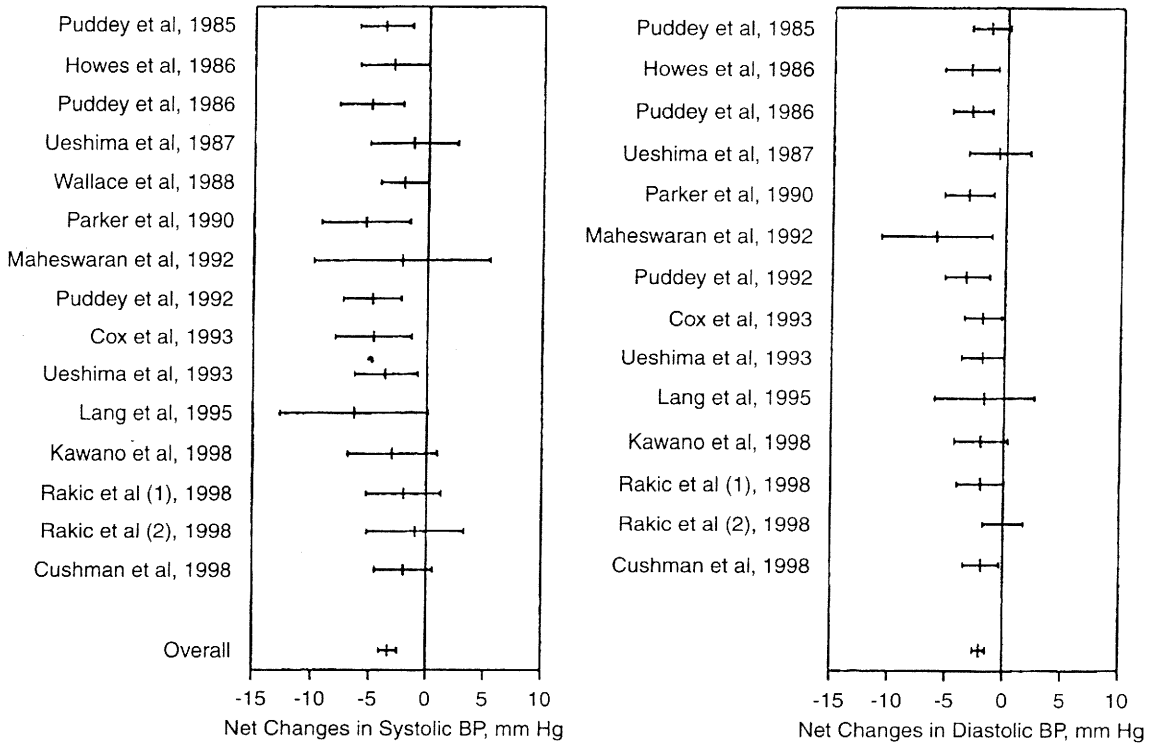

Figure 3 Average net changes in systolic and diastolic BPs and corresponding 95% CIs related to alcohol reduction intervention in 15 randomized controlled trials (adopted from Xin *et al.*[72] with permission).

dized conditions in Japanese men with hypertension.[73] After several days of the control period, the subjects consumed 1 ml kg^{-1} of alcohol with an evening meal for 7 days. Evening BP values decreased for several hours after alcohol consumption on both days 1 and 7, whereas morning BP was unchanged on day 1 but increased on day 7. The average 24-h BP was lower on day 1 and was the same on day 7 compared with the control period. A short-term repeated intake of alcohol therefore causes biphasic changes in BP without altering the average 24-h BP, at least in Japanese men.

We also examined the effect of a 4-week period of unrestricted alcohol consumption and that of alcohol restriction on the 24-h BP in hypertensive patients in a randomized crossover study.[74] The average level of daily alcohol intake was 66 ml in the unrestricted period and 11 ml in the restricted period. The daytime BP was 3/2 mm Hg higher in the unrestricted period than in the restricted period, but the nighttime BP was 4/2 mm Hg lower in the former (Figure 4). The average 24-h BP was comparable between the two periods. These effects of alcohol resulted in changes in the dipping pattern of the 24-h BP. Half of those who did not show a dip in BP during the restricted period changed and showed a dip in the unrestricted period, and half of those that showed a dip showed an extreme dip during this period.

On the other hand, Minami *et al.*[75] observed reductions in the daytime (−3.4 mm Hg) and 24-h (−3.2 mm Hg) systolic BPs after 3 weeks of alcohol restriction in Japanese men. In their study, daytime, nighttime and 24-h diastolic BPs did not change with alcohol reduction (−1.1, +2.1 and −0.3 mm Hg, respectively).

The effect of alcohol on the 24-h BP may differ between Orientals and Caucasians. Howes *et al.*[76] observed that short-term alcohol intake increased BP variability without changing the average BP in Australian subjects. However, Rakic *et al.*[77] showed that the average 24-h BP increased significantly after 4 weeks of alcohol consumption in Australian men. In their study, the average 24-h systolic BP was 2–3 mm Hg higher and the nighttime BP was not lower in the unrestricted compared with the restricted period. It was also reported that the average 24-h BP decreased after abstinence in alcoholic patients.[78]

In a systematic review, McFadden *et al.*[79] analyzed clinical trials that examined the BP after a period of sustained alcohol intake. In this review, the pressor effect of alcohol was evident in non-ABPM studies, but not in ABPM studies. An early effect of reducing the BP and a later effect of raising the BP led to smaller differences in the net effect of alcohol on BP values in ABPM studies.

We also studied changes in morning and evening home BP measurements during each of the 4 weeks of unrestricted consumption and restriction in hypertensive patients.[80] In this study, the morning BP increased by 4.4 mm Hg but late evening BP decreased by 7.4 mm Hg at the end of the unrestricted alcohol intake period. The pressor effect was significant from week 2, whereas the depressor effect was evident from day 1. These results indicate that the status of alcohol intake influences the morning–evening BP difference, and that slow pressor mechanism(s) are involved in alcohol-induced BP elevation.

It is therefore clear that alcohol consumption contributes to hypertension, and alcohol restriction decreases the daytime BP. It should be noted, however, that the effects of alcohol on BP vary according to the level and duration of consumption and the time from the last drink. Alcohol seems to exert a marked influence on circadian BP variation, whereas its influence on the average 24-h BP seems to be small. The mechanisms of the pressor effects of alcohol have not been fully clarified; however, changes in vascular reactivity and sympathetic nerve activity related to intermittent alcohol withdrawal seem to be more important than the direct actions of alcohol. Deficiencies in

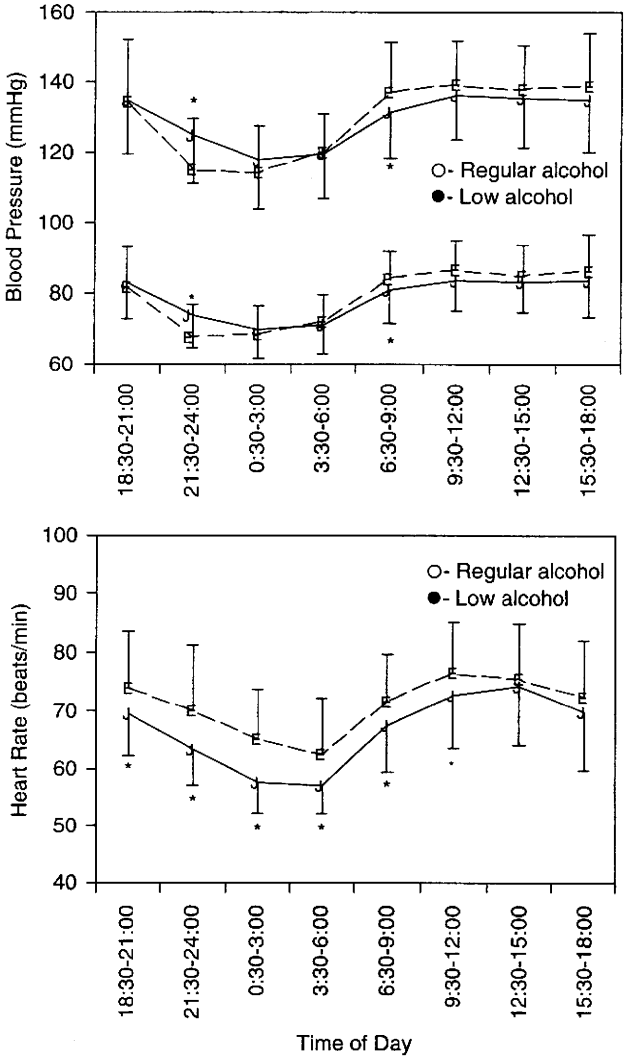

Figure 4 Profile of 24-h BP and heart rate at the end of the regular-alcohol period and the low-alcohol period in hypertensive patients. $*P<0.05$ between the two periods (adopted from Kawano *et al.*[74] with permission).

magnesium and calcium may contribute to BP elevation after chronic alcohol consumption. Increases in the caloric intake through consuming alcoholic beverages and elevated salt intake associated with drinking may also be involved in alcohol-related hypertension.

Experimental studies

Many experimental studies have examined the effect of chronic administration of alcohol on BP; however, the results have been inconsistent.

Strickland and Wooles,[81] reported an elevation of BP after ethanol administration (5–20% in drinking water) for 4 weeks in rats. In their study, the plasma level of norepinephrine was decreased in ethanol-fed animals. Vasdev *et al.*[82] observed BP elevation after 1 week during the administration of ethanol (5–10%) to Wister Kyoto rats. They noted increases in the concentration of platelet intracellular calcium ions and the uptake of calcium in the aorta. Hsieh *et al.*[53] identified increases in BP and intracellular calcium ions and a decrease in intracellular magnesium ions after 4 weeks of ethanol administration (15%) to rats. They suggested a role of magnesium deficiency in ethanol-induced hypertension as the BP elevation was attenuated by magnesium supplementation. Puddey *et al.*[83] reported a BP elevation of ~10 mm Hg and decreases in the level of phospholipids and the ratio of unsaturated/saturated fatty acids in the aorta and kidney after the chronic administration of alcohol to rats. Harada *et al.*[84] also observed increases in the BP and platelet-free calcium concentration with ethanol consumption (15%) in Wister Kyoto rats.

In some studies, the BP did not change after the chronic administration of alcohol to animals. Abdel-Rahman[85] reported that the BP increase was not different between ethanol-fed (5–20% in drinking water) spontaneously hypertensive (SH) rats and control SH rats during a 13-week observation period. The depressor effect of clonidine, however, was reduced in the ethanol-fed SH rats, suggesting a change in the neural regulation of BP.

Several studies have shown that chronic alcohol intake decreases BP in animals. Howe *et al.*[86] reported that BP values in alcohol-fed (5–20% in drinking water) Wister Kyoto, SH and stroke-prone SH rats were lower than those of respective control rats during a 6-month observation period. Hatton *et al.*[87] observed a BP decrease during chronic ethanol administration (36% in a liquid diet) for 18 weeks in Wistar rats. The vasoconstrictor response of resistant arteries to norepinephrine was enhanced and the vasodilator response to alcohol was attenuated in their study. Beilin *et al.*[88] reported a decrease in the resting BP of Wister Kyoto and SH rats after 12 weeks of ethanol administration (20% in drinking water), although cardiovascular reactivity to noise-related stress was augmented in the ethanol-fed SH rats. El-Mas and Abdel-Rahman[89] also observed a lower BP in freely moving, ethanol-fed (5% in a liquid diet) rats compared with control rats based on telemonitoring of the BP.

The reasons for the inconsistent results in experimental studies are not clear, but cannot be explained by differences in daily doses of alcohol administration. The periods of alcohol administration, however, are generally longer in studies showing BP reduction than those showing BP elevation. The timing of BP measurement may be important, such as in clinical studies. Crandall *et al.*[90] administered 30% alcohol twice daily (7–8 g kg^{-1}) for 10 weeks to rats and examined the levels of BP and blood alcohol. In their study, BP was normal at the time of the peak blood alcohol level but was elevated at 24 h after alcohol consumption, when alcohol was not detected in the plasma. Their results suggest that alcohol-induced hypertension is not because of its direct action but to alcohol withdrawal.

The harmful effects of large doses of alcohol, such as cardiac dysfunction, have been shown in experimental studies.[91] Schlicht *et al.*[92] however, reported that the lifespan of SH rats was prolonged by the chronic administration of ethanol. These observations are important, as both the adverse effects of a large amount of alcohol and the beneficial effects of a moderate amount on cardiovascular disease and total mortality have been shown in large-scale epidemiological studies.[8–12]

Interaction with antihypertensive drugs

Alcohol interacts with several antihypertensive agents. Experimental studies have shown that alcohol attenuates the effect of centrally acting antihypertensive drugs such as clonidine.[86] Heavy drinking is recognized as one of the factors responsible for resistant hypertension. The interaction between alcohol and antihypertensive drugs and the hypertensive effect of alcohol may have a role in alcohol-related resistant hypertension. In addition, heavy drinkers often show poor adherence to both pharmacological treatment and lifestyle modifications. Habitual drinkers taking antihypertensive drugs are also prone to morning hypertension.[93]

Table 2 Moderation of alcohol consumption recommended by hypertension treatment guidelines

JNC-7 (2003)	*ESH-ESC 2007*	*JSH 2009*
Men: ≦2 drinks per day (≦30 ml per day)	Men: ≦20–30 g per day	Men: ≦20–30 ml per day
Women, light weight person: ≦1 drink per day (≦15 ml per day)	Women: ≦10–20 g per day	Women: ≦10–20 ml per day

Expressed as amount of ethanol. ESH-ESC 2007, European Society of Hypertension–European Society of Cardiology guidelines;[5] JNC-7, Joint National Committee 7th report (Chobanian *et al.*[4]); JSH 2009, Japanese Society of Hypertension guidelines (Ogihara *et al.*[6]).

The combination of alcohol and antihypertensive drugs may also lead to a marked BP reduction. This phenomenon has been known; however, few clinical studies have addressed this interaction. It is possible that sympatholytic drugs augment the depressor effect of alcohol, as alcohol-induced hypotension is associated with the reflex activation of the sympathetic nervous system.[7] In our studies, alcohol and a beta blocker, propranolol, additively lowered the nighttime BP,[35] whereas alcohol and an alpha blocker, prazosin, synergistically acted to lower the BP in hypertensive patients.[94] It has also been reported that alcohol enhances the depressor effect of the calcium antagonist felodipine.[95] Changes in the type and timing of antihypertensive medication along with the moderation of alcohol consumption should be considered to treat hypertensive patients with a drinking habit.

Hypertension treatment guidelines

As an excess consumption of alcohol is a risk factor for hypertension, all hypertension treatment guidelines recommend the moderation of alcohol intake as a part of lifestyle modifications for the management of hypertension (Table 2). The 7th report of the Joint National Committee in the United States (JNC-7) recommends the limitation of daily alcohol consumption to no more than two drinks (30 ml) for most men and to no more than one drink (15 ml) for women and light-weight men.[4] According to the European guidelines (European Society of Hypertension–European Society of Cardiology guidelines, ESH–ESC 2007), the upper limit is 20–30 g per day for men and 10–20 g per day for women. The Japanese guidelines provide similar recommendations (20–30 ml per day for men and 10–20 ml per day for women). Of note, 600 ml of beer or 250 ml of wine contains about 30 ml of ethanol.

These recommendations put forward by the guidelines are appropriate because small doses of alcohol exert little adverse effects on BP and the cardiovascular system. There are, however, some concerns regarding the efficacy of alcohol restriction on BP because the effect of alcohol on average 24-h BP levels seems to be very small. In our studies, salt restriction and weight reduction substantially decreased the BP for 24 h, but the effect of alcohol restriction on average 24-h BP was not significant.[96–98] As light drinking has beneficial effects on the cardiovascular system, as described later, abstinence from alcohol should not be imposed on hypertensive individuals except for patients with special conditions.

ALCOHOL AND CARDIOVASCULAR DISEASE

Cardiac disease

Heart failure. A heavy alcohol intake is associated with cardiac hypertrophy and the risk of cardiomyopathy and heart failure.[8,36,37] It has been shown that the total consumption of alcohol is positively related to a left ventricular mass and is negatively associated with the ejection fraction in asymptomatic alcoholic subjects.[37] Recent epidemiological studies, however, have shown that moderate alcohol consumption is associated with a lower risk of heart failure.[99,100] Klatsky *et al.*[101] reported that heavy drinkers had an increased risk of heart failure because of noncoronary artery disease, whereas alcohol drinking was inversely related to the risk of heart failure because of coronary artery disease. Heavy drinking therefore seems to increase the risk of heart failure but light-to-moderate drinking may decrease the risk, probably because of its favorable association with coronary artery disease.

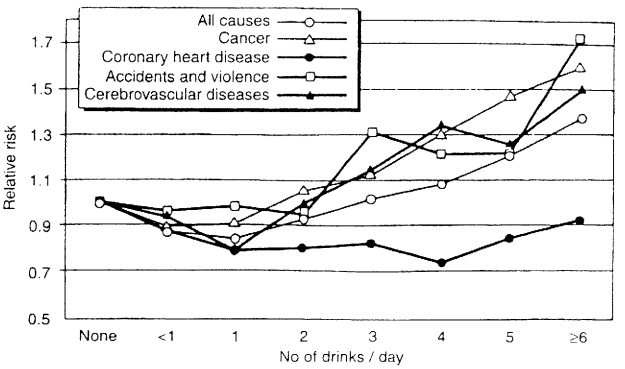

Figure 5 Alcohol consumption and relative risk of death over 12 years in American Cancer Society prospective study of 276 802 men aged 40–59 (adopted from Boffetta *et al.*[105] with permission).

Arrhythmia. Alcohol intake is associated with the risk of tachyarrhythmia, such as ventricular and supraventricular premature contractions and atrial fibrillation.[8,102–104] In the Danish Diet, Cancer and Health study, moderate-to-heavy consumption of alcohol was associated with an increased risk of atrial fibrillation.[103,104] Such alcohol-induced arrhythmia often occurs after binge drinking. Activation of the sympathetic nervous system and a decrease in the serum potassium level after drinking may trigger this arrhythmia.[3] Cardiac functional and structural changes because of chronic alcohol consumption also seem to have a role in arrhythmia.[37]

Coronary heart disease. Alcohol seems to have a beneficial effect on coronary heart disease.[8,9] It has been shown that the risk of myocardial infarction is 20–50% lower in habitual compared with nondrinkers.[9,105–108] This risk reduction is dose-dependent up to the level of moderate drinking, but further risk reduction has not been observed in heavy drinkers (Figure 5). A U-shaped relationship has been observed between the level of alcohol consumption and degree of coronary calcification in a general population.[109] In some studies, such as the Japan Collaborative Cohort Study,[110] the beneficial effect of alcohol on coronary heart disease was modest and not significant (Table 3).

The mechanisms behind the inverse association of alcohol with coronary heart disease have not been fully clarified. The alcohol-induced increase in HDL cholesterol, however, seems to be the most important mechanism.[107,111] The inhibitory effect of alcohol on blood coagulation also contributes to the lower risk of myocardial infarction.[107] In addition, it has been shown that moderate alcohol consumption is associated with a lower plasma level of C-reactive protein, suggesting an anti-inflammatory action of alcohol.[112] The weak effect of alcohol on the average 24-h BP may also have a role as a high BP is a strong risk factor for coronary heart disease.[74,79]

Red wine contains polyphenols that act to prevent atherosclerosis because of their antioxidant effect. Several studies have shown that

Table 3 Mortality from stroke, coronary heart disease and total cardiovascular disease by alcohol consumption category in men in the Japan Collaborative Cohort Study (adopted from Ikehara *et al.*[110] with modification)

			Ethanol intake, g per day			
	Nondrinkers	*Ex-drinkers*	*0.1–22.9*	*23.0–45.9*	*46.0–68.9*	*≧69.0*
Person–years	96423	25919	78478	101256	90000	41588
Total stroke	200	126	114	168	173	83
Age-adjusted HR	1.00	1.93[a]	0.91	0.98	1.46[a]	1.89[a]
Multivariable HR	1.00	1.90[a]	0.95	0.96	1.39[a]	1.71[a]
Hemorrhagic stroke	55	31	41	52	60	37
Age-adjusted HR	1.00	1.80[a]	1.09	1.02	1.51[a]	2.30[a]
Multivariable HR	1.00	1.79[a]	1.16	1.02	1.47	2.16[a]
Ischemic stroke	126	88	60	101	95	37
Age-adjusted HR	1.00	2.12[a]	0.80	0.99	1.44[a]	1.60[a]
Multivariable HR	1.00	2.11[a]	0.81	0.94	1.34[a]	1.39
Coronary heart disease	116	56	71	90	65	33
Age-adjusted HR	1.00	1.50[a]	0.94	88	0.87	1.16
Multivariable HR	1.00	1.35	0.96	0.82	0.76	0.95
Total cardiovascular disease	487	282	269	379	342	162
Age-adjusted HR	1.00	1.77[a]	0.88	0.90	1.16[a]	1.47[a]
Multivariable HR	1.00	1.66[a]	0.90	0.87	1.07	1.28[a]

Abbreviation: HR, hazard ratio.
[a]Significant vs. nondrinkers.

people who mainly drink red wine have a lower risk of cardiovascular disease than those who drink other kinds of alcoholic beverage.[113,114] It is suggested, however, that the low incidence of myocardial infarction in habitual drinkers is largely attributed to the effect of alcohol itself.[115]

Cerebrovascular disease

The relationship between alcohol consumption and total cerebrovascular disease is generally J-shaped, although it differs according to subtypes of stroke [105,116] (Figure 5). It is clear that alcohol is a risk factor for hemorrhagic stroke. A positive linear relationship has been observed between the level of alcohol consumption and risk of brain or subarachnoid hemorrhage.[8,110,116,117] Actions on the BP and blood coagulation system seem to be underlying mechanisms for this adverse influence of alcohol.

On the other hand, the relationship between alcohol consumption and the risk of ischemic stroke has been found to be J- or U-shaped.[110,116–118] The low risk in light drinkers seems to be due to the lower degree of atherosclerosis and the inhibition of blood coagulation, as in the case of ischemic heart disease. The increased risk in heavy drinkers is probably related to increases in the level and variability of the BP, hemoconcentration because of dehydration and thromboembolism associated with alcohol-induced atrial fibrillation. Regarding alcoholic beverage types, wine drinkers seem to have a lower risk of ischemic stroke.[119,120]

The favorable association of light-to-moderate drinking with the risk of ischemic stroke seems to be more apparent in Caucasians than in Japanese, although the results of epidemiological studies have been inconsistent in both populations. The racial differences may be related to variation in the frequencies of stroke subtypes. Atherothrombotic brain infarction is common in Caucasians, whereas lacunar stroke is more common in Japanese.

Several studies have examined the relationship between alcohol intake and subclinical findings on magnetic resonance imaging of the brain in general populations. In the Cardiovascular Study, a U-shaped relationship was observed between alcohol consumption and white matter abnormalities. Moreover, moderate drinking was also associated with a lower risk of lacunar infarction compared with abstainers.[121] Such risk reduction with moderate drinking, however, was not observed in the ARIC study, and an increased level of alcohol intake was associated with brain atrophy.[122]

Peripheral arterial disease and atherosclerosis

As light-to-moderate consumption of alcohol seems to act to suppress the progression of atherosclerosis, it may also have a favorable influence on peripheral arterial disease. The Edinburgh Artery Study supported the protective effect of alcohol, as there was a positive association between the level of alcohol intake and the ankle brachial index.[123] In the Physicians' Health Study, habitual drinkers showed a 26% lower incidence of peripheral arterial disease compared with nondrinkers after adjustment for confounding factors.[124] Similar results were also shown in the Framingham Heart Study and the ARIC study.[125,126]

Regarding the association of alcohol and carotid atherosclerosis, an inverse relationship was noted in the Lausanne Stroke Registry.[127] On the other hand, there was no significant association between alcohol intake and the carotid artery thickness in the ARIC study.[128] A U- or J-shaped relationship was observed between the level of alcohol intake and severity of carotid atherosclerosis in the Bruneck Study and the Study of Health in Pomerania.[129,130] Although the results of epidemiological studies have been inconsistent, light-to-moderate consumption seems to inhibit the development of carotid atherosclerosis.

Several studies examined the relationship between alcohol consumption and arterial stiffness by measuring the pulse wave velocity.

Sierksma *et al.*[131] identified a U-shaped relationship between alcohol consumption and the aortic pulse wave velocity. van den Elzen *et al.*[132] observed an inverse relationship between the alcohol intake and pulse wave velocity in young men and women. On the other hand, Kurihara *et al.*[133] reported that the brachial–ankle pulse wave velocity was elevated in heavy drinkers. These studies also support the favorable effect of moderate and the harmful effect of heavy drinking on large arteries.

Cardiovascular mortality and total mortality

As described earlier, alcohol seems to exert both beneficial and adverse effects on cardiovascular diseases. The relationship between alcohol consumption and total cardiovascular mortality has been shown to be J-, U- or L-shaped. A J-shaped relationship was observed in the Japan Collaborative Cohort Study; however, the beneficial effect of light-to-moderate drinking was not significant.[110] On the other hand, in very large longitudinal studies conducted by the American Cancer Society, a U-shaped relationship was observed in the original study,[105] and the relationship was L-shape (nondrinkers showed the highest cardiovascular mortality) in Study II.[10] In a meta-analysis conducted by Di Castelnuovo *et al.*,[114] a light-to-moderate consumption of wine or beer was associated with lower cardiovascular risk. A drinking habit, particularly wine consumption, has been shown as a part of a lifestyle associated with low cardiovascular risk.[134,135] It has also been suggested that the risk reduction associated with alcohol consumption is low in individuals without cardiovascular risk factors but is high in those with a marked cardiovascular risk. Taken together, light-to-moderate alcohol consumption seems to decrease cardiovascular mortality, whereas heavy drinking may result in poor cardiovascular outcomes compared with abstainers.

Alcohol is also related to several cancers, liver disease, psychiatric and neurological disorders and injury, and it seems to influence total mortality. A J- or U-shaped relationship has been observed between the level of alcohol intake and total mortality.[10,11,105,136,137] It has been suggested that all-cause mortality is the lowest among subjects who consume about one drink per day. In the American Cancer Society Prospective Study II, total mortality was lower in drinkers than in nondrinkers.[10] It has also been shown that wine drinkers have a lower mortality rate than drinkers who avoid wine.[136] In a meta-analysis of 34 studies including more than one million individuals, a J-shaped relationship was found between alcohol consumption and total mortality.[11] In this analysis, low levels of alcohol intake (one to two drinks per day for women and two to four drinks per day for men) were inversely associated with total mortality, although higher levels of alcohol increased mortality. Those findings suggest that a light-to-moderate intake of alcohol decreases but heavy consumption increases total mortality compared to nondrinking.

CONCLUSIONS

Alcohol has complex effects on the cardiovascular system. It is clear that alcohol consumption is related to hypertension, and therefore the restriction of alcohol intake is recommended in the management of hypertension. Alcohol and its metabolites, however, also exhibit a vasodilatory action, and the BP usually decreased after alcohol ingestion, especially in Orientals who show alcohol flush. Mechanisms for the pressor action of alcohol have not been completely clarified; however, an increase in the vascular sensitivity, activation of the sympathetic nervous system and depletion of magnesium and calcium may be involved. The depressor action of alcohol is due to a decrease in systemic vascular resistance that may be related to the attenuation of vascular sensitivity and production of nitric oxide. The pressor effect of alcohol consumed in the evening is apparent during the day, but its effect on average 24-h BP seems to be very small. It should be mentioned that casual BP measurement may lead to overestimating the hypertensive effect of alcohol.

Alcohol seems to exert both harmful and beneficial effects on cardiovascular disease. An excessive intake of alcohol is associated with increased risks of heart failure, arrhythmia and hemorrhagic stroke and causes an increase in total mortality. Light-to-moderate drinkers, however, show lower rates of atherosclerosis and lower risks of coronary heart disease, heart failure, ischemic stroke, peripheral artery disease and cardiovascular and total mortality compared with nondrinkers. As the aim of the management of hypertension is the prevention of cardiovascular disease and premature death, moderation of alcohol intake is to be recommended to hypertensive patients, but abstinence from alcohol should not be insisted on unless there are specific indications for it.

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Blood Pressure and Medication During Long-Term Antihypertensive Therapy Based on Morning Home SBP in Hypertensive Patients: Hypertension Control Based On Home Systolic Pressure (HOSP) Substudy

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ABSTRACT

We examined blood pressure (BP) and medication over 5 years in 80 hypertensive patients who participated in the Hypertension Control Based on home systolic pressure (HOSP) study that compares effects of strict and mild control of morning home systolic blood pressure (SBP) as well as amlodipine- and losartan-based regimens. Average morning home SBP after 5 years was 126 mmHg in the strict control group and 135 mmHg in the mild control group. The strict control group and the losartan group required more combination therapy than the other groups. These results show that long-term strict control of morning BP is feasible. Amlodipine appears to be more effective in controlling morning BP than losartan when the medication is administered alone in the morning.

KEYWORDS: hypertension; home blood pressure (BP); antihypertensive therapy; amlodipine; losartan

INTRODUCTION

It has been shown that home blood pressure (BP) is superior to office BP in the prediction of hypertension-related organ damage and prognosis (1–4). It is also known that systolic BP (SBP) is more closely associated with cardiovascular prognosis than diastolic BP (DBP) in the middle-aged and elderly population (5,6). In addition, BP shows circadian variation with morning rise, and cardiovascular events such as stroke and myocardial infarction occur most frequently in the morning (7,8).

We have been conducting a clinical trial based on morning home SBP named HOSP (Hypertension Control Based on Home Systolic Pressure) study (9,10). The HOSP study is a multicenter, randomized trial with two target levels of morning home SBP and two initial drugs in middle-aged and elderly patients with essential hypertension. The HOSP pilot study started in 2000 (9), and the main study was launched in 2003 (10). There are also several substudies that examine target organ damage and other clinical parameters at the National Cardiovascular Center (11).

Recent guidelines recommend strict BP control in the management of hypertension (12,13), although the control of hypertension in treated patients is still suboptimal (14,15). Self-measurement of BP at home is now popular in Japan, and the Japanese guidelines appreciate its usefulness in the diagnosis and management of hypertension (13). However, there are few studies that have assessed the feasibility of long-term strict control of home BP in hypertensive patients. In the present study, we examined the level of home BP and antihypertensive medication in patients who participated in the HOSP pilot study at our institute to assess the feasibility of strict control of home BP for 5 years.

METHODS

Subjects

The study subjects were 80 hypertensive patients who participated in the HOSP pilot study at the National Cardiovascular Center. The inclusion criteria of the HOSP pilot study were patients with essential hypertension, aged 40–79 years old, without cardiovascular complications, and with office and morning home SBP

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TABLE 1 Characteristics and baseline data of study participants

Patient characteristics	
Number, sex	N = 80 (men 39, women 41)
Age (years)	64 ± 9
Dyslipidemia (%)	67
Diabetes mellitus (%)	7
Hyperuricemia (%)	9
Drinking habits (%)	42
Smoking habits (%)	5
ECG-LVH (%)	26
Microalbuminuria (%)	38
Baseline data	
Office blood pressure (mmHg)	159 ± 12 / 95 ± 9
Morning home blood pressure (mmHg)	150 ± 10 / 91 ± 9*
Evening home blood pressure (mmHg)	143 ± 15 / 87 ± 10 **,+

Abbreviation: ECG-LVH - left ventricular hypertrophy on electrocardiogram.
*P < 0.05; **P < 0.01 vs. office blood pressure;+P < 0.05 vs. morning home blood pressure.

of 140–199 mmHg during a drug-free period (9). Characteristics of the study participants a shown in Table 1. Sixty-six patients (82%) were previously treated and the remaining 14 patients (18%) were never treated. Written informed consent was obtained for all participants prior to the study.

Protocol

The study protocol was approved by the Institutional Review Board and the Ethical Committee of the National Cardiovascular Center. The HOSP pilot study is an open, randomized study with 2 × 2 factorial design. Patients were treated according to prespecified target levels of morning home SBP, and either a calcium channel blocker (CCB) amlodipine or an angiotensin receptor blocker (ARB) losartan was used as an initial agent. After a 4 week drug-free period, subjects were randomly assigned to strict control (target morning home BP <130 mmHg) or mild control (130–139 mmHg) group, and to amlodipine (2.5–5 mg once daily in the morning) or losartan (25–50 mg once daily in the morning) group. Several other classes of antihypertensive drugs (diuretics, beta-blockers, and alpha-blockers) were added if morning home BP was not controlled after a 3-month monotherapy period. If home BP did not reach the target level despite multiple combination therapies, the daily dose of amlodipine and losartan was increased to 10 mg and 100 mg, respectively. The treatment period was 5 years if possible.

Measurements

Office BP was measured twice in the sitting position by a physician using a mercury sphygmomanometer at every visit (2 weeks–2 months interval). Home BP was measured three times in the early morning (before breakfast) and late evening (before going to bed) in the sitting position almost everyday by patients using an automated validated device (Omron HEM-757, Omron Corp., Kyoto, Japan). Serum biochemical parameters and urinary albumin excretion were measured at the baseline period and during the treatment period.

Statistical Analysis

Office and home BPs at the baseline period (drug-free period) and at 3 months, 1 year, 3 years, and 5 years of the treatment period were used for statistical analysis. Office BP records at two visits (four records) were averaged, and home BP records for 3 days before the two visits (18 morning records and 18 evening records) were averaged. All data were expressed as mean ± SD. A student's *t*-test, a chi-square test, and an analysis of variance were applied where appropriate. The data were analyzed using the StatView software (version 5.0, Abacus Concept Inc., Berkeley, CA). P values less than 0.05 were considered statistically significant.

RESULTS

Average baseline office BP, morning home BP, and evening home BP are shown in Table 1. Office BP was significantly higher than home BPs, and morning home BP was significantly higher than evening home BPs. Follow-up data were obtained in 78 patients (98%) at year 1 and in 59 patients (74%) at year 5. The main reason of loss of follow-up was relocation of the subjects.

Figure 1 shows the time course of morning home SBP in strict and mild control groups. There were significant differences in home SBP between the two groups throughout the 5 years of treatment period. Although the average SBP level did not reach the target level in either strict control group (134 ± 11 mmHg) or mild control group (141 ± 12 mmHg) at month 3 (end of monotherapy period), morning home SBP achieved the target level in both groups from year 1 to year 5. The average level of morning home SBP was 126 mmHg in

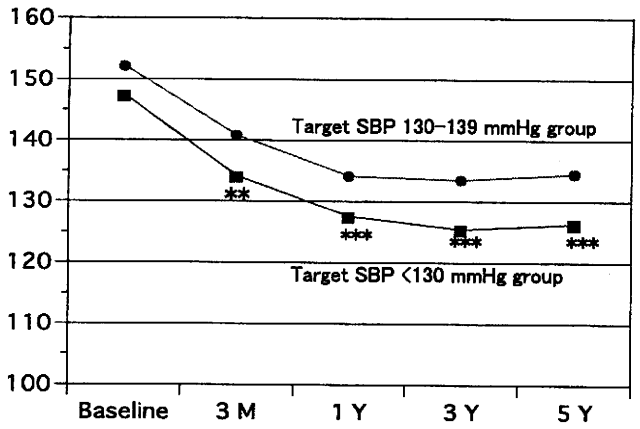

FIGURE 1 Time course of morning home systolic blood pressure (SBP) in strict and mild control groups. **P < 0.01; ***P < 0.001 between groups.

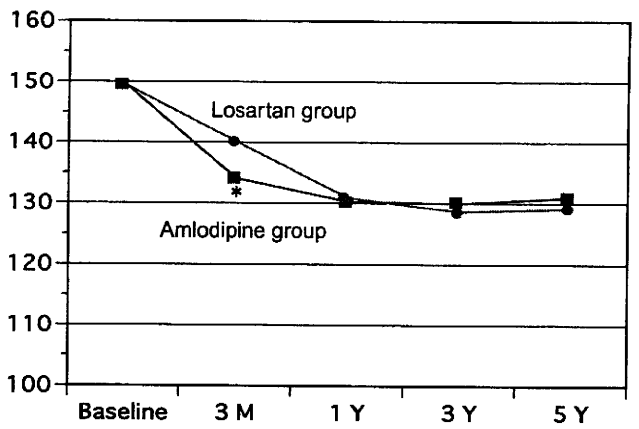

FIGURE 2 Time course of morning home SBP in amlodipine and losartan groups. *P < 0.05 between groups.

the strict control group and 135 mmHg in the mild control group at year 5 (Table 2). Office SBP and evening home SBP were also significantly lower in the strict control group than in the mild control group at the end of the treatment period.

Figure 2 shows the time course of morning home SBP in amlodipine and losartan groups. Morning home SBP was significantly lower in the amlodipine group (134 ± 8 mmHg) than in the losartan group (140 ± 13 mmHg) at the end of the monotherapy period (month 3); however, the difference disappeared from year 1 to year 5. At the end of the treatment period, there were no significant differences in office BP and home BPs between the two groups (Table 2).

The rate of target BP achievement is shown in Table 3. The achievement rate was about 80% in a whole group and in each subgroup. About half of the patients required combination therapy, and the average number of antihypertensive drugs in all subjects was 1.7 (Table 3). Beta blockers were most frequently used in the amlodipine group, while diuretics were the most common concomitant drugs in the losartan group. The rate of combination therapy and the number of antihypertensive drugs were significantly higher in the strict control group than in the mild control group. The losartan group also needed more combination therapy and antihypertensive drugs than the amlodipine group.

DISCUSSION

In the present study, morning home SBP achieved target levels for 5 years in the strict control group as well as in the mild control group with antihypertensive therapy using amlodipine or losartan as an initial agent. This result indicates that long-term control of home SBP to less than 130 mmHg is feasible in the treatment of hypertension.

It is well known that hypertension is an important risk factor for cardiovascular disease (5,6,16), and antihypertensive treatment is effective in improving the cardiovascular prognosis in hypertensive patients (17). The control of hypertension in treated patients,

TABLE 2 Office and home blood pressures after 5 years

	All subjects (n = 59)	Mild control group (n = 28)	Strict control group (n = 31)	Amlodipine group (n = 31)	Losartan group (n = 28)
Office SBP (mmHg)	138 ± 13	142 ± 12	135 ± 13*	137 ± 13	140 ± 13
Morning home SBP (mmHg)	130 ± 7	135 ± 6	126 ± 5***	131 ± 7	129 ± 7
Evening home SBP (mmHg)	127 ± 10	131 ± 10	123 ± 7***	130 ± 8	124 ± 11
Office DBP (mmHg)	81 ± 8	81 ± 8	80 ± 7	81 ± 8	80 ± 8
Morning home DBP (mmHg)	81 ± 7	82 ± 7	80 ± 7	82 ± 7	80 ± 8
Evening home DBP (mmHg)	77 ± 8	77 ± 8	77 ± 8	79 ± 7	75 ± 8

Abbreviations: SBP - systolic blood pressure; DBP - diastolic blood pressure.
*P < 0.05; ***P < 0.001 vs. mild control group.

TABLE 3 Rate of target blood pressure achievement and combination therapy and number of antihypertensive drugs after 5 years

	All subjects	Mild control group	Strict control group	Amlodipine group	Losartan group
Target BP achievement (%)	79	81	77	82	75
Combination therapy (%)	46	29	61*	32	61+
Number of antihypertensive drugs	1.7 ± 0.8	1.4 ± 0.7	1.9 ± 0.9*	1.5 ± 0.7	1.9 ± 0.9+
Concomitant antihypertensive drugs					
Diuretics (n)	17	6	11	4	13
Beta blockers (n)	17	4	13	8	9
Alpha blockers (n)	5	1	4	2	3

*$P < 0.05$ vs. mild control group; +$P < 0.05$ vs. amlodipine group.

however, is still not satisfactory despite wide distribution of many antihypertensive drugs (14,15) and a recommendation of strict BP control by guidelines (12,13). The level of home BP is generally lower than that of office BP, and recent guidelines adopted 135/85 mmHg as a criterion for hypertension by home BP (12,13). However, the control of home BP in treated patients also appears to be suboptimal in general practice. In the Japan Home Versus Office Blood Pressure Measurement Evaluation (J-HOME) study, the average level of home BP in treated patients was 140/82 mmHg and 60% of patients were not adequately controlled (18). Our results suggest that a stepped care approach based on home BP is effective in the control of home and office BPs.

It has been shown that home BP is more closely related to target organ damage and cardiovascular prognosis than office BP (1–4,19). However, antihypertensive trials have been based on office BP measured during daytime. Although there are several studies that examined BP control by home BP monitoring (20,21), there are few trials that assess the effects of different target levels of home BP on cardiovascular prognosis in hypertensive patients. Ongoing HOSP main study (10) and hypertension objective treatment based on measurement by electrical devices of blood pressure (HOMED-BP) study (22) will provide valuable information about the optimal level of home BP in the management of hypertension.

In the present study, morning home SBP was significantly lower in the amlodipine group than in the losartan group at the end of the monotherapy period, and the amoldipine group needed less combination therapy and antihypertensive drugs than the losartan group at the end of follow-up. However, the effect of both drugs on evening home BP at the monotherapy period was comparable. These results indicate that mor'ning administration of amlodipine is more effective in reducing morning BP than morning administration of losartan.

The different effects of amlodipine and losartan on morning BP appear to be related to their pharmacokinetic profiles rather than the mechanisms of antihypertensive action. The plasma half-life of losartan after oral administration is 1.5–2 h although that of active metabolite E-3174 is longer (4–5 h) (23). On the other hand, the plasma half-life of amlodipine is 33–50 h, and is much longer than that of losartan (24). It has been shown that the effect of losartan on morning home BP was smaller than other ARBs, and its morning/evening ratio is about 0.5 (25). We and others observed sustained the BP-lowering effect of amlodipine for 24 h with the trough/peak ratio of 0.85–0.9 by ambulatory BP monitoring (24,26). Therefore, amlodipine is superior to losartan in 24-hour BP control with once daily administration.

However, losartan appears to be superior to amlodipine regarding the renal protective effect. In a HOSP substudy, the reduction in urinary albumin excretion was significant in the losartan group but not in the amlodipine group (11). A similar renoprotective effect of losartan in comparison to amlodipine was also shown in patients with chronic kidney disease and hypertension in a Japan Losarton Therapy intended for the global renal protection in hypertensive patients (JLIGHT) study (27).

In conclusion, long-term control of morning home SBP to less than 130 mmHg is feasible in most hypertensive patients. Amlodipine appears to be more effective than losartan in lowering morning BP with once daily administration in patients with hypertension when the medication is administered alone in the morning.

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ORIGINAL ARTICLE: BIOLOGY

Association of carotid atherosclerosis with genetic polymorphisms of the klotho gene in patients with hypertension

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Aim: Previous studies suggest that klotho gene polymorphisms may be associated with atherosclerosis, but did not assess the relationship between klotho gene polymorphisms and atherosclerosis parameters such as carotid artery intima-media thickness (IMT). Here, we studied whether *klotho* single nucleotide polymorphisms (SNP) were associated with carotid atherosclerosis.

Methods: All subjects were Japanese. Eight-hundred and fifty-three patients with hypertension (465 men and 388 women) in the outpatient clinic and 1783 subjects from the general population (821 men and 962 women) attending health check-ups were analyzed in the present study. We measured mean IMT of the common carotid artery to evaluate carotid atherosclerosis. Four single nucleotide polymorphisms (SNP) (rs7323281; intron1, rs5644481; exon4, rs3752472; exon3, rs650439; intron4) of *klotho* were selected as representative SNP in haplotype blocks.

Results: Multivariate logistic regression analysis adjusted by confounding factors showed a significant association of rs650439 with carotid atherosclerosis in hypertensive patients (TT vs TA vs AA, $P < 0.01$; TT + TA vs AA, $P < 0.01$). By ANCOVA considering confounding factors, rs650439 was also significantly associated with mean IMT (TT + TA vs AA, $P = 0.04$) in the hypertensive population. However, there was no significant association between *klotho* SNP and carotid IMT in the general population. Compared to the general population, the subject group with hypertensive patients clearly had more atherosclerosis risk factors.

Conclusion: Only in hypertensive patients was *klotho* rs650439 strongly associated with mean IMT thickening of the common carotid artery. Therefore, *klotho* SNP (rs650439) may

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influence on the progression of carotid atherosclerosis in patients with hypertension. **Geriatr Gerontol Int 2010; 10: 311–318.**

Keywords: carotid atherosclerosis, klotho, single nucleotide polymorphism.

Introduction

Myocardial infarction and stroke originating from atherosclerosis are the main causes of death in middle-aged and elderly people. The prevention of atherosclerosis is important to maintain the health of these groups of people. Defects in klotho gene expression in mice result in a syndrome that is similar to human aging, including arteriosclerosis, osteoporosis, infertility, emphysema and ectopic calcification.[1] In *klotho*-deficient mice, Mönckeberg-type arteriosclerosis, which is seen in aged humans, was observed from the aorta to small arterioles,[1] and impairments in angiogenesis and vasculogenesis were additionally observed.[2] Endothelial cell dysfunction has been suggested as the initiating process in the development and progression of atherosclerosis. Endothelium-dependent vasodilation in response to acetylcholine is attenuated in the aorta and arterioles from *klotho*-deficient mice, and this endothelial dysfunction was prevented by parabiosis between wild-type mice and heterozygously *klotho*-deficient mice.[3] Subsequent studies in humans have identified a functional variant of *klotho*, dubbed KL-VS, that is associated with longevity[4] and early-onset occult coronary artery disease (CAD).[5] Furthermore, a recent study by Arking *et al.*[6] reported the association of KL-VS with high-density lipoprotein (HDL) levels, systolic blood pressure (SBP) and an increased risk of stroke, which suggests an association of *klotho* with atherosclerosis in white subjects. In the Japanese population, the G-395A polymorphism in the promoter region of human *klotho* was reported to be associated with bone density,[7] cognitive impairment[8] and CAD.[9]

We have previously reported that klotho gene delivery suppresses oxidative stress in mice,[10] and that klotho protein reduced H_2O_2-induced apoptosis and senescence in vascular cells.[11] Furthermore, we recently reported that klotho protein suppresses tumor necrosis factor-α-induced expression of adhesion molecules in the endothelium and monocyte adhesion to endothelium cells.[12] Atherosclerosis is thought to be a chronic inflammatory disease initiated and perpetuated by a variety of cardiovascular risk factors.[13] Thus, our experimental findings suggest that klotho protein plays a protective role in the pathogenesis of atherosclerosis.

Previous studies suggest that klotho gene polymorphisms may be associated with atherosclerosis. However, previous studies did not assess the relationship between klotho gene polymorphisms and atherosclerosis parameters such as carotid artery intima-media thickness (IMT). Here, we studied whether *klotho* single nucleotide polymorphisms (SNP) were associated with carotid atherosclerosis in Japanese using IMT measured by ultrasonography.

Methods

Subjects and DNA samples

Study subjects consisted of patients with hypertension and a general population participating in the so-called "Suita Study", which is a cohort study for cardiovascular diseases at the National Cardiovascular Center. The characteristics of patients with hypertension were as follows. Subjects with hypertension included 953 patients with hypertension (522 men and 431 women, average age 65.1 ± 10.5 years) recruited from the Division of Hypertension and Nephrology at the National Cardiovascular Center. Ninety-two percent of subjects (880 subjects) had essential hypertension, and the remaining 8% had secondary hypertension. The criteria for hypertension were a SBP greater than 140 mmHg or a diastolic blood pressure (DBP) greater than 90 mmHg or both, or the use of antihypertensive agents. Subjects from the general population were people who had visited the National Cardiovascular Center every 2 years for general health checkups. Age, SBP, DBP, body mass index (BMI), percentage of current smokers, percentage of current drinkers, and prevalence of hypertension and diabetes mellitus were significantly higher in men than in women. Total cholesterol, HDL cholesterol, and percentage of hyperlipidemia were significantly higher in women than in men. For both populations, in addition to performing a routine blood examination that included lipid profiles, glucose levels, blood pressure, anthropometric measurements, a physician or nurse administered questionnaires covering personal history of cardiovascular diseases, including angina pectoris, myocardial infarction and stroke. Smoking was defined as current smoking, past smoking and never. All subjects were Japanese. Because of a lack of clinical data or unsuccessful sequencing and genotyping, 853 patients with hypertension (465 men and 388 women) and 1783 subjects from the general population (821 men and 962 women) were analyzed in the present study.

DNA samples were obtained with written informed consent and the protocol was approved by the Ethical Review Committee of the National Cardiovascular

Center. Genomic DNA was isolated from peripheral blood leukocytes with an NA-3000 nucleic acid isolation system (Kurabo, Osaka, Japan).[14]

Sequencing and genotyping of klotho SNP

Sequencing and genotyping have been described previously.[15] Briefly, we attempted to sequence the promoter region and all exons of *klotho* in 96 Japanese patients with hypertension. All exons with their flanking sequences and approximately 1.6 kb of the promoter region were directly sequenced with an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA) using four sets of primers. Information about the primers and polymerase chain reaction (PCR) conditions is available on request. The obtained sequences were examined for the presence of variations using sequencer software (Gene Codes, Ann Arbor, MI, USA), followed by visual inspection. The A of the ATG of the initiator Met codon was designated nucleotide +1. The nucleotide sequence (GenBank accession ID NT_004671) was used as a reference sequence. We selected and genotyped four SNP (rs7323281; intron1, rs5644481; exon4, rs3752472; exon3, rs650439; intron4 on website, db SNP http://www.ncbi.nlm.nih.gov/projects/SNP/) selected as representative SNP of haplotype blocks and a minor allele frequency (MAF) of more than 0.05 obtained by direct sequencing. These four SNP genotyped in the present study were registered in the National Center for Biotechnology Information (NCBI) database, but other SNP identified by direct sequencing were novel (Supporting Information Table S1). We checked the Tag SNP on the conditions of r^2 more than 0.5 and MAF more than 0.05 from 45 Japanese in the HapMap database. In consequence, eight SNP were hit. In the present study, we directly sequenced the promoter, all exons and its adjacent intron in 96 Japanese. On the other hand, the HapMap database consists of full sequencing data. The difference of SNP number between HapMap and the present study for the covering area of the klotho gene would be mainly due to this reason. We think that the coding region would be more important than the intron area. The TaqMan PCR method was used for genotyping as previously described.[16] All clinical data, sequencing and genotyping results were anonymous.

Evaluation of carotid atherosclerosis

Carotid ultrasonography was used to measure mean IMT (m-IMT) to evaluate atherosclerosis as previously described.[17,18] Briefly, ultrasonography of both carotid arteries was performed with a high-resolution Duplex scanner (SSA-250A; probe, SMA-736S mechanical sector scanner; Toshiba, Tokyo, Japan) for B-scans. All measurements were performed by two trained sonographers who were unaware of the subjects' clinical data. We defined carotid atherosclerosis as m-IMT of 1.1 mm or more.

Statistical analysis

Values are expressed as the mean ± standard deviation The distribution of genotypes between groups with an m-IMT of 1.1 mm or more and groups with an m-IMT of less than 1.1 mm was analyzed by χ^2-test analysis. Differences in variables between hypertensive patients and the general population were also assessed by Student's *t*-test and χ^2-test analysis. Hardy–Weinberg equilibrium (HWE) was assessed by χ^2-test analysis.

In multivariable models, we considered atherosclerosis-associated risk factors and drug treatment. Multiple logistic analysis and ANCOVA were performed with confounders including age, sex, BMI, diabetes, hyperlipidemia, hypertension, chronic kidney disease (CKD), smoking status and information about taking drugs. CKD was defined as an estimated glomerular filtration rate of less than 60 mL/min per 1.73 m^2. Information about taking drugs included antihypertensive, lipid-lowering and hypoglycemic drugs. The adjusted odds ratios (OR) are given with the 95% confidence intervals (CI). All analyses except ANCOVA were performed with JMP statistical software ver. 8 and ANCOVA was performed with Dr SPSS II statistical software ver. 11.0.1. Statistical significance was established at $P < 0.05$.

Using HapMap database

We used HapMap database (http://hapmap.jst.go.jp) to assess SNP located in the same haplotype block with rs650439. Our data sources was Japanese, HapMap Data Rel 24/phasell Nov08, on NCBI B36 assembly, dbSNP b126.

Results

Study population

The characteristics of the subjects are shown in Table 1. Hypertensive patients had higher risk factors than the general population, for example, rate of male sex, BMI, blood pressure, prevalence of diabetes, prevalence of hyperlipidaemia, lower HDL cholesterol and CKD. The average m-IMT was higher in hypertensive patients than in the general population. In hypertensive patients, 184 subjects (21.6%) were classified as carotid atherosclerosis, and 90.6%, 30.1% and 8.1% subjects of this group were taking antihypertensive, lipid-lowering and antidiabetic drugs, respectively. On the other hand, in there general population there were only 70 subjects (3.9%) with carotid atherosclerosis, and 24.8%, 13.6%

Table 1 Characteristics of the hypertensive patients and general population

	Hypertensive ($n = 853$)	General ($n = 1783$)	*P*-value
Age, mean ± SD	65.2 ± 10.4	64.8 ± 11.2	0.3357
Sex (% male)	54.5	46.0	<0.0001
BMI, mean ± SD (kg/m^2)	23.9 ± 4.49	22.7 ± 3.1	<0.0001
SBP, mean ± SD (mmHg)	139.5 ± 17.5	130.1 ± 19.7	<0.0001
DBP, mean ± SD (mmHg)	82.4 ± 10.6	78.1 ± 10.4	<0.0001
Hypertension (%)	100	42.4	<0.0001
Diabetes (%)	24.2	8.64	<0.0001
HbA1c, mean ± SD (%)	5.68 ± 0.83	5.5 ± 0.7	<0.0001
Hyperlipidaemia (%)	62.1	42.1	<0.0001
HDL-C, mean ± SD (mg/dl)	51.9 ± 15.8	60.1 ± 15.6	<0.0001
Smoking Status (%)			<0.0001
Current	11.8	17.3	0.0002
Past	35.2	24.4	<0.0001
Never	53.0	58.3	0.0128
CKD (%)	30.7	14.1	<0.0001
eGFR, mean ± SD (mL/min per 1.73 m^2)	69.2 ± 27.1	77.8 ± 19.2	<0.0001
mean IMT, mean ± SD (mm)	0.84 ± 0.18	0.82 ± 0.13	0.0033
Number of subjects with IMT thickening (%)	184 (21.6%)	70 (3.9%)	<0.0001

SD, standard deviation; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high density lipoprotein cholesterol; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; IMT, intima-media thickness.

and 4.5% subjects were taking antihypertensive, lipid-lowering and antidiabetic drugs, respectively.

Association of klotho SNP with carotid atherosclerosis in hypertensive populations

The genotype frequencies of all analyzed polymorphisms were consistent with HWE. The relations between genotypes and atherosclerosis for the hypertensive group were analyzed in additive, dominant and recessive models. There were some significant associations in this analysis (Supporting Information Table S2). The rs650439 in *klotho* was significantly associated with carotid atherosclerosis (TT vs TA vs AA; $\chi^2 = 7.49$, $P = 0.02$, TT + TA vs AA [recessive model]; $\chi^2 = 6.33$, $P = 0.01$). The rs3752472 SNP was also associated with carotid atherosclerosis (CC + TC vs TT [dominant model]; $\chi^2 = 4.41$, $P = 0.04$). The other two SNP were not significantly associated with carotid atherosclerosis (data not shown).

The relationships between the two SNP (rs650439, rs3752472) and carotid atherosclerosis analyzed by multiple logistic analysis with confounders including age, sex, BMI, diabetes, hyperlipidaemia, CKD, smoking status and taking drugs are shown in Table 2. In this analysis, rs650439 was significantly associated with carotid atherosclerosis (TT vs TA vs AA, $P < 0.01$; TT + TA vs AA; $P < 0.01$), but rs3752472 was not significant (CC + TC vs TT, $P = 0.26$). ANCOVA was performed to assess the associations between SNP and the m-IMT value. rs650439 was only significantly associated with the m-IMT value (TT + TA vs AA, $P = 0.04$; Table 3). The other SNP were not significantly associated with the m-IMT.

Association of klotho polymorphisms with carotid atherosclerosis in the general population

Unlike the hypertensive group, rs650439 and rs3752472 were not significantly associated with carotid atherosclerosis on χ^2-test analysis (Supporting Information Table S2) and multiple logistic regression analysis adjusted by age, sex, BMI, diabetes, hyperlipidaemia, hypertension, smoking status, CKD and drug treatment (data not shown). The other two SNP, rs7323281 and rs564481, were not associated with carotid atherosclerosis either (data not shown). There were no significant associations between the m-IMT value and *klotho* SNP analyzed by ANCOVA (Table 4). We performed the subgroup analysis by ANCOVA in normotensive and hypertensive subjects of the general population. There were no significant associations between *klotho* SNP and m-IMT values, even in rs650439 (Table 5).

Discussion

Cardiovascular diseases (CVD) such as myocardial infarction and stroke are the main cause of human

Table 2 Multiple logistic regression analysis[‡] for carotid atherosclerosis and klotho gene polymorphism in the hypertensive patients

	Odds ratio	95% confidence interval	*P*-value
rs650439(TT + TA vs AA)			<0.01
TT + TA/AA	1.688	1.178–2.442	
rs650439 (TT vs TA vs AA)			<0.01
TT/AA	1.221	0.665–2.176	
TA/AA	1.825	1.254–2.677	
rs3752472 (CC + CT vs TT)			0.26
CC + CT/TT	0.347	0.044–2.263	
rs3752472 (CC vs CT vs TT)			0.18
CC/TT	0.332	0.042–2.173	
TC/TT	0.474	0.057–3.232	

[‡]Adjusted for age, sex, body mass index, smoking, diabetes, hyperlipidaemia, chronic kidney disease (estimated glomerular filtration rate <60mL/min per 1.73 m^2) and drug treatments.

Table 3 ANCOVA[‡] of mean IMT and klotho gene polymorphism in the hypertensive patients

SNP	Genotype	Mean IMT	95% confidence interval	*P*-value
rs650439	TT	0.844 ± 0.017	0.811–0.877	0.11
	TA	0.853 ± 0.009	0.836–0.870	
	AA	0.827 ± 0.009	0.809–0.844	
rs650439	TT + TA	0.851 ± 0.008	0.836–0.866	0.04
	AA	0.826 ± 0.009	0.809–0.844	
rs3752472	CC	0.837 ± 0.006	0.825–0.850	0.31
	CT	0.855 ± 0.015	0.825–0.885	
	TT	0.924 ± 0.076	0.776–1.074	
rs3752472	CC + CT	0.840 ± 0.006	0.828–0.852	0.26
	TT	0.925 ± 0.076	0.776–1.074	

IMT, intima-media thickness; SNP, single nucleotide polymorphism. [‡]Adjusted for age, sex, body mass index, smoking, diabetes, hyperlipidaemia, chronic kidney disease (estimated glomerular filtration rate <60 mL/min per 1.73 m^2), and drug treatments.

mortality. Atherosclerosis plays a pivotal role in the pathogenesis of CVD, so prevention of atherosclerosis is the main goal of both clinicians and researchers. Mice deficient in klotho gene expression exhibit a syndrome resembling premature human aging, including atherosclerosis.[1] Previous studies have suggested an association of the klotho gene polymorphisms with atherosclerotic disease in white and Japanese populations.[6] However, these previous studies did not measure atherosclerosis parameters such as carotid IMT.

In the present study, we evaluated the relationship between representative SNP in *klotho* and carotid atherosclerosis directly evaluated by ultrasonography using two different subjects: patients with hypertension and subjects from a general population in Japan. We genotyped four SNP (rs7323281; intron1, rs5644481; exon4, rs3752472; exon3, rs650439; intron4) selected as representative SNP from haplotype blocks obtained by the direct sequencing for *klotho* in 96 individuals. Arking *et al.* reported a functional variant of the klotho gene polymorphism (KL-VS) associated with aging[4] and early onset of CAD.[5] Imamura *et al.* reported that G-395A in a promoter region in *klotho* may be associated with CAD in Japan.[9] However, in the present study, we did not identify F352V or C370S (KL-VS) in exon2 or G-395A in the *klotho* promoter region by direct sequencing for human *klotho*.

In our multivariate logistic analysis, rs650439 (intron4) was strongly associated with carotid atherosclerosis in hypertensive patients ($P < 0.01$). In ANCOVA,

Table 4 ANCOVA† of mean IMT and klotho gene polymorphism in the general population

SNP	Genotype	Mean IMT	95% confidence interval	*P*-value
rs650439	TT	0.827 ± 0.007	0.813–0.841	0.19
	TA	0.816 ± 0.004	0.809–0.824	
	AA	0.825 ± 0.004	0.818–0.833	
rs650439	TT + TA	0.819 ± 0.003	0.812–0.826	0.21
	AA	0.825 ± 0.004	0.818–0.833	
rs3752472	CC	0.823 ± 0.003	0.818–0.829	0.35
	CT	0.813 ± 0.007	0.801–0.827	
	TT	0.802 ± 0.030	0.744–0.861	
rs3752472	CC + CT	0.822 ± 0.003	0.817–0.827	0.52
	TT	0.802 ± 0.030	0.744–0.861	

IMT, intima-media thickness; SNP, single nucleotide polymorphism. †Adjusted for age, sex, body mass index, smoking, hypertension, diabetes, hyperlipidaemia, chronic kidney disease (estimated glomerular filtration rate <60 mL/min per 1.73 m^2) and drug treatments.

Table 5 ANCOVA† of mean IMT and rs650439 in the hypertensive patients and the general population

SNP	Genotype	Mean IMT	95% confidence interval	*P*-value
Hypertensive	TT + TA	0.851 ± 0.008	0.836–0.866	0.04
	AA	0.826 ± 0.009	0.809–0.844	
General with hypertension	TT + TA	0.860 ± 0.006	0.849–0.871	0.11
	AA	0.874 ± 0.006	0.861–0.887	
General without hypertension	TT + TA	0.788 ± 0.004	0.780–0.796	0.76
	AA	0.790 ± 0.005	0.781–0.800	

IMT, intima-media thickness; SNP, single nucleotide polymorphism. †Adjusted for age, sex, body mass index, smoking, hypertension, diabetes, hyperlipidaemia, chronic kidney disease (estimated glomerular filtration rate <60 mL/min per 1.73 m^2) and drug treatments.

rs650439 was also significantly associated with the m-IMT value (TT + TA vs AA, $P = 0.04$; Table 3). On the other hand, in these analyses, the significant association of rs650439 was not observed in the general population. We have performed a linear regression analysis between genotypes and IMT as a continuous variable. There was a significant correlation of genotypes in rs650439 with IMT after adjusted confounding factors, only in hypertensive patients (TT + TA vs AA; $P = 0.04$). These results suggested that SNP of *klotho* may affect the progression of carotid atherosclerosis in the subjects with hypertension. However, there was no significant association of SNP of *klotho* with carotid atherosclerosis in the general population. In order to assess this discrepancy, we performed the subgroup analysis in normotensive and hypertensive subjects of a general population, however, *klotho* rs650439 was also not associated with IMT in this subgroup analysis (Table 5). The number of subjects with IMT of 1.1 or more was small ($n = 41$) even in hypertensive subjects of the general population. Although HWE were not significantly different among number of genotypes in subjects with or without IMT of 1.1 or more, allele frequency was quite different from the NCBI database. We consider these may be reasons that *klotho* rs650439 was not associated with IMT in hypertensive subjects of the general population.

Comparing the backgrounds in hypertensive patients and the general population, the hypertensive group clearly had higher risk factors such as SBP, prevalence of diabetes, hyperlipidaemia and CKD than the general population (Table 1). Moreover, risk factors for atherosclerosis such as diabetes, dyslipidemia and CKD were obviously of higher prevalence in hypertensive patients compared to the general population with hypertension (Supporting Information Table S3). Thus, we suppose

that *klotho* gene polymorphisms may be influencing additively on progression of atherosclerosis induced by classical risk factors.

Previous studies reported that patients with multiple risk factors have elevated oxidative stress. For example, patients with metabolic syndrome may have elevated oxidative stress[19] in their cardiovascular systems. Total body fat and waist circumference have been demonstrated to be positively associated with oxidative stress-mediated endothelial dysfunction[20] and vascular endothelial cell nicotinamide adenine dinucleotide phosphate oxidase activity.[21] Furthermore, several studies demonstrated that human endothelial and smooth muscle cells incubated with high glucose concentrations upregulate oxidative stress.[22,23] From these aspects, hypertensive patients might have higher oxidative stress than general population in this study. We have previously reported that *klotho* gene delivery upregulates manganese superoxide dismutase protein expression and suppresses the oxidative stress *in vivo*.[10] We have also reported that klotho protein reduced H_2O_2-induced apoptosis and senescence in vascular cells,[11] suggesting that it can protect endothelial cells from oxidative stress. Kuro-o *et al.* reported that klotho protein inhibits insulin signals and promotes FOXO activity, inducing superoxide dismutase production.[24,25] These previous reports suggest that klotho may have an antioxidative stress function. Therefore, we can speculate that the protective effect of klotho protein would be strongly expressed in a hyper-oxidative stress state, and that hypertensive patients with multiple risk factors may be more sensitive to reduction of klotho protein function. This may be the reason why the *klotho* rs650439 was associated with carotid atherosclerosis only in hypertensive patients.

Klotho protein is known to regulate calcium and phosphate metabolism,[26] and the abnormality of calcium and phosphate level affects progression of carotid atherosclerosis. Therefore, we investigated the relationship between SNP in *klotho* and serum level of calcium and phosphate. However, there were no significant differences in serum calcium and phosphorus levels among each genotype (data not shown). Thus, we suppose that carotid atherosclerosis may not be due to systemic abnormality of calcium and phosphorus metabolism.

The SNP in *klotho*, rs650439, is located in intron 4. According to this study, we speculate that rs650439 may modulate klotho protein function. One possible mechanism is that rs650439 may induce a splicing abnormality leading to a change in klotho protein construction or function. Another possible mechanism is that other functional SNP affecting *klotho* expression or function may be located in the same haplotype block as rs650439. We assessed the SNP located in the same haplotype block as rs650439 using the HapMap database. Almost all SNP were in intron1, and the rest of the SNP closely linked with rs650439 were in intron3, exon4 and near the 3'-terminal. There were no SNP located in the promoter region. rs648202, the only SNP located in a coding region (exon4), was synonymous (Ala to Ala). The influence of rs650439 on *klotho* function could not be elucidated in this study. Further studies are needed to clarify the functional role of rs650439 in *klotho*.

Although the difference of genetic backgrounds plays a key role in the pathophysiology of atherosclerosis, the effect of only one SNP would be limited. Thus, the other gene SNP which were associated with the progression of atherosclerosis might affect the results of the present study, and the interaction between *klotho* rs650439 and gene polymorphisms in other atherosclerosis-related genes may be leading to the multiplier effects.

In conclusion, this study is the first to reveal that the SNP in *klotho* is associated with carotid atherosclerosis in patients with hypertension. Further studies are needed to clarify the functional role of *klotho* rs650439 on the progression of atherosclerosis.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Identified single nucleotide polymorphisms of *klotho* by direct sequencing.
Table S2. Genotype distributions of each polymorphism in the hypertensive and general population.
Table S3. Comparison of subjects' backgrounds in the hypertensive group and the general population with hypertension.

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