDNA synthesis. Reverse transcription was performed using a SuperScript

Pre-amplification System (Gibco-BRL, Gaithersburg, MD, USA) with random oligonucleotide primers. The following primers were used: ICAM-1 forward 5′-CCGGAAGGTGTATGAACTGA-3′, reverse 5′-GGCAG CGTAGGGTAAGGTT-3′; VCAM-1forward 5′-GGCAGAGTACGCAAACACT T-3′, reverse 5′-GGCTGTAGCTCCCCGTTAG-3′; E-selectin forward 5′-GCCTTGAATCAGACGGAAGC-3′, reverse 5′-TGATGGGTGTTGCGGTT TC-3′; MCP-1 forward 5′- CAAACTGAAGCTCGCACTCTC-3′, reverse 5′- GCTGCAGATTCTTGGGTTGTG-3′; PECAM forward 5′-CAAAGACAACC CCACTGAAGAC-3′, reverse 5′-CGCAATGATCAAGAGAGCAATG-3′; P-selectin forward 5′-AGACAGGCCACCGAATATGAG-3′, reverse 5′-GGCC GTCAGTCGAGTTGT-3′; and GAPDH forward 5′-GGAGAAGGCTGGG GCTCAT-3′, reverse 5′-TGATGGCATGGACTGTGGTC-3′. A typical reaction (50 μL) contained 1 of 50 of reverse transcription (RT)-generated cDNA and 200 nM of primer in 1× SYBR Green RealTime Master Mix (Toyobo, Tokyo, Japan) buffer. The PCRs were carried out in a LineGene system (BioFlux, Tokyo, Japan) under the following conditions: 95°C for 5 min, followed by 40 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s.

2.7 Adhesion assay under static conditions

THP-1 cells were labelled with BCECF-AM (Calbiochem, San Diego, CA, USA), placed on a confluent HUVEC monolayer (1×10^4 per well) in a 96-well plate (1×10^5 THP-1 cells per well), and allowed to adhere for 10 min. After non-adherent cells were removed, the fluorescent intensity of adhered and total cells applied to the well was measured with a fluorescence plate reader (Fluoroskan Asent FL, GMI, Inc., Ramsey, MN, USA). The ratio of adherent to total cells was expressed as adhesion (%).

2.8 Statistical analysis

Data are presented as the mean ± SEM. Multiple comparisons were evaluated by ANOVA followed by Fisher's protected least significant difference test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Cilostazol activates AMP-activated protein kinase in human umbilical vein endothelial cells

Treatment of HUVEC with cilostazol resulted in time-dependent activation of AMPK, as monitored by phosphorylation of AMPK and its down-stream target, ACC (*Figure 1A*). Phosphorylation of AMPK with cilostazol was not affected by co-treatment with an adenylate cyclase inhibitor SQ 22536 (*Figure 1B*). A cell-permeable cAMP analogue pCTP-cAMP (100 μM) did not induce AMPK phosphorylation, and had no effect on cilostazol-induced AMPK phosphorylation (*Figure 1B*). Thus, cilostazol activates AMPK independent of cAMP in vascular endothelial cells. AMPK is controlled by upstream kinases, which have been identified as LKB1 or CaMKKβ.[19] Both LKB1 and CaMKKβ are expressed in HUVEC (data not shown). In order to assess whether CaMKKβ or LKB1 might act as an AMPK kinase (AMPKK) in cilostazol-treated cells, we used a siRNA approach to knock down the expression of LKB1 or CaMKKβ. Compared with the results following transfection using control siRNA, cilostazol-induced AMPK activation was significantly reduced in cells treated with CaMKK siRNA (siC1 or siC2), but not in cells treated with LKB1 siRNA (siL1 or siL2) (*Figure 1C*). We also examined whether other PDE3 inhibitors activate AMPK. Neither milrinone nor vesnarinone could induce AMPK activation (*Figure 1D*).

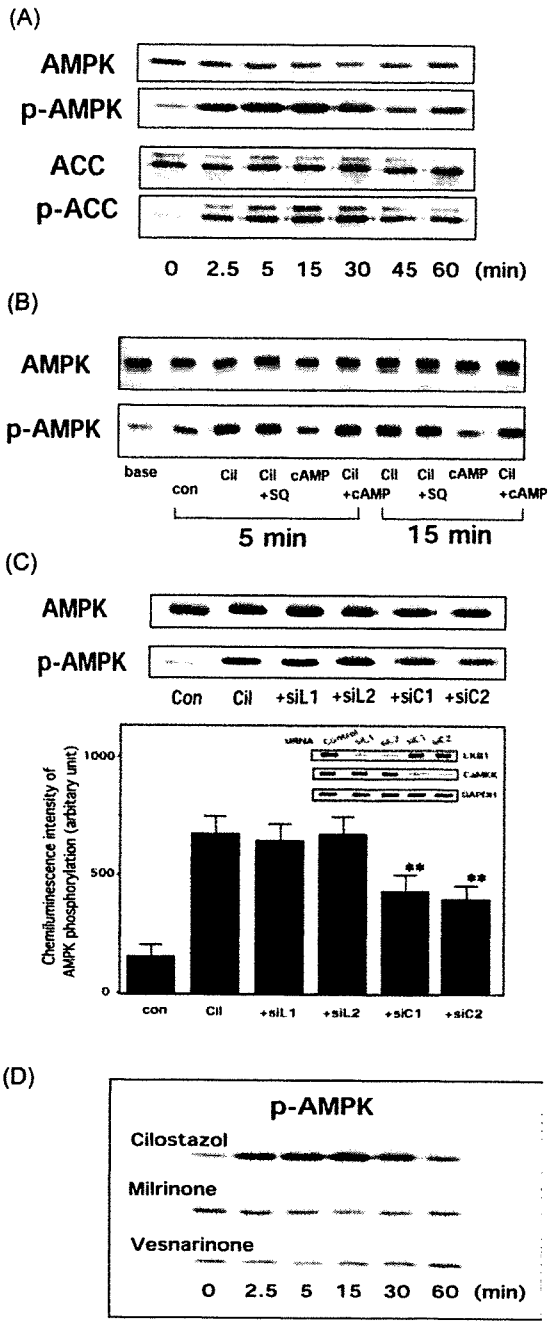

Figure 1 (*A*) Cilostazol activates AMP-activated protein kinase (AMPK) in vascular endothelial cells. Human umbilical vein endothelial cells (HUVEC) were treated with cilostazol (100 μM) for the indicated time periods before lysis, after which each cell lysate sample was probed with antibodies specific for phosphorylated forms of AMPK and acetyl-CoA carboxylase (ACC). (*B*) HUVEC were treated with cilostazol (100 μM) alone or in the presence of an adenylate cyclase inhibitor SQ 22536 (10 μM) or a cell-permeable cyclic AMP (cAMP) analogue pCTP-cAMP (100 μM). After 5 and 15 min of incubation, the cells were lysed and p-AMPK was analysed. Three independent studies showed similar results. (*C*) Cilostazol activates AMPK, which was significantly attenuated in HUVEC transfected with CaMKKβ siRNA (siC1 or siC2: 10 nM) but not with LKB1 siRNA (siL1 or siL2: 10 nM). Inset (lower figure): 48 h after cells were transfected with control siRNA, siL1, siL2, siC1, or siC2, the mRNA levels of LKB1, CaMKKβ, and GAPDH were determined. (*D*) Cilostazol, but not milrinone or vesnarinone, activates AMPK in vascular endothelial cells. HUVEC were treated with cilostazol (100 μM), milrinone (100 μM), or vesnarinone (100 μM) for the indicated time periods before lysis, after which each cell lysate sample was probed with antibodies specific for phosphorylated AMPK.

3.2 Cilostazol inhibits NF-κB activation and inhibits vascular cell adhesion molecule-1, E-selectin, intercellular adhesion molecule-1, monocyte chemoattractant protein-1, and PECAM-1 mRNA induction

We initially examined the effect of incubation of cilostazol with TNFα for 2 h on NF-κB activation in SVEC4 cells. TNFα induced a 7-fold increase in NF-κB-mediated reporter gene expression. Cilostazol dose-dependently suppressed TNFα-elicited activation of NF-κB (*Figure 2A*). We then examined the effect of siRNA specific for AMPKα1 on cilostazol-induced NF-κB inhibition, which was partially but significantly attenuated in AMPKα1 siRNA-transfected cells, compared with cells transfected with control siRNA (*Figure 2A*).

We also measured p50 and p65 in nuclear extracts from untreated HUVEC and from those treated with TNFα in the presence (30 or 100 μM) and absence of cilostazol. Both p50 and p65 markedly increased 30 min after stimulation with TNFα, from very low levels. This increase was dose-dependently inhibited by cilostazol (*Figure 2B*).

Incubation for 24 h with TNFα substantially induced the gene expression of VCAM-1, E-selectin, ICAM-1, and MCP-1. Induction of TNFα-induced gene expression was markedly suppressed by co-treatment with an NF-κB inhibitor, BAY11-7082, which is known selectively and irreversibly to inhibit cytokine-induced IκB phosphorylation,[20] suggesting that induction of these genes may be NF-κB-dependent (data not shown). Cilostazol significantly inhibited TNFα-induced gene expression (*Figure 2C*). We also examined the effect of cilostazol on TNFα-induced gene expression of PECAM-1 and P-selectin, adhesion molecules relevant in platelet–endothelium interaction. PECAM-1 mRNA was substantially induced by TNFα, which was clearly inhibited by cilostazol. Although P-selectin mRNA was modestly induced by TNFα, cilostazol did not affect the induction of P-selectin gene expression (*Figure 2C*).

3.3 Cilostazol inhibits adhesion of monocytic cells via suppressing NF-κB activation

Treating HUVEC with TNFα for 4 h significantly increased THP-1 cell adhesion. Pretreatment with cilostazol inhibited the TNFα-induced adhesion of THP-1 cell to HUVEC in a dose-dependent manner (*Figure 3A*). An NF-κB inhibitor BAY11-7082 markedly inhibited TNFα-induced THP-1 cell adhesion to HUVEC (*Figure 3A*). We then examined the effect of siRNA specific for AMPKα1 on cilostazol-induced inhibition of THP-1 cell adhesion, which was significantly attenuated in AMPKα1 siRNA-transfected cells, compared with cells transfected with control siRNA (*Figure 3B*).

3.3 TNFα stimulates IκB phosphorylation by inducing IκB kinase activity, while cilostazol inhibits TNFα-induced IκB kinase activity and IκB phosphorylation

We first determined whether TNFα-induced NF-κB activation might occur through phosphorylation and subsequent degradation of IκB. To determine whether TNFα might induce IκBα phosphorylation in HUVEC, western blot analysis using the antiphospho-Ser32 of IκBα antibody was performed. TNFα was observed to induce IκB phosphorylation within 15 min,

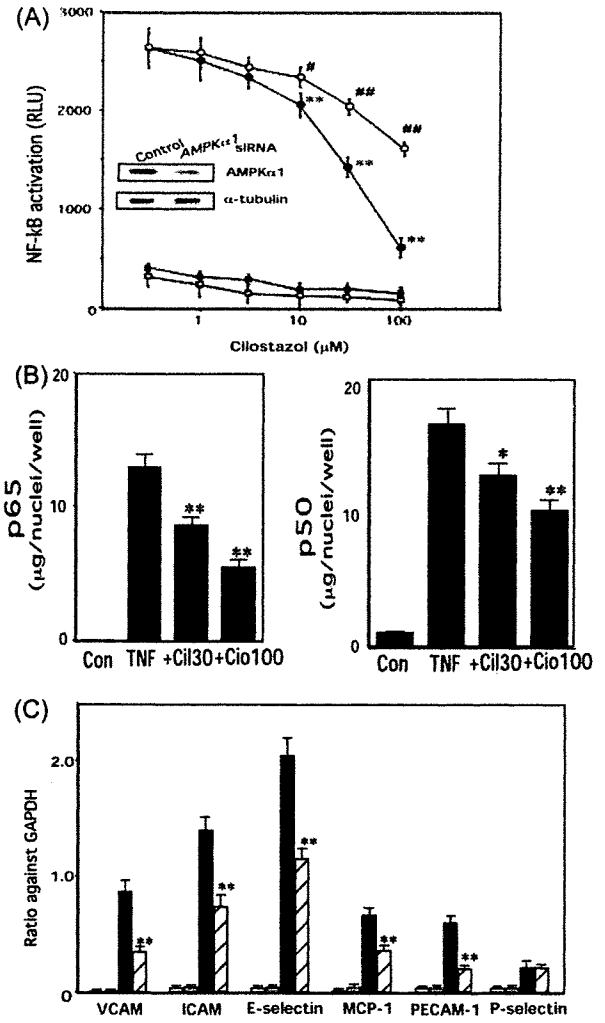

Figure 2 (*A*) Cilostazol inhibits tumour necrosis factor alpha (TNFα)-induced nuclear factor (NF)-κB-activation in SVEC4 cells. Cilostazol dose-dependently suppressed TNFα-activated NF-κB-dependent transcriptional activity, which was significantly attenuated in cells transfected with AMP-activated protein kinase (AMPK) siRNA (10 nM). Closed (control scrambled siRNA) and open squares (AMPK siRNA) represent the results in the absence of TNFα, whereas closed (control scrambled siRNA) and open circles (AMPK siRNA) represent the results in the presence of TNFα ($n = 6$). Inset: Cells were transfected with AMPKα1 or control siRNA for 48 h, after which the protein levels of AMPKα1 were determined by western blot analysis. Results represent the means ± SEM ($n = 4$). $^{**}P < 0.01$ vs. NF-κB activity in the absence of cilostazol, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. NF-κB activity in the presence of cilostazol. (*B*) Human umbilical vein endothelial cells (HUVEC) were stimulated with TNFα in the presence or absence of cilostazol (Cil30: 30 μM, Cil100: 100 μM) for 30 min. NF-κB p65 or p50 subunits were quantified within nuclear extracts using a transcription factor assay kit. Results represent the means ± SEM ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$. (*C*) Effects of cilostazol on TNFα-induced vascular cell adhesion molecule-1 (VCAM-1), E-selectin, intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), PECAM-1, and P-selectin mRNA expression in HUVEC. Cilostazol (30 μM) significantly inhibited VCAM-1, E-selectin, ICAM-1, MCP-1, and PECAM-1 mRNA levels. White bars: control, grey bars: control treated with cilostazol, black bars: TNFα, hatched bars: TNFα treated with cilostazol. Data represent the means ± SEM ($n = 4$) and are expressed as a ratio of GAPDH. $^{**}P < 0.01$ compared with the value of TNFα.

and decreased levels of phospho-IκBα were observed at 60 min (*Figure 4A*). The blot was then re-probed with anti-IκB antibody, producing evidence of significant degradation within 15–30 min. After this, IκB synthesis was

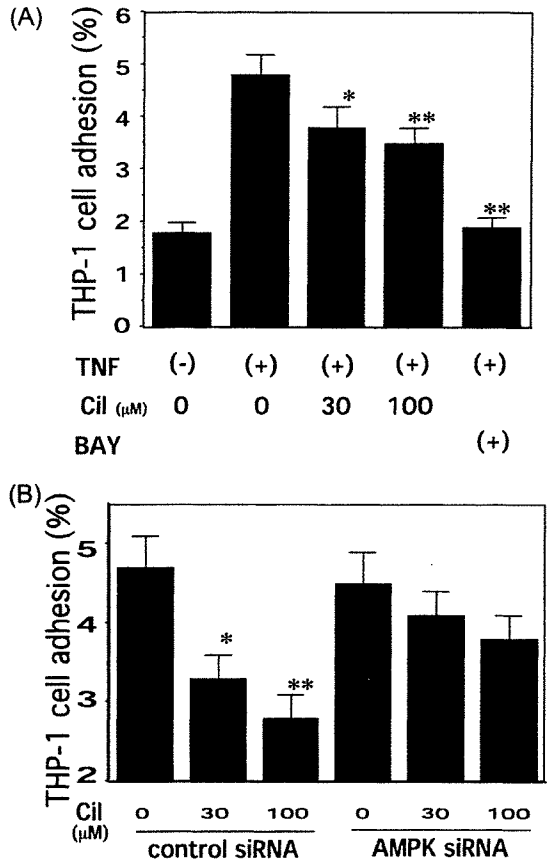

Figure 3 (*A*) Human umbilical vein endothelial cells (HUVEC) were pre-treated with cilostazol (30 or 100 μM) for 30 min and then incubated in the presence of tumour necrosis factor alpha (TNFα), and static adhesion assays were performed. The effect of BAY11-7082 (10 μM) on TNFα-induced cell adhesion was also examined. (*B*) Cilostazol dose-dependently suppressed TNFα-activated THP-1 cell adhesion that was significantly attenuated in HUVEC transfected with AMPK siRNA (10 nM). $n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$.

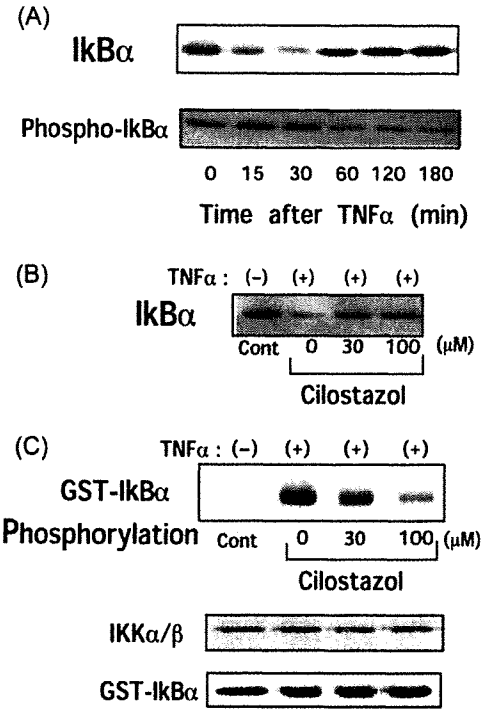

Figure 4 (*A*) Human umbilical vein endothelial cells (HUVEC) were incubated with tumour necrosis factor alpha (TNFα) for 0–180 min. The cells were lysed and subjected to western blot analysis using anti-IκB-α and anti-phospho-IκB-α antibodies. (*B*) The effect of cilostazol on IκB-α degradation in HUVEC. Cells were incubated for 30 min with cilostazol (30 and 100 μM), followed by TNFα for 30 min. Cells were then lysed and subjected to western blot analysis using anti-IκBα antibody. (*C*) The effect of cilostazol on IKK activity in HUVEC. Cells were incubated for 30 min with cilostazol (30 and 100 μM), followed by TNFα for 15 min. Cells were then lysed and immunoprecipitated with anti-IKKα/β antibody and used for kinase assay using recombinant IκBα as a substrate. Note that equal band densities for IKKα/β and GST-IκBα were observed. Three independent studies showed similar results.

re-activated, possibly by NF-κB, at 60 min (*Figure 4A*). Next, the effect of cilostazol on TNFα-induced IκBα degradation was determined 30 min after exposure to TNFα. Cilostazol partially inhibited TNFα-induced IκBα degradation (*Figure 4B*). A radiolabelled phosphorylated GST-IκBα-specific band was detected in TNFα-treated cells, while it was undetectable in untreated cells, thus demonstrating induction of IKK activity by TNFα (*Figure 4C*). IKK activity was dose-dependently inhibited by the treatment of the cells with cilostazol (*Figure 4C*). The remaining half of the immunoprecipitated samples were analysed by western blot analysis using anti-IKKα/β antibody, which showed identical expression levels of IKK, confirming expression of IKK in these cells. Identical amounts of GST-IκB were also detected when an equal volume of kinase reaction mixture was loaded into the SDS–PAGE column, followed by western blot analysis using anti-IκB antibody (*Figure 4C*).

3.4 AMP-activated protein kinase inhibition restored and cyclic AMP enhanced cilostazol-induced inhibition of nuclear factor-κB activation by tumour necrosis factor alpha

An AMPK inhibitor compound C restored cilostazol-induced inhibition of NF-κB activation by TNFα. This NF-κB activation by TNFα was completely inhibited by the NF-κB inhibitor BAY11-7082 (*Figure 5A*). A cell-permeable cAMP analogue pCTP-cAMP dose-dependently suppressed NF-κB activation by TNFα (*Figure 5B*). Although an adenylate cyclase inhibitor SQ 22536 had no effect on cilostazol-induced inhibition of NF-κB activation by TNFα, pCTP-cAMP enhanced cilostazol-induced inhibition of NF-κB activation by TNFα (*Figure 5C*).

4. Discussion

In the present study, we demonstrated that cilostazol inhibits TNFα-induced NF-κB activation in vascular endothelial cells. Cilostazol inhibited the NF-κB-dependent gene expression of various inflammatory and cell adhesion molecules, including VCAM-1, E-selectin, ICAM-1, and MCP-1. We demonstrated AMPK activation by cilostazol in HUVEC and examined whether this might be associated with the inhibition of cytokine-induced NF-κB activation. Transfection of AMPKα1 siRNA, which caused marked inhibition of AMPKα1 expression, significantly attenuated cilostazol-induced inhibition of NF-κB activation by TNFα in endothelial cells. An AMPK inhibitor compound C also restored cilostazol-induced inhibition of NF-κB activation by TNFα. An AMPK activator AICAR was observed to suppress

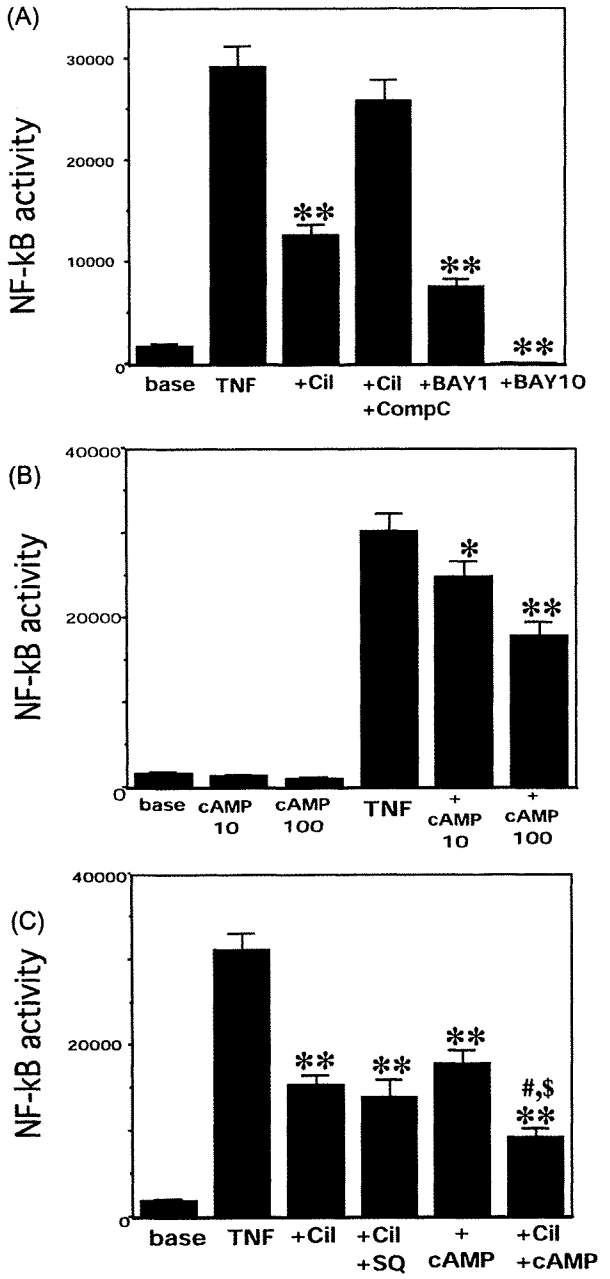

Figure 5 (*A*) An AMP-activated protein kinase (AMPK) inhibitor compound C (Comp C: 1 μM) restored cilostazol-induced inhibition of NF-κB activation by tumour necrosis factor alpha (TNFα) in SVEC4 cells. This NF-κB activation by TNFα was completely inhibited by the NF-κB inhibitor BAY11-7082 (BAY1 or BAY10: 1or 10 μM). (*B*) Cyclic AMP inhibited TNFα-elicited NF-κB activation. A cell-permeable cyclic AMP analogue pCTP-cAMP dose-dependently suppressed NF-κB activation by TNFα in SVEC4 cells (cAMP 10 or cAMP 100: pCTP-cAMP 10 or 100 μM). (*C*) Cilostazol and cAMP additively inhibited TNFα-elicited NF-κB activation. Although an adenylate cyclase inhibitor SQ 22536 (10 μM) had no effect on cilostazol-induced inhibition of NF-κB activation by TNFα, a cell-permeable cAMP analogue pCTP-cAMP (100 μM) enhanced cilostazol-induced inhibition of NF-κB activation by TNFα in SVEC4 cells. Results represent the means ± SEM ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. NF-κB activity by TNFα. $^{\#}P < 0.05$ vs. Cil, $^{\$}P < 0.05$ vs. cAMP.

cytokine-induced NF-κB activation in vascular endothelial cells.[21] These data suggest that AMPK activation by cilostazol may be responsible for the inhibition of NF-κB activation.

Although we did not perform a strict kinase assay for AMPK, it is likely that cilostazol activates AMPK since the extent of AMPK phosphorylation at Thr-172 strongly reflects its activity,[22] and since phosphorylation of the AMPK consensus substrate, ACC, at Ser-79 was observed. It remains elucidated how cilostazol activates AMPK, but our data that the down-regulation of CaMKKβ using RNA interference modestly but significantly inhibited cilostazol-induced AMPK activation, suggest that CaMKKβ might at least partly mediate the effect of cilostazol on AMPK activation. This ability of AMPK activation appears to be specific for cilostazol among PDE3 inhibitors since milrinone or vesnarinone could not activate AMPK in HUVEC.

We demonstrated that cilostazol inhibits the expression of pro-inflammatory and adhesion molecule genes by blocking phosphorylation and subsequent degradation of IκB-α. These data suggest that cilostazol might suppress TNFα-induced NF-κB activation prior to IκB phosphorylation. We further demonstrated the stimulation of IκB-α phosphorylation by TNFα through the induction of IKK activity, and inhibition of IKK activity and TNFα-induced IκB-α phosphorylation by cilostazol. Thus, cilostazol-activated AMPK may suppress NF-κB activation by inhibiting IKK activity in vascular endothelial cells. It has been reported that AICAR attenuates LPS-induced activation of NF-κB via down-regulation of IkB kinase α/β activity in glial cells.[23] This could be the same mechanism as we showed in this study in vascular endothelial cells, suggesting that AMPK activation may inhibit cytokine-induced NF-κB activation by suppressing IKK activity.

We investigated adenosine uptake using [^{3}H]adenosin in HUVEC. As shown in Supplementary material online, *Figure S1*, the adenosine uptake was dose-dependently inhibited by cilostazol in HUVEC. The increased plasma levels of adenosine due to cilostazol-induced inhibition may play a vasculo-protective role *in vivo*. We also examined the effect of cilostazol treatment on cAMP and cGMP levels in HUVEC (see Supplementary material online, *Table S1*). cGMP levels were low at the basal level, and the increase by cilostazol was also modest. However, this observation could be more evident *in vivo*, because cAMP elevating substances exist *in vivo*, and also NO increases cGMP levels *in vivo*. Thus, this observation could be considered as another underlying mechanism for cilostazol to play a protective role on vascular endothelial cells.

Cilostazol is a selective inhibitor of PFE3 by which it may increase intracellular cAMP and activate protein kinase A (PKA), thereby inhibiting platelet aggregation and inducing peripheral vasodilation *in vivo*.[2,24] Thus, we examined whether cAMP might be associated with cilostazol-induced activation of AMPK. We found that phosphorylation of AMPK with cilostazol was not affected by co-treatment with an adenylate cyclase inhibitor SQ 22536 and that a cell-permeable cAMP analogue pCTP-cAMP did not cause AMPK phosphorylation, and had no effect on cilostazol-induced phosphorylation of AMPK. Thus, AMPK activation by cilostazol independent of cAMP appears to protect against endothelial inflammation.

Although cAMP did not affect cilostazol-induced AMPK activation, we confirmed that elevated levels of cAMP inhibit cytokine-induced NF-κB activation in cultured endothelial cells. However, cilostazol alone, unless adenylate cyclase activators are also present, induces a modest

Correspondence

Influence of Statins on Glucose Tolerance in Patients with Type 2 Diabetes Mellitus: Subanalysis of the Collaborative Study on Hypercholesterolemia Drug Intervention and their Benefits for Atherosclerosis Prevention (CHIBA Study)

Koutaro Yokote, Yasushi Saito, and CHIBA study investigators

Department of Clinical Cell Biology and Medicine, Chiba University Graduate School of Medicine, Chiba, Japan

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Key words; Statin, Diabetes mellitus, Glycoalbumin, Hypercholesterolemia, Glycemic control

In the previous issue of J Atheroslcer Thromb[1], Yamakawa *et al.* showed a significant increase in glycohemoglobin A1c (HbA1c) level in diabetic patients treated with atorvastatin, but not in patients given pitavastatin or pravastatin. Although the finding is intriguing, their study could not exclude bias because it was retrospective and recruited subjects only at a single hospital.

Here we report the results of glycemic control subanalysis in a multicenter prospective comparative study of pitavastatin and atorvastatin (CHIBA study). In the original study, 204 Japanese patients with hypercholesterolemia recruited at 39 medical facilities were randomized to receive either 2 mg pitavastatin or 10 mg atorvastatin daily for 12 weeks, and the percent change from baseline in lipid parameters after 12 weeks of treatment was evaluated[2]. Among 99 patients with type 2 diabetes included in the study, 45 patients (23 on pitavastatin and 22 on atorvastatin) at 16 centers were subjected to the evaluation of parameters related to glucose tolerance. Serum lipid levels at the baseline were similar between groups. No changes were made in types or doses of medications used to treat diabetes or hypertension, or in lifestyle guidance throughout the study period. After 12 weeks of statin treatment, serum glycoalbumin had significantly increased by 0.67 ± 1.31% compared to the baseline in the atorvastatin group ($p = 0.026$), whereas no significant difference was observed in the pitavastatin group (**Table 1**). In the atorvastatin group, but not in pitavastatin group, the HbA1c level also tended to increase ($p = 0.098$). Since glycoalbumin is known to reflect glycemic control over a shorter period of time than HbA1c, only glycoalbumin might have detected a slight glycemic change during a treatment period of 12 weeks in this relatively small study. There were no significant changes in fasting plasma glucose, insulin or the homeostasis model assessment ratio, but all of these values tended to decrease in the pitavastatin group.

To date, adverse effects of atorvastatin on glucose tolerance have not been clearly demonstrated in clinical studies carried out in Western countries[3]; however, several reports have suggested the deterioration of glycemic control with atorvastatin use for 3 to 4 months in Japanese diabetic patients as referred to by Yamakawa *et al.*[1]. Recently, a PROVE-IT substudy has shown that high-dose atorvastatin treatment is associated with worse glycemic control[4]. An *in vitro* study demonstrated that atorvastatin near the physiological concentrations suppressed glucose transporter GLUT4 expression in 3T3-L1 adipose cells[5]. Since it has been suggested that the insulin-secreting capacity of pancreatic β-cells is lower in Japanese than Caucasians[6], even a slight negative influence on insulin sensitivity in the peripheral tissue may affect glycemic control in Japanese patients. Previous reports, together with our findings suggest that atorvastatin, but not pitavastatin, affects glycemic control in some Japanese diabetic patients. Larger clinical trials are required to draw a conclusion.

Address for correspondence: Koutaro Yokote, Department of Clinical Cell Biology and Medicine, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
E-mail: kyokote@faculty.chiba-u.jp
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Table 1. Change in fasting plasma glucose, glycohemoglobin A1c, insulin, glycoalbumin levels and homeostasis model assessment ratio in diabetic patients treated with statins

					Baseline vs. Week 12	PTV vs. ATV	
		Baseline	Week 12	Absolute change	*p*-Value	*p*-Value	95% C.I.
Fasting plasma glucose (mg/dL)	PTV	135.4±22.9	131.4±18.5	−4.04±18.6	0.289	0.234	−1.594-25.304
	ATV	130.6±22.9	138.4±28.5	7.81±28.3	0.163		
Glycohemoglobin A1c (%)	PTV	6.8±0.7	6.8±0.7	−0.03±0.4	0.690	0.389	−0.056-0.387
	ATV	6.6±0.6	6.7±0.8	0.13±0.5	0.098		
Insulin (mU/mL)	PTV	12.6±10.3	10.6±5.5	−1.98±7.4	0.231	0.315	−0.826-6.578
	ATV	7.9±3.8	8.8±5.6	0.89±3.7	0.290		
Glycoalbumin (%)	PTV	18.7±3.1	19.1±3.0	0.34±1.3	0.218	0.691	−1.471-2.650
	ATV	19.0±3.4	19.7±3.8	0.67±1.3	0.026		
Homeostasis model assessment ratio	PTV	4.4±4.3	3.5±1.9	−0.91±3.1	0.192	0.368	−0.262-2.714
	ATV	2.5±1.1	2.8±1.6	0.32±1.2	0.238		

Data are presented as the mean±SD. Pitavastatin (PTV, $n=23$), atorvastatin (ATV, $n=22$)

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Cardiorespiratory Fitness as a Quantitative Predictor of All-Cause Mortality and Cardiovascular Events in Healthy Men and Women

A Meta-analysis

Satoru Kodama, MD, PhD
Kazumi Saito, MD, PhD
Shiro Tanaka, PhD
Miho Maki, MS
Yoko Yachi, RD, MS
Mihoko Asumi, MS
Ayumi Sugawara, RD
Kumiko Totsuka, RD
Hitoshi Shimano, MD, PhD
Yasuo Ohashi, PhD
Nobuhiro Yamada, MD, PhD
Hirohito Sone, MD, PhD

Context Epidemiological studies have indicated an inverse association between cardiorespiratory fitness (CRF) and coronary heart disease (CHD) or all-cause mortality in healthy participants.

Objective To define quantitative relationships between CRF and CHD events, cardiovascular disease (CVD) events, or all-cause mortality in healthy men and women.

Data Sources and Study Selection A systematic literature search was conducted for observational cohort studies using MEDLINE (1966 to December 31, 2008) and EMBASE (1980 to December 31, 2008). The Medical Subject Headings search terms used included *exercise tolerance, exercise test, exercise/physiology, physical fitness, oxygen consumption, cardiovascular diseases, myocardial ischemia, mortality, mortalities, death, fatality, fatal, incidence,* or *morbidity*. Studies reporting associations of baseline CRF with CHD events, CVD events, or all-cause mortality in healthy participants were included.

Data Extraction Two authors independently extracted relevant data. CRF was estimated as maximal aerobic capacity (MAC) expressed in metabolic equivalent (MET) units. Participants were categorized as low CRF (<7.9 METs), intermediate CRF (7.9-10.8 METs), or high CRF (≥10.9 METs). CHD and CVD were combined into 1 outcome (CHD/CVD). Risk ratios (RRs) for a 1-MET higher level of MAC and for participants with lower vs higher CRF were calculated with a random-effects model.

Data Synthesis Data were obtained from 33 eligible studies (all-cause mortality, 102 980 participants and 6910 cases; CHD/CVD, 84 323 participants and 4485 cases). Pooled RRs of all-cause mortality and CHD/CVD events per 1-MET higher level of MAC (corresponding to 1-km/h higher running/jogging speed) were 0.87 (95% confidence interval [CI], 0.84-0.90) and 0.85 (95% CI, 0.82-0.88), respectively. Compared with participants with high CRF, those with low CRF had an RR for all-cause mortality of 1.70 (95% CI, 1.51-1.92; $P<.001$) and for CHD/CVD events of 1.56 (95% CI, 1.39-1.75; $P<.001$), adjusting for heterogeneity of study design. Compared with participants with intermediate CRF, those with low CRF had an RR for all-cause mortality of 1.40 (95% CI, 1.32-1.48; $P<.001$) and for CHD/CVD events of 1.47 (95% CI, 1.35-1.61; $P<.001$), adjusting for heterogeneity of study design.

Conclusions Better CRF was associated with lower risk of all-cause mortality and CHD/CVD. Participants with a MAC of 7.9 METs or more had substantially lower rates of all-cause mortality and CHD/CVD events compared with those with a MAC of less 7.9 METs.

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CORONARY HEART DISEASE (CHD) is a major cause of disability and premature death throughout the world.[1] Epidemiological studies have demonstrated an inverse association between physical fitness and the incidence of CHD or all-cause mortality in healthy or asymptomatic participants. Physical fitness is typically expressed as cardiorespiratory fitness (CRF) and is assessed by exercise tolerance testing[2]; however, it is rare for clinicians to consider CRF when evaluating future risk of CHD.[3]

A major reason for lack of consideration of CRF as a marker of CHD risk may be that the quantitative association of CRF for cardiovascular risk is not well established. The degree of risk reduc-

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Author Affiliations are listed at the end of this article. **Corresponding Author:** Hirohito Sone, MD, PhD, Department of Internal Medicine, University of Tsukuba Institute of Clinical Medicine, 3-2-7 Miya-machi, Mito, Ibaraki 310-0015, Japan (hsone@md.tsukuba.ac.jp).

Clinical Review Section Editor: Mary McGrae McDermott, MD, Contributing Editor. We encourage authors to submit papers for consideration as a Clinical Review. Please contact Mary McGrae McDermott, MD, at mdm608@northwestern.edu.

tion associated with each incremental higher level of CRF, the criteria for low CRF, and the magnitude of risk associated with low CRF have been inconsistent among studies. Our goal of this meta-analysis was to systematically review the quantitative relationship between CRF and all-cause mortality and CHD or cardiovascular disease (CVD) events in healthy individuals.

METHODS

Search Strategy

The meta-analysis was conducted according to the checklist of the Meta-analysis of Observational Studies in Epidemiology.[4] We performed a systematic literature search of MEDLINE (1966 to December 31, 2008) and EMBASE (1980 to December 31, 2008) for observational cohort studies. Three search themes were combined using the Boolean operator *and*. The first keywords were related to CRF (combined exploded versions of the Medical Subject Headings [MeSH] as follows: *exercise tolerance* OR *exercise test* OR *exercise/physiology* OR *physical fitness* OR *oxygen consumption*); the second keywords were related to the outcome of this meta-analysis (combined unexploded version of MeSH [*cardiovascular diseases*] or the exploded version of MeSH [*myocardial ischemia*]) or the following text words (*mortality* OR *mortalities* OR *death* OR *fatality* OR *fatal* OR *incidence** OR *event** OR *morbidity*); and the third keywords were related to risk estimates (combined text words as follows: *regression analysis* OR *regression model** OR *statistical regression** OR *logistic regression** OR *logit regression** OR *logistic model** OR *logit model** OR *Cox model* OR *hazard model* OR *odds ratio** OR *ORs* OR *relative odds* OR *risk ratio** OR *relative risk** OR *RRs*). We also included studies published in non-English language. In addition, we searched the reference lists of all identified relevant publications.

Inclusion and Exclusion Criteria

We included papers if (1) CRF was assessed by an exercise stress test; (2) the association of CRF with all-cause mortality and with CHD or CVD was evaluated; (3) CRF could be assessed as maximal aerobic capacity (MAC), expressed in units of metabolic equivalents (METs), which is defined as the ratio of intensity of physical activity to that of sitting at rest; and (4) risk ratios (RRs) and their corresponding 95% confidence intervals (CIs) relating to each category of MAC were reported or could be calculated. We excluded studies that were intended only for patients having a specific disease that presented a major risk factor, such as diabetes, hypertension, and familial hypercholesteremia, as well as studies that included patients with CHD or chronic heart failure.

To avoid double counting of a cohort, study selection was limited to a single set of results when multiple publications were available for a single observational study. The first priority for selection was the study with the longest follow-up and the second was the study with full cohort analysis covering the largest number of participants among articles from a single cohort. We conducted 2 separate meta-analyses for risk of all-cause mortality and CHD or CVD in relation to CRF. When an individual study provided data on both CHD or myocardial infarction (MI) and CVD,[5-7] priority for data abstraction was given to CVD because CVD is more comprehensive than CHD and MI. Similarly, if data on both events and deaths were provided,[6,8,9] priority was given to events.

We combined CHD and CVD into 1 outcome (CHD/CVD), which included studies whose outcome was a CVD event, CVD death, CHD event, or CHD death, because the number of eligible studies included was limited. Although criteria for the end point in CHD varied from study to study, the end points that we specified as CHD outcome in our meta-analysis were (1) death from MI; (2) death from CHD including MI; and (3) a CHD event, a term which meant either death from CHD, sudden cardiac death, occurrence of nonfatal CHD, or nonfatal MI. Additionally, we included studies whose outcome was either CVD death (ie, encompassing death from cardiovascular causes other than CHD) or CVD events (ie, lumping together fatal and nonfatal CVD).

Data Abstraction

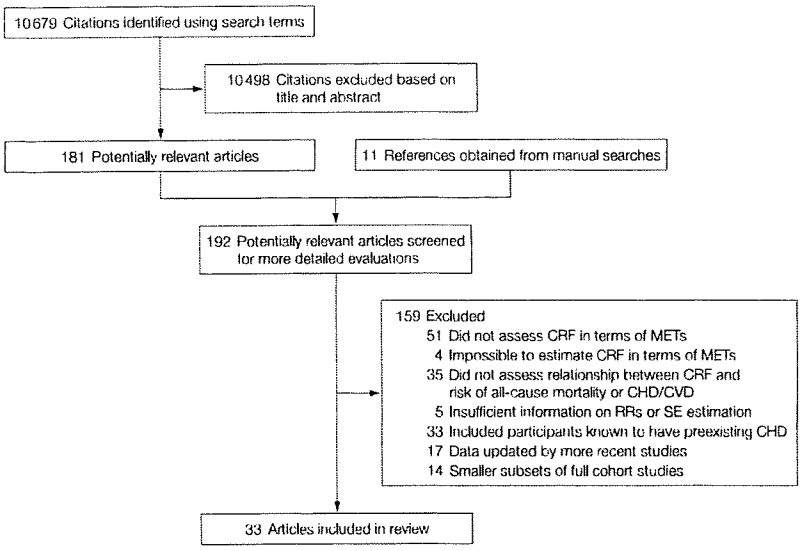
Data abstracted were the first author's name, year of publication, country of origin, specific outcomes, duration of follow-up, methods for outcome assessment, instrument or methods for measurement of CRF, whether maximal exercise testing (defined as instructing participants to continue exercise until their maximal workload) was conducted, mean of participants' age, proportion of men, number of participants and number of new cases (ie, deaths or events) during the observational periods, adjusted variables, and whether participants with abnormal electrocardiogram findings (ie, ST elevation/depression) during exercise testing were included. Two of our investigators (S. Kodama and H. Sone) independently reviewed each published paper and extracted relevant information. Any disagreement was resolved by consensus.

In studies using CRF as a categorical variable, we standardized all reported RRs into comparison of the risk of the lower CRF group with that in the higher CRF group. Therefore, when the lowest CRF group was referent, we converted the reported RR into its reciprocal. When a study provided several RRs, such as unadjusted and adjusted RRs, the most completely adjusted RR was used. The standard error (SE) of each RR was derived from 95% CIs or *P* values. If data related to RR and its corresponding SE were not provided, their value was directly calculated using data on the number of participants (P) and new cases (C) of risk and the reference (ref) groups in each comparison, using the equation:

$$RR = [(C_{risk}/P_{risk})/(C_{ref}/P_{ref})],\ SE^2 = [(1/C_{risk})-(1/P_{risk})]+[(1/C_{ref})-(1/P_{ref})].$$

The MAC was calculated from the exercise workload at the termination of exercise testing and relative exercise intensity (ie, proportion of the workload to MAC). The exercise workload was converted into MET units (1 MET corresponds to 3.5 mL/min/kg of oxygen consumption [$\dot{V}O_2$]), according to the Metabolic Calculation Handbook by

Figure 1. Selection of Articles for Meta-analysis



CHD indicates coronary heart disease; CRF, cardiorespiratory fitness; CVD, cardiovascular disease; METs, metabolic equivalents; and RRs, risk ratios.

the American College of Sports Medicine.[10] Relative exercise intensity was estimated using a linear equation according to Swain et al[11]:

heart rate at exercise/maximal heart rate = 0.64 × ($\dot{V}O_2$ at exercise/maximal $\dot{V}O_2$).

For some specific exercise stress tests, the MAC was directly estimated using the prediction equation determined by a previous validation study for each protocol of the exercise test (the Balke treadmill test,[12,13] the modified Bruce test,[14] and the Canadian Home Fitness test[15]).

When exposure was expressed as a range, we converted it into point estimates expressed as average exposure using the midpoint of the range except for the lowest and highest fit group. If data on the average value were not available, it was estimated by the assumption that the MAC levels of the study population had a normal distribution using the mean value and its SD of each study sample. This assumption is consistent with a prior study.[16] However, if the SD was not available, we assumed that its value equaled 2 METs, according to the statement of the American Heart Association.[17]

After converting all exposures into MET units, we additionally adjusted MET units for age and sex. According to a Statement for Healthcare Professionals From the American Heart Association,[17] we assumed that the MAC is 2 METs lower in women than in men and that for each year of aging, it decreased by 0.1 MET based on a prior study.[18] Finally, we represented CRF as the adjusted MAC under the assumption that all participants were 50-year-old men in the analyses described below.

Dose-Response and Categorical Analyses

We first performed dose-response analyses by summarizing how much risk reduction could be predicted per incremental increase in CRF. The study-specific RR for each higher MET (corresponding to 1-km/h higher running/jogging speed) in MAC, if not reported, was estimated by regressing the natural logarithm of the RR (lnRR) according to each CRF category against its corresponding mean MAC value, using the method described by Greenland and Longnecker.[19]

We then performed categorical analyses to summarize the risk of all-cause mortality and CHD/CVD for low CRF. We assigned every RR reported in each study to 1 of the following 3 comparisons based on the CRF level of risk and reference group: (1) low vs high CRF, (2) low vs intermediate CRF, and (3) intermediate vs high CRF. This method is based on a previous meta-analysis of the relationship between activity level and stroke risk.[20] For studies that presented risk estimates for more than 2 CRF categories, the ranges of the adjusted MAC of the lowest, highest, and in-between categories defined by each study were 5.5 to 7.8, 11.0 to 15.2, and 7.9 to 10.7 METs, respectively; except that in 2 studies,[21,22] the second highest category of CRF was more than 11.0 METs and, in 1 study,[7] the highest category of CRF was 10.6 METs.

To avoid overlap of the CRF range of the 3 categories, we defined low, intermediate, and high CRF as less than 7.9 METs, 7.9 to 10.8 METs, and 10.9 METs or more, respectively. Consequently, we could assign every RR in each study to 1 of the 3 predefined subgroups with 2 exceptions. In 2 studies,[21,22] the mean MAC values for both the highest and the second highest category were the same as the high CRF category (defined by ≥10.9 METs). Therefore, RR data for comparison between 2 CRF categories could not be included in our categorical analysis for these 2 studies.

Statistical Analysis

The pooled RRs for a 1-MET higher level of MAC and the lower CRF in comparison with the higher CRF within each of the 3 comparisons were estimated by using a fixed-effects or random-effects model.[23] If significant heterogeneity of RRs that was tested by calculating the I^2 statistic[24] was present, we chose the pooled estimates from the random-effects model because it is better than the fixed-effects model and it explains between-study heterogeneity.

To examine the effect of study characteristics on risk reduction per 1-MET higher level of MAC, sensitivity analyses were conducted for the possible confounders (mean age [≥50 years or not], sex [only men or not], adjustment for smoking [yes or no], adjustment for multiple confounders, defined as adjustment