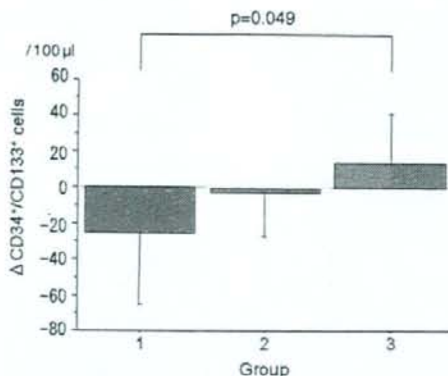

Fig 2. Number of delta CD34+/133+ cells between 10 days after onset of acute myocardial infarction (AMI) and 3 months after AMI onset in each patient group. Group 1 (n=10) exercised <120 min/week, group 2 (n=6) exercised between 120 and 239 min/week, and group 3 (n=7) exercised >240 min/week. The number of delta CD34+/133+ cells between 10 days and 3 months after AMI onset significantly increased in group 3 compared with group 1.

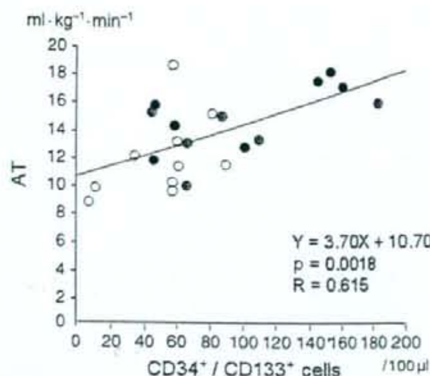

Fig 3. Correlation between oxygen consumption at the anaerobic threshold (AT) and the number of CD34+/133+ cells at 3 months after acute myocardial infarction. The number of CD34+/133+ cells significantly correlated with oxygen consumption at the AT (p=0.0018). Open circles represent for group 1, closed gray circles for group 2 and closed black circles for group 3.

eral vascular tonus and an increase in muscular oxygen uptake during exercise. Exercise training and an increase in the number of circulating CD34+/133+ cells might synergistically improve exercise capacity. Exercise capacity is a strong predictor of mortality in patients with cardiovascular diseases.[19] The number of circulating EPC has been reported to predict the occurrence of cardiovascular events and death from cardiovascular causes.[20,21]

Numaguchi et al have reported that the capability of EPC to differentiate (ie, quality of EPC) influences the degree of LV functional improvement and infarct size reduction.[21] The quality and quantity of EPCs may be both important for the salvage of infarcted myocardium.[21] In the present study, however, no difference was seen in LV functional improvement and future cardiac event between the high exercise group and low exercise group.

We used FACS to measure the number of circulating cells that were double-positive for CD34 and CD133, as an indicator of the number of probable EPC. Initial studies by Asahara et al[1] and Shi et al[22] demonstrated that EPC can be grown from peripheral blood mononuclear cells or from purified populations of CD34+ or CD133+ hematopoietic cells. EPC are reportedly characterized by coexpression of the hematopoietic marker CD34 and endothelial marker proteins, such as VEGF receptor-2 (VEGFR-2), von Willebrand factor, VE-cadherin, CD146, and CD31; however, the classification of EPC according to their FACS characteristics remains controversial.[1,7,23,24] In our pilot study, however, the number of circulating CD34+, 133+ and VEGFR-2+ cells counted by FACS was 0/100 µl in 52 of 80 patients with ischemic heart disease (data not shown). Findings by Inoue et al suggest that a small subgroup of CD34+/133+ cells can differentiate into vascular smooth muscle cells, as well as vascular endothelial cells, especially during the acute phase of AMI.[7] In the present study, late luminal loss tended to be higher in patients with a large number of CD34+/133+ cells 10 days after AMI onset.

Recent reports indicate that exercise induces mobilization of EPCs[11–13] and improvement of vascular dysfunction.[25] One of the mechanisms by which exercise training increases the number of EPCs is that exercise increases vascular wall shear stress, resulting in increases in vascular endothelial nitric oxide synthetase (eNOS) expression, AKT-mediated eNOS phosphorylation and, consequently, NO production. Laufs et al demonstrated that NO plays an important role in the VEGF-mediated regulation of EPC.[11] Sandri et al have reported that an ischemic stimulus is needed to induce significant release of EPC from the bone marrow.[26] As well as in the present study, previous studies showed that exercise was sufficient to increase the level of circulating EPC; that is, no critical ischemic stimulus was needed.[11,13] These differences may be due to patient background, study protocol and the cell markers used to identify EPC. In the present study, we collected control blood samples from patients 10 days after onset of AMI. High EPC levels in those samples may be due to inflammation resulting from AMI and vascular injury resulting from stenting. Exercise training maintained a high level of circulating CD34+/133+ cells in groups 2 and 3 during the 3 months following onset of AMI.

It has been reported that exercise training reduces stent restenosis,[27] although there was no reduction in late luminal loss in the present study. The different results may be explained by different exercise intensities and different exercise sessions (ie, session was supervised or non-supervised). Although EPC mobilization induced by exercise training suppressed intimal hyperplasia in a mouse carotid artery injury model, the increase in the number of CD34+/133+ cells induced by exercise training in the present study was lower than the increase observed in the animal model.[11] Such a small increase over a 3-month period may be insufficient to prevent stent restenosis. A newly developed stent coated with anti-CD34 antibody can recruit EPC to the stent site.[28] The EPC mobilized by exercise training may be able to differentiate into vascular endothelial cells. Therefore, the combination of exercise training and stents coated with anti-CD34 antibody appears to be a promising approach to further reducing stent restenosis. In the present study, there was no correlation between CD34+/133+ cells and HbA_{1c} levels because of the narrow range of HbA_{1c} levels (4.8–6.7). In a previous report, diabetes mellitus was a signifi-

cant predictor for a reduced CD34+ cell count.[29] This difference may be due to the severity of the diabetes.

Study Limitations

The first limitation of the present study is that a randomized protocol with supervised exercise sessions was not used because of ethical concerns. However, all patients recorded their daily exercise in their exercise diary. Therefore, the present study provides real-world data regarding daily exercise training after AMI in Japan. The second limitation is that we used surface markers, CD34+/133+, to identify probable EPC. Unfortunately, we did not confirm the identity of EPC by immunofluorescent staining for isolectin B4 followed by labeling with DiI-acetylated in cultured mononuclear cells.

In conclusion, increased daily exercise increased the number of circulating bone marrow-derived probable EPC 3 months after onset of AMI, and it appears to positively contribute to exercise capacity but not to a reduction of restenosis after bare metal stent implantation.

Acknowledgments

An abstract of this work was presented at the Annual Scientific Session of the European Society of Cardiology, on September 1, 2005 (Eur Heart J 2005). We thank Wilfred Y. Fujimoto for his helpful comments regarding the study's results, and Sachimi Jinbo for her technical assistance.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Sports, Science and Technology (No. 17790496). From January 2007, the author Takanori Yasu has been at the Department of Clinical Pharmacology & Therapeutics, University of the Ryukyus, Graduate School of Medicine.

References

1. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; **275:** 964–967.
2. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003; **348:** 593–600.
3. Yoshioka T, Ageyama N, Shibata H, Yasu T, Misawa Y, Takeuchi K, et al. Repair of infarcted myocardium mediated by transplanted bone marrow-derived CD34+ stem cells in a nonhuman primate model. *Stem Cells* 2005; **23:** 355–364.
4. Tatsumi T, Ashihara E, Yasui T, Matsunaga S, Kido A, Sasada Y, et al. Intracoronary transplantation of non-expanded peripheral blood-derived mononuclear cell promotes improvement of cardiac function in patients with acute myocardial infarction. *Circ J* 2006; **70:** 1017–1025.
5. Wollert KC, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, et al. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: The BOOST randomized controlled clinical trial. *Lancet* 2004; **364:** 141–148.
6. Schachinger V, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Holschermann H, et al; REPAIR-AMI Investigators. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. *N Engl J Med* 2006; **355:** 1210–1221.
7. Inoue T, Sata M, Hikichi Y, Sohma R, Fukuda D, Uchida T, et al. Mobilization of CD34-positive bone marrow-derived cells after coronary stent implantation: Impact on restenosis. *Circulation* 2007; **115:** 553–561.
8. Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, et al. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation* 2001; **103:** 2776–2779.
9. Manson JE, Greenland P, LaCroix AZ, Stefanick ML, Mouton CP, Oberman A, et al. Walking compared with vigorous exercise for the prevention of cardiovascular events in women. *N Engl J Med* 2002; **347:** 716–725.
10. Hakim AA, Petrovitch H, Burchfiel CM, Ross GW, Rodriguez BL, White LR, et al. Effect of walking on mortality among nonsmoking retired men. *N Engl J Med* 1998; **338:** 94–99.
11. Laufs U, Werner N, Link A, Endres M, Wassmann S, Jurgens K, et al. Physical training increases endothelial progenitor cells, inhibit neointima formation, and enhances angiogenesis. *Circulation* 2004; **109:** 220–226.
12. Rehman J, Li J, Parvathaneni L, Karlsson G, Panchal VR, Temm CJ, et al. Exercise acutely increases circulating endothelial progenitor cells and monocyte/macrophage-derived angiogenic cells. *J Am Coll Cardiol* 2004; **43:** 2314–2318.
13. Steiner S, Niessner A, Ziegler S, Richter B, Seidinger D, Pleiner J, et al. Endurance training increases the number of endothelial progenitor cells in patients with cardiovascular risk and coronary artery disease. *Atherosclerosis* 2005; **181:** 305–310.
14. Masuda H, Kalka C, Takahashi T, Yoshida M, Wada M, Kobori M, et al. Estrogen-mediated endothelial progenitor cell biology and kinetics for physiological postnatal vasculogenesis. *Circ Res* 2007; **101:** 598–606.
15. Strehlow K, Werner N, Berweiler J, Link A, Dirnagl U, Priller J, et al. Estrogen increases bone marrow-derived endothelial progenitor cell production and diminishes neointima formation. *Circulation* 2003; **107:** 3059–3065.
16. Ikeda N, Yasu T, Nishikimi T, Nakamura T, Kubo N, Kawakami M, et al. N-terminal pro-atrial natriuretic peptide and exercise prescription in patients with myocardial infarction. *Regul Pept* 2007; **141:** 154–158.
17. Beaver WL, Wasserman K, Whipp BJ. A new method for detecting anaerobic threshold by gas exchange. *J Appl Physiol* 1986; **60:** 2020–2027.
18. Yasu T, Katsuki T, Ohmura N, Nakada I, Owa M, Fujii M, et al. Delayed improvement of skeletal muscle metabolism and exercise capacity in patients with mitral stenosis following immediate hemodynamic amelioration by percutaneous transvenous mitral commissurotomy. *Am J Cardiol* 1996; **77:** 492–497.
19. Amundsen BH, Wisloff U, Slordahl SA. Exercise training in cardiovascular diseases. *Tidsskr Nor Laegeforen* 2007; **127:** 446–448.
20. Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, et al. Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med* 2005; **353:** 999–1007.
21. Numaguchi Y, Sone T, Okumura K, Ishii M, Morita Y, Kubota R, et al. The impact of the capability of circulating progenitor cell to differentiate on myocardial salvage in patients with primary acute myocardial infarction. *Circulation* 2007; **114:** I-114–I-119.
22. Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, et al. Evidence for circulating bone marrow-derived endothelial cells. *Blood* 1998; **92:** 362–367.
23. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, et al. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *J Clin Invest* 2001; **108:** 391–397.
24. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001; **89:** E1–E7.
25. Green DJ, Walsh JH, Maiorana A, Best MJ, Taylor RR, O'Driscoll JG. Exercise-induced improvement in endothelial dysfunction is not mediated by changes in cardiovascular risk factors: Pooled analysis of diverse patients populations. *Am J Physiol Heart Circ Physiol* 2003; **285:** H2679–H2687.
26. Sandri M, Adams V, Gielen S, Linke A, Lenk K, Krankel N, et al. Effects of exercise and ischemia on mobilization and functional activation of blood-derived progenitor cells in patients with ischemic syndromes. *Circulation* 2005; **111:** 3391–3399.
27. Belardinelli R, Paolini I, Cianci G, Piva R, Georgiou D, Purcaro A. Exercise training intervention after coronary angioplasty: The ETICA trial. *J Am Coll Cardiol* 2001; **37:** 1891–1900.
28. Aoki J, Serruys PW, Beusekom H, Ong AT, McFadden EP, Sianos G, et al. Endothelial progenitor cell capture by stents coated with antibody against CD34. *J Am Coll Cardiol* 2005; **45:** 1574–1579.
29. Eizawa T, Ikeda U, Murakami Y, Matsui K, Yoshioka T, Takahashi M, et al. Decrease in circulating endothelial progenitor cells in patients with stable coronary artery disease. *Heart* 2004; **90:** 685–686.

nature publishing group

See COMMENTARY page 603

Association of Body Mass Index With Cognitive Function in Elderly Hypertensive Japanese

Kenichi Sakakura[1,2], Satoshi Hoshide[3], Joji Ishikawa[3], Shin-ichi Momomura[1], Masanobu Kawakami[1], Kazuyuki Shimada[3] and Kazuomi Kario[3]

BACKGROUND
As hypertension, obesity, and leanness are reported to be associated with poor cognitive function, it is possible that obesity or leanness in hypertensive patients may also be associated strongly with poor cognitive function.

METHODS
We recruited 184 elderly hypertensive patients comprising 93 very elderly (aged ≥80 years) and 91 younger elderly (aged 61–79 years) subjects. A mini-mental state examination (MMSE) and 24-h ambulatory blood pressure monitoring (ABPM) were performed in all participants. Patients were classified as either lean, normal physique, or obese according to the body mass index (BMI) quartile. The prevalence of poor cognitive function, total MMSE score, and MMSE subscores were compared between the groups.

RESULTS
The prevalence of poor cognitive function, total MMSE score, and MMSE subscore attention/calculation were significantly different between the groups both in the total study population and in the very elderly patients. The multiple logistic regression model showed that leanness was a significant determinant of poor cognitive function in both the total study population (odds ratio (OR) 2.54, 95% confidence interval (CI) 1.13–5.73, $P = 0.02$) and the very elderly patients (OR 3.94, 95% CI 1.31–11.82, $P = 0.01$). Obesity was not a significant determinant in either the total study population, very elderly, or younger elderly groups.

CONCLUSION
While obesity in hypertensive elderly patients was not associated with poor cognitive function, leanness in hypertensive elderly patients was, especially in the very elderly.

Am J Hypertens 2008; **21**:627-632 © 2008 American Journal of Hypertension, Ltd.

Poor cognitive function is a serious medical and social problem. It is well known that high blood pressure (BP) affects cognitive function in elderly subjects.[1,2] Obesity, a well-established risk factor for cardiovascular diseases and target organ damage, is also associated with poor cognitive function.[3,4] Therefore, as both hypertension and obesity are known to be associated with poor cognitive function, it is possible that obesity in hypertensive patients may be associated strongly with poor cognitive function. However, the association between obesity and hypertension with poor cognitive function has not been fully investigated in the elderly population.

An association between a low body mass index (BMI) and poor cognitive function has also been reported,[5] with studies showing that lean hypertensive patients had a higher mortality rate and incidence of stroke.[6,7] Hence, leanness in combination with hypertension may also be associated with poor cognitive function in the elderly.

We hypothesized that either obesity or leanness in combination with hypertension may be associated with poor cognitive function in the elderly population. The purpose of this study was to investigate the relationship between obesity or leanness and poor cognitive function in hypertensive elderly patients.

METHODS

Subjects. We previously recruited 202 elderly patients to investigate the relationship and difference between ambulatory BP variability and cognitive function and quality of life in the younger elderly and the very elderly.[8] The inclusion criteria of this previous study were ambulatory patients in the Kiwa Public Clinic (Kiwa, Mie Prefecture, Japan) under stable management for various chronic diseases, such as hypertension, hyperlipidemia, diabetes, or osteoporosis. The exclusion criteria for participants in the study were: (i) an inability to walk into the clinic; (ii) difficulty providing informed consent; (iii) a cardiovascular event such as a stroke or myocardial infarction within the preceding 3 months; (iv) an active malignancy; and (v) chronic renal failure and dialysis treatment. Of these 202 elderly patients, 184 had hypertension defined by one or more of the following criteria: an ambulatory daytime BP ≥135/85 mm Hg, an ambulatory night time BP ≥120/75 mm Hg, or the use of antihypertensive medications. The remaining 18 patients were nonhypertensive and were excluded from this study. The 184 elderly patients enrolled in this study

[1]Cardiovascular Division, Department of Integrated Medicine I, Saitama Medical Center, Jichi Medical University School of Medicine, Saitama, Japan; [2]Kiwa Public Clinic, Kumano, Japan; [3]Division of Cardiovascular Medicine, Department of Medicine, Jichi Medical University School of Medicine, Tochigi, Japan. Correspondence: Kenichi Sakakura (ZXH03005@nifty.ne.jp)

Received 5 December 2007; first decision 10 January 2008; accepted 6 March 2008; advance online publication 3 April 2008. doi:10.1038/ajh.2008.157

© 2008 American Journal of Hypertension, Ltd.

consisted of 93 very elderly (aged ≥80 years) and 91 younger elderly (aged 61–79 years) subjects. Antihypertensive treatment was continued in the patients who were taking medication before the study. The study was approved by the Institutional Review Board of the Kiwa Public Clinic. Written informed consent was obtained from all participants before the study.

Twenty-four-hour ambulatory BP monitoring (ABPM). Twenty-four-hour ABPM was performed using the A&D TM-2431 device (A&D, Tokyo, Japan), which uses an oscillometric method. This device has been validated clinically according to the protocol of the British Hypertension Society.[9] The readings were obtained automatically at 30-min intervals for 24 h. The participants were asked to continue their routine activities during the period of ABPM, except for bathing and driving a car. All data editing was carried out exclusively using computerized criteria with the following rejection criteria being used to reduce bias: pulse <30/min or >200/min; diastolic BP <40 mm Hg or >160 mm Hg; systolic BP (SBP) <60 mm Hg or >280 mm Hg; and SBP–diastolic BP <10 mm Hg or >150 mm Hg. All 184 patients had >39 valid measurements in the total of 48 ambulatory BP measurements. The average 24-h SBP was then calculated.

Cognitive function. To investigate cognitive function, we carried out a mini-mental state examination (MMSE) in all participants.[10] The duration between the 24-h ABPM and the MMSE was <6 months. The MMSE values range from 0 to 30. The total MMSE score (0–30) and five MMSE subscores (orientation, registration, attention/calculation, recall, and language) were analyzed.[11] Various cutoff points distinguishing between poor and normal cognitive function have been used. Most studies classified subjects with a score ≤23 as having poor cognitive function.[12–14] Therefore, we defined poor cognitive function as patients with an MMSE score of ≤23 points. We also defined dementia as patients with an MMSE score of ≤17.[12]

Definition of leanness, normal physique, or obesity in hypertensive patients. BMI was calculated as weight in kilograms divided by height in square meters. We defined the hypertensive patients as lean if their BMI was in the lowest quartile (Q1), normal physique if their BMI was in the middle quartile (Q2 and Q3), and obese if their BMI was in the highest quartile (Q4). The Japan society for the study of obesity defines obesity as a BMI ≥25 kg/m^2 and leanness as a BMI <18.5 kg/m^2,[15] whereas the national institutes of health defines obesity as a BMI ≥30 kg/m^2.[16] If we analyzed our study data using these definitions, the number of lean subjects defined as a BMI ≤18.5 kg/m^2 was only 12 (6.5% of total patients). On the other hand, the number of lean patients defined as quartiles of the BMI was 46 (25% of total patients). Therefore, we

Table 1 | Clinical characteristics of the hypertensive, elderly patients groups as either lean, normal physique, or obese

	Total population (n = 184)	Lean (BMI 14.5–20.3) (n = 46)	Normal physique (BMI 20.4–24.7) (n = 92)	Obese (BMI 24.7–32.5) (n = 46)	P value for trend
Age (years)	78.1 ± 7.5	79.8 ± 7.8	78.3 ± 7.7	76.1 ± 6.4	0.06
Female sex (%)	79.3	82.6	78.3	78.3	0.82
BMI (kg/m^2)	22.8 ± 3.1	19.0 ± 1.3	22.6 ± 1.2	26.9 ± 2.0	<0.001
Mean 24-h SBP (mm Hg)	136 ± 15	137 ± 18	137 ± 14	133 ± 15	0.31
Mean 24-h DBP (mm Hg)	76 ± 7	76 ± 7	77 ± 7	77 ± 7	0.97
Mean pulse pressure (mm Hg)	60 ± 12	61 ± 14	61 ± 11	57 ± 10	0.11
Current smoking habit (%)	6.0	4.3	8.7	2.2	0.27
Diabetes mellitus (%)	13.6	10.9	14.1	15.2	0.81
Hyperlipidemia (%)	35.3	26.1	33.7	47.8	0.08
Metabolic syndrome (%)	13.6	0	0	54.3	<0.001
Statin (%)	21.7	19.6	20.7	26.1	0.7
Antihypertensive medication (%)	83.2	73.9	82.6	93.5	0.04
Calcium channel blocker (%)	59.2	52.2	58.7	67.4	0.33
Beta blocker (%)	10.9	17.4	7.6	10.9	0.22
ACE inhibitor (%)	16.8	13	17.4	19.6	0.69
ARB (%)	22.3	15.2	22.8	28.3	0.32
Alpha blocker (%)	8.7	8.7	9.8	6.5	0.81
Diuretics (%)	13.6	10.9	15.2	13	0.78
History of angina pectoris (%)	7.6	15.2	4.3	6.5	0.07
History of myocardial infarction (%)	3.3	6.5	3.3	0	0.21
History of stroke (%)	9.8	6.5	10.9	10.9	0.69

Data are expressed as mean ± s.d. or percentage. One way analysis of variance test for parametrical data and χ^2 test for categorical data.
ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure.

classified BMI based on quartiles in our study. Obese hypertensive patients who had hyperlipidemia and/or diabetes mellitus were defined as having the metabolic syndrome.

Statistical analysis. Parametrical data such as age, BMI, and the mean 24-h SBP were compared using one-way analysis of variance, while nonparametrical data such as the MMSE score were compared using the Kruskal–Wallis test. The Bonferroni test and Steel–Dwass test were performed as post-hoc analysis of parametric and nonparametric data, respectively. Categorical data such as female sex, poor cognitive function, a current smoking habit, and diabetes mellitus were compared using the χ^2 test. Multiple logistic regression analysis was performed to identify the determinants of poor cognitive function after controlling for known covariates. In this model, poor cognitive function was used as the dependent variable and all known covariates which were age, sex, 24-h SBP, calcium channel blockers (CCBs), angiotensin-converting enzyme (ACE) inhibitors, and angiotensin receptor blockers (ARBs) served as the independent variables. CCBs, ACE inhibitors, and ARBs were used as covariates, because these medications have been reported to affect cognitive function.[17–19] Odds ratios (ORs) and the 95% confidence interval (CI) were calculated. All analyses were performed using statistical software, SPSS 13.0/Windows (SPSS, Chicago, USA) and Ekuseru-Toukei 2006 (Social Survey Research Information, Tokyo, Japan). A P value <0.05 was considered significant.

RESULTS

Patients' characteristics

The patients' background characteristics are summarized in **Table 1**. The age of the total study population ranged between 61 and 94 years. The mean MMSE score and median MMSE

Table 2 | Poor cognitive function, MMSE total score, and MMSE subscore in hypertensive, elderly patients grouped as either lean, normal physique, or obese

	Lean (BMI 14.5–20.3 kg/m²)	Normal physique (20.4–24.7 kg/m²)	Obese (24.7–32.5 kg/m²)	*P* value for trend
Total patients (n = 184)	n = 46	n = 92	n = 46	
Poor cognitive function (MMSE score ≤23 points) (%)	56.5	30.4	32.6	0.009
Dementia (MMSE score ≤17 points) (%)	13.0	2.2	0	0.003
Total MMSE score (points)	23.2 ± 4.6	24.9 ± 3.7	25.3 ± 3.0	0.04
MMSE subscore orientation (0–10 points)	8.7 ± 1.7	9.0 ± 1.4	9.2 ± 0.9	0.31
MMSE subscore registration (0–3 points)	2.9 ± 0.4	3.0 ± 0.1	3.0 ± 0.0	0.6
MMSE subscore attention/calculation (0–5 points)	1.9 ± 1.8†	2.5 ± 1.8	2.6 ± 1.8	0.03
MMSE subscore recall (0–3 points)	1.9 ± 0.9	2.1 ± 0.9	2.2 ± 0.7	0.24
MMSE subscore language (0–9 points)	7.8 ± 1.3	8.3 ± 1.0	8.3 ± 0.9	0.09
Very elderly patients (aged ± ≥80 years) (n = 93)	n = 28	n = 46	n = 19	
Poor cognitive function (MMSE score ≤23 points) (%)	78.6	43.5	57.9	0.01
Dementia (MMSE score ≤17 points) (%)	21.4	4.3	0	0.01
Total MMSE score (points)	21.3 ± 4.5*	23.8 ± 4.3	23.6 ± 2.8	0.03
MMSE subscore orientation (0–10 points)	8.1 ± 1.9	8.5 ± 1.7	8.7 ± 1.0	0.45
MMSE subscore registration (0–3 points)	2.9 ± 0.6	3.0 ± 0.1	3.0 ± 0.0	0.71
MMSE subscore attention/calculation (0–5 points)	1.1 ± 1.1*	2.3 ± 1.9	1.9 ± 1.6	0.02
MMSE subscore recall (0–3 points)	1.7 ± 1.0	2.0 ± 0.9	2.1 ± 0.8	0.42
MMSE subscore language (0–9 points)	7.4 ± 1.4	8.0 ± 1.1	8.0 ± 1.2	0.19
Younger elderly patients (aged 61–79 years) (n = 91)	n = 18	n = 46	n = 27	
Poor cognitive function (MMSE score ≤23 points) (%)	22.2	17.4	14.8	0.81
Dementia (MMSE score ≤17 points) (%)	0	0	0	
Total MMSE score (points)	26.3 ± 2.9	26.0 ± 2.6	26.5 ± 2.6	0.76
MMSE subscore orientation (0–10 points)	9.6 ± 0.6	9.5 ± 0.7	9.6 ± 0.6	0.95
MMSE subscore registration (0–3 points)	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	1
MMSE subscore attention/calculation (0–5 points)	3.1 ± 2.0	2.7 ± 1.7	3.1 ± 1.9	0.61
MMSE subscore recall (0–3 points)	2.2 ± 0.6	2.2 ± 0.8	2.3 ± 0.6	0.72
MMSE subscore language (0 to 9 points)	8.4 ± 0.8	8.6 ± 0.7	8.6 ± 0.5	0.79

Kruskal–Wallis test for non parametrical data and χ^2 test for categorical data. Post hoc analysis was performed with Steel–Dwass test.
MMSE, mini-mental state examinations.
*P < 0.05 for lean vs. normal physique. †P < 0.05 for lean vs. obese.

Table 3 | Multiple logistic regression models for poor cognitive function

Independent variables	OR	95% CI	*P* value
Model 1: total patients (n = 184)			
Lean	2.54	1.13–5.73	0.02
Age (10 years increase)	3.88	2.28–6.63	<0.001
24-h SBP (10 mm Hg increase)	1.37	1.08–1.75	0.01
Female sex	0.81	0.32–2.04	0.65
Calcium channel blockers	0.79	0.38–1.66	0.54
ACE inhibitors	1.38	0.50–3.78	0.53
ARBs	0.48	0.18–1.23	0.13
Model 2: very elderly (n = 93)			
Lean	3.94	1.31–11.82	0.01
Age (10 years increase)	3.97	0.74–21.26	0.11
24-h SBP (10 mm Hg increase)	1.30	0.96–1.76	0.09
Female sex	1.48	0.43–5.10	0.53
Calcium channel blockers	0.91	0.35–2.38	0.84
ACE inhibitors	0.83	0.16–4.22	0.82
ARBs	0.47	0.15–1.54	0.21
Model 3: Younger elderly (n = 91)			
Lean	1.68	0.41–6.79	0.47
Age (10 years increase)	2.47	0.68–8.88	0.17
24-h SBP (10 mm Hg increase)	1.36	0.84–2.21	0.21
Female sex	0.38	0.10–1.48	0.16
Calcium channel blockers	0.56	0.16–1.90	0.35
ACE inhibitors	1.60	0.40–6.41	0.51
ARBs	0.50	0.09–2.82	0.43

The dependent variable was poor cognitive function (MMSE score ≤23 points) in all three models. All independent variables were adjusted in one step.
ACE, angiotensin-converting enzyme; ARBs, angiotensin receptor blockers; CI, confidence interval; OR, odds rate; SBP, systolic blood pressure.

score were 24.6 ± 3.9 and 25 points, respectively. A comparison of the patients' backgrounds grouped as lean, normal physique, and obese is shown in **Table 1**. None of the pairwise contrasts (other than BMI) were significant. The prevalence of the metabolic syndrome in obese patients was 54.3%.

Leanness, normal physique, and obesity in hypertensives

Poor cognitive function, total MMSE score, and MMSE subscore were compared in the hypertensive patients grouped as either lean, normal physique, or obese (**Table 2**). In the total 184 patients, the prevalence of poor cognitive function, total MMSE score, and MMSE subscore attention/calculation were significantly different in the three BMI groups (poor cognitive function: P = 0.009 for trend, total MMSE score: P = 0.04 for trend, MMSE subscore attention/calculation: P = 0.03 for trend). In the 93 very elderly patients, the prevalence of poor cognitive function, total MMSE score, and MMSE subscore attention/calculation were also significantly different between the three groups (poor cognitive function: P = 0.01 for trend, total MMSE score: P = 0.03 for trend, MMSE subscore attention/calculation: P = 0.02 for trend). However, in the 91 younger elderly patients, there was no significant difference between the groups.

A multiple logistic regression analysis was performed to verify whether leanness or obesity was a determinant of poor cognitive function in hypertensive patients after controlling for age, sex, 24-h SBP, CCBs, ACE inhibitors, and ARBs. Leanness was a significant determinant of poor cognitive function in the total patients (OR 2.54, 95% CI 1.13–5.73, P = 0.02) and in the very elderly patients (OR 3.94, 95% CI 1.31–11.82, P = 0.01), but not in the younger elderly patients (**Table 3**). Obesity was not a significant determinant of cognitive function in either the total patients (OR 1.26, 95% CI 0.54–2.90, P = 0.59), the very elderly (OR 1.75, 95% CI 0.55–5.62, P = 0.35), or the younger elderly (OR 0.83, 95% CI 0.22–3.08, P = 0.78). We also performed multiple logistic regression analysis to verify whether the metabolic syndrome was a determinant of poor cognitive function after controlling for age, sex, 24-h SBP, CCBs, ACE inhibitors, and ARBs. The metabolic syndrome was not a significant determinant of cognitive function in either the total patients (OR 1.12, 95% CI 0.40–3.09, P = 0.83), the very elderly (OR 3.43, 95% CI 0.68–17.25, P = 0.14), or the younger elderly (OR 0.20, 95% CI 0.02–1.87, P = 0.16).

DISCUSSION

Leanness in hypertensive patients was associated with poor cognitive function in the elderly, especially in the very elderly aged ≥80 years. On the other hand, obesity or the metabolic syndrome in elderly hypertensive patients was not associated with poor cognitive function.

Comparison with previous studies

Stamler *et al.* reported that lean, hypertensive subjects had an increased mortality rate compared with hypertensive subjects with higher BMIs.[6] Similarly, Wassertheil-Smoller *et al.* reported that elderly, lean, hypertensive patients were more prone to stroke than elderly hypertensive subjects with higher BMIs.[7] These studies imply that leanness in hypertensives is not a benign condition. However, there have been few reports on the association between leanness in hypertensive patients and poor cognitive function.

Cognitive function is impaired in lean but not obese hypertensive elderly subjects

Although previous studies reported that obesity was associated with poor cognitive function,[4,20,21] there was no relationship between obesity and poor cognitive function in the hypertensive elderly patients in our study. The cause of the difference between our and the earlier studies may be the age of the study population. Unlike our study, the population of these other studies focused mainly on middle-aged subjects. Taken together, these results suggest that obesity in mid-life may lead to future poor cognitive function.[4,20,21] On the other hand, leanness or a reduction in BMI is reported to be risk factors for poor cognitive function or dementia in the elderly.[5,22]

Therefore, obesity may be associated with future poor cognitive function in middle age and accordingly may no longer be associated with poor cognitive function in the elderly.

The metabolic syndrome has recently received considerable attention and an association between the syndrome and poor cognitive function has been reported.[23,24] As a consequence, we pay attention to poor cognitive function in patients with the metabolic syndrome. However, a recent study reported that the metabolic syndrome may have a protective effect on cognitive decline in the very elderly >85 years.[25] The reason why the metabolic syndrome was not associated with poor cognitive function in our study may be that our study population included a large number of very elderly patients ≥80 years. Our study suggests that we should also pay attention to leanness in hypertensive elderly patients, especially in the very elderly.

As some antihypertensive medications such as CCBs, ACE inhibitors, and ARBs have been reported to have a protective effect against cognitive decline,[17–19] it is possible that differences in these medications between the groups may have affected the variation we observed in cognitive function between the groups. To address this confounding factor, we used CCBs, ACE inhibitors, and ARBs as variables in the multiple logistic regression analysis. However, as we did not have a sufficient number of subjects treated with each class of antihypertensive medications, the association between antihypertensive medication and cognitive function may have been underestimated.

The mechanism of poor cognitive function in lean hypertensive patients

The mechanism of poor cognitive function in lean hypertensive patients is not clear. However, it is well known that hypertension affects cognitive function in elderly subjects.[1,2] Several mechanisms linking hypertension and poor cognitive function have been proposed. There is evidence that white matter medullary arterioles are vulnerable to hypertension, and that tortuosity of these arterioles is related to the severity of hypertension.[26] A higher level of vascular lesions and neuritic plaques have also been shown to be associated with elevated levels of BP in an autopsy study.[27] It is likely that these organic brain lesions associated with hypertension may be the cause of poor cognitive function.

On the other hand, the mechanism linking poor cognitive function and leanness is not fully understood. As leanness is associated with target organ damage and cardiovascular events such as stroke, cardiovascular death, and non-cardiovascular death,[7,28,29] it is possible that poor cognitive function may be caused by organic brain lesions such as silent cerebral infarction or deep white matter hyperintensity due to leanness. In our study, pulse pressure in lean hypertensive patients was marginally higher than that in obese hypertensives. Therefore, arterial stiffness may be an underlying pathway of cognitive decline in lean hypertensive patients. Decreasing body weight may also be an early marker of declining health or even neurodegenerative changes in the brain.[30] Therefore, leanness associated with hypertension may be a strong risk factor of poor cognitive function as a consequence of the combined effects of both these clinical characteristics.

The MMSE subscore for attention/calculation in lean hypertensive patients was lower than in either normal physique or obese hypertensive patients. Our results suggest that brain attention/calculation function may be vulnerable to a combination of hypertension and leanness.

Another explanation may be that leanness is an effect rather than a cause of poor cognitive function. For example, dementia such as Alzheimer's disease may change eating habits and lead to a loss of body weight.[31]

Study limitations

Because this study was a cross-sectional design, we were unable to resolve cause-and-effect relationships. A prospective cohort study on the relationship between leanness and poor cognitive function in hypertensive elderly subjects is necessary to elucidate these relationships.

There would also have been some differences between leanness and underweight, and such differences may have affected cognitive function. However, only body weight and height were measured in our study. Because other data such as abdominal circumference, serum albumin, and body fat percentage were not available, we could not address the possible differences between leanness and underweight.

Conclusion

While obesity or the presence of the metabolic syndrome was not associated with poor cognitive function in elderly hypertensive patients, leanness in combination with hypertension was related to reduced cognitive function in elderly subjects, especially the very elderly.

Disclosure: The authors declared no conflict of interest.

1. Forette F, Boller F. Hypertension and the risk of dementia in the elderly. *Am J Med* 1991; 90:145–195.
2. Peila R, White LR, Masaki K, Petrovitch H, Launer LJ. Reducing the risk of dementia: efficacy of long-term treatment of hypertension. *Stroke* 2006; 37:1165–1170.
3. Gorospe EC, Dave JK. The risk of dementia with increased body mass index. *Age Ageing* 2007; 36:23–29.
4. Whitmer RA, Gunderson EP, Barrett-Connor E, Quesenberry CP Jr, Yaffe K. Obesity in middle age and future risk of dementia: a 27 year longitudinal population based study. *BMJ* 2005; 330:1360.
5. Buchman AS, Wilson RS, Bienias JL, Shah RC, Evans DA, Bennett DA. Change in body mass index and risk of incident Alzheimer disease. *Neurology* 2005; 65:892–897.
6. Stamler R, Ford CE, Stamler J. Why do lean hypertensives have higher mortality rates than other hypertensives? Findings of the Hypertension Detection and Follow-up Program. *Hypertension* 1991; 17:553–564.
7. Wassertheil-Smoller S, Fann C, Allman RM, Black HR, Camel GH, Davis B, Masaki K, Pressel S, Prineas RJ, Stamler J, Vogt TM. Relation of low body mass to death and stroke in the systolic hypertension in the elderly program. The SHEP Cooperative Research Group. *Arch Intern Med* 2000; 160:494–500.
8. Sakakura K, Ishikawa J, Okuno M, Shimada K, Kario K. Exaggerated ambulatory blood pressure variability is associated with cognitive dysfunction in the very elderly and quality of life in the younger elderly. *Am J Hypertens* 2007; 20:720–727.
9. Palatini P, Frigo G, Bertolo O, Roman E, Da Corta R, Winnicki M. Validation of the A&D TM-2430 device for ambulatory blood pressure monitoring and evaluation of performance according to subjects' characteristics. *Blood Press Monit* 1998; 3:255–260.
10. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res* 1975; 12:189–198.

11. Ahto M, Isoaho R, Puolijoki H, Laippala P, Sulkava R, Kivela SL. Cognitive impairment among elderly coronary heart disease patients. *Gerontology* 1999; 45:87–95.
12. Rait G, Fletcher A, Smeeth L, Brayne C, Stirling S, Nunes M, Breeze E, Ng ES, Bulpitt CJ, Jones D, Tulloch AJ. Prevalence of cognitive impairment: results from the MRC trial of assessment and management of older people in the community. *Age Ageing* 2005; 34:242–248.
13. Wilber ST, Lofgren SD, Mager TG, Blanda M, Gerson LW. An evaluation of two screening tools for cognitive impairment in older emergency department patients. *Acad Emerg Med* 2005; 12:612–616.
14. O'Connor DW, Pollitt PA, Hyde JB, Brook CP, Reiss BB, Roth M. Do general practitioners miss dementia in elderly patients? *BMJ* 1988; 297:1107–1110.
15. Kondo T, Kobayashi I, Murakami M. Effect of exercise on circulating adipokine levels in obese young women. *Endocr J* 2006; 53:189–195.
16. Janssen I, Katzmarzyk PT, Ross R. Body mass index, waist circumference, and health risk: evidence in support of current National Institutes of Health guidelines. *Arch Intern Med* 2002; 162:2074–2079.
17. Forette F, Seux ML, Staessen JA, Thijs L, Birkenhager WH, Babarskiene MR, Babeanu S, Bossini A, Gil-Extremera B, Girerd X, Laks T, Lilov E, Moisseyev V, Tuomilehto J, Vanhanen H, Webster J, Yodfat Y, Fagard R. Prevention of dementia in randomised double-blind plACEbo-controlled Systolic Hypertension in Europe (Syst-Eur) trial. *Lancet* 1998; 352:1347–1351.
18. Rozzini L, Chilovi BV, Bertoletti E, Conti M, Del Rio I, Trabucchi M, Padovani A. Angiotensin converting enzyme (ACE) inhibitors modulate the rate of progression of amnestic mild cognitive impairment. *Int J Geriatr Psychiatry* 2006; 21:550–555.
19. Zanchetti A, Elmfeldt D. Findings and implications of the Study on Cognition and Prognosis in the Elderly (SCOPE)—a review. *Blood Press* 2006; 15:71–79.
20. Rosengren A, Skoog I, Gustafson D, Wilhelmsen L. Body mass index, other cardiovascular risk factors, and hospitalization for dementia. *Arch Intern Med* 2005; 165:321–326.
21. Kivipelto M, Ngandu T, Fratiglioni L, Viitanen M, Kareholt I, Winblad B, Helkala EL, Tuomilehto J, Soininen H, Nissinen A. Obesity and vascular risk factors at midlife and the risk of dementia and Alzheimer disease. *Arch Neurol* 2005; 62:1556–1560.
22. Nourhashemi F, Deschamps V, Larrieu S, Letenneur L, Dartigues JF, Barberger-Gateau P. Body mass index and incidence of dementia: the PAQUID study. *Neurology* 2003; 60:117–119.
23. Razay G, Vreugdenhil A, Wilcock G. The metabolic syndrome and Alzheimer disease. *Arch Neurol* 2007; 64:93–96.
24. Vanhanen M, Koivisto K, Moilanen L, Helkala EL, Hanninen T, Soininen H, Kervinen K, Kesaniemi YA, Laakso M, Kuusisto J. Association of metabolic syndrome with Alzheimer disease: a population-based study. *Neurology* 2006; 67:843–847.
25. van den Berg E, Biessels GJ, de Craen AJ, Gussekloo J, Westendorp RG. The metabolic syndrome is associated with decelerated cognitive decline in the oldest old. *Neurology* 2007; 69:979–985.
26. Hiroki M, Miyashita K, Oda M. Tortuosity of the white matter medullary arterioles is related to the severity of hypertension. *Cerebrovasc Dis* 2002; 13:242–250.
27. Petrovitch H, White LR, Ross GW, Steinhorn SC, Li CY, Masaki KH, Davis DG, Nelson J, Hardman J, Curb JD, Blanchette PL, Launer LJ, Yano K, Markesbery WR. Accuracy of clinical criteria for AD in the Honolulu-Asia Aging Study, a population-based study. *Neurology* 2001; 57:226–234.
28. Higashi Y, Sasaki S, Nakagawa K, Kimura M, Noma K, Sasaki S, Hara K, Matsuura H, Goto C, Oshima T, Chayama K, Yoshizumi M. Low body mass index is a risk factor for impaired endothelium-dependent vasodilation in humans: role of nitric oxide and oxidative stress. *J Am Coll Cardiol* 2003; 42:256–263.
29. Kenchaiah S, Pocock SJ, Wang D, Finn PV, Zornoff LA, Skali H, Pfeffer MA, Yusuf S, Swedberg K, Michelson EL, Granger CB, McMurray JJ, Solomon SD. Body mass index and prognosis in patients with chronic heart failure: insights from the Candesartan in Heart failure: Assessment of Reduction in Mortality and morbidity (CHARM) program. *Circulation* 2007; 116:627–636.
30. Johnson DK, Wilkins CH, Morris JC. Accelerated weight loss may precede diagnosis in Alzheimer disease. *Arch Neurol* 2006; 63:1312–1317.
31. Greenwood CE, Tam C, Chan M, Young KW, Binns MA, van Reekum R. Behavioral disturbances, not cognitive deterioration, are associated with altered food selection in seniors with Alzheimer's disease. *J Gerontol A Biol Sci Med Sci* 2005; 60:499–505.

Journal of Cardiology (2008) 52, 24–29

ELSEVIER

JOURNAL of CARDIOLOGY
Official Journal of the Japanese College of Cardiology
www.elsevier.com/locate/jjcc

ORIGINAL ARTICLE

Determinants of in-hospital death in left main coronary artery myocardial infarction complicated by cardiogenic shock

Kenichi Sakakura (MD)[a], Norifumi Kubo (MD)[a,*], Shigemasa Hashimoto (MD)[b], Nahoko Ikeda (MD)[a], Hiroshi Funayama (MD)[a], Taishi Hirahara (MD)[a], Yoshitaka Sugawara (MD)[a], Takanori Yasu (MD, FJCC)[a], Junya Ako (MD)[c], Masanobu Kawakami (MD)[a], Shin-ichi Momomura (MD, FJCC)[a]

[a] *Division of Cardiovascular Medicine, Department of Integrated Medicine I, Jichi Medical University Saitama Medical Center, Omiya, Japan*
[b] *Department of Cardiovascular and Renal Medicine, Saga University Faculty of Medicine, Saga, Japan*
[c] *Division of Cardiovascular Medicine, Stanford University School of Medicine, Stanford, CA, USA*

Received 12 January 2008; received in revised form 17 February 2008; accepted 18 March 2008
Available online 19 June 2008

KEYWORDS
Left main;
Acute myocardial infarction;
Shock;
In-hospital death

Summary
Background: Acute myocardial infarction (AMI) due to left main coronary artery disease is associated with significantly elevated morbidity and mortality. The aim of this study was to identify the predictors of in-hospital death from left main AMI complicated by cardiogenic shock.
Methods: Clinical record review identified a total of 25 cases of left main AMI with cardiogenic shock. Patients' background characteristics, laboratory data, and angiographic findings were analyzed according to the in-hospital mortality.
Results: In this patient subset, in-hospital mortality (60%) was associated with a history of hypertension ($p = 0.02$) and a higher heart rate ($p = 0.02$). Furthermore, in-hospital mortality was also associated with a complete right bundle branch block (CRBBB) pattern in the admission ECG ($p = 0.01$) and low HCO_3^- ($p = 0.0004$). In stepwise logistic regression analysis, a CRBBB pattern (OR 48.59, 95% CI 1.34–1768.10, $p = 0.03$) and low HCO_3^- (OR 0.62, 95% CI 0.40–0.94, $p = 0.02$) were found to be independent predictors of mortality.

* Corresponding author at: Cardiovascular Division, Jichi Medical University Saitama Medical Center, 1-847 Amanuma, Omiya 330-8503, Japan. Tel.: +81 48 647 2111; fax: +81 48 648 5188.
E-mail address: kubonori@omiya.jichi.ac.jp (N. Kubo).

0914-5087/$ — see front matter © 2008 Japanese College of Cardiology. Published by Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jjcc.2008.03.008

Conclusions: Left main AMI with cardiogenic shock was associated with high in-hospital mortality. A CRBBB pattern in the ECG on admission and a low HCO_3^- concentration were significant independent predictors of in-hospital death.
© 2008 Japanese College of Cardiology. Published by Elsevier Ireland Ltd. All rights reserved.

Introduction

Acute myocardial infarction (AMI) due to left main coronary artery occlusion/stenosis is associated with significantly elevated morbidity and mortality [1,2]. Cardiogenic shock has been reported to be an independent predictor of in-hospital death in left main AMI [2,3]. However, information on the determinants of in-hospital death in left main AMI with cardiogenic shock has been limited [2,4–7]. Thus, the aim of this study was to identity predictors of in-hospital death in left main AMI with cardiogenic shock.

Methods

Study patients

Case record review identified patients that meet the following criteria: (1) persistent chest pain, elevation of creatine kinase (at least 2-fold increases from normal upper limit) and ST-segment elevation or depression in electrocardiograms compatible with acute myocardial infarction, (2) culprit lesion in the left main coronary artery confirmed by coronary angiography, (3) cardiogenic shock (systolic blood pressure of <90 mm Hg with sufficient preload for at least 30 min or the need for supportive measures such as catecholamines to maintain a systolic blood pressure of ≥90 mm Hg) and (4) time from symptom onset to hospital arrival <48 h.

Hypertension was defined as a blood pressure >140/90 mm Hg or treatment for hypertension before admission. Hyperlipidemia was defined as total cholesterol >220 mg/dl or treatment for hyperlipidemia. Diabetes mellitus was defined as hemoglobin A1c >6.5% or treatment for diabetes mellitus. Smoking was defined as having a current smoking habit. Pre-myocardial infarction angina was defined as at least one episode of typical chest pain <48 h before AMI. Left ventricular function was determined by initial echocardiography or left ventriculography. Left ventricular dysfunction was defined as a global ejection fraction <35%.

Hospital chart records, patients' history, initial laboratory data, initial electrocardiograms and echocardiograms and coronary angiograms were reviewed. We divided left main AMI patients with cardiogenic shock into two groups according to the presence or absence of in-hospital death. The study was performed in accordance with the ethical standards of the local ethics committee on human experimentation.

Coronary angiogram, acute intervention and mechanical support

Initial coronary angiograms were reviewed for analysis. Collateral vessel filling was defined as the presence of more than Rentrop-grade 2 collateral filling from dye injection into the right coronary artery [8]. Right coronary artery stenosis was defined as the presence of ≥50% diameter stenosis by visual estimation. We classified acute intervention as percutaneous transluminal coronary angioplasty (PTCA), coronary artery bypass grafting (CABG), PTCA and CABG, and coronary stenting (Stent). PTCA was defined as any percutaneous coronary intervention without the use of coronary stenting. When CABG was emergently performed following PTCA, this was classified as PTCA and CABG. The use of an intra-aortic balloon pump (IABP) and extracorporeal percutaneous cardiopulmonary support (PCPS) was also reviewed. Success of acute intervention was determined as successful revascularization by PTCA or Stent with achievement of Thrombolysis in Myocardial Infarction grade ≥2 flow, or successful revascularization with bypass grafting.

Statistical analysis

All analyses were performed using statistical software, SPSS 13.0/Windows (SPSS Inc., Chicago, USA). Categorical variables are presented as numbers (percentages) and compared with a χ^2 test or Fisher's exact test. Continuous variables are compared using an unpaired Student's *t*-test. Stepwise logistic regression analysis was performed to find the predictors of in-hospital death. The stepwise logistic regression model included independent variables found to significantly influence in-hospital outcomes in univariate analysis (defined as $p < 0.05$). The odds ratio (OR) and the 95% confidence interval (CI) were calculated. A *p*-value <0.05 was considered to indicate statistical significance.

Table 1 Comparison of patients characteristics according to in-hospital death

	In-hospital death NO $n=10$	In-hospital death YES $n=15$	P value
Age (year)	60.9 ± 8.0	57.2 ± 10.2	0.34
Male sex (%)	8 (80)	11 (73.3)	1.00
Body-mass index (kg/m^2)	22.5 ± 2.5	23.9 ± 1.5	0.13
History of hypertension (%)	0 (0)	7 (46.7)	0.02
History of hyperlipidemia (%)	4 (40)	1 (6.7)	0.12
History of Diabetes mellitus (%)	3 (30)	3 (20)	0.65
Current history of smoking (%)	6 (60)	8 (53.3)	1.00
Referral from other hospital (%)	7 (70)	11 (73.3)	1.00
Time from onset to arrival at hospital (hour)	7.5 ± 12.7	2.7 ± 2.2	0.27
Alert consciousness at arrival (%)	5 (50)	3 (20)	0.19
Systolic blood pressure at arrival (mm Hg)	77.1 ± 29.5	51.9 ± 32.7	0.06
Heart rate (min^{-1})	77.6 ± 20.8	107.5 ± 34.2	0.02
Pre-myocardial infarction angina (%)	4 (40)	2 (13.3)	0.18
Initial laboratory data			
White blood cell counts ($\times 1000\,mm^{-3}$)	12.4 ± 4.7	16.3 ± 8.0	0.18
Hemoglobin (mg/dl)	13.5 ± 1.6	13.6 ± 2.0	0.97
Peak creatine kinase (mg/dl)	7861 ± 6280	10314 ± 5440	0.33
Creatinine (mg/dl)	1.1 ± 0.3	1.4 ± 0.5	0.07
C-reactive protein (mg/dl)	2.2 ± 5.4	2.1 ± 5.0	0.95
pH	7.37 ± 0.09	7.22 ± 0.18	0.03
HCO_3^- (mequiv./l)	23.1 ± 4.6	15.4 ± 4.6	0.0004
Initial electrocardiogram			
Complete right bundle branch block pattern (%)	2 (20)	10 (76.9)	0.01
ST elevation in aVr (%)	9 (90)	11 (84.6)	1.00
Left ventricular dysfunction (%)	8 (80)	15 (100)	0.15
Coronary angiogram			
Left main total occlusion (TIMI = 0 flow) (%)	6 (60)	6 (40)	0.43
Collateral (%)	4 (40)	1 (6.7)	0.12
Right coronary artery stenosis (%)	2 (20)	3 (20)	1.00
Type of acute intervention			0.33
No acute intervention (%)	1 (10)	1 (6.7)	
PTCA (%)	0 (0)	4 (26.7)	
CABG (%)	0 (0)	1 (6.7)	
PTCA and CABG (%)	2 (20)	1 (6.7)	
Stent (%)	7 (70)	8 (53.3)	
Thrombectomy in percutaneous intervention	2 (20)	4 (26.7)	1.00
Simultaneous percutaneous intervention to right coronary artery stenosis	1 (10)	0 (0)	0.40
Successful acute intervention (%)	9 (90)	9 (60)	0.18
Mechanical hemodynamic support			
Intra-aortic balloon pump (%)	10 (100)	14 (93.3)	1.00
Percutaneous cardiopulmonary support (%)	2 (20)	11 (73.3)	0.02

All categorical variables except type of acute intervention were compared with a Fisher's exact test. Type of acute intervention was compared with a χ^2 test.

Results

Among 2532 AMI patients admitted to Omiya Medical Center, Jichi Medical University, from April 1991 to December 2006, a total of 50 cases with left main AMI were identified. Among them, a total of 25 cases with left main AMI with cardiogenic shock were identified and included in this study.

Table 1 shows a comparison of patient characteristics according to the in-hospital outcome. Fifteen patients (60%) had died during the hospitalization. In-hospital mortality was associated with a history

Table 2 Stepwise logistic regression for in-hospital death

	OR	95% CI	*p* value
Step 3 HCO_3^- (mequiv./l)	0.62	0.40–0.94	0.02
Complete right bundle branch block pattern (%)	48.59	1.34–1768.10	0.03

OR = Odds ratio, 95% CI = 95% confidence interval.
The independent variables in Step 1 model were history of hypertension, HCO_3^-, heart rate, and Complete right bundle branch block pattern. History of hypertension and heart rate were removed in Steps 2 and 3, respectively. Final solution could not be found in Steps 1 and 2, because maximum iterations had been reached.

of hypertension ($p = 0.02$) and a complete right bundle branch block (CRBBB) pattern in the admission ECG ($p = 0.01$). In addition, in-hospital mortality was associated with a lower pH ($p = 0.03$), a lower HCO_3^- concentration ($p = 0.0004$) and higher heart rate ($p = 0.02$).

Table 2 shows the results of stepwise logistic regression analysis. The independent variables in the Step 1 model were a history of hypertension, HCO_3^- concentration, heart rate and a CRBBB pattern. In the step 3 model, HCO_3^- concentration (OR 0.62, 95% CI 0.40–0.94, $p = 0.02$) and a CRBBB pattern (OR 48.59, 95% CI 1.34–1768.10, $p = 0.03$) were found to have significant associations with in-hospital death after adjustment for each covariates.

Discussion

This study showed a high mortality rate (60%) in patients with left main AMI and cardiogenic shock, similar to the mortality rate (81.3%) reported in a previous study [2]. In the present study, predictors of in-hospital mortality included lower HCO_3^- concentration and a CRBBB pattern in the 12-lead ECG.

Patient background

In-hospital mortality was associated with a history of hypertension. Of note, there were no patients with a history of hypertension in the survival group. Hypertension may increase the risk of target organ damage such as silent cerebral infarction, carotid artery stenosis, left ventricular hypertrophy or chronic renal failure [9–12]. These subclinical organ damages may have increased the vulnerability of patients to cardiogenic shock. Since this study is a retrospective analysis, we are unable to fully exclude patient selection bias.

Initial laboratory data

In the present study, in-hospital mortality was associated with higher heart rate, lower HCO_3^- concentration, and a CRBBB pattern in the admission ECG. A low HCO_3^- concentration was reported to be a determinant of poor prognosis in in-patients with cardiogenic shock that required cardiopulmonary support [13]. The HCO_3^- concentration is primarily decreased in cardiogenic shock accompanied by lactic acidosis. Our results support the possibility that a low HCO_3^- concentration on admission reflects the severity of cardiogenic shock due to left main MI as well as the severity of metabolic acidosis.

A CRBBB pattern in the 12-lead ECG was reported to be a determinant of poor prognosis in anterior wall AMI [14,15]. A histopathological study showed that the new occurrence of RBBB associated with acute anteroseptal myocardial infarction was explained by necrosis of the right bundle branch [16].

Our study confirmed that CRBBB pattern is a predictor of poor prognosis in left main AMI. This electrocardiographic finding may possibly reflect extensive myocardial necrosis [17].

Our study failed to show that left ventricular function was a predictor of survival in left main AMI. However, the majority of patients (92%) in this study showed left ventricular dysfunction, suggesting that the study was underpowered to determine the effect of left ventricular function on survival. Although C-reactive protein (CRP) levels were reported to be a predictor of poor prognosis [18], our study failed to show a difference in CRP levels between survivors and non-survivors. Generally, CRP levels are elevated from the onset of AMI. The difference in the time from onset to hospital arrival might affect CRP levels.

Coronary angiogram, acute intervention and mechanical support as predictors of in-hospital death

Collateral flow more than Rentrop-grade 2 was reported to have a protective effect on enzymatic infarct size in patients with AMI [19]. However, our results could not support the protective effect of

collateral flow. Other coronary angiographic findings including left main occlusion and right coronary artery stenosis were not significantly different between the two groups. With respect to therapeutic options, Marso et al. reported that stenting was associated with an improved clinical outcome in AMI [4], although the in-hospital mortality rate was not significantly different. Our retrospective study was not designed to compare different types of acute intervention. Larger studies would be needed to address this issue.

The use of PCPS was associated with higher mortality; however, this finding may require careful interpretation. The use of PCPS may have been necessitated by a poor circulatory status, rather than the cause of a poor clinical outcome.

Clinical implications

Electrocardiography and arterial blood gas sampling may help to identify a high-risk subset of patients with acute myocardial infarction due to left main coronary artery disease. Prompt and extra effort should be made for the treatment of such patients.

Study limitations

Since this study was a retrospective analysis at a single center, there may have been a patient selection bias. As the study population was small, we could not perform multiple logistic regression analyses with a large number of independent variables. Due to the severe and acute nature of the disease, we lacked complete access to the information on background characteristics such as history taking about past medical history in several patients.

Conclusions

Left main AMI with cardiogenic shock is associated with high in-hospital mortality (60%). Low HCO_3^- concentration and a CRBBB pattern in the admission ECG were significant independent predictors of in-hospital death.

References

[1] Neri R, Migliorini A, Moschi G, Valenti R, Dovellini EV, Antoniucci D. Percutaneous reperfusion of left main coronary disease complicated by acute myocardial infarction. Catheter Cardiovasc Interv 2002;56:31–4.

[2] Zeymer U, Vogt A, Zahn R, Weber MA, Tebbe U, Gottwik M, et al. Predictors of in-hospital mortality in 1333 patients with acute myocardial infarction complicated by cardiogenic shock treated with primary percutaneous coronary intervention (PCI). Results of the primary PCI registry of the Arbeitsgemeinschaft Leitende Kardiologische Krankenhausarzte (ALKK). Eur Heart J 2004;25:322–8.

[3] Hata M, Shiono M, Sezai A, Iida M, Yoshitake I, Wakui S, et al. Outcome of emergency conventional coronary surgery for acute coronary syndrome due to left main coronary disease. Ann Thorac Cardiovasc Surg 2006;12:28–31.

[4] Marso SP, Steg G, Plokker T, Holmes D, Park SJ, Kosuga K, et al. Catheter-based reperfusion of unprotected left main stenosis during an acute myocardial infarction (the ULTIMA experience) Unprotected Left Main Trunk Intervention Multi-center Assessment. Am J Cardiol 1999;83:1513–7.

[5] Hochman JS, Sleeper LA, Webb JG, Dzavik V, Buller CE, Aylward P, et al. Early revascularization and long-term survival in cardiogenic shock complicating acute myocardial infarction. JAMA 2006;295:2511–5.

[6] Hochman JS, Sleeper LA, Webb JG, Sanborn TA, White HD, Talley JD, et al. Early revascularization in acute myocardial infarction complicated by cardiogenic shock SHOCK Investigators. Should we emergently revascularize occluded coronaries for cardiogenic shock. N Engl J Med 1999;341:625–34.

[7] Picard MH, Davidoff R, Sleeper LA, Mendes LA, Thompson CR, Dzavik V, et al. Echocardiographic predictors of survival and response to early revascularization in cardiogenic shock. Circulation 2003;107:279–84.

[8] Rentrop KP, Cohen M, Blanke H, Phillips RA. Changes in collateral channel filling immediately after controlled coronary artery occlusion by an angioplasty balloon in human subjects. J Am Coll Cardiol 1985;5:587–92.

[9] Kario K, Pickering TG, Umeda Y, Hoshide S, Hoshide Y, Morinari M, et al. Morning surge in blood pressure as a predictor of silent and clinical cerebrovascular disease in elderly hypertensives: a prospective study. Circulation 2003;107:1401–6.

[10] Su TC, Jeng JS, Chien KL, Sung FC, Hsu HC, Lee YT. Hypertension status is the major determinant of carotid atherosclerosis: a community-based study in Taiwan. Stroke 2001;32:2265–71.

[11] Mancia G, Carugo S, Grassi G, Lanzarotti A, Schiavina R, Cesana G, et al. Prevalence of left ventricular hypertrophy in hypertensive patients without and with blood pressure control: data from the PAMELA population. Pressioni Arteriose Monitorate E Loro Associazioni. Hypertension 2002;39:744–9.

[12] Bidani AK, Griffin KA. Pathophysiology of hypertensive renal damage: implications for therapy. Hypertension 2004;44:595–601.

[13] Imasaka K, Masuda M, Oishi T, Shimizu I, Iwai T, Kajihara T, et al. Mechanical cardiac support system for patients with postcardiotomy cardiogenic shock: analysis of risk factors for survival. Jpn J Thorac Cardiovasc Surg 2004;52:163–8.

[14] Melgarejo-Moreno A, Galcerá-Tomás J, Garciá-Alberola A, Valdés-Chavarri M, Castillo-Soria FJ, Mira-Sánchez E, et al. Incidence, clinical characteristics, and prognostic significance of right bundle-branch block in acute myocardial infarction: a study in the thrombolytic era. Circulation 1997;96:1139–44.

[15] Ricou F, Nicod P, Gilpin E, Henning H, Ross Jr J. Influence of right bundle branch block on short- and long-term survival after acute anterior myocardial infarction. J Am Coll Cardiol 1991;17:858–63.

[16] Okabe M, Fukuda K, Nakashima J, Hiroki T, Arakanawa K, Kikuchi M. A quantitative histopathological study of right bundle branch block complicating acute anteroseptal myocardial infarction. Br Heart J 1991;65:317–21.
[17] Sgarbossa EB, Pinski SL, Topol EJ, Califf RM, Barbagelata A, Goodman SG, et al. Acute myocardial infarction and complete bundle branch block at hospital admission: clinical characteristics and outcome in the thrombolytic era GUSTO-I Investigators. Global utilization of streptokinase and t-PA [tissue-type plasminogen activator] for occluded coronary arteries. J Am Coll Cardiol 1998;31:105–10.
[18] Zairis MN, Manousakis SJ, Stefanidis AS, Papadaki OA, Andrikopoulos GK, Olympios CD, et al. C-reactive protein levels on admission are associated with response to thrombolysis and prognosis after ST-segment elevation acute myocardial infarction. Am Heart J 2002;144:782–9.
[19] Elsman P, Van't Hof AWJ, De Boer MJ, Hoorntje JCA, Suryapranata H, Dambrink JHE, et al. Role of collateral circulation in the acute phase of ST-segment-elevation myocardial infarction treated with primary coronary intervention. Eur Heart J 2004;25:854–8.

Available online at www.sciencedirect.com

ScienceDirect

Experimental Physiology – Research Paper

Hypotonicity reduces the activity of murine aquaporin-2 promoter induced by dibutyryl cAMP

Takako Saito[1], Tomoyuki Saito[1], Keizo Kasono[1], Hiroyuki Tamemoto[1], Masanobu Kawakami[1], Sei Sasaki[2] and San-e Ishikawa[1]

[1]Department of Medicine, Jichi Medical University Saitama Medical Center, 1-847 Amanuma, Omiya-ku, Saitama 330-8503, Japan
[2]Department of Nephrology, Tokyo Medical and Dental University, 1-5-45, Yushima Bunkyo, Tokyo 113-8519, Japan

The present study was undertaken to determine whether hypotonicity regulates the aquaporin-2 (AQP-2) gene *in vitro*. The 5′-flanking region of the AQP-2 gene contains the tonicity-response enhancer (TonE) promoter located between −570 and −560 bp, and another distinct hypertonicity-responsive region between −6.1 and −4.3 kb of the AQP-2 gene. The 5′-flanking region of murine AQP-2 gene up to −9.5 kb was cloned into a luciferase (Luc) reporter plasmid. The constructs, which have TonE and/or the hypertonicity-responsive region, together with the murine AQP-2 gene, were co-transfected into murine IMCD$_3$ cells. When the cells were co-transfected with the construct containing more than 1.1 kb of the 5′-flanking region of murine AQP-2 gene (−9.5AQP2, −6.1AQP2 and −1.1AQP2) and the AQP-2 gene, 24 h exposure to 5 μmol l^{-1} dibutyryl cAMP (DBcAMP) significantly increased the Luc activity by 2.3-fold in the isotonic medium (300 mosmol kg^{-1}). In the hypotonic medium (225 mosmol kg^{-1}), basal activity was not altered, and the response of Luc activity to 24 h exposure to 5 μmol l^{-1} DBcAMP was abolished. Similar findings were obtained in isosmotic, urea-supplemented medium (estimated tonicity, 225 mosmol kg^{-1}). The response of Luc activity to 5 μmol l^{-1} DBcAMP in the hypotonic medium was not affected in cells either transfected with 0.36 kb of the 5′-flanking region of AQP-2 or co-transfected with −1.1AQP2 and a dominant-negative TonE binding protein (pDNTonEBP). Pre-incubation of cells with 1 μmol l^{-1} SP600125, an inhibitor of c-Jun N-terminal kinase (JNK), restored the response of Luc activity to 5 μmol l^{-1} DBcAMP under hypotonic conditions. These findings may indicate that hypotonicity reduces the cAMP-induced AQP-2 promoter activity mediated via TonE by activating JNK kinase.

(Received 17 March 2008; accepted after revision 27 May 2008; first published online 30 May 2008)
Corresponding author S. Ishikawa: Department of Medicine, Jichi Medical University, Saitama Medical Center, 1-847 Amanuma Omiya-ku, Saitama, Saitama 330-8503, Japan. Email: saneiskw@jichi.ac.jp

Aquaporin-2 (AQP-2) is the arginine vasopressin (AVP)-regulated water channel of renal collecting duct cells (Fushimi *et al.* 1993; Sasaki *et al.* 1994). Activation of AVP V$_2$ receptors initiates cellular trafficking of AQP-2-bearing vesicles from cytosol to apical plasma membrane within minutes (short-term regulation; Saito *et al.* 1997; Christensen *et al.* 1998). In addition, AVP induces AQP-2 gene transcription mediated by cAMP-response element (CRE) located at −310 to −304 bp of the AQP-2 promoter, and regulates the abundance of AQP-2 protein (long-term regulation; Nielsen *et al.* 1993; Matsumura *et al.* 1997). Arginine vasopressin thus plays a crucial role in the on–off regulation of the cellular trafficking of AQP-2 and the synthesis of AQP-2 protein in collecting duct cells.

Hypertonicity directly regulates the transcriptional regulation of the AQP-2 gene independently of AVP. So far, it has been reported that there are at least two hypertonicity-responsive elements in the 5′-flanking region of the AQP-2 gene. One is a tonicity response enhancer (TonE) located at −570 to −560 bp (TGGAAATTTCTT) and the other is a hypertonicity-responsive region located between −6.1 and −4.3 kb, which is different from the nucleotide sequence of TonE (Storm *et al.* 2003; Kasono *et al.* 2005). In addition, hypertonic stimulation participates in the synergistic effect of cAMP-induced AQP-2 gene transcription (Kasono *et al.* 2005). Amongst the AQP family, it is evident that the expression of AQP-1, AQP-4 and AQP-9 are controlled

© 2008 The Authors. Journal compilation © 2008 The Physiological Society
DOI: 10.1113/expphysiol.2008.042663

Table 1. Composition of media

Component	Standard DMEM	NaCl-free DMEM	Hypotonic DMEM 175 mosmol kg^{-1}	200 mosmol kg^{-1}	225 mosmol kg^{-1}	250 mosmol kg^{-1}	275 mosmol kg^{-1}	Isosmotic (urea-supplemented) medium
$CaCl_2 \cdot 2H_2O$	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
$Fe(NO_3)_3 \cdot 9H_2O$	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002
$MgSO_4$	0.81	0.81	0.81	0.81	0.81	0.81	0.81	0.81
KCl	5.3	5.3	5.3	5.3	5.3	5.3	5.3	5.3
$NaHCO_3$	44	44	44	44	44	44	44	44
NaCl	109	0	37.5	50	62.5	70	82.5	62.5
NaH_2PO_4	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Glucose	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Urea	0	0	0	0	0	0	0	75
Osmolality (mosmol kg^{-1})	301.2 ± 0.29	99.7 ± 0.48	175.2 ± 0.48	200.2 ± 0.25	224.7 ± 0.48	250.5 ± 0.29	275.25 ± 0.48	299.7 ± 0.75

All values are in millimoles per litre except for osmolality. All the amino acids and vitamins are contained in all the media similar to that of standard DMEM.

by hypertonicity independently of the TonE sequence (Umenishi & Schrier, 2002; Arima *et al.* 2003).

In chronic AVP excess, as seen with the syndrome of inappropriate secretion of antidiuretic hormone (SIADH), the antidiuretic action of AVP is attenuated, thus producing some water diuresis (Gross *et al.* 1983; Ishikawa & Schrier, 2003). This state has been termed as renal 'escape' from AVP-induced antidiuresis. Recent *in vivo* studies have demonstrated that upregulation of AQP-2 mRNA and protein expression was significantly attenuated in SIADH rats compared with rats having an excess of AVP in the absence of volume expansion (Ecelbarger *et al.* 1997; Saito *et al.* 2001). Therefore, hypotonicity or volume expansion may directly regulate AQP-2 mRNA expression. In the present study, we determined that hypotonicity regulates the transcription of the AQP-2 gene in an *in vitro* model.

Methods

Cell culture

Mouse inner medullary collecting duct ($mIMCD_3$; CRL-2123) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The passage number of the cells was nine. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MI, USA) supplemented with 5 mmol l^{-1} D-glucose, penicillin G (100 units ml^{-1}), streptomycin (100 $\mu g\,ml^{-1}$) and 10% fetal bovine serum (FBS). To clarify the effects of hypotonicity, all experiments were performed in medium containing a low concentration of FBS (0.5%).

Preparation of hypotonic and isosmotic media

The control medium (300 mosmol kg^{-1}) was standard DMEM, which contains 109 mmol l^{-1} NaCl. A custom-made NaCl-free DMEM was prepared by Wako Biochemicals Co. (Tokyo, Japan). In comparison with standard DMEM, the NaCl-free DMEM has an identical composition of all constituents except for NaCl (see Table 1). Thus, Na^+ ions are present as the salts, e.g. $NaHCO_3$. Its measured osmolality by freezing point depression was 99.7 mosmol kg^{-1} (mean value). Sodium chloride at a concentration of 37.5, 50.0, 62.5, 75.0 or 87.5 mmol l^{-1} was added into the NaCl-free DMEM to make hypotonic DMEM with an osmolality of 175, 200, 225, 250 or 275 mosmol kg^{-1}, respectively (Table 1). Also, we made an isosmotic, but hypotonic urea-supplemented medium by addition of NaCl (62.5 mmol l^{-1}) and urea (75 mmol l^{-1}; Table 1). Urea is a permeable solute, so that the estimated tonicity remained as low as around 225 mosmol kg^{-1}.

Plasmid construction for reporter assay

At first, to make *Sfi*I and *Eco*RI sites in the multiple cloning site of pGL3-basic vector (Promega, Madison, WI, USA), annealed synthetic oligonucleotide fragments, 5′-TCGAGGGCCTGTACGGCCTCGCGAAGAATTCGAT-ATCA-3′ and 5′-AGCTTGATATCGAATTCTTCGCGAG-GCCGTACAGGCCC-3′, were inserted between the *Xho*I and *Hind*III restriction sites, and named a modified-pGL3-basic vector. As with our previous study (Kasono *et al.* 2005), the murine AQP-2 gene with a total of 14 kb of flanking sequence (∼10.5 kb from the 5′ side of the initiation codon and 3.5 kb from the 3′ side of the termination codon) was cloned into pSPORT-1 (Invitrogen, Tokyo, Japan) from overlapping λ-clones. In this study, all primers for polymerase chain reaction (PCR) amplifications of the murine AQP-2 promoter region were constructed according to the sequencing data of the murine AQP-2 gene reported by Nelson *et al.* (National Center for Biotechnology, National Institute

© 2008 The Authors. Journal compilation © 2008 The Physiological Society

of Health, accession number AY055468). Introduction of *Sfi*I and *Eco*RI sites were performed by PCR using upper primers 5′-ACTCGAATGGCCTGTACGGCCG-AAACATCACACTTAGGAAG-3′ (murine AQP-2 promoter from bp −9516 to −9497), 5′-ACTCGAATG-GCCTGTACGGCCTCATTCATTCATTACCCGTG-3′ (from bp −6041 to −6022) and 5′-ACTCGAATGG-CCTGTACGGCCTGAGGCAGCTCCATGGGGTA-3′ (from bp −356 to −337), and lower primer 5′-CTAGCCAGAATTCATGCTGCTCGGCCTTCTGAGCG-3′ (from bp 1 to −20). After cutting these PCR products with *Sfi*I and *Eco*RI, the fragments were cloned into the modified-pGL3-basic vector. These constructs contained 9.5, 6.1 and 0.36 kb of 5′-flanking regions of the AQP-2 promoter, and were named −9.5AQP2, −6.1AQP2 and −0.36AQP2, respectively. We also cut out 1.1 kb of the 5′-flanking region between the *Hin*dIII sites from the −9.5AQP2. In this 1.1 kb fragment, the 5′ site is located within the genomic sequence and the 3′ site is located in the multiple cloning site of vector. We inserted this 1.1 kb fragment into the *Hin*dIII sites of modified-pGL3-basic vector (−1.1AQP2). The sequences of all plasmids were confirmed by dideoxy sequencing.

A truncated TonE-binding protein (TonEBP) containing only the DNA-binding site (N terminal 472 amino acids) showed dominant-negative activity on TonE-mediated stimulation of reporter gene expression (Miyakawa *et al.* 1998; Woo *et al.* 2002). The truncated TonEBP cloned into the multiple cloning site of pcDNA3 vector, and named as pDNTonEBP, was kindly provided by H. M. Kwon, Johns Hopkins University, Baltimore, MD, USA.

Transient transfections

Murine $IMCD_3$ cells (5×10^4) were cultured in 24-well tissue culture plates (16 mm in diameter) and grown for 24 h in standard DMEM medium. The cells were transfected with 0.45 μg of DNA per well of the promoter vectors described above. Also, 0.45 μg of DNA per well of murine AQP-2 cDNA was inserted in the multiple cloning site of pcDNA3 vector, and co-transfected into the cells. For internal control of transfection, 0.1 μg of DNA per well of pRL-TK vector (Promega) was transfected into the cells. Transfections were carried out using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Transfection efficacy was determined by using the pGL3-green fluorescence protein (GFP) vector, in which GFP was inserted into the *Hin*dIII site of the modified pGL3-basic vector as described in our previous report (Kasono *et al.* 2005). The number of fluorescence-positive cells per microscopic field was counted and expressed as a percentage of the total cells observed. In these conditions, the transfection ratio was around 30%. The expression of AQP-2 was confirmed by Western blotting. Hypotonic stress was induced by the 225 mosmol kg^{-1} medium or the 300 mosmol kg^{-1} medium containing urea (Table 1), and the cells were cultured for an additional 48 h before the assay. There was no difference in cell numbers after 48 h of transfection between the control and hypotonic conditions (225 mosmol kg^{-1}). Standard DMEM was used for cells exposed to isotonic conditions. The p38 mitogen-activated protein (MAP) kinase inhibitor SB203580 (1 μmol l^{-1}), MAP kinase kinase (MEK) inhibitor U0126 (1 μmol l^{-1}), JNK inhibitor SP600125 (1 and 10 μmol l^{-1}; Wojtaszek *et al.* 1998) or phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (1 μmol l^{-1}; Promega), dissolved in dimethyl sulphoxide (DMSO), were added 1 h before the hypotonic conditions were induced. The DMSO was also added to the control cultures to ensure that all wells had the same concentration of 1 μmol l^{-1} DMSO.

Cell viability assay

We determined the effect of hypotonicity on cell shape and cell viability. To observe cell shape, a Diaphot inverse microscope (Nikon, Tokyo, Japan) was used. To determine cell viability, the CellTiter-Glo Luminescent cell viability assay (Promega) was used. Murine $IMCD_3$ cells (5×10^4) were cultured in 24-well tissue culture plates with the 300 mosmol kg^{-1} medium and grown for 24 h. The cells were transfected with pGL3-basic vector, pRL-TK vector and pcDNA3 vector. Twenty-four hours later, the medium was changed to the hypotonic medium. The cells were incubated in the hypotonic medium for an additional 48 h. After washing twice with PBS, 0.5 ml of serum-free hypotonic medium and CellTiter-Glo Reagent (500 μl $well^{-1}$) were added to each well. A 100 μl aliquot of the lysate was used for the assay using a TD-2020 luminometer (Turner Designs, Sunnyvale, CA, USA). Luminescence was calculated as a ratio compared with control wells containing isotonic medium.

Luciferase and protein assay

Luciferase (Luc) activities were measured after 48 h incubation. In some experiments, 5 μmol l^{-1} dibutyryl cAMP (DBcAMP; Sigma-Aldrich) was added to the medium 6 or 24 h before the Luc assay. Cell lysis buffer (100 μl $well^{-1}$) was added to each well. A 10 μl aliquot of the lysate was used for protein assay using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). The rest of the lysate was analysed for Luc activity using the Luciferase Assay System (Promega) and a TD-2020 luminometer (Turner Designs). Luciferase activity was standardized by the corresponding renilla luciferase activity in most cases and by the amount of protein when the treatments were performed in samples originating from the same transfection. Data are presented as fold induction, usually

© 2008 The Authors. Journal compilation © 2008 The Physiological Society

compared with the control wells cultured in the isotonic medium in the absence of DBcAMP.

Statistical analysis

The data are expressed as means ± S.E.M. Values of Luc activity were analysed by analysis of variance (two-way ANOVA) and Student's unpaired t test. A P value of less than 0.05 was considered significant.

Results

At first, we determined cellular viability when exposed to the hypotonic medium. Figure 1 shows the effects of hypotonic media on cell viability and cell shape in the mIMCD$_3$ cells. The cell viability assay is based on the ATP content, hence it measures metabolically active cells. As shown in Fig. 1*A*, the luminescence in media of 200 and 175 mosmol kg^{-1} was significantly lower than in the control medium (300 mosmol kg^{-1}), but was maintained at more than 80% of control values in the 225 mosmol kg^{-1} medium. As shown in Fig. 1*B*, rounded cells were evident in the 200 and 175 mosmol kg^{-1} media. Cells exposed to the 225 mosmol kg^{-1} medium had sufficient viability and normal morphology to be used in the present study. We also determined cell number and protein contents after exposure to hypotonic medium of 225 mosmol kg^{-1}. There were no differences in

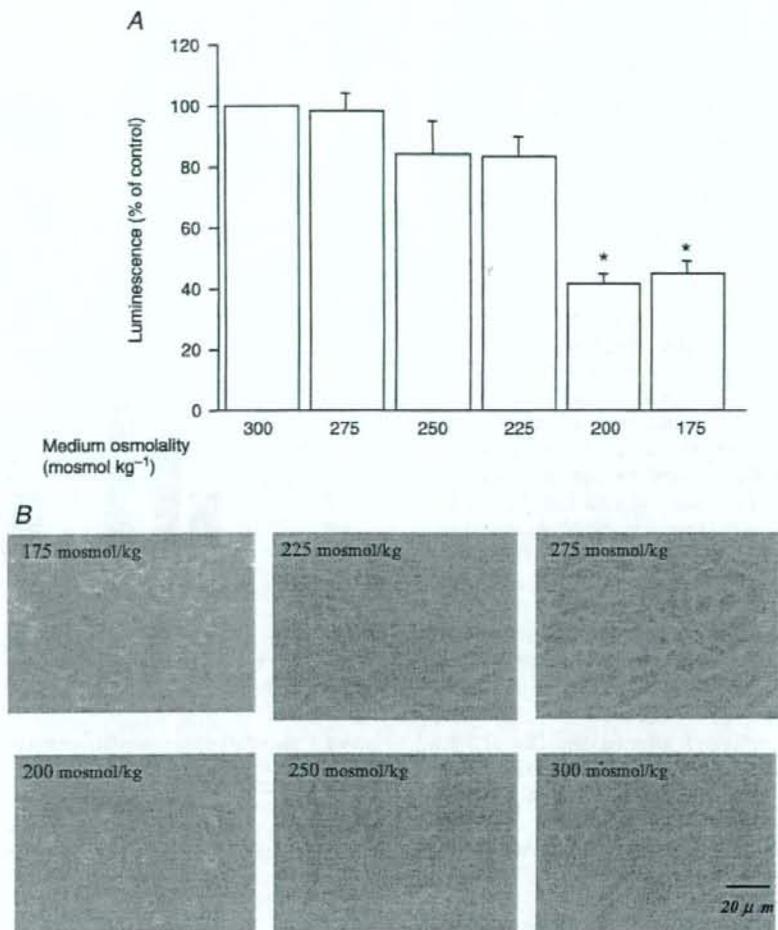

Figure 1. The effect of hypotonic media on cell viability (*A*) and cell shape (*B*) in the mIMCD$_3$ cells
A, to assess cell viability, transfected cells were incubated with isotonic (300 mosmol kg^{-1}) or hypotonic medium (175, 200, 225, 250, 275 mosmol kg^{-1}) for 48 h. $^*P < 0.05$ *versus* the vehicle group in the isotonic condition. Values are means ± S.E.M., $n = 4$. *B*, cell shape, visualized at magnification ×200.

© 2008 The Authors. Journal compilation © 2008 The Physiological Society

cell number ($2.33 \pm 0.41 \times 10^5$ *versus* $2.56 \pm 0.26 \times 10^5$, $n = 6$, n.s.) and protein contents ($2.65 \pm 0.21 \times 10^2$ *versus* $2.81 \pm 0.16 \times 10^2$ μg per well, $n = 6$, n.s.) between cultures in 225 and 300 mosmol kg^{-1} media.

In the present study, all experiments were performed in mIMCD$_3$ cells co-transfected with the vectors for the constructs and AQP-2 cDNA, because native mIMCD$_3$ cells do not express aquaporin-2. Figure 2 shows the effect of hypotonicity on DBcAMP-induced Luc activity in the mIMCD$_3$ cells co-transfected with −6.1AQP2 and murine AQP-2. In isotonic medium, the Luc activity significantly increased in a time-dependent manner in response to 5 μmol l^{-1} DBcAMP. In the hypotonic medium, the response to DBcAMP was markedly reduced. Similar results were obtained with the cells co-transfected with −1.1AQP2 and murine AQP-2 (data not shown).

The promoter of the murine AQP-2 gene contains the binding sites for CRE and TonEBP, and these elements are located at −310 to −304 bp (GACGTCA) and −570 to −560 bp (TGGAAATTTGT), respectively. Figure 3 shows the fold induction of Luc activities of several constructs induced by 5 μmol l^{-1} DBcAMP under isotonic and hypotonic conditions. A 24 h exposure of cells to 5 μmol l^{-1} DBcAMP significantly increased the fold induction of Luc activities of −9.5AQP2, −6.1AQP2 and −1.1AQP2 in isotonic media. However, inductions of Luc activities under hypotonic conditions were totally abolished despite stimulation with DBcAMP. These results suggested that the hypotonicity responsive region is located in 1.1 kb of the 5′-flanking region of AQP-2 gene. To evaluate whether this response is mediated via TonE, the study was also performed with −0.36AQP2, which contains the CRE but does not include TonE. In both the isotonic and the hypotonic medium, 5 μmol l^{-1} DBcAMP stimulated the Luc activity equally. That is to say, the inhibition of DBcAMP-induced Luc activity by hypotonicity disappeared. Moreover, hypotonic medium did not affect the increase in 5 μmol l^{-1} DBcAMP-induced Luc activity of −1.1AQP2 when the cells were co-transfected with pDNTonEBP (1 μg well^{-1}; Fig. 4).

Figure 5 depicts the effects of various substances on the AQP-2 promoter activity in cells co-transfected with −1.1AQP2 and murine AQP-2 gene in hypotonic medium. Neither 1 μmol l^{-1} SB203580 (p38 inhibitor) nor U0126 (MEK inhibitor) affected the Luc activity of −1.1AQP2. Also, 1 μmol l^{-1} wortmannin (PI3K inhibitor) did not alter the Luc activity in response to DBcAMP in the hypotonic conditions. However, 1 μmol l^{-1} SP600125, an inhibitor of JNK kinase, totally restored the response of Luc activity to 5 μmol l^{-1} DBcAMP in the hypotonic medium. At 1 μmol l^{-1}, SB203580, U0126, wortmannin or SP600125 did not affect the DBcAMP-induced Luc activity in the isotonic medium. Similar results were obtained with the co-transfection of −6.1AQP2 and murine AQP-2 (data not shown).

Whether osmolality or tonicity is important for modulating the Luc activity of AQP-2 promoter was examined. Figure 6 shows the effect of isosmolality (not isotonicity) on the Luc activity in the cells co-transfected with −1.1AQP2 and murine AQP-2 gene. In the isotonic conditions, exposure of cells to 5 μmol l^{-1} DBcAMP significantly increased the Luc activity in a time-dependent manner. In the hypotonic conditions, the DBcAMP-induced Luc activity was totally blunted. In the isosmotic, urea-supplemented medium (estimated

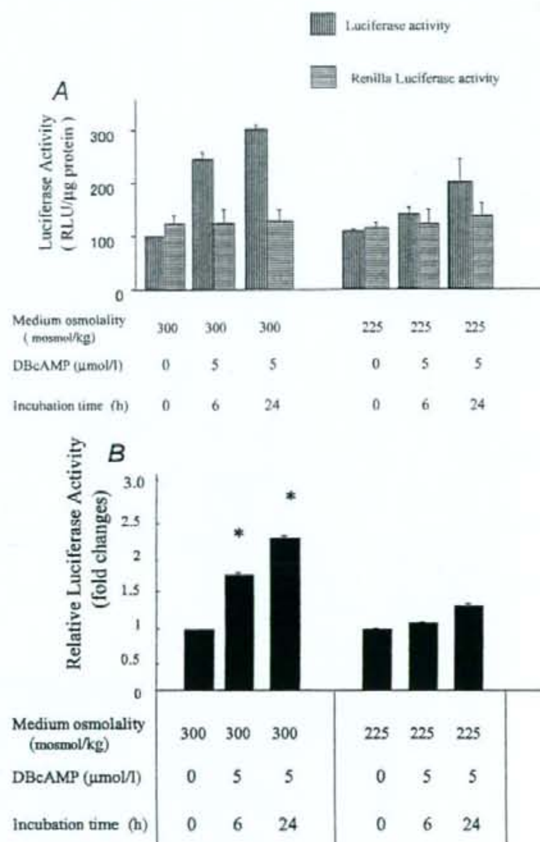

Figure 2. The effect of hypotonicity on the AQP-2 promoter activity when the IMCD$_3$ cells were transfected with −6.1AQP2 and murine AQP-2 gene

The transfected cells were incubated in the isotonic (300 mosmol kg^{-1}) or the hypotonic medium (225 mosmol kg^{-1}) for 48 h. Dibutyryl cAMP (5 μmol l^{-1}) was added to the cells cultured in the medium at time point of 24 or 42 h. *$P < 0.05$ *versus* the isotonic condition without DBcAMP. Values are means ± S.E.M. Each experiment was performed in quadruplicate, and the data are analysed from 3 independent experiments. *A*, results of luciferase and renilla luciferase activity are expressed as relative light units (RLU) normalized to protein concentrations. *B*, results are expressed as luciferase activity standardized to the corresponding renilla luciferase activity.

© 2008 The Authors. Journal compilation © 2008 The Physiological Society

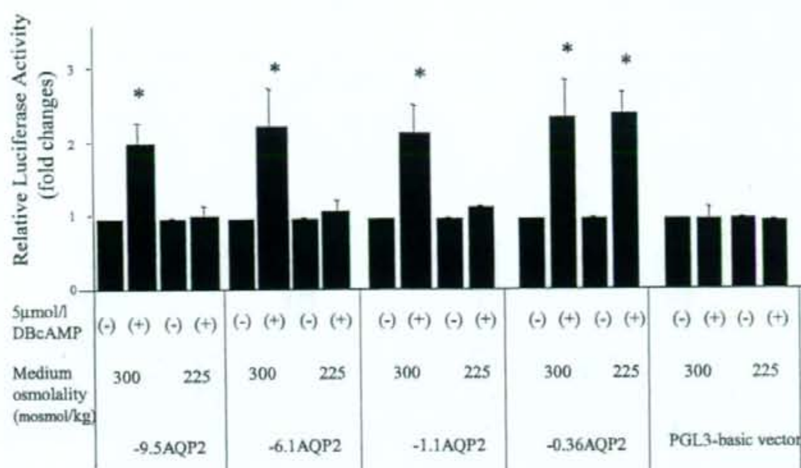

Figure 3. The effects of varying constructs of the 5′-flanking region of AQP-2 gene on the AQP-2 promoter activity
The −9.5AQP2, −6.1AQP2, −1.1AQP2, −0.36AQP2 or pGL3-basic vector was co-transfected with murine AQP-2 gene into $IMCD_3$ cells. They were incubated in the isotonic or hypotonic medium for 48 h. Dibutyryl cAMP (5 μmol l^{-1}) was added to the medium at 24 h. *$P < 0.05$ *versus* the DBcAMP (−) group in the isotonic conditions. Values are means ± S.E.M., $n = 6$.

tonicity, 225 mosmol kg^{-1}, Table 1), basal Luc activity remained unchanged and the response to 5 μmol l^{-1} DBcAMP was also totally blunted. Similar results were obtained with the co-transfection of −6.1AQP2 and murine AQP-2 gene. When the cells were co-transfected with −6.1AQP2 and murine AQP-2 gene, 24 h exposure to 5 μmol l^{-1} DBcAMP significantly increased the Luc activity by 2.2-fold in the isotonic medium. In the hypotonic medium, the fold induction of Luc activity was only 1.1-fold (n.s.). In the isosmotic, urea-supplemented (hypotonic) medium, the response of Luc activity to 24 h exposure of DBcAMP also disappeared.

Discussion

The present study shows that in $mIMCD_3$ cells that were co-transfected with −6.1AQP2 and AQP-2 gene, while DBcAMP stimulates Luc activity in isotonic media, it has

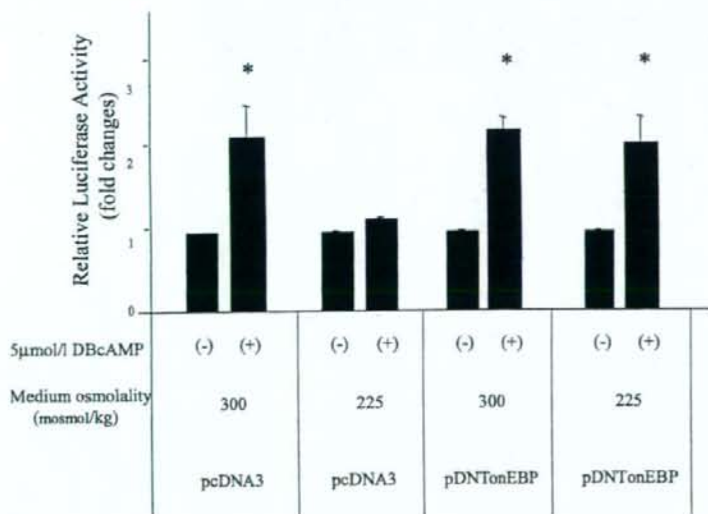

Figure 4. Effects of pDNTonEBP on hypotonicity-induced Luc activity of −1.1AQP2
The −1.1AQP2 (0.45 μg per well) was co-transfected with pcDNA3 (1.0 μg per well) or pDNTonEBP (1.0 μg per well) and incubated in the hypotonic medium for 48 h. *$P < 0.05$ *versus* the isotonic conditions without DBcAMP. Values are means ± S.E.M., $n = 6$.

© 2008 The Authors. Journal compilation © 2008 The Physiological Society