2.3. Statistical analysis

All data were reported on standardized forms, which were then entered into a database. Categorical variables among the groups were assessed by the chi-square test or Fisher's exact test. The mean levels of variables between two groups were compared by unpaired t-test or Mann–Whitney U-test. Comparison between more than two groups was done by Kruskal–Wallis test. Differences in serum lipid levels and IMT between baseline and after 24 months in each group were compared using paired t-test or Wilcoxon's signed-ranks test. Between group, mean percentage change from baseline to 24 months was compared using multiple regression analysis with the Bonferroni correction. Percentage change was calculated by the following formula: % change = (value at 24 months − baseline value)/baseline value × 100. $P < 0.05$ were considered statistically significant in all analyses.

3. Results

3.1. Baseline characteristics of the three groups by sex

In men, no significant differences in age, BMI, blood pressure, smoking, history, or IMT were found between the three groups. The TC, LDL-C and TG levels were significantly higher in groups A and B than in group C ($P < 0.01$), respectively. No significant difference in TC, LDL-C, or HDL-C level was found between groups A and B. HDL-C was significantly higher in group A than group C ($P < 0.01$) (Table 1).

In women, age, BMI, blood pressure, history of hypertension, serum cholesterol, TG and IMT were significantly higher in groups A and B than in group C ($P < 0.01$), respectively; whereas, smoking was significantly higher in group C than in groups A and B ($P < 0.01$), respectively. The TC, LDL-C, and IMT levels were significantly higher in group B than group A ($P < 0.01$).

3.2. Changes in variables at baseline and 24 months (Tables 2 and 3)

In men, no significant change in BMI was found in the A, B, or C group; however, a significant increase of BMI was found in the TC turning abnormal group ($P = 0.046$) (Table 2). The systolic blood pressure level significantly increased in groups A and C ($P = 0.019$ and $P < 0.001$), respectively, but were not changed in group B. No significant change in diastolic blood pressure level was found in any group over 24 months.

The TC and LDL-C levels significantly decreased over 24 months in groups A and B ($P < 0.001$), but in group A the TC level continued over 220 mg/dl throughout the 24 months. No significant change in the TC or LDL-C level was found in group C; however, a significant decrease in the TC and LDL-C level was found in the continuous TC normal group ($P < 0.001$) and a significant increase in TC level was found in the TC turning to abnormal group ($P < 0.001$). The HDL-C level significantly decreased over 24 months in group B ($P = 0.019$). In groups A and C, no significant change in HDL-C was found over 24 months. The TG level significantly decreased over 24 months in group B ($P = 0.012$). No significant change in TG was found in group A or C over 24 months.

The IMT level was significantly reduced after 24 months in groups A, B, and C ($P = 0.002$, $P = 0.002$, and $P < 0.001$), respectively. In group C, a significant decrease in IMT was found in the continuous TC normal group ($P < 0.001$), whereas no significant change was found in the TC turning abnormal group ($P = 0.481$).

In women, BMI was significantly decreased in groups A and B ($P < 0.001$ and $P = 0.033$), respectively, but was not changed in group C (Table 3). The systolic blood pressure level significantly increased in groups A and C ($P = 0.029$ and $P < 0.001$), respectively, but was not changed in group B. No significant change in diastolic blood pressure level was found in any group over 24 months.

The TC and LDL-C levels significantly decreased over 24 months in groups A and B ($P < 0.001$), but in group A the TC level over 24 months was continuously higher than 220 mg/dl. In group C, the TC and LDL-C levels were significantly increased ($P = 0.004$ and 0.036), respectively. Significant decreases in TC and LDL-C were found in the continuous TC normal group ($P = 0.047$ and 0.022), whereas significant increases in TC and LDL-C were found in the TC turning to abnormal group ($P < 0.001$). The HDL-C level significantly decreased over 24 months in groups A and B ($P = 0.041$ and $P < 0.001$), respectively. In group C, a significant increase in the HDL-C level was found in the TC turning abnormal group ($P = 0.006$). The TG level significantly increased over 24 months in group C ($P = 0.014$); however, this was only true for the continuous TC normal group ($P = 0.025$). No significant change in TG was found in groups A and B over 24 months of follow-up.

IMT was significantly reduced after 24 months of follow-up in groups A and B ($P < 0.001$). In group C, no significant change in IMT was found; however, a significant decrease in IMT was found in the continuous TC normal group ($P = 0.006$), whereas no significant change in IMT was found in the TC turning abnormal group ($P = 0.358$).

3.3. Mean percentage change of lipid and IMT levels between the baseline and 24 month values (Figs. 1 and 2)

In men, the TC and LDL-C change was significantly higher in group B (−21.4 and −23.3%) than in groups A (−6.8 and −10.1%) and C (−0.9 and −1.3%) ($P < 0.01$), respectively (Fig. 1A). The TC and LDL-C change was significantly higher in group A than in group C ($P < 0.01$).

Table 1
Baseline characteristics of the study groups by sex

	Men ($n = 398$)				Women ($n = 992$)			
	Group A ($n = 107$)	Group B ($n = 33$)	Group C ($n = 258$)	P-value	Group A ($n = 330$)	Group B ($n = 126$)	Group C ($n = 536$)	P-value
Age (years)	59.1 ± 9.7	60.6 ± 8.1	59.9 ± 12.4	0.264	59.0 ± 10.7[b]	62.2 ± 8.7[b]	52.6 ± 13.1	<0.001
Body mass index (kg/m^2)	23.7 ± 3.1	23.2 ± 3.5	23.2 ± 2.8	0.397	22.9 ± 3.4[b]	23.3 ± 3.4[b]	21.9 ± 2.9	<0.001
Blood pressure (mmHg)								
Systolic	131.8 ± 16.3	132.4 ± 17.2	132.3 ± 18.3	0.988	130.3 ± 19.6[b]	133.1 ± 19.6[b]	121.9 ± 19.2	<0.001
Diastolic	78.8 ± 10.3	77.3 ± 11.5	79.4 ± 10.4	0.550	77.4 ± 10.6[b]	78.3 ± 10.0[b]	73.0 ± 10.7	<0.001
Smoking (%)	80 (74.8)	25 (75.8)	207 (80.2)	0.477	35 (10.6)[b]	7 (5.6)[b]	81 (15.1)	<0.01
History (%)								
Hypertension	37 (34.6)	10 (30.3)	103 (39.9)	0.416	97 (29.4)[b]	40 (31.7)[b]	101 (18.8)	<0.001
Diabetes mellitus	15 (14.0)	4 (12.1)	21 (8.1)	0.216	18 (5.5)	4 (3.2)	20 (3.7)	0.388
Cardiovascular disease	4 (3.7)	0 (0)	16 (6.2)	0.24	9 (2.7)	3 (2.4)	6 (1.1)	0.200
Cerebrovascular disease	3 (2.8)	0 (0)	7 (2.7)	0.628	4 (1.2)	2 (1.6)	3 (0.6)	0.425
Serum cholesterol (mg/dl)								
Total	243.2 ± 19.4[b]	250.8 ± 23.7[b]	187.2 ± 21.7	<0.001	246.3 ± 23.2[a,b]	257.7 ± 31.4[b]	190.3 ± 20.2	<0.001
Low-density lipoprotein	157.1 ± 21.3[b]	159.5 ± 25.8[b]	109.0 ± 21.9	<0.001	157.6 ± 24.2[a,b]	168.8 ± 27.3[b]	110.8 ± 19.5	<0.001
High-density lipoprotein	58.7 ± 14.9[b]	58.5 ± 18.5	55.1 ± 12.6	0.125	65.4 ± 15.4[b]	67.8 ± 14.9	62.8 ± 13.2	<0.01
Serum triglycerides (mg/dl)	137.1 ± 68.8[b]	163.8 ± 94.1[b]	115.2 ± 62.9	<0.001	105.2 ± 47.8[b]	117.1 ± 59.7[b]	83.4 ± 48.6	<0.001
Intima-media thickness (mm)	0.88 ± 0.26	0.96 ± 0.30	0.88 ± 0.21	0.352	0.83 ± 0.18[a,b]	0.88 ± 0.19[b]	0.75 ± 0.16	<0.001

Data represents the mean value ± S.D. or number (%) of subjects. Group A, lifestyle modification; group B, lifestyle modification with lipid-lowering drug; group C, normal TC.

[a] $P < 0.01$, compared to group B.

[b] $P < 0.01$, compared to group C.

Table 2
Changes in variables from baseline to 24 months of follow-up in men

	Men ($n = 398$)			
	Baseline, mean ± S.D.	24 months, mean ± S.D.	Percentage change	*P*-value
Body mass index (kg/m^2)				
Group A	23.7 ± 3.1	23.8 ± 3.4	−0.4	0.523
Group B	23.2 ± 3.5	22.9 ± 3.4	−1.3	0.056
Group C	23.2 ± 2.8	23.2 ± 3.0	0.0	0.393
Continuous TC normal group	23.2 ± 2.9	23.2 ± 3.0	0.0	0.822
TC turning abnormal group	22.9 ± 2.7	23.4 ± 3.1	2.2	0.046
Systolic blood pressure (mmHg)				
Group A	131.8 ± 16.3	134.8 ± 17.7	2.3	0.019
Group B	132.4 ± 17.2	132.8 ± 17.0	0.3	0.957
Group C	132.3 ± 18.3	135.5 ± 20.1	2.4	<0.001
Continuous TC normal group	132.4 ± 18.2	135.6 ± 19.8	2.4	<0.001
TC turning abnormal group	131.6 ± 20.2	134.3 ± 24.3	2.1	0.355
Diastolic blood pressure (mmHg)				
Group A	78.9 ± 10.3	80.7 ± 11.6	2.3	0.069
Group B	77.4 ± 11.6	79.0 ± 11.9	2.1	0.513
Group C	79.4 ± 10.4	79.4 ± 12.0	0.0	0.982
Continuous TC normal group	79.3 ± 10.3	79.4 ± 12.2	0.1	0.926
TC turning abnormal group	79.6 ± 11.1	79.0 ± 9.5	−0.8	0.815
TC (mg/dl)				
Group A	243.2 ± 19.4	226.6 ± 24.9	−6.8	<0.001
Group B	250.8 ± 23.7	197.2 ± 25.5	−21.4	<0.001
Group C	187.2 ± 21.7	185.6 ± 25.6	−0.9	0.218
Continuous TC normal group	185.9 ± 21.7	181.5 ± 22.5	−2.4	<0.001
TC turning abnormal group	201.8 ± 14.6	231.0 ± 9.6	14.5	<0.001
LDL-C (mg/dl)				
Group A	157.1 ± 21.3	141.2 ± 24.2	−10.1	<0.001
Group B	159.5 ± 25.8	122.4 ± 26.2	−23.3	<0.001
Group C	109.0 ± 21.9	107.6 ± 23.6	−1.3	0.255
Continuous TC normal group	108.2 ± 23.7	104.1 ± 20.8	−3.8	<0.001
TC turning abnormal group	117.9 ± 21.0	146.5 ± 18.7	24.3	<0.001
HDL-C (mg/dl)				
Group A	58.7 ± 14.9	57.0 ± 14.0	−2.9	0.014
Group B	58.5 ± 18.5	50.4 ± 12.6	−13.8	0.019
Group C	55.1 ± 12.6	54.6 ± 13.3	−0.9	0.266
Continuous TC normal group	54.9 ± 12.5	54.0 ± 13.1	−1.4	0.734
TC turning abnormal group	57.6 ± 14.7	61.0 ± 13.7	5.9	0.136
TG (mg/dl)				
Group A	137.1 ± 68.8	142.4 ± 74.4	3.9	0.313
Group B	163.8 ± 94.1	122.3 ± 54.6	−25.3	0.012
Group C	115.2 ± 62.9	117.2 ± 67.9	1.7	0.608
Continuous TC normal group	113.8 ± 62.8	117.1 ± 69.1	2.9	0.384
TC turning abnormal group	131.7 ± 62.0	117.4 ± 53.3	−10.9	0.351
IMT (mm)				
Group A	0.88 ± 0.26	0.82 ± 0.16	−6.8	0.002
Group B	0.96 ± 0.30	0.80 ± 0.19	−16.7	0.002
Group C	0.88 ± 0.21	0.84 ± 0.16	−4.5	<0.001
Continuous TC normal group	0.89 ± 0.21	0.82 ± 0.17	−7.9	<0.001
TC turning abnormal group	0.87 ± 0.26	0.86 ± 0.12	−1.1	0.481

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; IMT, intima-media thickness. Group A, lifestyle modification, $n = 107$; group B, lifestyle modification with lipid-lowering drug, $n = 33$; group C, normal TC, $n = 258$; continuous TC normal group, $n = 237$; TC turning abnormal group, $n = 21$.

The HDL-C change was significantly higher in group B (−13.8%) than in groups A (−2.9%) and C (−0.9%) ($P < 0.05$), respectively. The TG change was significantly higher in group B (−25.3%) than in groups A (3.9%) and C (1.7%) ($P < 0.05$), respectively.

The IMT change was significantly higher in group B (−16.7%) than in group C (−4.5%) ($P < 0.05$) (Fig. 2A). No significant difference in the IMT change was found between groups A and B or between groups A and C over 24 months.

Table 3
Changes in variables from baseline to 24 months of follow-up in women

	Women ($n = 992$)			
	Baseline, mean ± S.D.	24 months, mean ± S.D.	Percentage change	*P*-value
Body mass index (kg/m^2)				
Group A	22.9 ± 3.4	22.7 ± 3.3	−0.9	<0.001
Group B	23.3 ± 3.4	23.1 ± 3.5	−0.9	0.033
Group C	21.9 ± 2.9	21.9 ± 3.0	0.0	0.198
Continuous TC normal group	21.8 ± 3.0	21.9 ± 3.0	0.5	0.236
TC turning abnormal group	22.3 ± 2.8	22.3 ± 2.8	0.0	0.616
Systolic blood pressure (mmHg)				
Group A	130.3 ± 19.6	131.9 ± 21.3	1.2	0.029
Group B	133.1 ± 19.6	131.8 ± 16.5	−1.0	0.247
Group C	121.9 ± 19.2	124.0 ± 19.8	1.7	<0.001
Continuous TC normal group	121.8 ± 19.3	123.6 ± 19.8	1.5	0.005
TC turning abnormal group	122.2 ± 18.4	127.0 ± 19.4	3.9	0.004
Diastolic blood pressure (mmHg)				
Group A	77.4 ± 10.6	77.8 ± 11.4	0.5	0.318
Group B	78.3 ± 10.0	76.8 ± 9.3	−1.9	0.076
Group C	73.0 ± 10.7	73.8 ± 10.9	1.1	0.063
Continuous TC normal group	72.8 ± 10.8	73.6 ± 10.8	−1.1	0.126
TC turning abnormal group	73.9 ± 10.1	75.2 ± 11.3	1.8	0.223
TC (mg/dl)				
Group A	246.3 ± 23.2	232.3 ± 27.6	−5.7	<0.001
Group B	257.7 ± 31.4	216.4 ± 31.0	−16.0	<0.001
Group C	190.3 ± 20.2	192.7 ± 26.4	1.3	0.004
Continuous TC normal group	187.4 ± 20.2	185.8 ± 21.0	−0.9	0.047
TC turning abnormal group	207.6 ± 9.1	234.6 ± 14.4	13.0	<0.001
LDL-C (mg/dl)				
Group A	157.6 ± 24.2	145.6 ± 28.2	−7.6	<0.001
Group B	168.8 ± 27.3	133.3 ± 32.4	−21.0	<0.001
Group C	110.8 ± 19.5	112.5 ± 24.5	1.5	0.036
Continuous TC normal group	108.7 ± 19.7	106.9 ± 20.5	−1.7	0.022
TC turning abnormal group	123.3 ± 12.4	146.3 ± 18.5	18.7	<0.001
HDL-C (mg/dl)				
Group A	65.4 ± 15.4	64.4 ± 15.4	−1.5	0.041
Group B	67.8 ± 14.9	62.6 ± 14.6	−7.7	<0.001
Group C	62.8 ± 13.2	62.7 ± 13.7	−0.2	0.618
Continuous TC normal group	62.3 ± 13.1	61.7 ± 13.4	−1.0	0.074
TC turning abnormal group	65.9 ± 13.6	68.7 ± 14.1	4.2	0.006
TG (mg/dl)				
Group A	105.2 ± 47.8	100.1 ± 49.7	−4.8	0.087
Group B	117.1 ± 59.7	111.9 ± 59.0	−4.4	0.208
Group C	83.4 ± 48.6	87.9 ± 55.2	5.4	0.014
Continuous TC normal group	82.0 ± 47.8	86.3 ± 53.6	5.2	0.025
TC turning abnormal group	91.7 ± 52.9	97.7 ± 63.3	6.5	0.319
IMT (mm)				
Group A	0.83 ± 0.18	0.79 ± 0.16	−4.8	<0.001
Group B	0.88 ± 0.19	0.81 ± 0.16	−8.0	<0.001
Group C	0.75 ± 0.16	0.74 ± 0.14	−1.3	0.055
Continuous TC normal group	0.75 ± 0.16	0.73 ± 0.14	−2.7	0.006
TC turning abnormal group	0.80 ± 0.15	0.80 ± 0.13	0.0	0.358

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; IMT, intima-media thickness. Group A, lifestyle modification, $n = 330$; group B, lifestyle modification with lipid-lowering drug, $n = 126$; group C, normal TC, $n = 536$; continuous TC normal group, $n = 460$; TC turning abnormal group, $n = 76$.

In women, the TC and LDL-C change was significantly higher in group B (−16.0% and −21.0%) than in groups A (−5.7% and −7.6%) and C (1.3 and 1.5%) ($P < 0.01$), respectively (Fig. 1B). The TC and LDL-C change was significantly higher in group A than in group C ($P < 0.01$). The HDL-C change was significantly higher in group B (−7.7%) than in groups A (−1.5%) and C (−0.2%) ($P < 0.05$), respectively. TG was significantly increased in

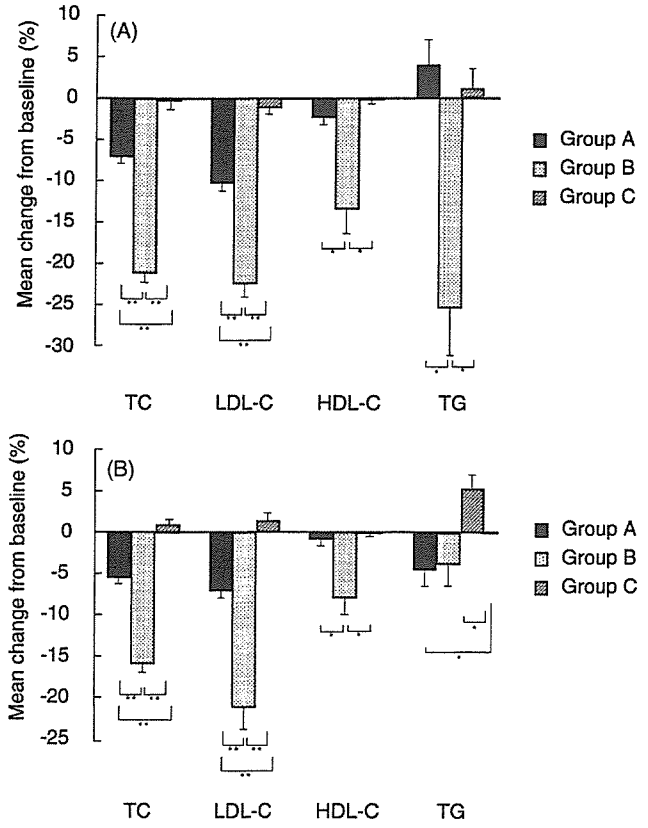

Fig. 1. (A) Mean percentage change in the serum lipid level of men over 24 months. Group A, lifestyle modification; group B, lifestyle modification with lipid-lowering drug; group C, normal TC: $^{**}P < 0.01$, $^{*}P < 0.05$. (B) Mean percentage change in the serum lipid level of women over 24 months. Group A, lifestyle modification; group B, lifestyle modification with lipid-lowering drug; group C, normal TC: $^{**}P < 0.01$, $^{*}P < 0.05$.

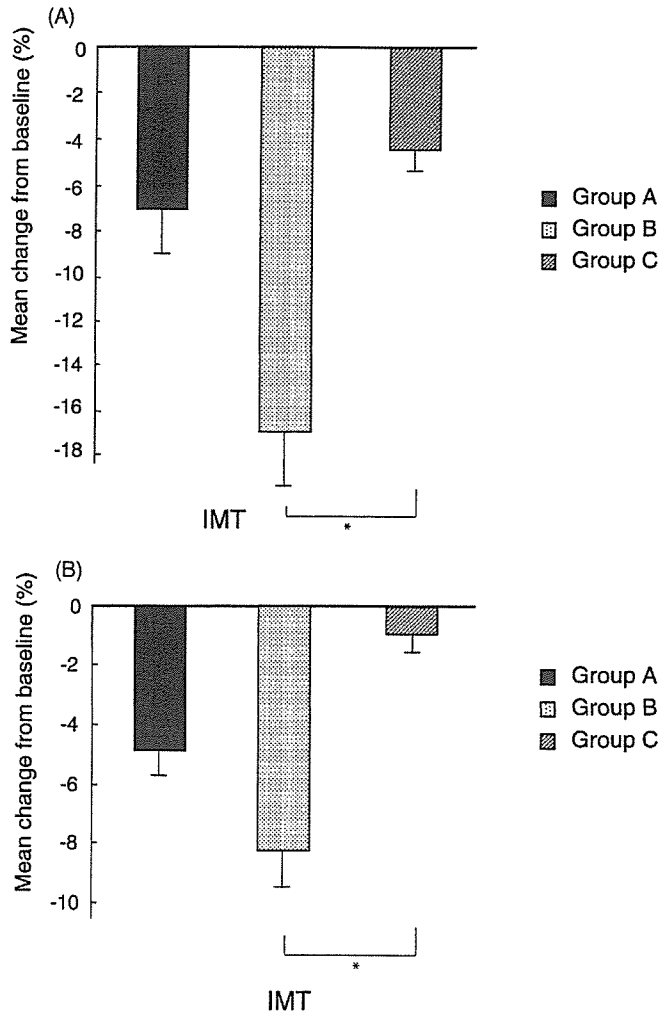

Fig. 2. (A) Mean percentage change in the IMT of men over 24 months. Group A, lifestyle modification; group B, lifestyle modification with lipid-lowering drug; group C, normal TC: $^{*}P < 0.05$. (B) Mean percentage change in the IMT of women over 24 months. Group A, lifestyle modification; group B, lifestyle modification with lipid-lowering drug; group C, normal TC: $^{*}P < 0.05$.

group C (5.4%), but decreased in groups A (−4.8%) and B (−4.4%) (group A or B versus C, $P < 0.05$).

The IMT change was significantly higher in group B (−8.0%) than in group C (−1.3%) ($P < 0.05$) (Fig. 2B). No significant difference in the IMT change was found between groups A and B or between groups A and C over 24 months.

4. Discussion

This large prospective study was designed to evaluate the effectiveness of various treatment regimens in subjects divided into three groups according to their cholesterol level at baseline and assessed by carotid IMT: (A) a lifestyle modification alone group; (B) a lifestyle modification with lipid-lowering drug therapy group; and (C) a control group. To our knowledge, this is the first evidence from a large-scale study to show that lifestyle modification alone can promote a significant reduction of carotid IMT over a 2 year period in men and women with hypercholesterolemia. Based on previous reports [2–4], a 'healthy lifestyle' (e.g., a well-balanced diet, regular physical exercise, smoking stoppage, and moderate alcohol intake) has become the backbone of consensus statements for primary prevention of cardiovascular disease. However, whether lifestyle changes, especially in diet and physical activity, can mediate a protective effect through a favorable influence on carotid atherosclerosis is still controversial.

Hodis et al. [15] reported that patients receiving lovastatin with dietary therapy had consistent reduction of carotid IMT as early as 1 year, whereas patients receiving only dietary therapy had a consistent increase of IMT at 2 and 4 years. The Fukuoka Atherosclerosis Trial [16] also showed that a diet therapy group had a significant reduction of LDL-C, but that IMT progression continued. On the other hand, Markus et al. [17] demonstrated that lifestyle modification, such as BMI reduction and dietary cholesterol intake reduction and quitting smoking, could reduce the annual rate of IMT progression. The results of studies of the effect of physical activity on IMT have been mixed, with a report of a protective

association with workplace activity [21], reduction in men but not women [22], and no relation [23]. Fields et al. [18] found that a multimodality traditional approach involving dietary, exercise, herbal food supplement, and stress reduction approaches could attenuate carotid atherosclerosis, particularly in those with marked cardiovascular risk. The differences in these results may be explained, at least in part, by differences in the populations or the study design, including lifestyle changes or the follow-up period. Our results indicate that comprehensive lifestyle modification can inhibit or reduce carotid IMT progression. It seems reasonable to suppose that a combination of an optimal dietary therapy and increased use of energy from fat through aerobic physical exercise might create a physiologic state that would be beneficial to the carotid arterial wall. If previous studies [15,16] had included more comprehensive lifestyle modification programs (e.g., diet, physical exercise, smoking stoppage, and weight control), the progression of carotid atherosclerosis may have been retarded even without the use of lipid-lowering drugs.

In our study, changes in the serum cholesterol level appeared to be correlated more closely with carotid IMT than did the changes in other lifestyle-related risk factors. Interestingly, the lifestyle modification alone group showed a significant regression of carotid IMT, even though the cholesterol level remained above the recommended level [24]. Schuler et al. [4] also found that patients engaging in regular physical exercise and consuming a low-fat diet over 1 year had a 10% reduction of the mean TC level, and that coronary atherosclerosis progressed in 23% of their patients, but was not changed in 45% and regressed in 32%, even though the TC level continued over 220 mg/dl, as in our lifestyle modification alone group. That a high serum cholesterol level is a major risk factor for the initiation and the development of atherosclerosis is beyond any doubt. However, our results showed that carotid IMT can be reduced by the reduction of TC, even though the TC level may remain above a level at which we would normally expect IMT to increase. Lifestyle changes should be part of any comprehensive treatment program for subjects with hypercholesterolemia, especially for low-risk subjects as were tested in our study, although the underlying mechanisms remain to be completely elucidated.

The reduction of carotid IMT was greater in the lifestyle modification with lipid-lowering drug group than in the lifestyle modification alone group in this study. Our results also showed that comprehensive lifestyle modification can reduce carotid IMT, even without the use of lipid-lowering drugs. From a medical cost standpoint, a comprehensive lifestyle modification program would obviously be the best first line of treatment for carotid atherosclerosis. Lifestyle intervention is safe and compatible with concurrent treatment for other conditions with atherosclerotic vascular risk, such as hypertension, diabetes, and obesity [18]. Moreover, atherosclerotic changes in carotid IMT have also become widely accepted as a marker of generalized atherosclerosis and have been associated with future cardiovascular and cerebrovascular events [8,10,11]. Therefore, the lifestyle modification of potential behavior-dependent factors may be a cost-effective way of preventing future vascular events.

In conclusion, our results suggested that comprehensive lifestyle modification can reduce carotid IMT and serum cholesterol levels and that cholesterol reduction provides benefit even when the TC level remains above that usually recommended.

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Carotid atherosclerosis and cardiovascular risk factors: a comparison of residents of a rural area of Okinawa with residents of a typical suburban area of Fukuoka, Japan

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Abstract

Areas of Japan are known worldwide for the longevity of their residents. Okinawa has the highest longevity in Japan and a low rate of death due to cardiovascular disease. We investigated carotid atherosclerotic (CA) risk factors in islands of I city in Okinawa prefecture and compared them with K town, a suburban area of Fukuoka prefecture in Kyushu, to determine the relationship between cardiovascular risk factors and carotid atherosclerosis. We investigated conventional cardiovascular risk factors in 1078 I city residents (375 men, mean age 63.7 and 703 women, mean age 60.0) in 2000 and 2364 K town residents (676 men, mean age 57.1 and 1688 women, mean age 53.0) in 1999. Carotid atherosclerosis was assessed by mean intima-media thickness (IMT) by B-mode ultrasound. The mean IMT was significantly lower in the residents of I city than in those of K town ($P < 0.05$). Total cholesterol (TC) and low-density-lipoprotein cholesterol (LDL-C) levels and smoking rate were also lower in I city than in K town. Body mass index (BMI) and triglyceride (TG) level were higher in I city than in K town. In I city, multiple regression analysis found independent relationships between carotid atherosclerosis and age, sex (male), hypertension, LDL cholesterol, high-density-lipoprotein cholesterol (HDL-C), and diabetes. The lower mean IMT is probably related to a lower lifetime burden of atherosclerotic risk factors, which may in turn be related to the longevity of Okinawa residents. BMI was not a cardiovascular risk factor, although LDL cholesterol was a common important risk factor.

Keywords: Okinawa; Fukuoka; Japan; Carotid arteries; Risk factors; Cross-sectional study

1. Introduction

Japan is known worldwide for the longevity of its residents. Okinawa is known for having the highest longevity in Japan and a low rate of death due to cardiovascular disease, including coronary heart disease and stroke, which are leading causes of death in many countries [1–3]. We thought, therefore, that an investigation of Okinawa residents would be useful for identifying factors that could be useful in the prevention of cardiovascular disease.

B-mode ultrasonography currently appears to provide the most accurate assessment of early atherosclerosis, allowing visualization and direct measurement of wall thickness. An increase in carotid artery intima-media thickness (IMT) has been associated with conventional cardiovascular risk factors [4–6], coronary heart disease, stroke, and atherosclerosis elsewhere in the arterial system [7–9]. IMT abnormalities are also predictive of cardiovascular prognosis [10,11] and improvement of carotid atherosclerosis (CA) has been reported to reduce cardiovascular events [12]. On the basis of these findings, carotid IMT measurement can be regarded as an indicator of generalized atherosclerosis. Moreover, non-invasive assessment of IMT makes ultrasonography ideal for screening and for serial studies [13].

Little study has been done of the relationship between cardiovascular risk factors and carotid atherosclerosis in Okinawa. We investigated carotid atherosclerotic risk factors in the general population of I city, located on an island in the eastern part of the Yaeyama district of Okinawa prefecture [14,15] and compared the results with K town, a typical

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Japanese suburban city in Fukuoka prefecture [16], to determine the relationship between cardiovascular risk factors and carotid atherosclerosis.

2. Materials and methods

2.1. Subjects

The studies were approved by the Ethics Committee of Kyushu University Hospital. Informed consent was obtained from all residents.

We investigated 1078 I city residents in 2000 and 2353 K town residents in 1999 without a history of cardiovascular disease, including coronary heart disease and stroke, who participated in free health examinations. Of the 1078 I city residents analyzed, 375 were men and 703 were women, aged 20–89. Of the 2364 K town residents analyzed, 676 were men and 1688 were women, aged 20–89. After exclusion for hyperlipidemia, hypertension, diabetes and obesity, 62 men and 205 women in I city and 184 men and 699 women in K town were categorized as healthy subjects.

2.2. Medical history

Excluded were subjects who had a history of coronary heart disease and/or stroke, refused ultrasound examination, did not return the questionnaire, or did not take the required blood examination. Altogether, 50 men and 81 women from among the 1209 subjects of I city and 46 men and 62 women from among the 2472 subjects from K town were excluded from this analysis. Height and weight were measured in light clothing and without shoes. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared. Systolic blood pressure and diastolic blood pressure were twice measured in the right arm with the subject in a sitting position after taking a short rest. Smoking behavior was assessed by questionnaire. Subjects were classified as smokers (current or past smokers) and non-smokers. Hypertension was defined as either systolic blood pressure $\geq$140 mmHg, diastolic pressure $\geq$90 mmHg, or treatment with antihypertensive medications. Hyperlipidemia was defined as either total cholesterol (TC) $\geq$220 mg/dl, triglyceride (TG) $\geq$150 mg/dl, or lipid-lowering drug administration. Diabetes was defined as a self-reported history of diabetes, a fasting plasma glucose (FPG) level $\geq$126 mg/dl, or the use of antidiabetic drugs. Obesity was defined as BMI $\geq$26.4 kg/m^2.

2.3. Assay methods

All blood samples were drawn between 9 and 12 a.m. after an overnight fast and stored at $-20\,^{\circ}$C until analysis. The following parameters were measured. Total cholesterol, high-density-lipoprotein cholesterol (HDL-C), triglyceride and fasting plasma glucose. Low-density-lipoprotein cholesterol (LDL-C) was calculated indirectly using the Friedewald formula.

2.4. Ultrasonographic measurement

High-resolution B-mode ultrasound examination was done with a 7.5 MHz mechanical sector transducer on the Aloka SSD-2000 (Aloka Co. Ltd., Tokyo, Japan) by three specially trained ultrasound technicians. All the medical histories and the assay results were blinded to the ultrasound technicians. Carotid IMT was measured at points 20, 25 and 30 mm proximal to the flow divider on the far wall of the right and left common carotid artery at the end of the diastolic phase. From this, mean IMT was determined for each individual. Mean IMT $\geq$1.1 mm was defined as abnormal IMT. Carotid atherosclerosis was defined as the presence of abnormal IMT.

2.5. Statistical analysis

Age, sex, BMI, total cholesterol level, LDL cholesterol level, HDL cholesterol level, triglyceride level, systolic blood pressure, diastolic blood pressure, diabetes and smoking rate were used as conventional risk factors and mean IMT was used to define the level of carotid atherosclerosis. Mean numerical variable comparison between I city and K town by age groups categorized 20–39, 40–49, 50–59, 60–69 and 70–89 was done by Student's t-test. Categorical variable comparison between I city and K town by age was done by the χ^2-test. Two way ANOVA was used for the means of numerical variables and Mantel Haenszel test was used to adjust categorical variables for age in comparison of I city and K town. Forward stepwise multiple logistic regression analysis was used to determine the independent risk factors for carotid atherosclerosis. P values <0.05 were considered statistically significant.

3. Results

Area profiles by age and sex are shown in Tables 1 and 2. Both male and female mean IMT was significantly lower in I city than in K town ($P < 0.05$, 0.05, respectively, two way ANOVA).

In comparison of I city and K town, the conventional risk factors total cholesterol, HDL cholesterol and LDL cholesterol were significantly lower in both male and female I city residents ($P < 0.05$, 0.05, 0.05 in males and $P < 0.05$, 0.05, 0.05 in females, respectively, two way ANOVA). Triglyceride level and BMI were significantly higher in both male and female I city residents ($P < 0.05$, 0.05 in males and $P < 0.05$, 0.05 in females, respectively, two way ANOVA). The difference in BMI between I city and K town increased as age increased. Systolic blood pressure was significantly lower in I city females ($P < 0.05$; two way ANOVA). The diabetes rate was significantly higher in male I city

Table 1
Characteristics of the male study population by age and area

	Age group					Total
	20–39	40–49	50–59	60–69	70–89	
Number						
I city	22	49	36	120	148	375
K town	91	110	116	229	130	676
BMI (kg/m^2)						
I city	23.6 (3.9)	25.0 (3.4)[a]	24.4 (3.2)	24.4 (2.6)[a]	23.5 (3.1)[a]	24.1 (3.1)[b]
K town	23.3 (3.8)	23.1 (2.8)	23.8 (2.6)	23.5 (2.9)	22.4 (2.9)	23.2 (3.0)
SBP (mmHg)						
I city	118.8 (13.6)	126.9 (15.0)[a]	127.3 (16.7)	132.4 (14.6)[a]	132.6 (17.2)[a]	130.4 (16.2)
K town	115.4 (11.9)	119.7 (15.1)	130.4 (17.2)	136.8 (18.0)	139.0 (18.8)	130.5 (19.0)
DBP (mmHg)						
I city	77.4 (10.5)[a]	83.2 (11.7)[a]	82.9 (12.2)	79.3 (8.8)	73.5 (10.7)[a]	77.8 (11.0)
K town	71.8 (9.7)	77.5 (11.7)	81.6 (11.1)	81.3 (11.3)	77.7 (9.8)	78.7 (11.3)
TC (mg/dl)						
I city	193.5 (32.2)	197.4 (33.7)	201.4 (29.5)[a]	194.1 (30.0)[a]	192.6 (28.3)[a]	194.6 (29.9)[b]
K town	201.8 (33.6)	208.1 (37.5)	215.0 (37.1)	203.5 (33.7)	204.0 (32.2)	206.1 (34.8)
HDL-C (mg/dl)						
I city	54.6 (12.5)	53.6 (12.7)	55.3 (10.9)	55.0 (12.9)	56.0 (13.9)	55.2 (13.0)[b]
K town	55.7 (15.0)	56.3 (13.2)	56.4 (14.6)	56.8 (14.7)	57.4 (14.1)	56.6 (14.4)
LDL-C (mg/dl)						
I city	110.7 (29.6)	110.6 (33.5)[a]	117.2 (26.8)[a]	113.1 (26.6)[a]	113.1 (26.1)[a]	113.0 (27.8)[b]
K town	121.3 (28.9)	126.3 (32.6)	129.9 (33.3)	122.2 (31.6)	124.6 (28.9)	124.5 (31.2)
TG (mg/dl)						
I city	141.4 (78.5)	165.9 (79.4)[a]	144.9 (76.3)	130.2 (70.8)	117.8 (66.5)	132.0 (72.7)[b]
K town	124.2 (68.3)	127.5 (68.7)	143.5 (90.4)	122.2 (65.8)	110.2 (55.9)	124.7 (70.3)
Diabetes rate (%)						
I city	4.5[c]	12.2	13.9	19.2[c]	12.2	14.1[d]
K town	0.0	1.8	10.3	10.9	14.6	8.6
Smoking rate (%)						
I city	54.5[c]	67.3[c]	36.1[c]	45.0[c]	49.3[c]	49.3
K town	78.0	87.3	75.9	74.7	89.2	80.2
Current						
I city	45.5	44.9	27.8	32.5	29.7	33.3
K town	63.7	49.1	39.7	28.8	30.8	39.1
Past						
I city	9.1	22.4	8.3[c]	12.5[c]	19.6[c]	16[d]
K town	14.3	38.2	36.2	45.9	58.5	41.1
Mean IMT (mm)						
I city	0.57 (0.14)[a]	0.68 (0.16)	0.74 (0.16)[a]	0.83 (0.18)[a]	0.93 (0.24)[a]	0.83 (0.22)[b]
K town	0.66 (0.13)	0.72 (0.13)	0.83 (0.19)	0.94 (0.20)	1.03 (0.26)	0.86 (0.23)

Values in parentheses are mean (S.D.). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HDL-C, high-density-lipoprotein cholesterol; LDL-C, low-density-lipoprotein cholesterol; TG, triglyceride.

[a] $P < 0.05$ between I city and K town in the same age group by Student's t-test.

[b] $P < 0.05$ between I city and K town adjusted for age by two way ANOVA.

[c] $P < 0.05$ between I city and K town in the same age group by χ^2-test.

[d] $P < 0.05$ between I city and K town adjusted for age by Mantel Henszel test.

residents ($P < 0.05$; Mantel Haenszel test) and the smoking rate was significantly lower in both male and female I city residents ($P < 0.05$, 0.05, respectively, Mantel Haenszel test).

Forward stepwise multiple logistic regression analysis (Table 3) showed age, high LDL cholesterol level, low HDL cholesterol level, sex (male), and diabetes to be independent risk factors for carotid atherosclerosis (mean IMT $\geq$ 1.1 mm) in I city ($P < 0.0001$, OR; 2.13, $P = 0.0005$, OR; 1.02, $P = 0.0012$, OR; 0.96, $P = 0.0023$, OR; 2.34, $P = 0.0064$, OR; 2.49, respectively). Age, high systolic blood pressure, high LDL cholesterol level, sex (male),

Table 2
Characteristics of the female study population by age and area

	Age group					Total
	20–39	40–49	50–59	60–69	70–89	
Number						
I city	73	111	110	199	210	703
K town	312	344	429	423	180	1688
BMI (kg/m^2)						
I city	21.5 (3.4)	23.5 (3.7)[a]	24.6 (3.5)[a]	24.7 (2.8)[a]	24.5 (3.5)[a]	24.1 (3.5)[b]
K town	20.9 (3.2)	22.1 (3.1)	22.4 (2.9)	23.2 (3.2)	22.2 (3.4)	22.2 (3.2)
SBP (mmHg)						
I city	107.2 (14.0)	116.3 (16.1)	123.9 (14.7)	127.5 (16.1)[a]	133.4 (15.9)[a]	124.8 (17.6)[b]
K town	108.1 (12.6)	115.9 (15.3)	127.9 (20.2)	134.3 (19.4)	136.7 (16.4)	124.4 (20.3)
DBP (mmHg)						
I city	66.3 (10.4)	73.0 (12.1)	76.2 (10.5)	75.5 (10.3)[a]	74.6 (10.0)	74.0 (10.9)
K town	66.6 (9.1)	71.2 (10.3)	77.6 (11.3)	78.6 (10.3)	75.8 (8.5)	74.3 (11.2)
TC (mg/dl)						
I city	174.4 (26.5)[a]	195.0 (32.8)	214.4 (31.9)[a]	216.2 (35.4)	214.3 (33.4)[a]	207.7 (35.6)[b]
K town	182.4 (29.1)	202.0 (32.7)	227.9 (38.2)	228.9 (35.8)	219.4 (29.9)	213.5 (38.5)
HDL-C (mg/dl)						
I city	64.0 (15.7)	60.6 (13.6)[a]	62.0 (13.9)[a]	55.0 (11.0)[a]	57.4 (13.2)[a]	58.6 (13.4)[b]
K town	64.6 (13.8)	65.5 (13.9)	66.1 (14.5)	62.2 (14.8)	52.7 (13.7)	64.4 (14.3)
LDL-C (mg/dl)						
I city	93.6 (25.4)[a]	113.9 (28.8)[a]	130.1 (28.1)[a]	133.5 (32.6)[a]	129.7 (30.9)	124.6 (32.5)[b]
K town	104.0 (25.8)	120.7 (30.7)	141.7 (34.1)	143.9 (33.1)	134.4 (28.2)	130.2 (34.6)
TG (mg/dl)						
I city	83.9 (56.2)[a]	102.6 (51.2)[a]	111.6 (62.8)	138.7 (66.1)[a]	135.9 (71.1)[a]	122.2 (66.7)[b]
K town	69.0 (37.6)	78.6 (39.6)	100.6 (57.8)	113.8 (57.7)	111.5 (60.8)	94.8 (54.4)
Diabetes rate (%)						
I city	0.0	3.6	3.6	7.5	10.5	6.4
K town	0.6	1.5	4.2	5.4	6.7	3.6
Smoking rate (%)						
I city	16.4	7.2[c]	5.5	2.5[c]	3.3[c]	5.4[d]
K town	26.6	21.5	10.3	8.3	9.4	15.0
Current						
I city	12.3	5.4	5.5	2.0	1.9	4.1[d]
K town	15.1	10.8	6.5	5.0	3.9	8.3
Past						
I city	4.1	1.8[c]	0.0[c]	0.5[c]	1.4[c]	1.3[d]
K town	11.5	10.8	3.7	3.3	5.6	6.7
Mean IMT (mm)						
I city	0.54 (0.10)[a]	0.64 (0.12)[a]	0.72 (0.13)[a]	0.80 (0.16)[a]	0.87 (0.19)[a]	0.75 (0.19)[b]
K town	0.61 (0.11)	0.69 (0.12)	0.78 (0.15)	0.89 (0.17)	0.95 (0.18)	0.77 (0.19)

Values in parentheses are mean (S.D.). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HDL-C, high-density-lipoprotein cholesterol; LDL-C, low-density-lipoprotein cholesterol; TG, triglyceride.

[a] $P < 0.05$ between I city and K town in the same age group by Student's t-test.

[b] $P < 0.05$ between I city and K town adjusted for age by two way ANOVA.

[c] $P < 0.05$ between I city and K town in the same age group by χ^2-test.

[d] $P < 0.05$ between I city and K town adjusted for age by Mantel Henszel test.

high triglyceride level, and high diastolic blood pressure were independent risk factors for carotid atherosclerosis (mean IMT $\geq$ 1.1 mm) in K town ($P < 0.0001$, OR; 2.56, $P < 0.0001$, OR; 1.03, $P < 0.0001$, OR; 1.01, $P < 0.0001$, OR; 2.09, $P = 0.0115$, OR; 1.00, $P = 0.0478$, OR; 0.98, respectively).

On analysis of healthy subjects (Table 4), the mean IMT of female I city residents was significantly lower than that found in K town residents ($P < 0.05$; two way ANOVA). The mean IMT of male I city residents was lower in each age group than was found in K town males, but there was no significant difference by two way ANOVA.

Table 3
Forward stepwise multiple logistic regression analysis of CA (mean IMT 1.1 mm) by age, sex, BMI, TC, HDL-C, LDL-C, TG, SBP, DBP, and diabetes rate

Variable	Coefficient	Odds ratio	95% CI of OR		*P* value
I city					
Age (year)	0.7559	2.13	1.63	2.77	0.0001
LDL-C (mg/dl)	0.0153	1.02	1.01	1.02	0.0005
HDL-C (mg/dl)	−0.0368	0.96	0.94	0.99	0.0012
Male (yes/no)	0.8487	2.34	1.35	4.05	0.0023
Diabetes (yes/no)	0.9500	2.59	1.35	4.94	0.0064
K town					
Age (year)	0.9413	2.56	2.12	3.1	<0.0001
SBP (mmHg)	0.0252	1.03	1.01	1.04	<0.0001
LDL-C (mg/dl)	0.0110	1.01	1.01	1.02	<0.0001
Male (yes/no)	0.7348	2.09	1.48	2.93	<0.0001
TG (mg/dl)	0.0032	1.00	1.00	1.01	0.0115
DBP (mmHg)	−0.0213	0.98	0.96	1.00	0.0478

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HDL-C, high-density-lipoprotein cholesterol; LDL-C, low-density-lipoprotein cholesterol; TG, triglyceride; CA, carotid atherosclerosis (mean IMT 1.1 mm).

4. Discussion

The present cross-sectional study is the first to use ultrasonography to survey carotid atherosclerosis in a sub population of Okinawa residents. We showed that the intima-media thickness of I city residents was significantly lower than that of K town residents. Previous research reported that residents of Okinawa had a low rate of death due to cardiovascular disease [1–3]. According to the Japanese Ministry of Health, Labour and Welfare, mortality by coronary heart disease and stroke in Okinawa prefecture were lower than Fukuoka prefecture. In 1999, the mortality rates (per 100,000 population) for coronary heart disease and stroke were 45.9 and 58.7, respectively, in Okinawa prefecture and 53.3 and 95.0, respectively, in Fukuoka prefecture. The difference in IMT was probably related to the low rate of death due to cardiovascular disease in Okinawa.

The serum total cholesterol, LDL cholesterol and HDL cholesterol, and smoking rate were lower in the residents of I city than in K town residents and the BMI, triglyceride level and diabetes rate were higher. On multiple regression analysis, age, male sex and LDL cholesterol were independent risk factors for carotid atherosclerosis in both I city and K town. These results show LDL cholesterol to be one of the most important biochemical risk factors for atherosclerosis. In the present study, although, the LDL cholesterol level was lower in I city than in K town, the HDL cholesterol level was lower and the triglyceride level was higher in I city than in K town. In 1985, Hosaki et al. [17] reported that the residents of Okinawa had a higher serum HDL cholesterol level and a lower LDL cholesterol level than residents of Tokyo, and that this was related to the lower cardiovascular mortality seen in Okinawa. Also implicated was the possibility of a relationship between the simple Okinawan low-fat diet composed of rice, sweet potatoes, soybeans, vegetables, seaweed, pork and fish. Changes in dietary habits in Okinawa related to the American influence after World War II and the necessity to establish better dietary habits to improve health were reported [18]. The present study indicates that delicate changes in serum lipoproteins may be gradually occurring with changes in dietary habits in the rural areas of Okinawa. The mean IMTs of the younger age groups (20–39 groups) in Okinawa, however, were still lower than those of the corresponding age groups in Fukuoka. This may indicate that

Table 4
Characteristics of healthy subjects

	Age group					Total
	20–39	40–49	50–59	60–69	70–89	
Male						
Number						
I city	7	6	5	18	26	62
K town	48	38	27	46	25	184
Mean IMT						
I city	0.59 (0.16)	0.63 (0.11)	0.74 (0.11)	0.75 (0.22)[a]	0.91 (0.19)	
K town	0.63 (0.10)	0.70 (0.13)	0.81 (0.16)	0.92 (0.24)	1.00 (0.20)	
Female						
Number						
I city	56	59	32	36	22	205
K town	260	210	121	74	34	699
Mean IMT						
I city	0.54 (0.10)[a]	0.62 (0.10)[a]	0.69 (0.11)[a]	0.79 (0.14)	0.85 (0.17)[b]	
K town	0.60 (0.11)	0.68 (0.11)	0.75 (0.14)	0.84 (0.19)	0.93 (0.15)	

Values with parentheses are mean (S.D.). Healthy subjects are defined as subjects after exclusion of those with hyperlipidemia, hypertension, diabetes and obesity.

[a] $P < 0.05$ between Okinawa and Fukuoka in the same age group by Student's *t*-test.

[b] $P < 0.05$ between Okinawa and Fukuoka adjusted for age by two way ANOVA.

not only dietary habits but some other important factors influence carotid IMT. Future observation is needed to clarify the significance of dietary habits on serum lipoproteins and carotid IMT in Okinawa.

Obesity has become a public health problem because of its increasing prevalence and the associated cardiovascular risk factors, including diabetes, hyperlipidemia and hypertension [19]. BMI is widely used as an index of obesity [20–23], and much attention has been paid to BMI as a risk factor for coronary heart disease [24,25]. However, BMI was not a significant risk factor for carotid atherosclerosis in either area. Despite the higher BMI in I city than in K town, the residents of I city had a lower mean IMT, lower rate of cardiovascular disease and the highest longevity in Japan. The high BMI in I city tended to be most notable in the older age groups. High BMI may not be influential as a causal factor of cardiovascular disease. Some studies have reported that higher body weight in older persons is negatively correlated with the death rate [26–28]. That a higher BMI, as found in I city, has a beneficial effect for older persons and is involved in the low rate of cardiovascular disease is possible. In addition, BMI indicates neither the regional fat composition nor muscle or bone mass, even though previous studies have reported that abdominal fat as indicated by waist-to-hip circumference, computed tomography, or ultrasonography is more closely associated with atherosclerosis and cardiovascular risk factors than overall body fat as indicated by BMI [29–34]. Furthermore, some studies have reported the possibility of differences in the relationship between BMI and cardiovascular risk by race, ethnicity, and geographical area [35,36]. Residents of Okinawa are racially and ethnically different than mainland Japanese. Obesity as a risk factor for cardiovascular disease, therefore, needs to be evaluated more carefully in Japan. Further study of obesity will be needed to determine how the amount and prevalence of body fat is related to cardiovascular risk factors in Japan and Okinawa.

The average mean IMT in all age groups was also lower in I city than in K town among healthy subjects. This indicates the possibility that factors other than conventional risk factors were related to the lower IMT in Okinawa. Some environmental factors such as food, climate, or lifestyle may be related to this phenomenon. Genetic factors also may be important. Some studies have reported relationship between genetic factors, such as the endothelial nitric oxide synthase locus, LDL receptor locus, and GPIIIa polymorphism [37–39], and atherosclerosis. Studies of twins suggested a familial risk of coronary artery disease correlated with genetic factors, not merely a common familial environment [40]. To our knowledge, there are no reports of the relationship between genetics and cardiovascular diseases in Okinawa. Further studies will be needed to clarify this issue.

In conclusion, carotid IMT was low in I city. This is possibly related to the low rate of death due to cardiovascular disease and to residents of Okinawa having the highest longevity in Japan. Lower LDL cholesterol and other factors such as lifestyle, diet, genetics, climate and the environment in Okinawa may also be related to this phenomenon. Further research in Okinawa is necessary for the prevention of cardiovascular disease and to clarify the risk factors.

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Constitutive nitric oxide production in bovine aortic and brain microvascular endothelial cells: a comparative study

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Vascular endothelium constitutively generates nitric oxide (NO) in large vessels and induces a relaxation of smooth muscle cells. However, little is known about the production of NO in microvessels, where smooth muscle layers are thin or absent. In this study, we have compared the constitutive production of NO in bovine brain microvascular endothelial cells (BBECs) with that in bovine aortic endothelial cells (BAECs). ATP, acetylcholine (ACh) and A23187 induced Ca^{2+} transients both in BBECs and BAECs. In contrast, although ATP and A23187 evoked a similar degree of $[Ca^{2+}]_i$ increase in both types of cell, they failed to induce NO production in BBECs, as measured with an NO-sensitive fluorescent dye DAF-2, whereas in BAECs there was an increase in DAF-2 fluorescence. Hypotonic stress induced ATP release and subsequent NO production in BAECs, but not in BBECs. We have developed an *in vitro* model vessel system that consists of aortic smooth muscle cells embedded in a collagen gel lattice and overlaid with endothelial cells. Precontracted gels showed relaxation in response to ACh, when BAECs were overlaid. However, ACh-induced relaxation was not observed in BBEC-overlaid gels. Expression of eNOS protein as well as cellular uptake of L-[^{3}H]arginine were significantly lower in BBECs than in BAECs. These results indicate that Ca^{2+}-dependent NO production is at an undetectable level in BBEC, for which at least two factors, i.e. low levels of eNOS expression and L-arginine uptake, are responsible.

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Nitric oxide (NO) plays various physiological and pathological roles in variety of cell types (Moncada *et al.* 1991). It is well documented that vascular endothelium constitutively generates NO in response to the elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Ca^{2+} binds to calmodulin thereby stimulating endothelial NO synthase (eNOS) to produce NO, in combination with other cofactors such as NADPH and tetrahydrobiopterin (Lopez-Jaramillo *et al.* 1990). Endothelium-derived NO induces a relaxation of vascular smooth muscle cells, and prevents atherosclerosis and cell adhesion (Moncada *et al.* 1991). However, this view has been mainly developed in larger conducting vessels, and the relatively greater importance of endothelium-derived hyperpolarizing factor (EDHF) rather than NO has been suggested in smaller resistance arteries (Garland *et al.* 1995). Furthermore, a neural rather than an endothelial source of NO has been considered to regulate vascular flow in rat mesenteric arterioles (Kashiwagi *et al.* 2002).

It has been reported in cultured human cerebral microvascular endothelial cells that an exogenously applied NO donor inhibits endothelin-1-induced Ca^{2+} transients and down-regulates actin reorganization (Chen *et al.* 2003). Other reports have also shown that eNOS protein is expressed in rat brain microvascular endothelium and its expression level is altered by pathophysiological stimuli such as oestrogen (McNeill *et al.* 1999), perinuclear EP3 receptor stimulation (Gobeil *et al.* 2002) or angiotensin II (Yamakawa *et al.* 2003). However, if NO is constitutively generated by eNOS in cerebrocortical microvascular endothelium, it would affect the functions of neighbouring neurones directly or by changing cerebrocortical blood flow. Actually there has been no direct evidence reported so far showing the constitutive production of NO in cerebral microvascular endothelium. Furthermore, a recent report has shown that control of cerebral microcirculation is obtained by neurone-to-glia signals but not by vascular signals (Zonta *et al.* 2003).

The aim of this study was to clarify whether NO is constitutively generated in bovine brain microvascular endothelial cells (BBECs) or not. We have used two methods to detect NO production in BBECs and bovine aortic endothelial cells (BAECs). Firstly we measured the intracellular NO production with an NO-sensitive fluorescent dye, DAF-2 (Kojima *et al.* 1998). Secondly, we have developed a novel method to detect cultured endothelium-dependent vasorelaxation. We have previously reported that vascular smooth muscle cells embedded in collagen gels show contraction in response to Ca^{2+} mobilizing stimuli (Kimura *et al.* 2002). We therefore considered that the endothelial functions could be examined by overlaying cultured endothelium onto smooth muscle cell-embedded collagen gel. The results obtained indicate that Ca^{2+}-dependent NO production is detectable using these methods in BAECs, but not in BBECs.

Methods

Cell culture

Thoracic aortas and brains of 1-year-old calves were obtained from the local slaughterhouse. BAECs were scraped off from the intima with the edge of a razor (Oike *et al.* 2000). BBECs were prepared following a Percoll gradient separation method as previously described (Kimura *et al.* 1998b). Both BAECs and BBECs were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories, Linz, Austria). Cells from primary cultures were subcultured at a split ratio of 1 : 3, and the harvested subcultured cells were used for the present experiments. Cells were grown on coverslips, which were coated with collagen type IA (Nitta Gelatin Inc., Osaka, Japan), for measuring $[Ca^{2+}]_i$ and NO production. Endothelial identification of BAECs was confirmed by the specific uptake of fluorescence-labelled acetylated low-density lipoprotein (Dil-Ac-LDL) as previously reported (Kimura *et al.* 2001a). BBECs exhibited immunohistochemical staining for antifactor VIII antibody, indicating their endothelial nature. Endothelial cells obtained from 12 aortas and four brains were used in the present study.

Bovine aortic smooth muscle cells (BASMCs) from thoracic aortas were cultured in DMEM with 10% FBS by the explant method as previously described (Kimura *et al.* 2002). Cells grown to confluency were harvested by trypsin digestion and stored at –80°C after one-step subculture. Smooth muscle α-actin was stained to confirm that the cells retained the nature of smooth muscle cells (not shown). The cells were embedded in collagen gel lattice for the gel contraction assay as described below. Smooth muscle cells from five aortas were used in the present study.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured from non-confluent BBECs and BAECs with fura-2 using an Attofluor digital fluorescence microscopy system (Atto Instruments, Rockville, MD, USA), as previously described (Oike *et al.* 2000).

Measurement of intracellular production of NO

For the measurement of NO with DAF-2, an NO-sensitive fluorescent dye (Kojima *et al.* 1998), non-confluent cells grown on coverslips were incubated with a diacetylated form of DAF-2 (10 μM, Daiichi Pure Chemicals, Co. Ltd, Tokyo, Japan) for 20 min at room temperature and for a subsequent 20 min at 37°C. DAF-2 was excited at a wavelength of 490 nm and emitted fluorescence at a wavelength of 515 nm was measured with an Attofluor fluorescence microscopy system. Since DAF-2 has single excitation and single emission wavelengths, conversion of DAF-2 fluorescence into intracellular NO concentrations is impossible (Kojima *et al.* 1998). However, since DAF-2 fluorescence increases almost linearly with NO concentration (Kojima *et al.* 1998), we have expressed the DAF-2 fluorescence relative to its initial values. Because NOS generates O_2^- instead of NO in the absence of L-arginine (Xia *et al.* 1998), we added an excess concentration of L-arginine (3 mM) to all solutions used for NO measurement, except for the experiment with N^{ϖ}-nitro-L-arginine methyl ester (L-NAME)-treated cells, where L-NAME (0.1 mM) was added during the last 30 min of the DAF-2 incubation period.

Gel contraction assay

Endothelial NO production was also assessed in an *in vitro* model vessel, which consists of a BASMC-embedded collagen gel and an overlaid endothelium. Cultured BASMCs were re-suspended in DMEM containing 0.2% collagen type IA at a density of 4×10^5 cells ml^{-1}, poured into a 24-well culture plate and allowed to form a gel for 10 min at 37°C, as previously described. DMEM with 10% FBS was then poured on to the gel. After culturing for 24 h at 37°C, BBECs or BAECs were overlaid on the gel at a density of 2×10^4 cells cm^{-2}.

After culturing for a further 24 h at 37°C, the gel was used for the contraction assay (see Fig. 4*Ba*). The lateral surface of the gel was carefully detached from the culture well with a fine needle. The culture plate was then placed on a hotplate (MP-10 Dm; Kitazato Supply, Shizuoka, Japan) and kept at 37°C. The gel images were captured with a digital camera (QV-800SX, Casio, Tokyo, Japan) every 1 min throughout the experiment. Contraction of the gel was then evaluated by measuring its surface area with image analysis software (Adobe Photoshop, Adobe Systems Inc., USA).

Measurement of ATP release with luciferase bioluminescence

For the measurement of the extracellular ATP concentration ($[ATP]_o$), BAECs and BBECs were seeded on 96-well culture plates at densities of 5000 cells well^{-1}. After culturing for 3 days, $[ATP]_o$ was measured using luciferin–luciferase chemiluminescence as previously described (Oike *et al.* 2000).

Western blotting of eNOS protein expression

Expression of eNOS protein in BAECs and BBECs was assessed by chemiluminescence Western blotting. Cells were lysed with 2% SDS and the lysate was electrophoresed through 7.5% polyacrylamide gel. Western blot analysis for eNOS protein was then performed using antieNOS polyclonal antibody (StressGen Biotechnologies Co., San Diego, CA, USA) and a chemiluminescence system (SuperSignal West Dura, Pierce Co., Rockford, IL, USA). Emitted chemiluminescence was detected and analysed with a lumino image analyser (FAS-1000, Toyobo, Osaka, Japan).

L-[^{3}H]arginine uptake

Measurement of cellular uptake of L-[^{3}H]arginine (Amershan, Uppsala, Sweden) was performed as previously reported (Nelin *et al.* 2001) with slight modifications. BBECs and BAECs were seeded on 6-well culture plates at a density of 25 000 cells well^{-1}. After culturing for 3 days, the cells were washed three times with Hanks' balanced salt solution (HBSS, Sigma, St Louis, MO, USA). To determine total L-[^{3}H]arginine uptake, 1 ml of HBSS with 1 μCi ml^{-1} L-[^{3}H]arginine was placed on each well. Non-specific uptake of L-[^{3}H]arginine was determined with HBSS containing 1 μCi ml^{-1} L-[^{3}H]arginine and 10 mM unlabelled L-arginine. After 15 min of incubation at 37°C, the cells were washed three times with ice-cold HBSS, and lysed with 1 ml well^{-1} of 0.2 N NaOH. Aliquots were added to scintillation cocktail and radioactivity was quantified with a liquid scintillation spectrometer (LSC3500, Aloka Co., Tokyo, Japan).

Drugs and solutions

Krebs solution used in the present study contained (mM); NaCl 132.4, KCl 5.9, $CaCl_2$ 1.5, $MgCl_2$ 1.2, glucose 11.5, Hepes 11.5, and pH was adjusted to 7.4 by NaOH. Hypotonic solution (–30%) was prepared by adding distilled water to Krebs solution at a water : Krebs ratio of 3 : 7. We have previously shown that alterating the ionic composition of Krebs solution by adding water does not influence the cellular Ca^{2+} responsiveness (Oike *et al.* 2000). All drugs used in the present study were purchased from Sigma.

Statistics

Pooled data were expressed as means ± S.E.M. values. Statistic significance was examined with Student's unpaired *t* test. A probability below 0.05 ($P < 0.05$) was considered to show a significant difference.

Results

Effects of ATP and A23187 on NO production in BAECs and BBECs

We have previously reported that ATP induced Ca^{2+} transients both in BBECs and BAECs, but the concentration–response relationship in BBECs was shifted to higher concentrations than that in BAECs (Kimura *et al.* 1998*a*, 2000*b*). This was confirmed in this study; i.e. ATP (1 μM) induced Ca^{2+} oscillations in Krebs solution in BAECs (Fig. 1*Aa*), and a similar Ca^{2+} response was obtained with 10 μM ATP in BBECs (Fig. 1*Ab* and *c*). The Ca^{2+} ionophore A23187 (1 μM) also induced $[Ca^{2+}]_i$ elevation in both cell types (Fig. 1*Ba* and *b*). The net increments in 1 μM A23187-induced Ca^{2+} transients were not significantly different in BAECs and BBECs (Fig. 1*Bc*).

We then evaluated Ca^{2+}-dependent NO production in BAECs and BBECs using DAF-2. We have previously demonstrated that Ca^{2+} mobilization evoked by ATP or A23187 induced an increase in NO production in BAECs (Koyama *et al.* 2002). As shown in Fig. 2*A*, a solution exchange alone did not induce any apparent change in DAF-2 fluorescence up to 20 min in both BAECs and BBECs, even in the presence of 3 mM L-arginine. In BAECs, ATP (1 μM) induced a gradual increase in

DAF-2 fluorescence (○, Fig. 2*B*), which was inhibited by pretreatment with 0.1 mM L-NAME (Fig. 2*D*), suggesting that DAF-2 fluorescence was properly linked to cellular NO production. In contrast, BBECs did not show any increase in DAF-2 fluorescence in response to 10 μM ATP (●, Fig. 2*B*), even though this concentration of ATP induced Ca^{2+} transients in BBECs (Fig. 1*A*). A23187 also induced an increase in DAF-2 fluorescence in BAECs, but not in BBECs (Fig. 2*C*). These results are summarized in Fig. 2*D*, and indicate that ATP and A23187 do not induce NO production in BBECs.

Effects of hypotonic stress on NO production in BAECs and BBECs

We have previously reported that hypotonic stress (HTS), as an example of mechanical stress, induces NO production in a Ca^{2+}-dependent manner due to ATP release in BAECs (Kimura *et al.* 2000*a*; Oike *et al.* 2000). So we then compared the HTS-induced, ATP-mediated NO production in BAECs and BBECs. HTS (−30%) induced ATP release in BAECs as previously reported (Oike *et al.* 2000). $[ATP]_o$ was elevated to 55.8 ± 5.9 nM after being exposed to HTS for 10 min ($n = 14$), whereas it was 28.8 ± 3.2 nM when the cells were kept in isotonic solution for the same period ($n = 14$, Fig. 3*A*). In contrast, a HTS-induced increase in $[ATP]_o$ was not observed in BBECs; i.e. $[ATP]_o$ was 25.6 ± 2.5 nM in isotonic solution ($n = 13$) and 28.1 ± 2.3 nM in hypotonic solution ($n = 13$, $P > 0.05$ *versus* isotonic). As expected, HTS induced an increase in DAF-2 fluorescence in BAECs, but not in BBECs (Fig. 3*B*).

Endothelium-dependent relaxation of smooth muscle cell-embedded collagen gels

To further investigate NO production in BBECs and BAECs, we have developed a novel method for detecting cultured endothelium-dependent vasorelaxation. Endothelial cells were overlaid onto a BASMC-embedded collagen gel lattice as shown in the cartoon (Fig. 4*Ba*) so that endothelium-derived substances could affect the gel contraction.

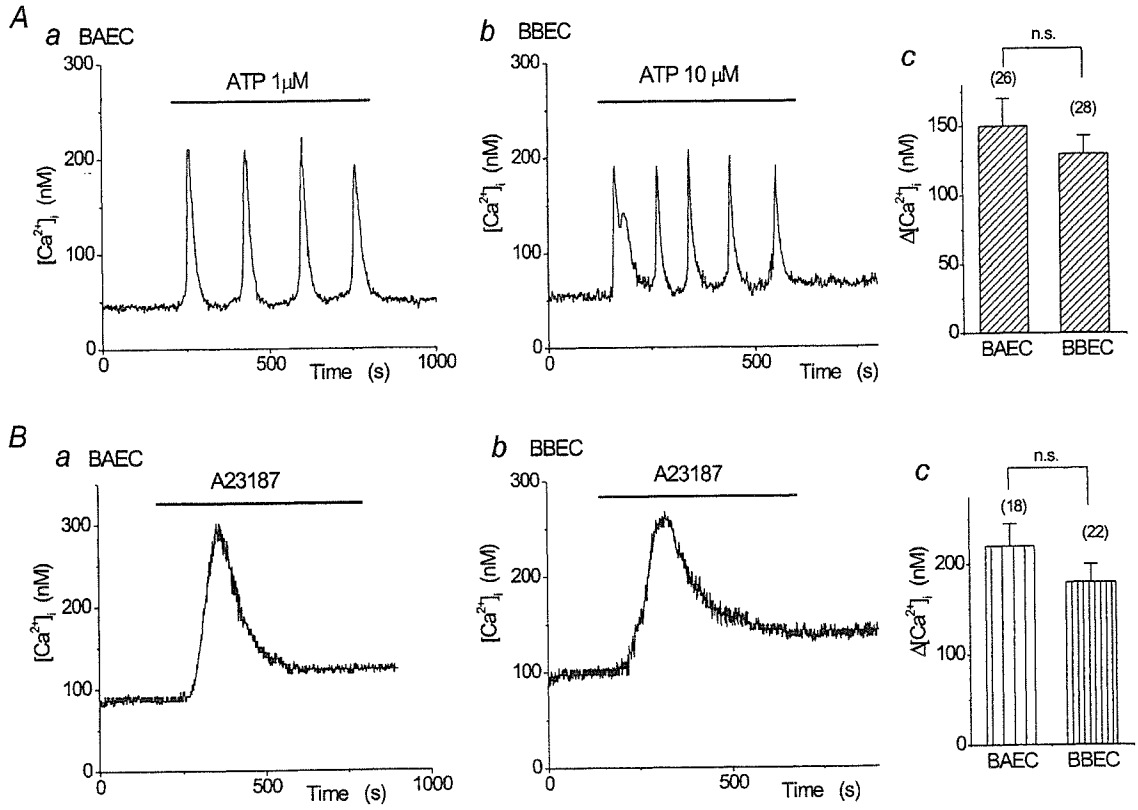

Figure 1. Ca^{2+} transients induced by ATP and A23187 in bovine aortic (BAECs) and brain microvascular (BBECs) endothelial cells

A, representative traces of ATP-induced Ca^{2+} oscillations in BAECs (*a*) and BBECs (*b*). Similar levels of net $[Ca^{2+}]_i$ elevation ($\Delta[Ca^{2+}]_i$) were obtained with 1 μM and 10 μM ATP in BAECs and BBECs, respectively (*c*). *B*, A23187 (1 μM) induced Ca^{2+} transients in both BAECs (*a*) and BBECs (*b*) to a similar degree (*c*). n.s., $P > 0.05$.

In this assay, we used the classical endothelial agonist ACh (Furchgott & Zawadzki, 1980) as a Ca^{2+} mobilizing agent. ACh (10 μM) induced Ca^{2+} transients in BAECs (Fig. 4*Aa*). The same concentration of ACh (10 μM) induced smaller Ca^{2+} responses in BBECs (not shown), and a similar Ca^{2+} response was obtained with a higher concentration of ACh (100 μM, Fig. 4*Ab*).

BASMC-embedded collagen gel lattices showed a rapid contraction in response to noradrenaline (NAd, 1 μM) both in BAEC- and BBEC-overlaid gels (Fig. 4*Bb* and *c*, ○). Pretreatment with L-NAME did not affect the NAd-induced contraction both in BAEC- and BBEC-overlaid gels (Fig. 4*Bb* and *c*, ●), thereby suggesting that NAd does not evoke NO production in these overlaid endothelia. Subsequent application of 10 μM ACh induced a relaxation of the precontracted gels in BAEC-overlaid gels (Fig. 4*Bb*, ○). The relaxation was not observed in the absence of overlaid BAECs (not shown) and was significantly inhibited when the gel was pretreated with L-NAME (Fig. 4*Bb*, ●), thereby indicating that the relaxation of the gel was due to BAEC-derived NO. In contrast, when BBECs were overlaid on to a BASMC-embedded collagen gel, the precontracted gel did not show relaxation in response to 100 μM ACh (Fig. 4*Bc*, ○).

Therefore, these results indicate that $[Ca^{2+}]_i$ elevation leads to the release of a significant amount of NO in BAECs but not in BBECs.

Western blotting of eNOS protein expression in BAECs and BBECs

To explore the cellular mechanisms responsible for lower NO production in BBECs, we then examined the expression of eNOS protein in BAECs and BBECs with

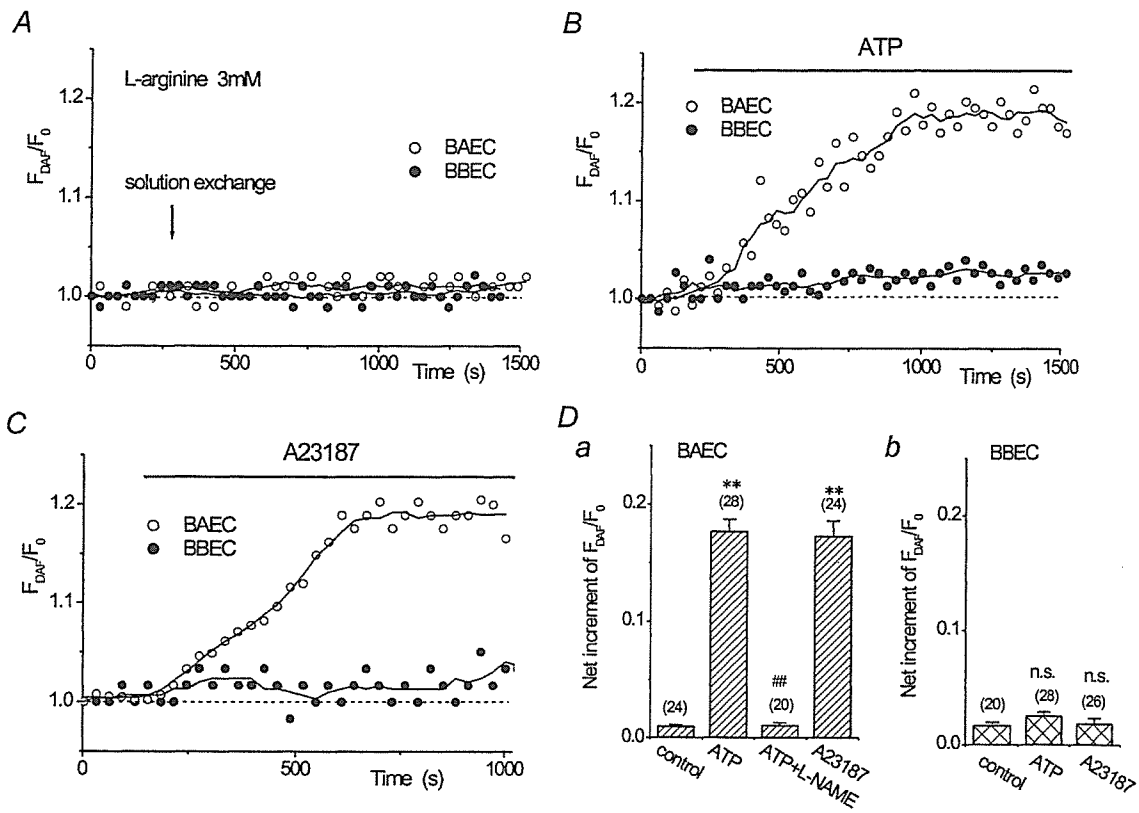

Figure 2. Effects of ATP and A23187 on NO production, assessed with DAF-2, in BAECs and BBECs
A, solution exchange alone did not induce an increase in DAF-2 fluorescence in both BAECs (○) and BBECs (●). Representative results are shown. DAF-2 fluorescence (F_{DAF}) is expressed relative to its initial value (t = 0, F_0). Circles are the actual values of DAF-2 fluorescence and continuous lines were drawn by averaging the adjusting five points. Measurements were performed in the presence of 3 mM L-arginine. *B*, ATP (1 μM) induced an increase in DAF-2 fluorescence in BAECs (○), whereas 10 μM ATP did not show any apparent increase in DAF-2 fluorescence in BBECs (●). Representative results are shown. *C*, A23187 (1 μM) also increased DAF-2 fluorescence in BAECs (○) but not in BBECs (●). Representative results are shown. *D*, net increment of relative DAF-2 fluorescence at 10 min after solution exchange (control) or the application of ATP or A23187 in BAECs (*a*) and BBECs (*b*). Note that L-NAME (0.1 mM) inhibited ATP-induced DAF-2 fluorescence. $^{**}P < 0.01$ *vs.* control. ## $P < 0.01$ *vs.* ATP alone. n.s., $P > 0.05$ *vs.* control.

Western blotting. Though expression of eNOS protein was observed in BBECs, BAECs showed much a denser band of eNOS (Fig. 5*A*). Densitometric analysis revealed that the expression level of eNOS protein relative to that of the housekeeping β-actin protein was 0.133 ± 0.006 in BAECs ($n = 6$) but 0.092 ± 0.006 in BBECs ($n = 6$, Figs 5*B*; $P < 0.01$).

Cellular L-[^{3}H]arginine uptake in BBECs and BAECs

To further examine the possible cause of the reduction of NO production in BBECs, we finally examined the cellular L-arginine uptake in BAECs and BBECs. As shown in Fig. 6, uptake of L-[^{3}H]-arginine over 15 min was significantly lower in BBECs than in BAECs (BAECs, 930.6 ± 28.4 d.p.m. (μg protein)$^{-1}$; BBECs, 638.4 ± 15.7 d.p.m. (μg protein)$^{-1}$, $n = 5$ for both cell types, $P < 0.01$).

Discussion

We have previously reported that ATP induces Ca^{2+} oscillations in BAECs and BBECs with different concentration–response relationships (Kimura *et al.* 1998*a*, 2000*b*). The present study showed that Ca^{2+} transients induced by 1 μM ATP in BAECs were similar to those induced by 10 μM ATP in BBECs (Fig. 1*A*). Furthermore, the Ca^{2+} ionophore A23187 (1 μM) induced Ca^{2+} transients in both BAECs and BBECs (Fig. 1*B*). In spite that the similar degree of $[Ca^{2+}]_i$ elevation was induced by ATP and A23187 in BAECs and BBECs, these agents induced an increase in DAF-2 fluorescence only in BAECs (Fig. 2), thereby suggesting that $[Ca^{2+}]_i$ elevation does not lead to detectable NO production in BBECs. In addition, HTS induced NO production in BAECs but not in BBECs (Fig. 3*B*), so we suppose that mechanical stresses that can be mimicked by HTS do not evoke NO production in BBECs. We have previously shown that HTS-induced NO production is due to endogenous ATP release (Kimura *et al.* 2000*a*), which was also absent in BBECs (Fig. 3*A*). Anion channels (Sabirov *et al.* 2001; Hisadome *et al.* 2002) and vesicular exocytosis (Bodin & Burnstock, 2001) have been suggested as components of the mechanical stress-induced ATP release pathway, and tyrosine kinases and the RhoA/Rho-kinase cascade have been reported as cellular mechanisms for HTS-induced ATP release (Koyama *et al.* 2001). Therefore it can be speculated that BBECs lack some of these or any other as yet unknown mechanisms that are involved in HTS-induced ATP release.

The absence of detectable Ca^{2+}-dependent NO production in BBECs may contradict previous reports

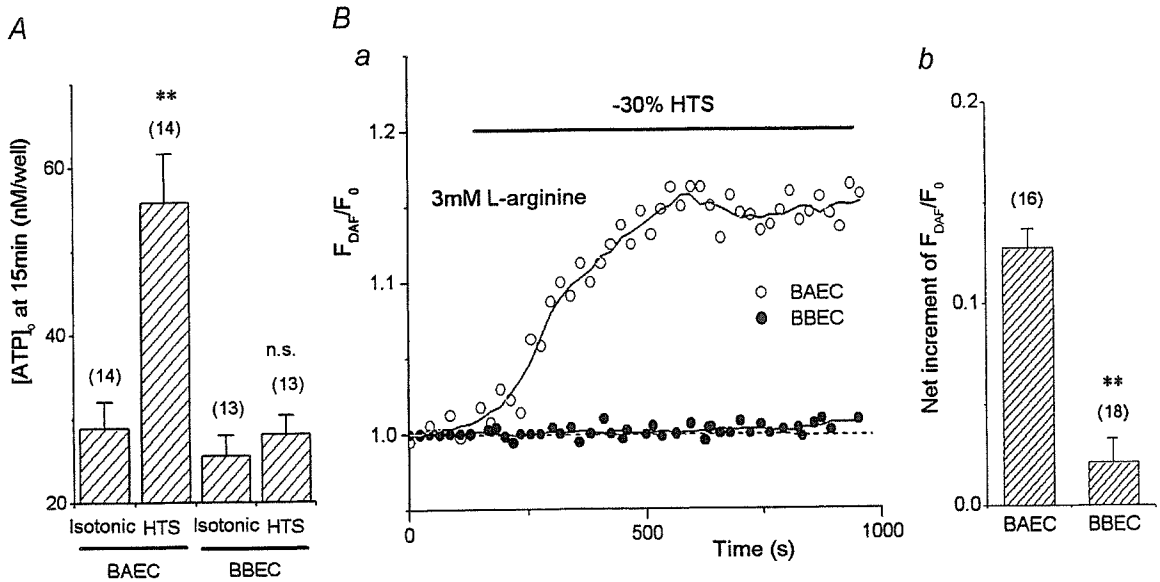

Figure 3. Effects of hypotonic stress (HTS) on ATP release and subsequent NO production in BAECs and BBECs

A, HTS (–30%) induced an increase in $[ATP]_o$ in BAECs but not in BBECs. Luciferin chemiluminescence was measured for 10 min, and converted into corresponding $[ATP]_o$ with $[ATP]_o$–chemiluminescence standard curves obtained in each solution. $^{**}P < 0.01$ *vs.* BAEC isotonic. n.s., $P > 0.05$ *vs.* BBEC isotonic. *Ba*, gradual increase in DAF-2 fluorescence was evoked by HTS in BAECs (○) but not in BBECs (●) in the presence of 3 mM L-arginine. Representative results are shown. *Bb*, Statistical analysis of net increment of relative DAF-2 fluorescence at 10 min after starting HTS. $^{**}P < 0.01$ *vs.* BAECs.