the pacing rate to the maximum rate, thus allowing for 1:1 capture in each rabbit. At the end of 4 weeks of ventricular tachypacing, open chest electrophysiological studies were performed under anesthesia and ventilated mechanically with isoflurance-containing room air (0.5% per 2 L/min) as described previously.[26] After electrophysiological evaluation, both atria were harvested for biologic and histological analyses.

Western Blotting

On postoperative day, tissue samples obtained on operative day 28 were homogenized lysis buffer containing 20 mmol/L Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mmol/L NaCl, 0.5% deoxycholic acid, 1 mmol/L sodium orthovanadate, and protease inhibitor mixture (Sigma). The protein content was determined by using the Bradford method. The same amount of protein (40 μg) was loaded per lane and separated using denaturing 10% polyacrylamide gels. The membranes were probed with antibodies to CatK,[27] to AT1R (Santa Cruz Biotechnology), to gp91phox (BD Biosciences), and to total p38MAPK and phosphorylated p38MAPK (p-p38MAPK) (Cell Signaling Technology, Inc). Bands were visualized using chemiluminescence (ECL Western Blotting Detection Kit, Amersham Biosciences).

Immunohistochemistry

On postoperative day 28, transverse sections (5-μm thickness) of atrial tissues were stained with rabbit polyclonal antibody to CatK (dilution, 1:100) as described previously.[27] As a negative control, the primary antibody was replaced by nonimmune immunoglobulin G (Vector). Azan Mallory staining was applied to evaluate fibrotic deposition in rabbit atria. We set a threshold to automatically compute the blue pixel area for the histochemical stain and then computed the ratio (percent) of the positively stained area to the total cross-sectional atrial free-wall area using BZ-II Analyzer, Exe 1.42 software (Keyence). Three random microscopic fields (×400) from 8 independent cross sections of atrial free-wall (24 fields) in each animal were quantified and averaged for each animal.

Assay of Superoxide Production

Specific NADPH oxidase activity of total homogenates of fresh left atrial tissue was measured with the use of a lucigenin-based enhanced chemiluminescence assay as described.[20] A low lucigenin concentration (5 mmol/L) was used to minimize artifactual O_2^- production attributable to redox cycling. In brief, homogenate protein (1 mg) lysis buffer (1 mL; 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L ethylene glycol tetraacetic acid, and 1% Triton X-100) was transferred to an assay tube, and NADPH and dark-adapted lucigenin were added to final concentrations of 100 and 5 mmol/L, respectively, immediately before the measurement of chemiluminescence. All assays were performed in triplicate. The chemiluminescence signal was sampled every minute for 12 minutes using a tube luminometer (20/20; Turner Biosystems), and the respective background counts were subtracted from the experimental values.

Cell Culture and Simulation

Neonatal rat atrial myocytes from 1-day-old Wistar rats were isolated as described previously[28] and cultured in a mixture (50:50, v/v) of Dulbecco's modified Eagle's medium and Ham's F-12 (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics.[11] After 24 hours of culture, the cells (5×10^4/well in 12-well plates) were pretreated with or without various reagents for appropriate amounts of time, and the cells were subjected to the related biologic assays.[11]

Gene Expression Assay

Total RNA was isolated cultured cells and was subjected to reverse transcription with a PCR Core kit (Applied Biosystems). The resulting cDNA was subjected to quantitative real-time PCR analysis with targeted gene primers and with use of the ABI 7300 Real-Time PCR System under the following conditions: 50°C (2 minutes) for UNG incubation, 94°C (10 minutes) for Ampli*Taq* Gold activation, 95°C (15 seconds), and 59°C (1 minute) for 40 cycles as previous described.[27] The amount of each mRNA was normalized against the corresponding amount of glyceraldehyde-3-phosphate dehydrogenase mRNA.

Assay of Collagenolytic Activity

Total protein (100 μg) from the extracts of cells and atrial tissues was incubated with 500 μg/mL fluorescein-labeled type I collagen (Molecular Probes Inc) for 6 hours. Reactions were performed in the absence or presence of several protease inhibitors at indicated concentrations as described previously.[29]

Statistical Analysis

Summary descriptive statistics for continuous parameters are presented as mean±SD values. Categorical variables were compared among study groups by using the χ^2 test. Student's *t* test (for comparison of continuous parameters between 2 groups) or 1-way ANOVA (for comparison of continuous parameters among 3 or more groups), followed by Tukey's post-hoc test, was used to test significant differences. Cystatin C and high-sensitive C-reaction protein

concentrations were logarithmically transformed because the data showed a skewed distribution. If the homogeneity of the variance assumption was violated, the nonparametric Kruskal–Wallis test was used instead. The factors that related at the $P<0.1$ level were selected as independent variable candidates for multiple logistic regression analysis and were used to evaluate the independent contribution of clinical parameters to AF. Correlation coefficients were calculated using linear regression analysis. In animal and in cell experiments, we performed the text related to ensuring normality for the tests with very small sample sizes. StatFlex (version 6.0; Artech) was used for all statistical analysis. *P* values of <0.05 were considered statistically significant.

Results

All Patients

The baseline clinical and demographic features of the study population are shown in Table 1. The AF group was older and had more men than did the control group ($P<0.001$). There were no differences between the AF and control groups in potential causal factors. AF patients had taken more β-blockers than had the control subjects ($P=0.02$). Patients with AF had significantly ($P<0.05$) larger LADs and worse ejection fractions than did control subjects.

Compared with controls, patients with AF had significantly ($P<0.001$) higher plasma CatK, interleukin-1β, ICTP, and I-PINP levels and lower I-PINP:ICTP ratios than did control subjects. Compared with controls, AF patients had higher levels of hemoglobin A1c ($P<0.001$), high-sensitive C-reactive protein ($P=0.04$), and atrial natriuretic peptide ($P<0.001$), whereas there were no significant differences in potassium or in low-density and high-density lipoproteins. In all subjects, univariate regression analysis revealed that there was a positive correlation between CatK and ICTP ($r=0.3$, $P<0.0001$; Figure 1A) and LAD ($r=0.4$, $P<0.0001$; Figure 1B).

PAF Versus PeAF

The baseline characteristics of the PAF and PeAF groups are presented in Table 2. Patients with PeAF had larger LADs and worse ejection fractions than did PAF patients ($P<0.05$). With the exception of age, the percentage of women, chronic heart failure, and use of statin treatments, there were no significant differences between the 2 groups. As expected, the PeAF group had increased levels of plasma high-sensitive C-reactive protein and atrial natriuretic peptide ($P<0.05$ for both) compared with patients with PAF. In addition, the levels of CatK, ICTP, and interleukin-1β were higher and the I-PINP:ICTP ratio lower among subjects in the PeAF group compared with those in the PAF group ($P<0.05$ for all comparisons; Figure 2).

Table 1. Patient Characteristics

	AF (n=209)	Control (n=112)	*P* Value
Demographic Characteristics			
Age, y	60.1±10.9	54.8±10.7	<0.001
Female, %	23.0	42.9	<0.001
Body mass index, kg/m^2	23.7±3.4	23.0±2.8	0.09
Smokers, %	30.1	23.2	0.25
Causal factors			
Hypertension, %	45.5	38.4	0.26
Diabetes mellitus, %	20.1	12.5	0.09
Ischemic heart disease, %	8.1	3.6	0.12
Chronic heart failure, %	7.2	4.5	0.34
Echocardiography			
Left atrial diameter, mm	39.0±6.9	32.3±4.9	<0.001
LV ejection fraction, %	61.8±8.4	77.1±6.9	0.002
Blood examination			
Potassium, mEq/L	4.3±0.4	4.2±0.4	0.48
LDL, mg/dL	118.4±27.8	121.2±29.3	0.40
HDL, mg/dL	55.7±14.2	57.4±13.3	0.32
Hemoglobin A1c, %	5.6±0.7	5.2±0.7	<0.001
ANP, pg/mL	70.9±70.9	27.3±29.9	<0.001
hs-CRP, mg/dL	0.14±0.4	0.06±0.1	0.04
IL-1β, pg/mL	14.3±7.0	10.9±7.9	<0.001
I-PINP:ICTP	13.7±7.7	25.1±19.2	<0.001
ICTP, ng/mL	3.8±2.7	1.5±1.3	<0.001
CatK, ng/mL	13.1±6.7	6.1±3.9	<0.001
Medications			
ACEIs, %	4.3	8.0	0.17
ARBs, %	30.1	26.8	0.53
CCBs, %	20.1	17.0	0.50
β-Blockers, %	29.7	8.9	0.02
Statins, %	20.1	22.3	0.64
Antiarrhythmic drugs, %	100	0	

Values are expressed as mean±SD. AF indicates atrial fibrillation; LV, left ventricular; LDL, low-density lipoprotein; HDL, high-density lipoprotein; ANP, atrial natriuretic peptide; hs-CRP, high-sensitivity C-reactive protein; IL, interleukin; I-PINP, intact procollagen type I N-terminal propeptide; ICTP, carboxyl-terminal telopeptide of type I collagen; CatK, cathepsin K; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CCB, calcium channel blocker.

Biomarkers for Prediction of AF

Table 3 shows the results of the multiple logistic regression analyses to assess the factors (included at the $P<0.1$ level in

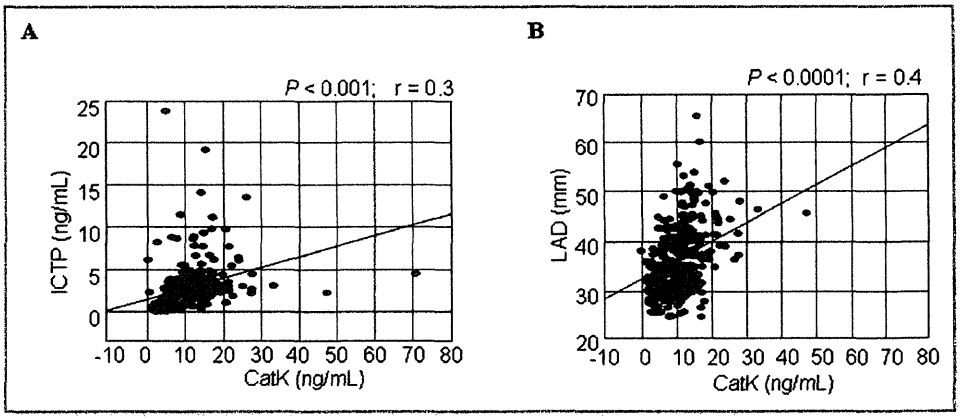

Figure 1. Correlations between plasma levels of CatK and ICTP (A) and (B) LAD. In all patients, there were positive correlations between plasma levels of cathepsin (CatK) and carboxyl-terminal telopeptide of type I collagen (ICTP) and left atrial diameter (LAD).

Table 1) that independently contributed to AF. According to the multiple logistic regression analyses, only LAD (odds ratio, 1.19; 95% CI, 1.06 to 1.36; $P<0.05$), CatK (odds ratio, 1.27; 95% CI, 1.10 to 1.39; $P<0.01$), and I-PINP:ICTP (odds ratio, 0.79; 95% CI, 0.69 to 0.90; $P<0.001$) were significantly associated with AF.

Inhibitory Effect of AT1R Antagonism on CatK Expression and Atrial Remodeling With AF

Immunostaining showed that the expression of CatK was markedly increased throughout the atrial tissue of AF rabbits, with apparent expression in the myocytes, and this change was inhibited by olmesartan (Figure 3A). As shown in Figure 3B, collagenolytic activity was higher in AF rabbits than in controls, and this change was more sensitive to a specific CatK inhibitor (CatK-II) and the broad-spectrum cysteine protease E64 than to GM6001, an inhibitor of matrix metalloproteinases (Figure 3B). Olmesartan reduced the amount of increased collagenolytic activity in the atrial extract of AF rabbits (0.56±0.09 versus 0.94±0.19 fluorescence intensity, $P<0.01$).

AF rabbits had increased levels of NADPH oxidase activity (Figure 3C) and of an NADPH oxidase subunit, gp91phox protein (Figure 3D; $P<0.01$). As shown in Figure 3D and 3E, Western blotting revealed that the levels of CatK, AT1R, and p-p38MAPK proteins were greater in AF rabbits than in controls ($P<0.01$). All of these changes were reversed with olmesartan ($P<0.01$). In addition, rabbits with AF had significantly higher atrial tissue Ang II levels than did control rabbits (78.3±6.0 versus 36.2±4.8 pg/100 mg, $P<0.01$), and this effect was also reduced by olmesartan (45.1±3.7 versus 78.3±6.0, $P<0.05$). The duration of AF was 11.8±0.2 seconds in ventricular-tachypaced rabbits, whereas AF was not induced in nonpaced controls (Figure 4A). The duration of AF was significantly reduced in olmesartan-treated rabbits (3.2±0.1 seconds) compared with ventricular tachypacing–only rabbits ($P<0.01$). Furthermore, olmesartan significantly suppressed atrial fibrosis compared with the control group (3.9±0.6 versus 9.8±1.6%, $P<0.05$) (Figure 4B and 4C).

Quantitative real-time PCR demonstrated that both H_2O_2 and Ang II significantly ($P<0.05$) enhanced CatK mRNA expression and that olmesartan reduced CatK expression in response to Ang II in cultured rat neonatal atrial myocytes (Figure 5A). Ang II enhanced the collagenolytic activity in cells extracts, and this effect was sensitive to CatK-II and E64 (Figure 5B). Western blots showed olmesartan-mediated reductions in the level of Ang II–induced CatK, gp91phox, and p-p38MAPK (Figure 5C). Furthermore, Ang II–induced CatK gene expression was suppressed ($P<0.001$) by olmesartan as well as by NADPH oxidase inhibitor apocynin and p38 inhibitor SB202190 (Figure 5D). Furthermore, apocynin markedly enhanced MAPK inhibitor–mediated action.

Discussion

Plasma Biomarkers of CatK and Collagen Turnover and AF

Several studies have examined the effect of myocardial collagen turnover on the pathogenesis of AF and the outcome of AF ablation.[26,30,31] Multiple lines of evidence indicate that CatK is the most abundant and important cysteinyl enzyme synthesized by the cardiovascular system and that it is relevant to cardiovascular disorders, including atherosclerosis,[6–8] osteoarthritis,[32] and heart failure.[27] To the best of our knowledge, this is the first study to show that patients with AF had higher levels of plasma CatK than did control subjects. In agreement with this observation, CatK levels and CatK-related

Table 2. Patient Characteristics

	Control (n=112)	PAF (n=146)	PeAF (n=63)	ANOVA (*P* Value)
Demographic characteristics				
Age, y	54.8±10.7	61.1±10.6*	57.8±11.3*‡	0.04
Female, %	42.9	27.4*	12.7†‡	<0.001
Body mass index, kg/m^2	23.0±2.8	3.5±3.5	24.0±3.1	0.21
Smoker, %	23.2	28.1	34.9	0.31
Causal factors				
Hypertension, %	38.4	46.6	42.9	0.48
Diabetes mellitus, %	12.5	21.9	15.9	0.47
Ischemic heart disease, %	3.6	7.5	9.5	0.25
Chronic heart failure, %	4.5	3.4	15.9†‡	0.002
Echocardiography				
Left atrial diameter, mm	32.3±4.9	37.2±6.1	43.1±7.0†‡	<0.001
LV ejection fraction, %	77.1±6.9	63.5±7.6*	58.0±9.0†‡	<0.001
Blood examination				
Potassium, mEq/L	4.6±0.4	4.2±1.5	4.3±1.3	0.06
LDL, mg/dL	121.3±29.3	120.0±26.5	114.7±30.4	0.24
HDL, mg/dL	57.4±13.3	55.9±15.1	55.3±11.8	<0.001
Hemoglobin A1c, %	5.2±0.7	5.6±0.6*	5.6±0.8*	0.89
hs-CRP, mg/dL	0.06±0.1	0.14±0.1*	0.16±0.2†‡	0.02
ANP, pg/mL	27.3±29.9	59.5±69.9*	97.3±66.4†‡	<0.001
Cystatine C, mg/L	0.9±0.3	1.0±0.2	1.1±0.9	0.23
Medications				
ARBs or ACEIs, %	34.8	37.7	27.0	0.08
CCBs, %	17.0	19.9	20.6	0.05
β-Blockers, %	8.9	30.8*	27.0*	0.01
Statins, %	22.3	25.3	7.9†‡	0.01

Values are expressed as mean±SD. PAF indicates paroxysmal atrial fibrillation; PeAF, persistent atrial fibrillation; LV, left ventricular; LDL, low-density lipoprotein; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; ANP, atrial natriuretic peptide; ARB, angiotensin II receptor blocker; ACEI, angiotensin-converting enzyme inhibitor; CCB, calcium channel blocker.

*P<0.05 compared with control value.

†P<0.01 compared with control values.

‡P<0.05 compared with value for PAF vs PsAF patients.

collagenolytic activity were increased substantially in the atrial tissue of rabbits with tachypacing-induced AF. Univariate regression analysis showed a positive correlation between plasma ICTP and CatK levels. Moreover, multivariable logistic regression analysis clearly showed that CatK and the I-PINP:ICTP ratio were independent predictors of AF. Coupled with several recent studies showing increased serum levels of several Cats (S and L) in association with ischemic heart disease,[8,14] our findings indicate that elevated plasma levels of CatK with a collagen metabolism–related index (I-PINP:ICTP ratio) can serve as a novel marker of AF and a noninvasive method of documenting the mechanisms of atrial fibrosis in AF.

Patients initially presenting with PAF often exhibit disease progression and eventually develop PeAF.[1] Although the exact pathophysiological mechanisms remain unclear, the persistence of AF is thought to result from atrial remodeling.[3,30] Increasing evidence suggests that atrial fibrosis, which has a slower time course than does AF, may be involved in the development and recurrence of AF.[1,30] In the current study, CatK and ICTP (a marker of collagen degradation) levels were higher, but the I-PINP:ICTP ratio was lower, in the PeAF group than in the PAF group. Interestingly, CatK levels were positively correlated with LAD, and patients with PeAF had larger LADs than did those with PAF. These findings imply that—in addition to the duration of arrhythmia—impaired

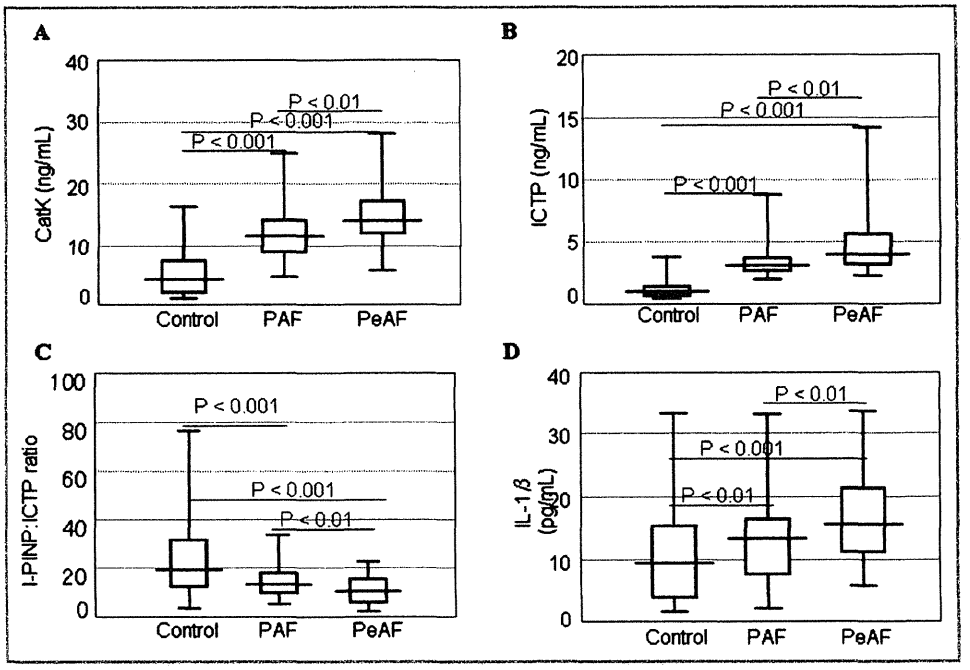

Figure 2. Box plot depiction of the differences in the levels of plasma CatK, I-PINP, ICTP, and IL-1β. Plasma levels of CatK, I-PINP, ICTP, and IL-1β. Levels of (A) CatK and (B) ICTP gradually increased, whereas (C) the I-PINP:ICTP ratios decreased from control subjects to patients with PAF to subjects with PeAF. D, IL-1β levels in control subjects differed from those in both PAF and PeAF patients. Boxes represent the median (black line), 25th percentile, and 75th percentile of observed data; whiskers show the 5th and 95th percentiles of each group. Values are expressed as mean±SD. **P*<0.01 vs control; †*P*<0.01 vs PAF. CatK indicates cathepsin K; ICTP, carboxyl-terminal telopeptide of type I collagen; IL, interleukin; I-PINP, intact procollagen type I of N-terminal propeptide; PAF, paroxysmal atrial fibrillation; PeAF, persistent atrial fibrillation.

Table 3. Independent Predictors of AF According to Multivariable Logistic Regression Analysis

	Odds Ratio Estimate	95% CI	*P* Value
LAD	1.19	1.06 to 1.36	<0.05
CatK	1.27	1.10 to 1.39	<0.01
I-PINP/ICTP ratio	0.79	0.69 to 0.90	<0.001

AF indicates atrial fibrillation; LAD, left atrial diameter; CatK, cathepsin K; I-PINP, intact procollagen type I N-terminal propeptide; ICTP, carboxyl-terminal telopeptide of type I collagen.

balance between collagen synthesis and metabolism may be one of the initiating factors for AF. However, it should be noted that there are also other initiating factors such as the pulmonary veins for the initiation and maintenance of AF.[30]

Ang II Inhibition Alleviates Structural Remodeling Related to AF

Many of the Ang II–induced actions in intracellular signaling transduction pathways that regulate gene expression are mediated by the activation and nuclear translocation of MAPKs.[33,34] We have shown that Ang II promotes p38MAPK phosphorylation and CatK expression in cultured rat neonatal atrial myocytes and that these effects are reversed with olmesartan. An inhibitor of p38MAPK inhibited Ang II–induced CatK gene expression. In vivo studies, AF rabbits exhibited substantial protein expression of CatK accompanied by increased atrial levels of Ang II, AT1R, and phosphorylated p38MAPK proteins, and these changes were reversed by olmesartan. Thus, AT1R antagonism appears to attenuate CatK expression through the AT1R-p38MAPK–dependent signaling pathway in the atrial tissues of AF rabbits. Recently, it was reported that the Cat inhibitor E64d prevented hypertensive cardiac remodeling and dysfunction in a Dahl rat model.[11] In this study, olmesartan attenuated the duration of AF and atrial fibrosis in tachypacing rabbits. Furthermore, olmesartan reduced the increase in collagenolytic activity in the atrial tissue of AF rabbits. The data from enzyme assays demonstrated that increased collagenolytic activity in both atrial tissues and neonatal atrial myocyte–conditioned medium was attenuated by a CatK-specific inhibitor as well as a broad-spectrum Cat inhibitor. Coupled with several recent clinical trials showing the prevention of AF with Ang II inhibition,[22,24] these findings indicate that the attenuation of AT1R/p38MAPK-dependent

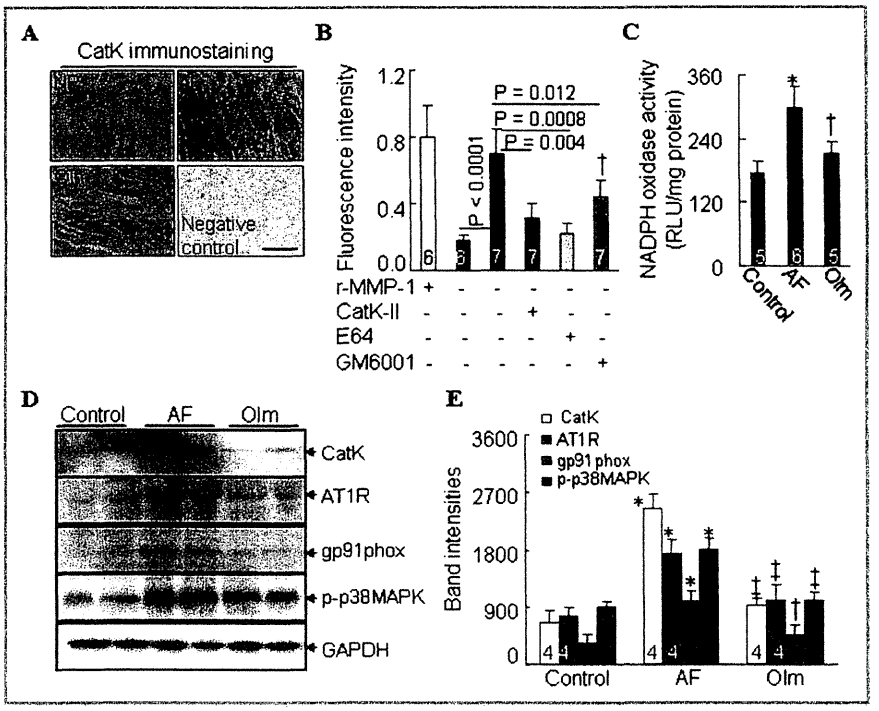

Figure 3. CatK protein expression and NADPH oxidase activity in nonpaced control (NP), ventricular tachypacing (VTP), and ventricular tachypacing plus administration of olmesartan (Olm) rabbits. A, Representative images for CatK immunostaining in the atrial tissues of NP, VTP, Olm rabbits and negative controls (without primary antibody). B, ELISAs of collagenolytic activity in untreated atrial tissues or in those treated with a broad-spectrum inhibitor of Cats (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane [E64], 20 μmol/L; Molecular Probes), a CatK-specific inhibitor (CatK-II, 10 μmol/L), and an inhibitor of matrix metalloproteinases (GM6001, 10 μmol/L; both from Calbiochem). Recombinant matrix metalloproteinase 1 (rMMP-1) was included as a positive control. C, Chemiluminescence showing NADPH oxidase activity in atrial tissues from 3 groups. D, Representative Western blots and (E) quantitative data showing the levels of CatK, AT1R, gp91phox, p-p38MAPK, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in left atrial tissue from rabbits. Analyzed animal numbers indicated on related bars. Scale bars indicate 50 μm. Values are expressed as mean±SEM. *P<0.01 vs NP; †P<0.01, ‡P<0.001 vs VTP. AF indicates atrial fibrillation; AT1R, angiotensin type 1 receptor; CatK, cathepsin K; NADPH, nicotinamide adenine dinucleotide phosphate.

CatK expression and activity by Ang II inhibition could represent a novel mechanism for the protection of structural remodeling-related AF, at least in an animal model. In addition, the data from our study and the findings of other researchers[35,36] suggest that the inhibition of matrix metalloproteinases also contributes to collagen metabolism in atrial fibrosis. To attain a deeper understanding of the importance of CatK participation in this context, additional

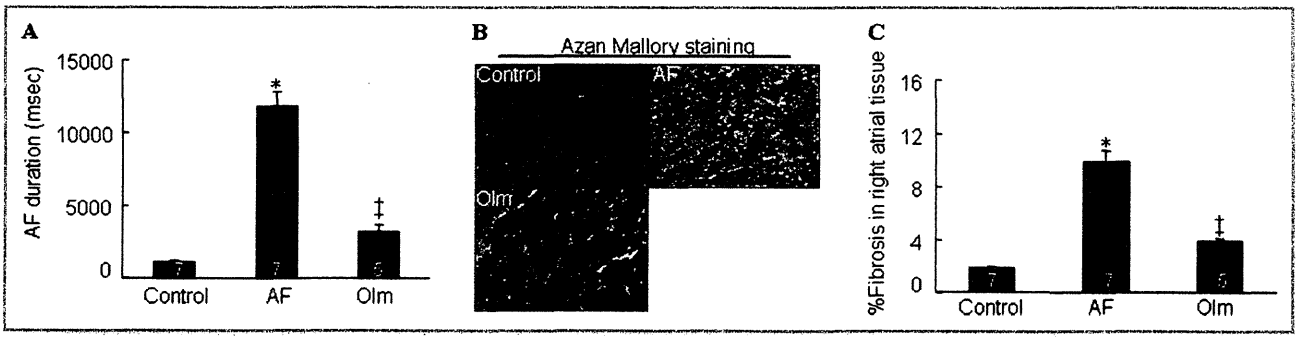

Figure 4. A, Duration of AF in NP, VTP, and olmesartan (Olm)-treated rabbits. B, Representative images and quantitative data show interstitial fibrosis in the right atrial tissues of 3 experimental groups. Analyzed animal numbers indicated on related bars. Scale bars indicate 100 μm. Values are expressed as mean±SEM. *P<0.01 vs control; †P<0.01 vs VTP. AF indicates atrial fibrillation; NP, nonpaced control; VTP, ventricular tachypacing.

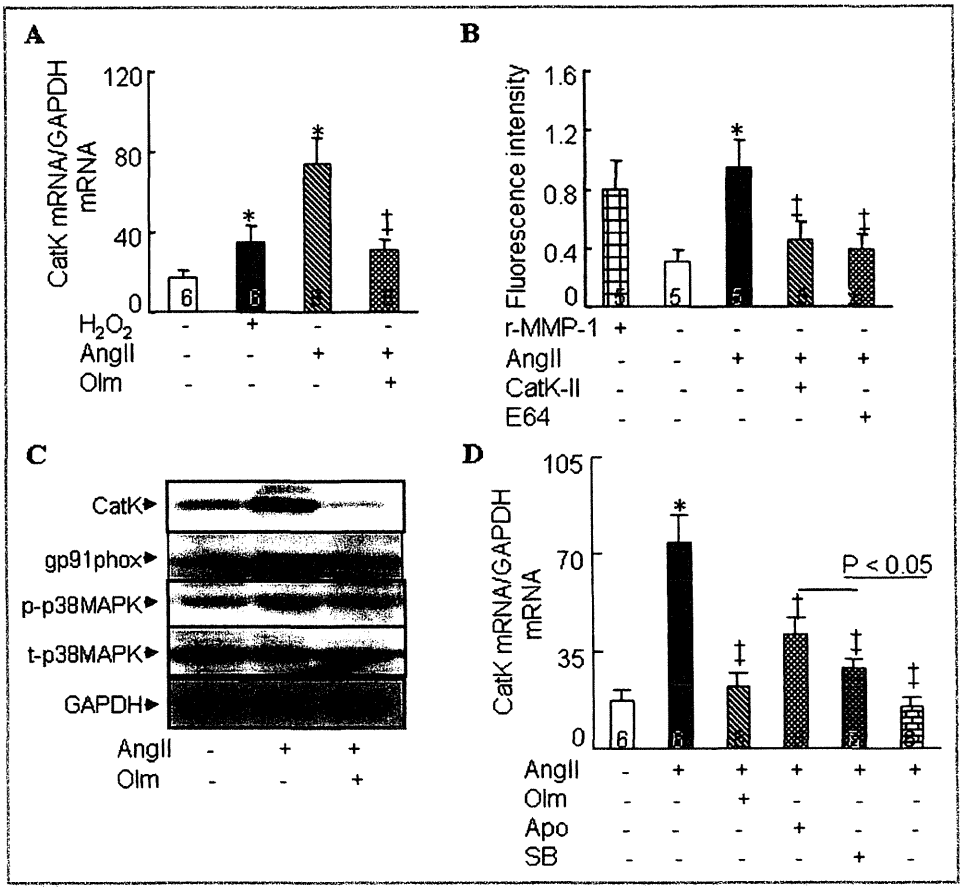

Figure 5. Regulation of CatK expression in cultured rat neonatal atrial myocytes. A, Quantitative real-time PCR assays showing the expression of CatK mRNA levels in cultured cells treated with and without olmesartan (Olm, 1 μmol/L) in the presence of Ang II (1 μmol/L) or H_2O_2 (100 μmol/L) for 24 hours. B, Immunofluorescence shows the collagenolytic activity induced by Ang II in cells left untreated or treated with CatK-II (10 μmol/L) and E64 (10 μmol/L). C, Representative Western blots showing the levels of CatK, gp91phox, p-p38, and t-p38 induced by Ang II in cultured neonatal atrial myocytes untreated or treated with Olm (p-p38MAPK and t-p38MAPK levels for 30 minutes; CatK and gp91phox levels for 24 hours). D, Quantitative PCR shows Ang II–mediated CatK mRNA expression in cells left untreated or treated with Olm (1 μmol/L), apocynin (Apo, 100 μmol/L), SB202190 (SB, 10 μmol/L), or Apo+SB for 24 hours. Analyzed numbers indicated on related bars. Scale bars indicate 50 μm. Values are expressed as mean±SEM. *P<0.05 vs control; †P<0.01, ‡P<0.001 vs corresponding control. Ang indicates angiotensin II; CatK, cathepsin K; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; rMMP1, recombinant matrix metalloproteinase 1.

experiments using animals with conditional gene knockout may be necessary.

Cross-talk Between NADPH Oxidase and Ang II/AT1R Signaling Pathway

NADPH oxidase has been implicated in the pathogenesis of cardiovascular disease.[37,38] Our data show that the abundance and activity of the NADPH oxidase and its subunit gp91phox markedly increased in the atria from rabbits with AF. In vitro, we observed that both Ang II and H_2O_2 enhanced CatK expression and activity in cultured neonatal atrial myocytes. Furthermore, NADPH oxidase inhibition with apocynin showed an inhibitory effect on CatK expression in response to Ang II. Because Ang II inhibition attenuated CatS expression and activation via the reduction of NADPH oxidase activity in cultured macrophages,[6] we propose that superoxide generation by NADPH oxidase, through "cross-talk" with the Ang II signaling pathway, can regulate the proteolytic activity of CatK as well as contribute to the pathophysiology of AF. This notion is supported by current and previous findings that AT1R antagonism attenuated atrial and ventricular remodeling and fibrosis associated with NADPH oxidase–derived superoxide production and CatK expression and activation.[11,20] It is noteworthy that apocynin enhanced SB202190-mediated inhibitory effects on CatK expression in neonatal atrial myocytes. This effect raises the possibility that NADPH-oxidase–derived O_2^- signaling directly affects CatK expression independent of the AT1R/p38MAPK signaling pathway.

Study Limitations

Several limitations of the present study should be pointed out. First, the small number of participants with and without AF limited the power of this study to prove relationships and differences and limited our power to conduct subgroup analysis by PAF and PeAF. Second, the plasma markers of CatK and collagen turnover are not atrial tissue specific. In addition, patients with cardiomyopathy, myocardial infarction, congenital heart disease, congestive heart failure, or valvular heart diseases and those receiving hemodialysis were excluded. It is unclear how their exclusion may have influenced the present results. Third, despite our efforts to match the control and AF groups, the AF patients were older and included more men. However, the levels of CatK and collagen-turnover markers were higher in patients with PeAF than in those with PAF, even though the PeAF group was younger and included more men, indicating that age- and sex-associated differences did not influence the interpretation of our findings. In addition, LAD size and the frequency of β-blocker intake were higher in the AF group than in the control group. Furthermore, a potential impact of these variations on CatK expression was excluded by using multivariable and stepwise logistic models. Fourth, it will also be necessary to investigate cardiovascular events as clinical outcomes in future follow-up studies. Fifth, further study will be also necessary to evaluate dose-dependent beneficial effects of olmesartan on CatK expression and atrial remodeling in rabbit model.

Clinical Implication and Conclusions

Our observations show that increased plasma CatK levels are closely linked to the presence of AF and to increased levels of collagen turnover. Therefore, measurement of the circulating CatK level could provide useful information for the evaluation of atrial remodeling in patients with AF. In addition, our findings confirm and extend earlier work[19] linking the AT1R/p38MAPK signaling pathway and the activation of NADPH oxidase with AF. We showed that increases in CatK expression and activity were accompanied by local atrial changes in Ang II/AT1R signaling pathway activation in the atrium with AF. The antagonism of AT1R-mediated beneficial effects on atrial fibrotic remodeling–related AF are likely attributable, at least in part, to the attenuation of CatK expression and activation induced by the AT1R/p38MAPK-dependent and -independent signaling pathway activations.

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Disclosures

None.

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Ⅳ. 研究成果の刊行物・別刷

LETTERS TO THE EDITOR

RESEARCH

RELATIONSHIP BETWEEN LIGHT-INTENSITY PHYSICAL ACTIVITY AND COGNITIVE FUNCTION IN A COMMUNITY-DWELLING ELDERLY POPULATION—AN 8-YEAR LONGITUDINAL STUDY

To the Editor: There is considerable evidence suggesting the beneficial effects of moderate to vigorous physical activity (MVPA) and the negative effects of sedentary behavior on health outcomes.[1,2] A meta-analysis of 15 prospective studies showed a significant inverse relationship between high levels of physical activity and risk of cognitive decline in cognitively normal older adults,[3] but the contribution of light-intensity physical activity and sedentary time to cognitive function is less well known.

Light-intensity physical activity, which includes activities such as washing dishes, ironing, and other routine domestic or occupational tasks,[4] is the predominant determinant of variability in total daily energy expenditure.[5] Light-intensity physical activity is particularly important for older adults because they tend to spend a greater portion of their day performing light-intensity physical activity than any other age group.[6]

The purpose of the present study was to examine the relationship between light-intensity physical activity and sedentary time and cognitive decline independent of MVPA in a community-dwelling population aged 60 and older.

Participants were 550 (289 men, 261 women) adults aged 60 and older who completed the second (April 2000 to May 2002) and sixth (April 2008 to May 2010) wave of examinations of the National Institute for Longevity Sciences—Longitudinal Study of Aging (NILS-LSA)[7] in Aichi, Japan.

Trained interviewers asked subjects about time spent in physical activity for the past 12 months using a questionnaire developed by the Japanese Lifestyle Monitoring Study Group.[8] The questionnaire captured the duration of light-intensity physical activity, MVPA, and sedentary time determined by metabolic equivalent (MET) scores (a multiple of the resting metabolic rate) reported in the literature.[9]

Cognitive function was assessed using the Mini-Mental State Examination (MMSE). A decline of at least three points in MMSE score from baseline to follow-up was considered meaningful from a clinical point of view.[10]

Education level (>9, ≤9 yr), smoking status (current, former, never), occupation (working or not), depressive symptoms (Center for Epidemiologic Studies Depression

Table 1. Incidence and Odds of Significant Cognitive Decline During the Follow-Up Period According to Quartile of Light-Intensity Physical Activity and Sedentary Time per Day (N = 550)

	Second	Third	Highest	
Light Intensity and Sedentary Time	**Odds Ratio (95% Confidence Interval)**			***P* for Trend**
Light-intensity physical activity time per day (1.6–2.9 METs)				
Time, hours	1.4–2.3 (138)	2.4–3.6 (137)	≥3.7 (139)	
Model 1	0.59 (0.30–1.16)	0.60 (0.30–1.20)	0.50 (0.25–0.99)	.06
Model 2	0.58 (0.28–1.21)	0.53 (0.25–1.12)	0.39 (0.19–0.83)	.02
Model 3	0.58 (0.28–1.20)	0.53 (0.25–1.12)	0.39 (0.18–0.83)	.02
Working				
Yes (n = 155)	0.35 (0.08–1.58)	0.35 (0.09–1.41)	0.37 (0.10–1.47)	.18
No (n = 395)	0.63 (0.26–1.53)	0.61 (0.23–1.60)	0.26 (0.09–0.71)	.01
Education, years				
≤9 (n = 189)	0.30 (0.09–0.97)	0.50 (0.15–1.67)	0.26 (0.07–0.94)	.08
>9 (n = 361)	0.84 (0.32–2.26)	0.52 (0.19–1.43)	0.44 (0.17–1.18)	.07
Sedentary time (≤1.5 METs)				
Time, hours	11.5–13.0 (137)	13.1–14.2 (137)	≥14.3 (139)	
Model 1	1.45 (0.77–2.73)	1.22 (0.65–2.26)	1.97 (1.01–3.86)	.09
Model 2	1.47 (0.74–2.89)	1.37 (0.69–2.70)	2.66 (1.18–5.98)	.03
Model 3	1.57 (0.78–3.16)	1.51 (0.74–3.07)	3.03 (1.29–7.14)	.02
Working				
Yes (n = 155)	2.65 (0.79–8.85)	0.80 (0.27–2.39)	2.04 (0.55–7.65)	.66
No (n = 395)	1.13 (0.45–2.88)	1.85 (0.70–4.88)	3.74 (1.21–11.58)	.02
Education, years				
≤9 (n = 189)	2.67 (0.85–8.34)	1.99 (0.66–5.99)	3.90 (1.02–14.94)	.07
>9 (n = 361)	1.48 (0.58–3.79)	1.32 (0.52–3.38)	2.73 (0.87–8.55)	.12

The lowest quartile was used as a reference. Cognitive decline indicated as change of at least three points on the Mini-Mental State Examination. Model 1 was controlled for age, sex, and educational level; Model 2 was controlled as in Model 1 with further control for smoking status, self-rated health, Center for Epidemiological Studies—Depression Scale, sleep duration, whether participant was working, hypertension, myocardial infarction, hyperlipidemia, diabetes mellitus, stroke, and rheumatoid arthritis. Model 3 was controlled as in Model 2 with further control for moderate- to vigorous-intensity physical activity time. Subgroups were controlled as in Model 3 without control for educational level (education group) and occupational level (occupation group). METs = metabolic equivalents of a task.

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Scale (CES-D) score ≥16, <16), body mass index (BMI), self-rated health, sleep duration, and history of medical conditions (hypertension, myocardial infarction, hyperlipidemia, diabetes mellitus, stroke, rheumatoid arthritis) were based on answers to the questionnaire and interview.

For all logistic regression analyses, Model 1 was adjusted for age, sex, and educational level. Model 2 was further adjusted for BMI, initial MMSE score, smoking status, self-rated health, CES-D score, education level, sleep duration, occupation, hypertension, myocardial infarction, hyperlipidemia, diabetes mellitus, stroke, and rheumatoid arthritis. Model 3 was further adjusted for MVPA time. The odds of cognitive decline relative to light-intensity physical activity and sedentary time was also analyzed in subgroups of subjects (educational level ≤9 vs >9 years, working vs not). All analyses were performed using SAS version 9.1.3 (SAS Institute, Inc., Cary, NC).

There were 96 cases of cognitive decline over a mean follow-up of 8 years in the 550 subjects. Compared with the lowest quartile, the odds of developing cognitive decline were lower (Model 1: odds ratio (OR) = 0.50, 95% confidence interval (CI) = 0.25–0.99; Model 2: OR = 0.39, 95% CI = 0.19–0.83; Model 3: OR = 0.39, 95% CI = 0.18–0.83) in the highest quartile of light-intensity physical activity time, and the dose-related response was significant in Models 2 and 3 (P for trend = .02 for each). In contrast, those in the highest quartile of overall sedentary time were more likely to show cognitive decline than those in the lowest quartile (Table 1). This tendency was also found in subjects who were not working and with lower education, regardless of multivariate control.

This study provides longitudinal evidence associations between light-intensity physical activity time and overall sedentary time and cognitive decline over an 8-year period in community-dwelling adults aged 60 and older. This association remained after controlling for covariates including baseline health status and MVPA time.

These findings have important implications because it may be easier for older adults to increase light-intensity physical activity than to increase vigorous or moderate structured exercise training. This could be particularly important for older adults who tend to spend more time participating in light-intensity physical activity than in more-vigorous activities. Light-intensity physical activity interventions may also be more likely to succeed across a variety of settings, including the workplace.

SungChul Lee, PhD
Atsumu Yuki, PhD
Yukiko Nishita, MD
Chikako Tange, PhD
Department for Development of Preventive Medicine, Center for Development of Advanced Medicine for Dementia, National Center for Geriatrics and Gerontology, Morioka, Obu, Aichi, Japan

HeungYoul Kim, PhD
Department of Human Wellness, Tokaigakuen University, Ukigai-cho, Miyoshi, Aichi, Japan

Rumi Kozakai, PhD
Department of Sport Education, School of Lifelong Sport, Hokusho University, Hokkaido, Japan

Fujiko Ando, MD, PhD
Faculty of Health and Medical Sciences, Aichi Shukutoku University, Nagakute-cho, Aichi, Japan

Hiroshi Shimokata, MD, PhD
Department for Development of Preventive Medicine, Center for Development of Advanced Medicine for Dementia, National Center for Geriatrics and Gerontology, Morioka, Obu, Aichi, Japan

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PLOS ONE

A Randomized Controlled Trial of Multicomponent Exercise in Older Adults with Mild Cognitive Impairment

Takao Suzuki[1]*, Hiroyuki Shimada[2], Hyuma Makizako[2], Takehiko Doi[2], Daisuke Yoshida[2], Kengo Ito[3], Hiroshi Shimokata[4], Yukihiko Washimi[5], Hidetoshi Endo[6], Takashi Kato[3]

1 Research Institute, National Center for Geriatrics and Gerontology, Obu, Aichi, Japan, 2 Section for Health Promotion, Department for Research and Development to Support Independent Life of Elderly, Center for Gerontology and Social Science, National Center for Geriatrics and Gerontology, Obu, Aichi, Japan, 3 Department of Clinical and Experimental Neuroimaging, Center for Development of Advanced Medicine for Dementia, National Center for Geriatrics and Gerontology, Obu, Aichi, Japan, 4 Department for Development of Preventive Medicine, Center for Development of Advanced Medicine for Dementia, National Center for Geriatrics and Gerontology, Obu, Aichi, Japan, 5 Department of Cognitive Disorders, Hospital of National Center for Geriatrics and Gerontology, National Center for Geriatrics and Gerontology, Obu, Aichi, Japan, 6 Department of Comprehensive Geriatric Medicine, Hospital of National Center for Geriatrics and Gerontology, National Center for Geriatrics and Gerontology, Obu, Aichi, Japan

Abstract

Background: To examine the effect of multicomponent exercise program on memory function in older adults with mild cognitive impairment (MCI), and identify biomarkers associated with improvement of cognitive functions.

Methodology/Principal Findings: Subjects were 100 older adults (mean age, 75 years) with MCI. The subjects were classified to an amnestic MCI group (n = 50) with neuroimaging measures, and other MCI group (n = 50) before the randomization. Subjects in each group were randomized to either a multicomponent exercise or an education control group using a ratio of 1:1. The exercise group exercised for 90 min/d, 2 d/wk, 40 times for 6 months. The exercise program was conducted under multitask conditions to stimulate attention and memory. The control group attended two education classes. A repeated-measures ANOVA revealed that no group × time interactions on the cognitive tests and brain atrophy in MCI patients. A sub-analysis of amnestic MCI patients for group × time interactions revealed that the exercise group exhibited significantly better Mini-Mental State Examination ($p = .04$) and logical memory scores ($p = .04$), and reducing whole brain cortical atrophy ($p < .05$) compared to the control group. Low total cholesterol levels before the intervention were associated with an improvement of logical memory scores ($p < .05$), and a higher level of brain-derived neurotrophic factor was significantly related to improved ADAS-cog scores ($p < .05$).

Conclusions/Significance: The results suggested that an exercise intervention is beneficial for improving logical memory and maintaining general cognitive function and reducing whole brain cortical atrophy in older adults with amnestic MCI. Low total cholesterol and higher brain-derived neurotrophic factor may predict improvement of cognitive functions in older adults with MCI. Further studies are required to determine the positive effects of exercise on cognitive function in older adults with MCI.

Trial Registration: UMIN-CTR UMIN000003662

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* E-mail: suzutaka@ncgg.go.jp

Introduction

Alzheimer's disease (AD) places a considerable and increasing burden on patients, caregivers and society. The number of older adults living with AD is predicted to increase from the current 26.6 million to 106.2 million by 2050 globally. [1] The current standard of care for mild to moderate AD involves treatment with acetylcholinesterase inhibitors to improve cognitive function. The *N*-methyl-d-aspartate antagonist memantine has also been reported to improve cognitive function in patients with moderate to severe AD. [2] While these drugs improve the symptoms of AD, they do not have substantial disease-modifying effects. [3] Thus, attempts have been made to identify individuals at increased risk of AD, and to test interventions that might delay the progression of prodromal symptoms of dementia.

An association has been proposed between regular participation in physical activity, especially aerobic exercise, and a variety of cognitive benefits. [4,5,6,7,8] Several meta-analyses have reported that physical activity is associated with improvements in attention, processing speed, and executive function in older adults with and without cognitive impairments. [9,10,11] However, these studies produced some inconsistent findings, with some reporting

cognitive gains in memory function [10,11] and other study reporting equivocal results. [9]

Evidence from neuropsychological and neuroimaging studies has suggested that mild cognitive impairment (MCI) represents a clinical prodrome to degenerative dementias such as AD. [12] For example, a population-based study in Sweden reported that the relative risks of progression to dementia in a 3-year follow-up in subjects with mild, moderate, and severe cognitive impairment (without dementia), were 3.6, 5.4, and 7.0, respectively. [13] However, of the individuals with MCI, 11% remained stable, and 25% exhibited an improvement in cognitive function between baseline and follow-up observation. [13] This variation in MCI populations should be examined to facilitate the development of interventions for inhibiting the progression of dementia. Several randomized controlled trials (RCTs) have been conducted to investigate the effects of exercise or physical activity on cognitive function in older adults with MCI. [4,5,6,7,8] These studies have revealed the effects of exercise or physical activity on cognitive function, including executive function, in older adults with MCI. However, the effect of exercise on memory function in this population remains unclear.

The precise neurobiological mechanism for the improvement of cognitive functions remains unknown, however a large number of rodent studies suggest a central role of certain molecules such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor (IGF-1), and vascular endothelial growth factor (VEGF). The molecules have been shown to facilitate neurogenesis in the hippocampus, promote synaptic plasticity in the hippocampus and cerebral cortex, and angiogenesis and enhance growth and protection of neurovasculature. [14,15] In fact, some neuroimaging studies of human subjects revealed that aerobic exercise increased hippocampal volume, [16] and gray and white matter regions including the cingulate cortex, supplementary motor cortex, inferior frontal gyrus, and superior temporal gyrus. [17]

The present randomized trial was designed to test whether a 6-month supervised multicomponent exercise program could reduce the rate of cognitive decline, especially in memory function, and reduce the rate of brain volume decline among older adults with MCI. The multicomponent exercise program included aerobic exercise, muscle strength training, and postural balance retraining, because previous reviews suggested that combined aerobic exercise and strength training interventions improved attention and working memory to a greater extent than aerobic exercise alone. [11,18] We explored the biomarkers for identifying improvement of cognitive functions. Serum total cholesterol (T-cho), hemoglobin A1c (HbA1c), BDNF, and VEGF levels at baseline were used as potential predictors.

Methods

CONSORT checklist and the protocol for this trial is available as supporting information; see **Checklist S1** and **Protocol S1**.

Participants

Subjects in this study were recruited from two volunteer databases (n = 1,543), which included elderly individuals (65 years and over) selected either by random sampling or when they attended a medical check-up in Obu, Japan. Inclusion criteria specified that prospective participants were community-dwelling individuals aged 65 years and over. A total of 528 prospective participants with a Clinical Dementia Rating (CDR) of 0.5, or who complained of memory impairment, were recruited in the first round of eligibility assessments. Of these, 135 subjects satisfied the requirements of the second round of eligibility assessments, which included neuropsychological tests, which included language and memory tests, attention and executive function tests, clinical diagnosis, activities of daily living (ADL), educational level, and magnetic resonance imaging. Thirty-five subjects were excluded, meaning that a total of 100 subjects took part in the study (mean age, 75.4±7.1 years; 65–95 years, men n = 55, 51%). All subjects met the definition of MCI as per the Petersen criteria. [19] All MCI subjects had objective impairments in either episodic memory and/or executive functioning at least 1.5 standard deviations below the age-adjusted mean for at least one of the neuropsychological tests. Final classification of subjects was based on the above factors and consensus of a team of neuroscientists. Exclusion criteria included a CDR = 0, or a CDR of 1–3, a history of neurological, psychiatric, or cardiac disorders or other severe health issues, use of donepezil, impairment in basic activities of daily living (ADL), and participation in other research projects. Subjects were classified to an amnestic MCI group (aMCI) (n = 50) with neuroimaging measures, and other MCI group (n = 50) before the randomization. Then, the subjects in each group were randomly assigned to either a multicomponent exercise or an education control group using a ratio of 1:1. Participant characteristics at the beginning of the study are shown in **Table 1**. We confirmed that there were no significant differences in demographic characteristics, physical performance, or instrumental ADL levels between the exercise and control groups. Fifty subjects with aMCI (mean age, 76.0±7.1 years; 65–92 years, men n = 27, 54%) were selected from among the subjects to participate in a sub-analysis. All subjects included the aMCI group agreed to measure functional neuroimaging tests. This sub-analysis was limited to aMCI patients because aMCI is most likely to progress to AD.[20] Objective memory impairment to determine aMCI was defined as a lower memory score on the Wechsler Memory Scale-Revised (WMS-R) Logical Memory II. [21]

Ethics

The Ethics Committee of the National Center for Geriatrics and Gerontology approved the study protocol. The purpose, nature, and potential risks of the experiments were fully explained to the subjects, and all subjects gave written, informed consent before participating in the study. The subjects had the capacity to consent because they maintained general cognitive function and daily activities.

Interventions

The six-month, multicomponent exercise program included biweekly 90-minute sessions involving aerobic exercise, muscle strength training, postural balance retraining, and dual-task training. In addition, the exercise program included a focus on promoting exercise and behavior change. Two trained physiotherapists involved in geriatric rehabilitation conducted each intervention. Each exercise class contained 16–17 participants, and each supervised session began with a 10-min warm-up period and stretching exercise, followed by 20 min of muscle strength exercise. The subjects then practiced aerobic exercise, postural balance retraining, and dual-task training for 60 min. In the aerobic exercise and postural balance retraining, subjects underwent circuit training, including stair stepping, endurance walking, and walking on balance boards. The mean intensity of the aerobic exercise was approximately 60% of maximum heart rate which was similar to the intensity used in previous studies. [4,6] Eleven of the 40 classes during the six-month intervention period included approximately 20–30 minutes of consecutive outdoor walking. In the dual-task training sessions, subjects performed concurrent cognitive tasks during exercise. For example, the subjects in the

Table 1. Characteristics of the subjects.

	All subjects		aMCI subjects	
	Exercise (n=50)	Control (n=50)	Exercise (n=25)	Control (n=25)
Age, mean (SD), y	74.8 (7.4)	75.8 (6.1)	75.3 (7.5)	76.8 (6.8)
Men, No. (%)	25 (50.0)	26 (52.0)	13 (52.0)	14 (56.0)
Educational level, mean (SD), y	10.9 (2.8)	10.4 (2.4)	11.1 (2.4)	10.8 (2.7)
Diagnosis, No. (%)				
Hypertension (3*, 1†)	23 (46.9)	22 (45.8)	13 (52.0)	11 (45.8)
Heart disease (4*, 1†)	5 (10.2)	1 (2.1)	2 (8.0)	0 (0)
Diabetes Mellitus	8 (16.0)	3 (6.0)	5 (20.0)	3 (12.0)
Medication, 3 and over (2*, 1†)	22 (44.0)	19 (39.6)	10 (40.0)	11 (45.8)
Blood pressure, mmHg				
Systolic, mean (SD)	144.6 (21.6)	142.4 (19.4)	152.2 (21.0)	143.7 (21.3)
Diastolic, mean (SD)	74.6 (11.7)	75.1 (11.2)	77.3 (11.1)	74.3 (10.1)
Blood test				
Total cholesterol, mean (SD), mg/dL	211.7 (36.2)	200.5 (34.5)	212.6 (36.9)	202.8 (32.2)
HbA1c, mean (SD), %	5.6 (0.8)	5.4 (0.5)	5.6 (0.6)	5.4 (0.5)
BDNF, mean (SD), ng/mL	12.1 (10.0)	13.5 (10.4)	11.9 (11.3)	14.4 (12.2)
VEGF, mean (SD), pg/mL	97.6 (19.7)	103.5 (22.2)	95.9 (18.4)	96.7 (15.4)
Physical performances				
Grip strength, mean (SD), kg	24.7 (8.1)	23.5 (7.3)	25.2 (7.3)	23.1 (8.4)
One legged standing, mean (SD), s	34.6 (24.6)	31.2 (23.9)	34.0 (25.1)	29.3 (23.6)
Timed up & go, mean (SD), s	8.8 (2.5)	9.2 (2.1)	9.0 (2.2)	9.1 (2.0)
IADL subscale of TMIG index, mean (SD), score	4.8 (0.9)	4.9 (0.3)	5.0 (0.2)	4.9 (0.3)
GDS, mean (SD), score	3.8 (3.1)	3.3 (2.8)	3.0 (2.1)	2.6 (2.0)
Cognitive functions, score				
MMSE, mean (SD)	26.8 (2.3)	26.3 (2.7)	26.8 (1.8)	26.6 (1.6)
ADAS-cog, mean (SD)	6.0 (2.8)	6.5 (2.8)	6.3 (2.2)	6.8 (2.2)
WMS-LM I, mean (SD)	14.6 (6.9)	13.8 (7.4)	12.5 (5.9)	12.0 (4.9)
WMS-LM II, mean (SD)	10.5 (7.4)	9.4 (7.4)	8.2 (5.4)	6.9 (5.0)
Clinical subtype, No. (%)				
Amnestic MCI	34 (68.0)	37 (74.0)		
Non-amnestic MCI	16 (32.0)	13 (26.0)		
VSRAD				
MTA-ERC atrophy, mean (SD) (1*)	1.3 (0.9)	1.5 (1.0)	1.4 (1.0)	1.4 (1.0)
WBC atrophy, mean (SD) (1*)	7.3 (4.7)	8.3 (4.6)	7.9 (3.9)	7.4 (3.3)

Abbreviations: IADL subscale of TMIG index, instrumental activities of daily living subscale of Tokyo Metropolitan Institute of Gerontology index; GDS, Geriatric Depression Scale; MMSE, Mini-Mental State Examination; ADAS-cog, Alzheimer's Disease Assessment Scale-cognitive subscale; WMS, Wechsler Memory Scale; MCI, mild cognitive impairment. *missing value in all subjects. †missing value in the aMCI subjects.

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exercise group were asked to walk while inventing their own poem, as aerobic exercise. In the ladder training exercise, subjects learned to step in consecutive square segments, and were instructed to step as quickly and accurately as possible. Before and after each session of the program, physiotherapists conducted a health check of each subject. The physiotherapists and a well-trained instructor implemented risk management for accidents and other adverse events during the program. The subjects were instructed to carry out daily home-based muscle strength exercises and walking, which were self-monitored using a booklet and pedometer based on the concept of promoting exercise and behavior change. Attendance at each session was recorded and a transportation service was provided for participants, if necessary, to help subjects maintain their participation in the program.

Subjects in the education control group attended two education classes about health promotion during the 6-month study period. The class provided information regarding healthy diet, oral care, prevention of urinary incontinence, and health checks. However, the group did not receive specific information regarding exercise, physical activity, or cognitive health.

Outcomes

Cognitive Functions. The Mini-Mental State Examination (MMSE) [22] and Alzheimer's Disease Assessment Scale-Cognitive subscale (ADAS-cog) [23] were used to assess general cognitive function.

Modified versions of the logical memory subtest from the WMS-R [21] was used to assess memory function. In the WMS-R, two short stories (Story A and B) were read aloud to the subject, who was then instructed to recall details of the stories immediately (LM I, immediate recall) and after 30 min (LM II, delayed recall; each total recall score = 50). [21]

MRI. MRI was performed with a 1.5-T system (Magnetom Avanto, Siemens, Germany). Three-dimensional volumetric acquisition with a T1-weighted gradient echo sequence was then used to produce a gapless series of thin sagittal sections using a magnetization preparation rapid-acquisition gradient-echo sequence (repetition time, 1700 ms; echo time, 4.0 ms; flip angle 15°, acquisition matrix 256×256, 1.3-mm slice thickness).

In analysis of brain volume, we used the voxel-based specific regional analysis system for Alzheimer's disease (VSRAD), which enables the examination of atrophy of the bilateral medial temporal areas including the entorhinal cortex (MTA-ERC) using voxel-based morphometry. [24] The VSRAD has been shown to achieve high accuracy (87.8%) in discriminating patients in the very early stages of AD with MCI from normal control subjects using Z scores. [24] A previous VSRAD study reported that atrophy of the MTA-ERC exhibited a clear functional relationship with blood flow changes in the hippocampus, thalamus and temporal lobe, which were suggested to be closely related to inter-regional anatomical and physiological connections. [25]

Acquired MRI images were formatted to gapless, transaxial images, followed by extraction of the gray matter images using SPM2. Anatomical standardization was used to fit each individual brain to standard template MRIs in the common coordinate system of the MNI T1 MRI template. [26] The segmented gray matter images were then subjected to affine and nonlinear standardization using a template of prior gray matter. The anatomically standardized gray matter images were then smoothed again using an isotropic Gaussian kernel 12 mm in full width at half maximum, to determine the partial volume effect and create a spectrum of gray matter intensities. Gray matter intensities were equivalent to the weighted average of gray matter voxels located in the volume fixed by the smoothing kernel. Regional intensity was considered equivalent to gray matter concentration. We compared the gray matter image of each patient with the mean and standard deviation (SD) of gray matter images of healthy volunteers using voxel-by-voxel Z score analysis. In the final step, the Z score was calculated according to the following equation: (Z score = ((control mean) − (individual value))/control SD). The Z score thus reflected the degree of atrophy in bilateral MTA-ERC. Higher Z scores indicated clearer MTA-ERC atrophy. VSRAD also automatically measured the degree of atrophy in the whole brain cortices (WBC), including the hippocampus: if the Z-score was more than 2.0 within a voxel, the area was considered to exhibit atrophy. [24] Thus, the proportion of atrophic area in the whole brain (%) was measured in the following way: 100×([the number of voxels with Z-score≥2.0]/[the number of whole brain voxels]).

Biochemical measures3. T-cho, HbA1c, BDNF, VEGF receptor 1 (VEGFR1) were used as biomarkers. Blood samples were collected between 11 am and 4 pm in a non-fasting state. The blood samples were kept at room temperature for 30 min to allow for clotting, after which the samples were centrifuged for 15 min. Serum was then harvested and stored at −25 °C until analysis. Analyses were carried out centrally in one laboratory (Special Reference Laboratories, Tokyo, Japan). BDNF and VEGFR1 were measured with the Quantikine Human kit (R&D systems, Inc. Minneapolis, MN, USA). Coefficients of variation (CVs) of BDNF in intra-assay and inter-assay precision were 2.6 3.2 and 3.5 9.8, respectively. Those of VEGFR1 were 3.8 6.2 for intra-assay and 7.6 11.3 inter-assay precision.

Sample size

Since participants were selected on the basis of memory impairments, memory was considered the most important cognitive outcome in our study. Therefore, sample size calculations were based on AVLT data. [27] A previous study reported that a sample of 34 participants per group to detect a clinically relevant effect, with 80% power. [6] To allow for a dropout of 25%, the final sample size was 85 participants.

Randomization–Sequence generation

Subjects were randomly assigned after completion of baseline assessments. Subjects were classified to an amnestic MCI group (n = 50) with neuroimaging measures, and other MCI group (n = 50) before the randomization. The subjects in each group were randomized to either a multicomponent exercise or an education control group using a ratio of 1:1. The subjects were further randomized and dichotomized into two groups, an amnestic MCI group (n = 50) with neuroimaging measures, and other MCI group (n = 50).

Randomization–Implementation and concealment

After the baseline assessment, subjects were randomized using the option "random sample of cases" in IBM SPSS statistics software (Version 19; SPSS Inc., Chicago, IL, USA). A researcher who was not aware of the aims of the study performed the randomization procedure.

Blinding

Study personnel involved in the collection of outcome measures were blinded to the randomization assignment. Several trained speech therapists blinded to group status conducted the cognitive tests, and one speech therapist recalculated all of the results.

Statistical methods

Statistical analysis was performed using IBM SPSS statistics software. For the baseline comparisons between exercise and control groups for all subjects, and for the amnestic MCI (aMCI) sub-analysis, Pearson's method, together with Chi square analysis with Fisher's exact test was used to investigate the categorical data. Kolmogorov-Smirnov tests confirmed that all continuous variables followed a normal distribution. Basic characteristics of patients were compared between the two groups using *t*-tests.

A general linear model for repeated-measures analysis of variance (ANOVA) was used to determine the group difference for the cognitive tests and VSRAD measurements. Two time points were treated as the within-subjects factor (effect over time) and the differences between the exercise and control groups were treated as the between-subjects factor. When the repeated-measures ANOVA indicated that the group × time interaction was significant, tests of simple main effects were performed to determine which group or groups differed significantly across the intervention period. Alpha level of the post-hoc analyses were adjusted for the Bonferroni method, i.e. corrected alpha = .025.

Multiple logistic regression models were used to determine the predictors of improvements in cognitive function. Dependent variables were the cognitive tests which showed significant improvements in the comparison between before and after the intervention of all subjects. Based on the results from the cognitive tests, the subjects were dichotomized into two categories; the subjects who improved their cognitive test scores (improvement

group) and the subjects who showed no improvement, or who exhibited a deterioration in their cognitive test scores (no improvement group). Biochemical variables at baseline measurements were treated as independent variables. Covariates such as age, sex, educational level, and the intervention group were included in the logistic model.

The univariate analyses and repeated-measures ANOVA were performed with all subjects grouped together as well as with a subgroup that was limited to older adults with aMCI. The logistic regression analysis was performed to determine the predictors of improvement of cognitive functions in all subjects. All statistical significance tests were two-sided, and an alpha-level of .05 was considered statistically significant.

Results

Participant flow

Figure 1 shows the flow of participants from the time of screening to study completion at 6 months. Ninety-two (exercise group, n = 47) subjects completed the 6-month follow-up. Of the 50 aMCI subjects, 47 (94%) completed the 6-month follow-up. Two of the remaining 47 subjects in the exercise group (one male, one female) missed all exercise programs, but completed the examinations before and after the intervention. The two subjects were included in the following analyses. Mean adherence to the exercise program, including the remaining 47 subjects, was 85.9%, and 38 subjects (80.9%) in the exercise group attended our intervention program with greater than 80% adherence.

Baseline data

There were no significant differences in baseline characteristics between all subjects grouped together and the aMCI group alone (**Table 1**).

Participants analyzed

Our primary analysis of cognitive function included all patients who remained at the end of the study (total n = 92; exercise group, n = 47; control group, n = 45). A total of 90 subjects (exercise group, n = 46; control group, n = 44) completed MRI scanning. When the analyses were limited to subjects with aMCI, the exercise and control groups included 24 and 23 subjects in assessments of cognitive function and MRI, respectively.

Outcomes in all MCI subjects

Table 2 shows changes in cognitive scores over the 6-month period by group. There were main effects of time in ADAS-cog ($p = .01$), WMS-LM I ($p < .01$), WMS-LM II ($p < .01$), and WBC atrophy level ($p = .03$), although no main effects of group and group × time interactions were detected on the cognitive tests and brain atrophy (**Table 2**).

Outcomes in aMCI subjects

When the analyses were limited to subjects with aMCI, the repeated-measures ANOVA for MMSE showed a significant effect of group ($p = .03$) and there was a group × time interaction in MMSE ($p = .04$) indicating benefit of the exercise over time. Tests of simple main effects revealed that the control group decreased in MMSE score ($p = .015$) after intervention. A repeated-measures ANOVA showed a significant effect of time ($p < .01$) and group × time interaction ($p = .04$) in WMS-LM I. Tests of simple main effects showed that the exercise group exhibited better WMS-LM I ($p < .01$) scores compared to baseline, but not in the control group. The repeated-measures ANOVA for WMS-LM II ($p < .01$) and MTA-ERC atrophy ($p = .03$) showed a significant effect of time. However, there were no main effects of group and no group × time interactions. A repeated-measures ANOVA showed a significant group × time interaction ($p < .05$) in WBC atrophy level. There were no main effects of group or time. Tests of simple main effects revealed that the subjects in the control group showed increased WBC atrophy ($p = .01$) after intervention, compared with their baseline scores (**Table 2, Figure 2**).

Relationships between cognitive functions and biomarkers

Paired *t*-tests revealed significant improvements in ADAS-cog ($p = .01$), WMS-LM I ($p < .01$), and WMS-LM II scores ($p < .01$) after the intervention. Multiple logistic regression analysis revealed that low T-cho level before the intervention was associated with improvement in WMS-LM I (odds ratio (OR) 0.98, 95% confidence interval (95% CI) 0.96 1.00, $p = .02$). Higher BDNF level at baseline was significantly related to improvements in ADAS-cog (OR 1.07, 95% CI 1.02 1.13, $p = .01$) independent of age, sex, educational level, and intervention (**Table 3**).

Adverse events

Four subjects (exercise group, n = 2; control group, n = 2) experienced adverse events (hospitalization for illness). Falls (as a type of minor adverse event) over a 6-month period were reported by 23/90 (26%) of subjects, with no significant differences among groups. There were no other adverse events during exercise intervention for 6-months.

Discussion

Evidence of exercise on cognitive function

Older adults with MCI have been found to exhibit greater decreases in memory function than in other cognitive functions, relative to healthy older adults. [28] The enhancement of cognitive function, especially memory function, in individuals with MCI may play a crucial role in preventing the progression from MCI to AD in older adults. Klusmann et al. reported significant effects of a multifaceted exercise program on cognitive function, finding that a 6-month exercise program resulted in improvements in delayed story recall. [29] However, their sample consisted of healthy, well-functioning females without any signs of cognitive impairment. In addition, previous studies reported that aerobic exercise or other physical activity can increase executive function in older adults with cognitive impairments, but the effects of exercise on memory function in this population remain unclear. [4,5,6,7,8] To our knowledge, this is the first study to demonstrate an improvement in logical memory following multicomponent exercise training among older adults with aMCI. The exercise group showed significant differences not only in WMS-LM I scores, but also in MMSE scores compared to the control group in aMCI populations. Our intervention study extends the results of previous studies with healthy samples, indicating the potential for an increase in memory performance and maintenance of general cognitive function in subjects exhibiting signs of cognitive decline.

A meta-analysis of aerobic exercise and neurocognitive performance demonstrated that interventions combining aerobic exercise and strength training, similar to our program, improved attention, processing speed and working memory to a greater extent than aerobic exercise alone. [11] However, the mechanism underlying this improvement remains unclear. A previous study reported that subjects with MCI improved their episodic memory performance when they were exposed to a multifactorial cognitive intervention program that included dual-task attentional and memory training. [30] Dual-task deficit is recognized as a potential

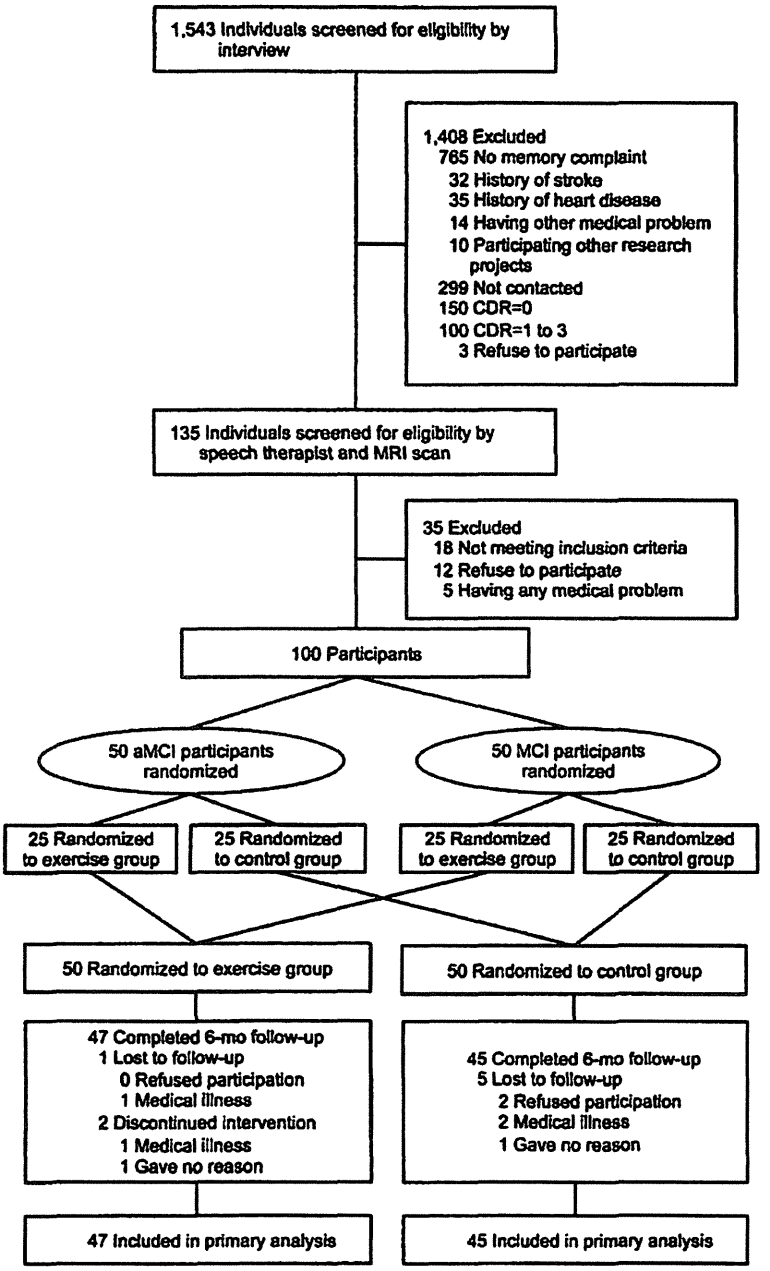

Figure 1. Subject flow diagram from initial contact through to study completion.
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early marker for dementia, [31,32] and dual-task-related changes in performance were greater in subjects with MCI compared with cognitively normal age-matched controls. [33,34] Our multicomponent program involved changes in cognitive load using dual-task stimulation and learning tasks. We believe that dual-task training may have a greater effect on various cognitive functions, for example, general and memory functions, than interventions that only focus on aerobic exercise. [7,10] However, the results from the present study do not provide direct evidence for the positive effect of dual-task training. Future studies are required to investigate the effects of dual-task training on cognitive function in the older adults with MCI.

Lautenschlager et al. reported that physical activity and behavioral intervention improved general cognition among adults with MCI. [4] The multicomponent exercise training in the current study also included encouragement for subjects to engage in more physical activity. Our results further support the notion that training involving physical activity can have a beneficial effect

Table 2. Comparison of Cognitive Function between the Exercise and Control Group.

	All subjects (n = 100)						aMCI subjects (n = 50)					
	Mean Difference From Baseline (95% CI) in All Subjects		P Value ANOVA for Repeated Measures			ES	Mean Difference From Baseline (95% CI) in aMCI Group		P Value ANOVA for Repeated Measures			ES
	Exercise Group (n = 47)	Control Group (n = 45)	Group	Time	Group × time interaction	r	Exercise Group (n = 24)	Control Group (n = 23)	Group	Time	Group × time interaction	r
MMSE	0.2 (−0.5, 0.9)	−0.3 (−1.1, 0.4)	0.18	0.79	0.32	0.11	0.3 (−0.8, 1.3)	−1.4 (−2.5, −0.3)	0.03	0.14	.04[b]	0.31
ADAS-cog	−0.8 (−1.4, −0.2)	−0.2 (−0.8, 0.4)	0.17	0.01	.16 (1)[c]	0.15	−1.2 (−2.1, −0.3)	−0.1 (−1.0, 0.8)	0.1	0.06	0.1	0.24
WMS-LM I	2.8 (1.4, 4.2)	1.0 (−0.5, 2.4)	0.29	<.01	0.08	0.19	3.8 (1.6, 5.9)	0.5 (−1.6, 2.7)	0.14	<.01	.04[a]	0.31
WMS-LM II	3.4 (2.0, 4.8)	1.9 (0.4, 3.4)[b]	0.28	<.01	0.15	0.15	3.8 (1.8, 5.7)	2.1 (0.1, 4.2)	0.11	<.01	0.26	0.17
MTA-ERC	0 (−0, 0.1)	0 (0, 0.1)	0.18	0.08	0.89	0.02	0.1 (0, 0.2)	0 (−0.1, 0.1)	0.91	0.03	0.27	0.17
WBC	0.1 (−0.4, 0.7)	0.7 (0.1, 1.2)	0.08	0.03	0.16	0.15	−0.1 (−0.8, 0.6)	0.9 (0.2, 1.6)	0.86	0.08	<.05[b]	0.29

Abbreviations: MMSE, Mini-Mental State Examination; ADAS-cog, Alzheimer's Disease Assessment Scale-cognitive subscale; WMS, Wechsler Memory Scale; MTA-ERC, medial temporal areas including the entorhinal cortex; WBC, whole brain cortices; ES, effect size.

[a]$p<.025$; significant differences before versus after intervention in the exercise group

[b]$p<.025$; significant differences before versus after intervention in the control group

[c]Missing value

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not only on memory function, but also on general cognitive function in people with aMCI. General cognitive function can be used to discriminate between people who progress to AD and those who do not. [35] Improvements of memory function and maintenance of general cognitive function suggest that multicomponent exercise can help prevent progression from MCI to AD. However, despite significant interactions, the effect sizes in general cognitive function and logical memory were small. Moreover, these interactions would not become significant if the p-values were adjusted for multiple comparisons. Further studies are required to determine the positive effects of exercise on cognitive function in older adults with MCI.

Relationship between exercise and brain atrophy

It is well established that structures in the medial temporal lobe, particularly the hippocampus and ERC, are essential for normal memory function. There is an emerging literature describing baseline structural MRI correlates of cognitive impairment in elderly adults with mild cognitive impairment (MCI) and Alzheimer's disease (AD). Some studies have identified relationships between aerobic exercise and increased brain volume [16,17] and functional connectivity between parts of the frontal, posterior, and temporal cortices [36] in healthy older adults. For example, Erickson et al. found that the hippocampus remains plastic in late adulthood and that a 1-year period of aerobic exercise was sufficient for enhancing volume. [16] Our 6-month multicomponent exercise program with MCI subjects revealed that exercise did not have a significant group × time interaction on MTA-ERC scores or WBC atrophy compared to the control group. However, there was significant group × time interaction in WBC atrophy level, when tested in a sub-analysis restricted to aMCI subjects. Post-hoc analyses revealed that the control group exhibited increased WBC atrophy after intervention, compared with their baseline scores. These results suggest that older adults with aMCI may exhibit high levels of plasticity in WBC atrophy. Further study is needed to establish our findings using large samples and detailed neuroimaging analysis.

Predictors of increasing of cognitive function

In the relationships between cognitive function and biochemical measures, low T-cho and high BDNF serum levels at baseline were associated with increased memory and general cognitive function in the MCI subjects, respectively. Serum lipoprotein levels may be a common and potentially modifiable risk factor for AD. [37] For example, a prospective study reported that lower serum levels of LDL and T-cho were associated with better cognitive performance and a lower risk of cognitive impairment in 1,037 women with cardiovascular disease. [38] Our finding extends knowledge about the relationships between T-cho and cognitive function to older adults with MCI. Animal studies have revealed that the structure and function of the hippocampus, a brain region critical for certain forms of cognition, is adversely affected by hyperlipidemia. (e.g. [39]) Abnormal lipid metabolism may be undesirable status for improvement cognitive functions, especially memory. Exercise is also a valid and feasible way to manage lipoprotein levels and regular activity may be potential strategies for preventing cognitive decline in elderly individuals. [40]

One of the main determinants of cell size is cell growth, which is modulated by certain growth factors, such as BDNF. The levels of BDNF-associated gene expression have been found to increase with physical activity. [14] BDNF expression has also been suggested to play a role in learning and synaptic plasticity. [41]

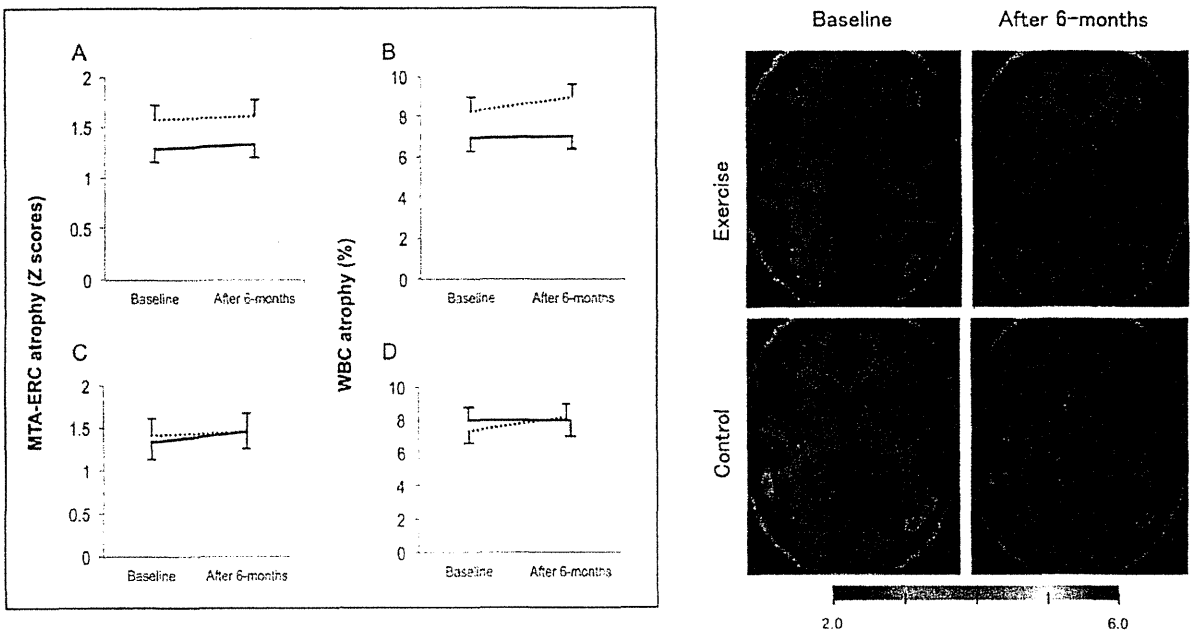

Figure 2. Change in MTA-ERC and WBC volumes in response to the 6-month intervention. Abbreviations: MTA-ERC, medial temporal areas including the entorhinal cortex; WBC, whole brain cortices. Left panel shows change in MTA-ERC and WBC volumes before and after the 6-month intervention. Solid and dashed lines indicate the exercise and control groups, respectively. Group mean differences and standard errors for MTA-ERC and WBC atrophy are shown in panels A and B, respectively, for all subjects. Panels C and D show mean differences and standard errors for MTA-ERC and WBC atrophy, respectively, for older adults with aMCI. The repeated-measures ANOVA revealed that there was a significant group × time interaction on WBC atrophy level ($p<.05$) in older adults with aMCI. Right panel shows typical images for VSRAD, indicated atrophy region, in subjects with aMCI in the exercise and control groups. The upper panel shows WBC atrophy in a man (81 years old) with aMCI who completed the 6-month exercise program. The rate of WBC atrophy decreased after the intervention (8.74% at baseline to 6.39% after the intervention). The lower panel shows WBC atrophy of a man (80 years old) with aMCI in the control group. The rate of WBC atrophy increased after the 6-month intervention period (7.19% at baseline to 10.48% after the intervention).
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The present results indicate that high serum BDNF levels have a beneficial effect on general cognitive function in older adults with MCI.

Limitations

The present study involved several limitations. The small sample size should be addressed by replication with a larger group of adults with MCI. Of the 135 potential subjects screened for eligibility in our study, 35 were excluded for not meeting inclusion criteria, refusal to participate, or medical reasons (**Figure 1**). This

Table 3. Predictors of Improvements in Cognitive Function.

	ADAS-cog	*P*	WMS-LM I	*P*	WMS-LM II	*P*
	OR (95% CI)		**OR (95% CI)**		**OR (95% CI)**	
Age, years	0.97 (0.91–1.05)	.44	0.95 (0.89–1.03)	.22	0.96 (0.90–1.04)	.34
Sex, women/men	1.00 (0.35–2.82)	1.00	0.74 (0.26–2.13)	.57	2.56 (0.85–7.66)	.09
Educational level, years	0.85 (0.70–1.04)	.11	0.93 (0.76–1.13)	.45	1.01 (0.83–1.22)	.96
Intervention, exercise group/control group	2.85 (1.10–7.37)	.03	2.27 (.90–5.72)	.08	1.98 (.77–5.12)	.16
T-cho, mg/dl	1.00 (0.98–1.02)	.96	**0.98 (0.96–1.00)**	**.02**	0.99 (0.97–1.01)	.18
HbA1c, %	0.53 (0.25–1.14)	.10	1.20 (0.57–2.53)	.64	0.61 (0.29–1.30)	.20
BDNF, ng/ml	**1.07 (1.02–1.13)**	**.01**	1.00 (0.95–1.05)	.94	1.02 (0.97–1.08)	.39
VEGFR1, pg/ml	0.99 (0.97–1.01)	.39	0.99 (0.96–1.01)	.32	1.00 (0.98–1.03)	.74

Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval; ADAS-cog, Alzheimer's Disease Assessment Scale-cognitive subscale; WMS, Wechsler Memory Scale; T cho, total cholesterol; HbA1c, hemoglobin A1c; BDNF, brain-derived neurotrophic factor (BDNF); VEGFR1, vascular endothelial growth factor receptor 1.
Missing values: ADAS-cog (n = 10), WMS-LM I (n = 9), WMS-LM II (n = 9)
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