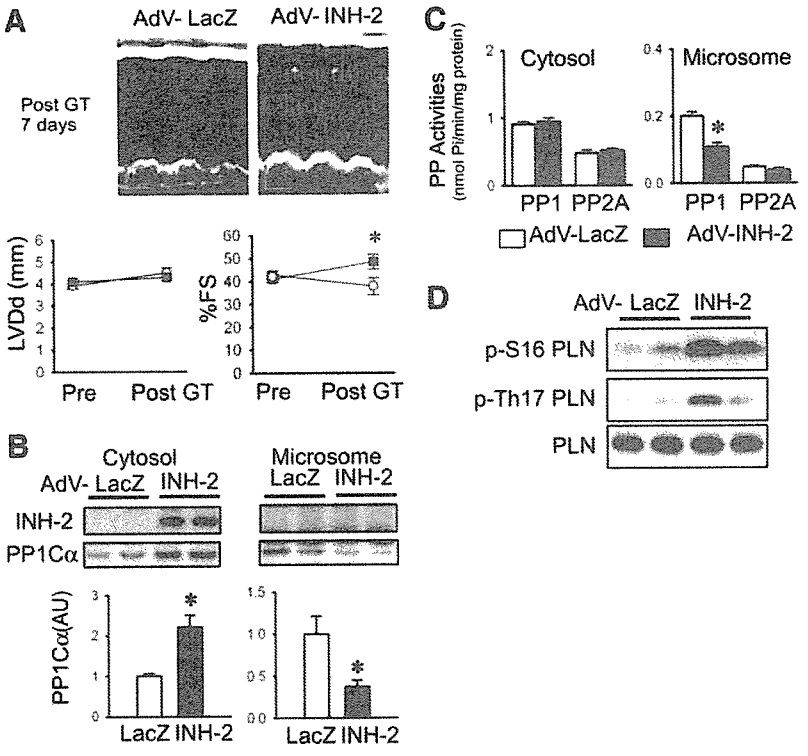

Figure 4. *In vivo* AdV-INH-2 gene transfer in normal hamsters. *A)* Representative echocardiographic M-mode images are shown for LacZ- and INH-2- treated hearts with quantitative data. The horizontal bar indicates 50 ms and the vertical bar indicates 2 mm. The LV end-diastolic dimension (LVDd) and percent fractional shortening (%FS) are shown before and 7 days after the gene-transfer in the AdV-LacZ (open circles, $n=5$) and AdV-INH-2 (solid squares, $n=6$) groups. $*P < 0.05$ compared with LacZ-treated control hamsters. *B)* Expression levels of INH-2 and PP1 catalytic subunits (PP1Cα) were determined in the LacZ- and INH-2-treated groups in the cytosol and microsomes. Representative blottings are shown with quantitative analysis. *C)* PP1 and PP2A activities are shown in the cytosol and the microsomes following the adenoviral gene transfer. $*P < 0.05$ compared with the LacZ group. *D)* Representative immunoblottings are shown for Ser16 PLN, Thr17 PLN, and total PLN in the LacZ- and INH-2-treated groups. INH-2 gene transfer significantly increased Ser16 PLN phosphorylation.

expression in the short term, we further tested the long-term effect of INH-2 gene delivery by AAV-mediated gene transfer, as described previously (19, 25). We performed AAV-mediated gene transfer of INH-2 at 14 wk of age, followed by serial assessment of cardiac function for 12 wk after the gene transfer by echocardiography and hemodynamic analysis. Comparison of the survival time course between the AAV-INH-2- and AAV-LacZ-treated groups was made during this 12 wk follow-up period. As shown in **Figure 7*A***, the transfection efficiency of AAV-LacZ was ~40% of the LV cross-sectional area, which was similar to that by adenoviral gene delivery. The expression of exogenous INH-2 was confirmed by immunoblotting in the cardiac homogenate as shown in Figure 7*B*. AAV-INH-2 gene transfer preserved %FS compared with the AAV-LacZ-treated group over the observation period (Fig. 7*C*). The left ventricular end-diastolic diameter showed decreasing tendency at 1 and 3 mo following gene transfer, but did not reach the level of statistical significance (data not shown). The maximum dP/dt at 26 wk of age in the AAV-INH-2 group also showed a tendency toward preservation ($P=0.051$) compared with the AAV-LacZ group, whereas LV peak systolic pressure and heart rate did not show any significant difference (Table 1). Other hemodynamic parameters also did not show statistical significance as summarized in Table 1. AAV-INH-2 gene transfer significantly decreased microsomal PP1 activity (Fig. 7*D*) and increased PLN phosphorylation at Ser16 (Fig. 7*E*), as observed in the short-term adenoviral gene transfer, although PKA activity showed no differences between in the AAV-INH-2-treated and the AAV-LacZ-treated groups (data not shown). In addition, there was significantly less interstitial fibrosis in INH-2-treated CM hamster hearts (Fig. 7*F*). It should be noted that the difference between the AAV-INH-2- and AAV-LacZ-treated groups in the hemodynamic data at 26 wk of age may have been biased toward dissipation. This is because 71% of the AAV-LacZ-treated animals had died before 26 wk of age, presumably because of the worsening cardiac function, and could not be assessed (see below).

In the Kaplan-Meier plot (Fig. 7*G*), the AAV-INH-2-treated group showed a 54% survival rate at 12 wk after the gene transfer, whereas the AAV-LacZ-treated CM hamsters exhibited a deleterious time course, ending with a 29% survival rate. The difference between these survival curves was statistically significant ($P=0.03$). These results clearly indicate that INH-2 gene transfer had a favorable effect on progressive HF in the long term.

DISCUSSION

In the present study, we demonstrated that *in vivo* gene transfer of INH-2 effectively prevented HF progression in the observation period of one week in adenoviral gene transfer and three months in AAV-mediated gene transfer. The INH-2 gene transfer not only preserved LV function but also reduced mRNA expression of BNP, a prognostic marker of HF in the short-term experiment. Furthermore, long-term expression of exogenous INH-2 also caused less progression of LV dysfunction and interstitial fibrosis, and the extended survival in CM hamsters. To our knowledge, this is the first report demonstrating the long-term therapeutic effect of PP1 inhibition in progressive HF using the high efficiency cardiac gene transfer approach (19, 20, 25).

Our biochemical characterization suggested that hy-

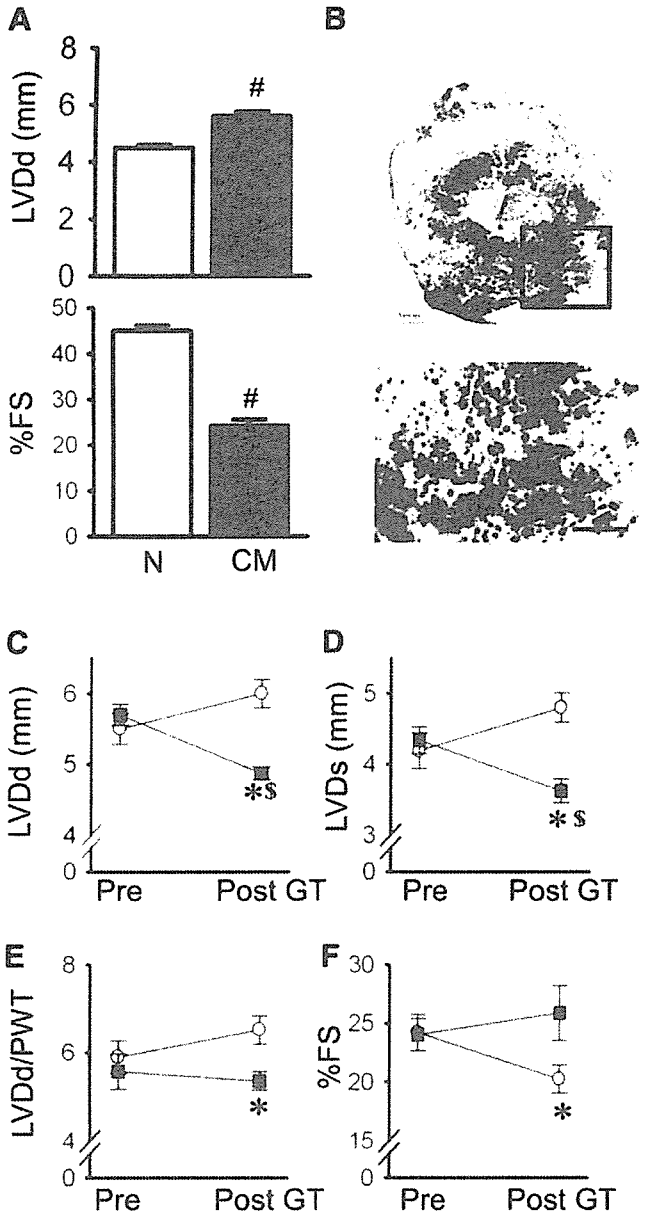

Figure 5. The effect of *in vivo* AdV-INH-2 gene transfer on LV systolic function in CM hamsters. *A)* LVDd and %FS at 14 wk of age are shown for normal (N) and cardiomyopathic (CM) hamsters (n=10 in each group). $^{\#}P < 0.05$ compared with normal hamsters. *B)* Representative Bluo-gal staining is shown at 7 days after AdV-LacZ gene transfer in a CM hamster. High magnification of the rectangular area is shown (*bottom*). *C)* The LV end-diastolic and systolic dimensions (LVDd, LVDs) are shown for CM hamsters at 7 days after the gene transfer (GT) of LacZ (open circles, n=8) or INH-2 (closed squares, n=8). The index of wall stress, LVDd/PWT and the changes of %FS are shown for the gene transfer of LacZ (open circles) and INH-2 (solid squares). $^{*}P < 0.05$ compared with LacZ-treated hamsters. $^{\$}P < 0.05$ compared with pre gene transfer.

peractivation of PKA and increase in phosphorylation of PLN at younger age in CM hamsters may represent the compensatory mechanism against deteriorating cardiac function. However, the compensatory mechanism eventually fails in the late phase, as demonstrated by rapid deterioration of cardiac function, which coincided with the increase in PP1 activity and decrease in phosphorylation of Ser16-PLN, even though PKA activity and cAMP content were still higher than those in normal hamsters. Because changes in PKA activities did not directly correlate with changes in PLN phosphorylation at the terminal stage of HF (i.e., 28 wk of age), it is suggested that defects in local regulation of PKA through a variety of A-kinase-anchoring proteins (AKAP) may also be critically involved in the phosphorylation balance in CM hamsters (29). In addition, local regulation of PP1C and its regulatory proteins may also participate in altered phosphorylation balance of phosphoproteins in the corresponding microdomains. The increase in phosphorylation of Ser16-PLN together

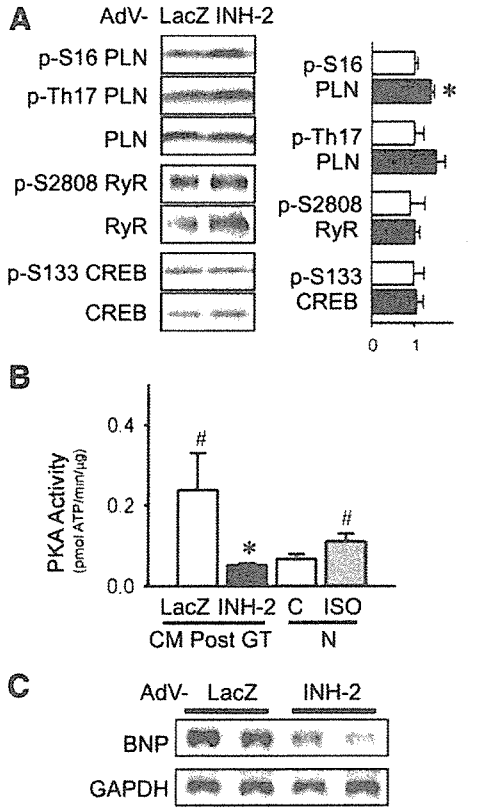

Figure 6. Effects of AdV-INH-2 gene transfer on biochemical characteristics in CM hamster hearts. *A)* Representative immunoblots are shown for the phosphorylation of Ser16-PLN (p-S_{16}PLN), Thr17-PLN (p-Th_{17}PLN), Ser2808-RyR (p-S_{2808}RyR) and Ser133-CREB (p-S_{133}CREB), along with the immunoblotting of the total levels of the corresponding proteins in LacZ-and INH-2-treated hamster LV homogenates. The ratios of p-Ser16 PLN/PLN, p-Th17 PLN/PLN, p-S2808 RyR/RyR, and p-S133 CREB/CREB (*right*) are shown relative to the LacZ-treated group, which is designated as 1 (n=6 in each group). *B)* Endogenous protein kinase A activities are shown in AdV-LacZ- or AdV-INH-2-treated groups, together with the value of baseline and maximally isoproterenol-stimulated state in age-matched normal hamster (n=6 in CM hamster groups, n=3 in normal hamster groups). *C)* Representative Northern blotting of BNP is shown after the gene transfer of LacZ or INH-2. GAPDH served as an internal control for the RNA loading (n=6 in each group). $^{*}P < 0.05$ compared with the LacZ group. $^{\#}P < 0.05$ compared with baseline of normal hamsters. Open and solid columns indicate the LacZ- and INH-2-treated group, respectively.

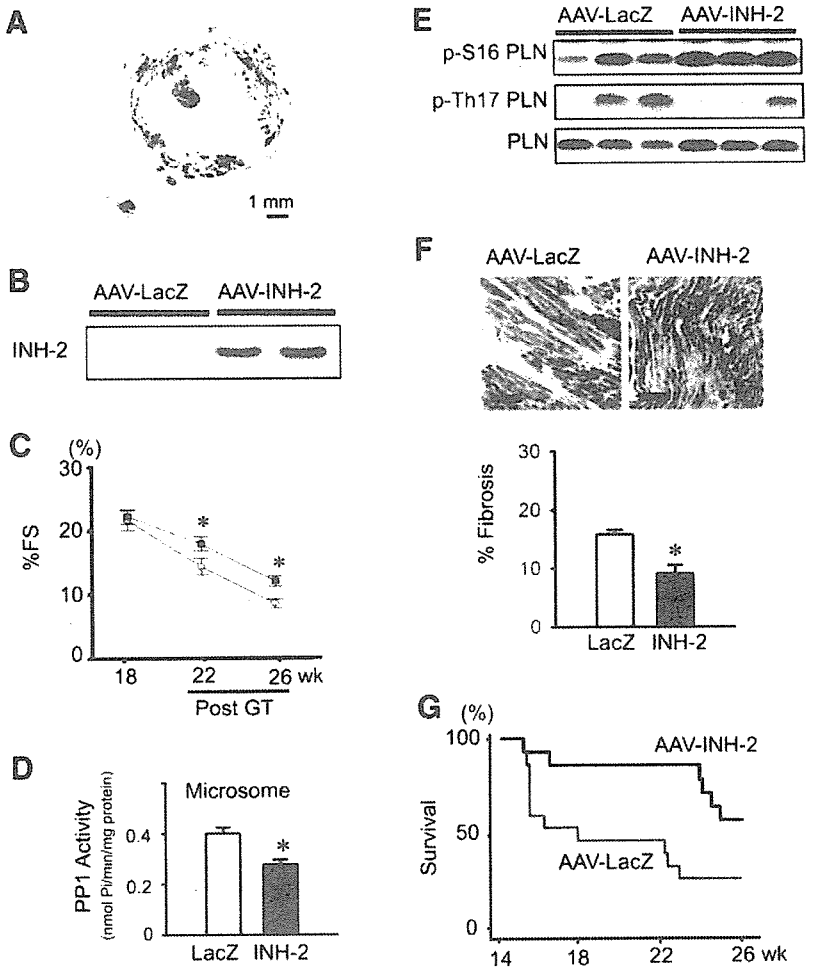

Figure 7. Effects of AAV-INH-2 gene transfer on cardiac function and survival time course in CM hamsters. *A)* Representative Bluo-gal staining is shown for the LV cross section at 12 wk after AAV-LacZ gene transfer. *B)* A representative immunoblot is shown for INH-2 at 12 wk after AAV-LacZ gene transfer. *C)* The time course of % fractional shortening (%FS) assessed by echocardiography is shown for the LacZ group (open circles) and INH-2 group (closed squares) gene transfer by AAV. $*P < 0.05$ compared with the LacZ group. *D)* Microsomal PP1 activity is shown after AAV-mediated gene transfer into hearts of the LacZ (n=5) and INH-2 groups (n=7). $*P < 0.05$ compared with the LacZ-treated group. *E)* Representative immunoblottings are shown for Ser16 PLN, Thr17 PLN, and total PLN in the LacZ-treated (n=4) and INH-2-treated (n=4) groups. *F)* Representative images are shown from tissue sections with Azan Mallory staining from the LacZ-treated (n=6) and INH-2-treated (n=7) CM hamsters. Quantification of the percent areas of fibrosis is shown in the lower panel. $*P < 0.05$ compared with the LacZ-treated group. *G)* The Kaplan-Meier plot is shown for the survival of CM hamsters after AAV-mediated gene transfer of LacZ (n=17) and INH-2 (n=13) (P=0.03).

with PKA activation during the transition to HF in the CM heart is somewhat different from previous reports (7, 8, 30). However, other studies have reported that the phosphorylation status of Ser16-PLN did not change in progressive HF (31, 32). As HF is a disease with multiple etiologies, which progresses through complicated biochemical alterations, it is expected that the phosphoprotein status of Ser16-PLN and PKA activity may differ depending on the etiology and/or the time point examined. Nonetheless, our data support the notion that hyperactive PP1 is an exacerbating factor against the compensatory mechanism of HF, because suppression of PP1 activity by INH-2 gene transfer showed beneficial effects in CM hamster where phosphorylation of PLN is still preserved compared with that in normal hamsters. Therefore, INH-2 gene transfer seems to support the compensatory mechanism in the failing heart, resulting in better cardiac function and survival.

Our data suggested that the beneficial effect of INH-2 gene delivery is, at least in part, attributable to the increase in phosphorylation of Ser16-PLN at Ser16, because inhibition of endogenous PLN has been reported to be beneficial in treating certain types of HF (19, 25, 33–35), including HF in the other hamster model of cardiomyopathy (i.e., the BIO14.6 strain model) (19). Interestingly, INH-2 gene transfer *in vivo* caused a decrease in microsomal PP1 activity, which may be explained by a translocation of the PP1Cα protein from the microsome to the cytosol. Increase in

TABLE 1. *Hemodynamic parameters in AAV-LacZ- and AAV-INH-2-transferred CM hamsters*

Hamster age	Heart Rate (bpm)	LVPSP (mmHg)	dP/dt max (mmHg/s)	dP/dt min (mmHg/s)	LVEDP (mmHg)	Tau (ms)
AAV-LacZ-treated CM hamsters (n = 5)						
12 wk after GT	317 ± 21	91 ± 7	4383 ± 383	−3265 ± 141	6.0 ± 3.7	11.0 ± 2.6
AAV-INH-2-treated CM hamsters (n = 7)						
12 wk after GT	321 ± 8	99 ± 4	5233 ± 273	−3870 ± 396	6.8 ± 3.7	10.3 ± 1.3

LVPSP, LV peak systolic pressure; LVEDP, LV end-diastolic pressure; dP/dt max, dP/dt min maximum and minimum of the first derivative of LV pressure; Tau(τ), time constant of LV pressure decay during isovolumic relaxation calculated from exponential fitting.

cytosolic PP1Cα in normal and CM hamster, increasing trend of cytosolic PP1 activity in CM hamster may also be explained by the function of INH-2 to stabilize PP1Cα in the cytosol, as suggested previously (15). Although the molecular mechanisms by which INH-2 induces a translocation of PP1C remain to be determined, the subsequent decrease in microsomal PP1 activity appeared to be well coupled with the increase in Ser16 PLN phosphorylation by INH-2 overexpression.

Intriguingly, INH-2 gene transfer normalized the hyperactive PKA in CM hamsters in the short-term experiment. This was interpreted that INH-2 augmented PLN phosphorylation, thereby enhancing cardiac contractility in the failing heart without PKA activation. Enhanced cardiac performance by INH-2 gene transfer relieved the hyperactive sympathetic tone and concomitant β-adrenergic signaling in these CM hamsters, thereby decreasing PKA activity in the heart. This interpretation is supported by the trend of a decrease in serum catecholamine levels in the I-2-treated CM hamsters. Although hyperactivation of the β-adrenergic system enhances cardiac output in the short term, it may be cardiotoxic over the long run, according to the failed clinical trials of phosphodiesterase III inhibitors (36) and *in vivo* transgenic animal studies (37, 38). In addition, sympathetic hyperactivation results in the increase in the peripheral vascular resistance, thereby increasing the afterload of the failing heart (1). Therefore, the enhanced cardiac function via PLN phosphorylation without activating PKA may explain the extended survival time in CM hamsters by INH-2 gene transfer.

The phosphorylation status of RyR appeared to be less affected in *in vivo* INH-2-treated hamsters, whereas *in vitro* adenoviral INH-2 gene transfer was associated with an increase in RyR phosphorylation, as well as an increase in PLN phosphorylation in the cardiomyocytes. This difference of the effect of INH-2 on RyR phosphorylation between *in vivo* and *in vitro* experiments may be explained by the extent of PP1 inhibition and the difference of PP1C expression levels between *in vitro* and *in vivo*, and local regulation of PKA and PP1 activities as discussed above (29). These findings suggest that PLN phosphorylation is more sensitive to the modulation of PP1 activity. This differential effect of INH-2 on RyR and PLN may be beneficial because hyperphosphorylation of RyR may cause Ca^{2+} leak leading to arrhythmogenicity (39) and cardiac dysfunction (40), although the effect of INH-2 gene transfer on arrhythmogenicity remains to be determined. It also remains to be determined whether INH-2 gene delivery causes some adverse effects, such as carcinogenesis or metabolic disorder, since systemic PP inhibition by chemicals has been known to be hazardous (41).

Recently, Pathak et al. (14) have demonstrated that transgenic PP1 inhibition by constitutively active INH-1 suppressed the LV remodeling in an aortic constriction model and prevented the progression of cardiac failure in aorta-banded rats in the short-term using adenoviral mediated *in vivo* gene transfer approach. Although the long-term benefit of PP1 inhibition by gene transfer was not evaluated in their study, their results are in agreement with the present study regarding the benefit of selective PP1 inhibition and the enhancement of PLN phosphorylation in the failing heart.

In conclusion, we have demonstrated that *in vivo* myocardial PP1 inhibition by inhibitor-2 up-regulated SR calcium handling through PLN phosphorylation and corrected the abnormally increased PKA activity, thereby ameliorating the long-term progression of HF in the cardiomyopathic hamster. PP1 inhibition in the sarcoplasmic reticulum and sarcolemma may constitute a new therapeutic target for the treatment of end-stage HF. FJ

We thank Hiromi Nakagawa, Tomoko Hozawa, Megumi Ohishi, and Rie Ishihara for technical assistance, and Tomoko Ohkusa for her maintenance of the CM hamster colony. This study was supported by grants from the Ministry of Education, Science and Technology of Japan [Nos. 14370228 (M.M.), 15590754 (Y.I.), 16209026 (M.M.)] and by Grants-in-Aid from the Japan Heart Foundation Molecular Cardiovascular Research Group (Y.I.), the Mitsubishi Pharma Research Foundation (M.M.), the Japan Cardiovascular Research Foundation (Y.I.), the Takeda Science Foundation (Y.I.), and the Kanae Foundation for Life & Socio-Medical Science (YI), and by a Grant from the Sankyo Pharmaceutical Inc. for the Department of Molecular Cardiovascular Biology, Yamaguchi University, School of Medicine.

REFERENCES

1. Cohn, J. N., Bristow, M. R., Chien, K. R., Colucci, W. S., Frazier, O. H., Leinwand, L. A., Lorell, B. H., Moss, A. J., Sonnenblick, E. H., Walsh, R., et al. (1997) Report of the National Heart, Lung, and Blood Institute Special Emphasis Panel on Heart Failure Research. *Circulation* **95,** 766–770
2. Wehrens, X. H., and Marks, A. R. (2004) Novel therapeutic approaches for heart failure by normalizing calcium cycling. *Nat. Rev. Drug Discov.* **3,** 565–573
3. Chien, K. R. (1999) Stress pathways and heart failure. *Cell* **98,** 555–558
4. Bers, D. M. (2002) Cardiac excitation-contraction coupling. *Nature* **415,** 198–205
5. Neumann, J., Boknik, P., Herzig, S., Schmitz, W., Scholz, H., Gupta, R. C., and Watanabe, A. M. (1993) Evidence for physiological functions of protein phosphatases in the heart: evaluation with okadaic acid. *Am. J. Physiol.* **265,** H257–H266
6. Marx, S. O., Reiken, S., Hisamatsu, Y., Jayaraman, T., Burkhoff, D., Rosemblit, N., and Marks, A. R. (2000) PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* **101,** 365–376
7. Huang, B., Wang, S., Qin, D., Boutjdir, M., and El-Sherif, N. (1999) Diminished basal phosphorylation level of phospholamban in the postinfarction remodeled rat ventricle: role of beta-adrenergic pathway, G(i) protein, phosphodiesterase, and phosphatases. *Circ. Res.* **85,** 848–855
8. Sande, J. B., Sjaastad, I., Hoen, I. B., Bokenes, J., Tonnessen, T., Holt, E., Lunde, P. K., and Christensen, G. (2002) Reduced level of serine(16) phosphorylated phospholamban in the failing rat myocardium: a major contributor to reduced SERCA2 activity. *Cardiovasc. Res.* **53,** 382–391
9. Boknik, P., Fockenbrock, M., Herzig, S., Knapp, J., Linck, B., Luss, H., Muller, F. U., Muller, T., Schmitz, W., Schroder, F., and Neumann, J. (2000) Protein phosphatase activity is increased in a rat model of long-term beta-adrenergic stimulation. *Naunyn Schmiedebergs Arch. Pharmacol.* **362,** 222–231
10. Gupta, R. C., Mishra, S., Rastogi, S., Imai, M., Habib, O., and Sabbah, H. N. (2003) Cardiac SR-coupled PP1 activity and expression are increased and inhibitor 1 protein expression is

decreased in failing hearts. *Am. J. Physiol. Heart Circ. Physiol.* **285,** H2373–H2381
11. Neumann, J., Eschenhagen, T., Jones, L. R., Linck, B., Schmitz, W., Scholz, H., and Zimmermann, N. (1997) Increased expression of cardiac phosphatases in patients with end-stage heart failure. *J. Mol. Cell Cardiol.* **29,** 265–272
12. Carr, A. N., Schmidt, A. G., Suzuki, Y., del Monte, F., Sato, Y., Lanner, C., Breeden, K., Jing, S. L., Allen, P. B., Greengard, P.,et al. (2002) Type 1 phosphatase, a negative regulator of cardiac function. *Mol. Cell. Biol.* **22,** 4124–4135
13. El-Armouche, A., Rau, T., Zolk, O., Ditz, D., Pamminger, T., Zimmermann, W. H., Jackel, E., Harding, S. E., Boknik, P., Neumann, J., and Eschenhagen, T. (2003) Evidence for protein phosphatase inhibitor-1 playing an amplifier role in beta-adrenergic signaling in cardiac myocytes. *FASEB J.* **17,** 437–439
14. Pathak, A., Del Monte, F., Zhao, W., Schultz, J. E., Lorenz, J. N., Bodi, I., Weiser, D., Hahn, H., Carr, A., Syed, F., et al. (2005) Enhancement of cardiac function and suppression of heart failure progression by inhibition of protein phosphatase 1. *Circ. Res.* **96,** 756–766
15. Kirchhefer, U., Baba, H. A., Boknik, P., Breeden, K. M., Mavila, N., Bruchert, N., Justus, I., Matus, M., Schmitz, W., Depaoli-Roach, A. A. et al.. (2005) Enhanced cardiac function in mice overexpressing protein phosphatase inhibitor-2. *Cardiovasc. Res.* **68,** 98–108
16. Tsubata, S., Bowles, K. R., Vatta, M., Zintz, C., Titus, J., Muhonen, L., Bowles, N. E., and Towbin, J. A. (2000) Mutations in the human delta-sarcoglycan gene in familial and sporadic dilated cardiomyopathy. *J. Clin. Invest.* **106,** 655–662
17. Karkkainen, S., Miettinen, R., Tuomainen, P., Karkkainen, P., Helio, T., Reissell, E., Kaartinen, M., Toivonen, L., Nieminen, M. S., Kuusisto, J., Laakso, M., and Peuhkurinen, K. (2003) A novel mutation, Arg71Thr, in the delta-sarcoglycan gene is associated with dilated cardiomyopathy. *J. Mol. Med.* **81,** 795–800
18. Ikeda, Y., Martone, M., Gu, Y., Hoshijima, M., Thor, A., Oh, S. S., Peterson, K. L., and Ross, J., Jr. (2000) Altered membrane proteins and permeability correlate with cardiac dysfunction in cardiomyopathic hamsters. *Am. J. Physiol. Heart Circ. Physiol.* **278,** H1362–H1370
19. Hoshijima, M., Ikeda, Y., Iwanaga, Y., Minamisawa, S., Date, M. O., Gu, Y., Iwatate, M., Li, M., Wang, L., Wilson, J. M., Wang, Y., Ross, J., Jr., and Chien, K. R. (2002) Chronic suppression of heart-failure progression by a pseudophosphorylated mutant of phospholamban via in vivo cardiac rAAV gene delivery. *Nat. Med.* **8,** 864–871
20. Ikeda, Y., Gu, Y., Iwanaga, Y., Hoshijima, M., Oh, S. S., Giordano, F. J., Chen, J., Nigro, V., Peterson, K. L., Chien, K. R., and Ross, J., Jr. (2002) Restoration of deficient membrane proteins in the cardiomyopathic hamster by in vivo cardiac gene transfer. *Circulation* **105,** 502–508
21. Mackintosh, K. (1993) serine/threonine phosphatase assay and purification. In *Protein Phosphorylarion: A Practical Approach* (Graham, H., H., ed.) pp. 167–195, Oxford University Press, New York.
22. Okuda, S., Yano, M., Doi, M., Oda, T., Tokuhisa, T., Kohno, M., Kobayashi, S., Yamamoto, T., Ohkusa, T., and Matsuzaki, M. (2004) Valsartan restores sarcoplasmic reticulum function with no appreciable effect on resting cardiac function in pacing-induced heart failure. *Circulation* **109,** 911–919
23. Reiken, S., Lacampagne, A., Zhou, H., Kherani, A., Lehnart, S. E., Ward, C., Huang, F., Gaburjakova, M., Gaburjakova, J., Rosemblit, N., Warren, M. S., He, K. L., Yi, G. H., Wang, J., Burkhoff, D., Vassort, G., and Marks, A. R. (2003) PKA phosphorylation activates the calcium release channel (ryanodine receptor) in skeletal muscle: defective regulation in heart failure. *J. Cell Biol.* **160,** 919–928
24. Aoki, H., Kang, P. M., Hampe, J., Yoshimura, K., Noma, T., Matsuzaki, M., and Izumo, S. (2002) Direct activation of mitochondrial apoptosis machinery by c-Jun N-terminal kinase in adult cardiac myocytes. *J. Biol. Chem.* **277,** 10244–10250
25. Iwanaga, Y., Hoshijima, M., Gu, Y., Iwatate, M., Dieterle, T., Ikeda, Y., Date, M. O., Chrast, J., Matsuzaki, M., Peterson, K. L., Chien, K. R., and Ross, J., Jr. (2004) Chronic phospholamban inhibition prevents progressive cardiac dysfunction and pathological remodeling after infarction in rats. *J. Clin. Invest.* **113,** 727–736
26. Yoshimura, K., Aoki, H., Ikeda, Y., Fujii, K., Akiyama, N., Furutani, A., Hoshii, Y., Tanaka, N., Ricci, R., Ishihara, T., Esato, K., Hamano, K., and Matsuzaki, M. (2005) Regression of abdominal aortic aneurysm by inhibition of c-Jun N-terminal kinase. *Nat. Med.* **11,** 1330–1338
27. Xiao, B., Jiang, M. T., Zhao, M., Yang, D., Sutherland, C., Lai, F. A., Walsh, M. P., Warltier, D. C., Cheng, H., and Chen, S. R. (2005) Characterization of a novel PKA phosphorylation site, serine-2030, reveals no PKA hyperphosphorylation of the cardiac ryanodine receptor in canine heart failure. *Circ. Res.* **96,** 847–855
28. Reiken, S., Wehrens, X. H., Vest, J. A., Barbone, A., Klotz, S., Mancini, D., Burkhoff, D., and Marks, A. R. (2003) Beta-blockers restore calcium release channel function and improve cardiac muscle performance in human heart failure. *Circulation* **107,** 2459–2466
29. Dodge-Kafka, K. L., Soughayer, J., Pare, G. C., Carlisle Michel, J. J., Langeberg, L. K., Kapiloff, M. S., and Scott, J. D. (2005) The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways. *Nature* **437,** 574–578
30. Bartel, S., Stein, B., Eschenhagen, T., Mende, U., Neumann, J., Schmitz, W., Krause, E. G., Karczewski, P., and Scholz, H. (1996) Protein phosphorylation in isolated trabeculae from nonfailing and failing human hearts. *Mol. Cell Biochem.* **157,** 171–179
31. Bohm, M., Reiger, B., Schwinger, R. H., and Erdmann, E. (1994) cAMP concentrations, cAMP dependent protein kinase activity, and phospholamban in non-failing and failing myocardium. *Cardiovasc. Res.* **28,** 1713–1719
32. Schwinger, R. H., Bohm, M., Schmidt, U., Karczewski, P., Bavendiek, U., Flesch, M., Krause, E. G., and Erdmann, E. (1995) Unchanged protein levels of SERCA II and phospholamban but reduced Ca^{2+} uptake and $Ca^{(2+)}$-ATPase activity of cardiac sarcoplasmic reticulum from dilated cardiomyopathy patients compared with patients with nonfailing hearts. *Circulation* **92,** 3220–3228
33. Minamisawa, S., Hoshijima, M., Chu, G., Ward, C. A., Frank, K., Gu, Y., Martone, M. E., Wang, Y., Ross, J., Jr., Kranias, E. G., et al. (1999) Chronic phospholamban-sarcoplasmic reticulum calcium ATPase interaction is the critical calcium cycling defect in dilated cardiomyopathy. *Cell* **99,** 313–322
34. Sato, Y., Kiriazis, H., Yatani, A., Schmidt, A. G., Hahn, H., Ferguson, D. G., Sako, H., Mitarai, S., Honda, R., Mesnard-Rouiller, L., et al. (2001) Rescue of contractile parameters and myocyte hypertrophy in calsequestrin overexpressing myocardium by phospholamban ablation. *J. Biol. Chem.* **276,** 9392–9399
35. Freeman, K., Lerman, I., Kranias, E. G., Bohlmeyer, T., Bristow, M. R., Lefkowitz, R. J., Iaccarino, G., Koch, W. J., and Leinwand, L. A. (2001) Alterations in cardiac adrenergic signaling and calcium cycling differentially affect the progression of cardiomyopathy. *J. Clin. Invest.* **107,** 967–974
36. Cruickshank, J. M. (1993) Phosphodiesterase III inhibitors: long-term risks and short-term benefits. *Cardiovasc. Drugs Ther.* **7,** 655–660
37. Antos, C. L., Frey, N., Marx, S. O., Reiken, S., Gaburjakova, M., Richardson, J. A., Marks, A. R., and Olson, E. N. (2001) Dilated cardiomyopathy and sudden death resulting from constitutive activation of protein kinase a. *Circ. Res.* **89,** 997–1004
38. Bisognano, J. D., Weinberger, H. D., Bohlmeyer, T. J., Pende, A., Raynolds, M. V., Sastravaha, A., Roden, R., Asano, K., Blaxall, B. C., Wu, S. C., et al. (2000) Myocardial-directed overexpression of the human beta(1)-adrenergic receptor in transgenic mice. *J. Mol. Cell Cardiol.* **32,** 817–830
39. Wehrens, X. H., Lehnart, S. E., Reiken, S. R., Deng, S. X., Vest, J. A., Cervantes, D., Coromilas, J., Landry, D. W., and Marks, A. R. (2004) Protection from cardiac arrhythmia through ryanodine receptor-stabilizing protein calstabin2. *Science* **304,** 292–296
40. Yano, M., Ono, K., Ohkusa, T., Suetsugu, M., Kohno, M., Hisaoka, T., Kobayashi, S., Hisamatsu, Y., Yamamoto, T., Noguchi, N., et al. (2000) Altered stoichiometry of FKBP12.6 versus ryanodine receptor as a cause of abnormal Ca(2+) leak through ryanodine receptor in heart failure. *Circulation* **102,** 2131–2136
41. Cohen, P. T. (2002) Protein phosphatase 1–targeted in many directions. *J. Cell Sci.* **115,** 241–256

Received for publication October 23, 2005.
Accepted for publication January 24, 2006.

Circ J 2006; **70:** 777–786

AT1 Receptor Antagonist Restores Cardiac Ryanodine Receptor Function, Rendering Isoproterenol-Induced Failing Heart Less Susceptible to Ca^{2+}-Leak Induced by Oxidative Stress

Takahiro Tokuhisa; Masafumi Yano; Masakazu Obayashi; Toshiyuki Noma; Mamoru Mochizuki; Tetsuro Oda; Shinichi Okuda; Masahiro Doi; Jinyao Liu; Yasuhiro Ikeda; Takeshi Yamamoto; Tomoko Ohkusa; Masunori Matsuzaki

Background The Ca^{2+} regulatory proteins in the sarcoplasmic reticulum (SR) play a key role in the pathogenesis of heart failure. In the present study the effect of chronic β-receptor-stimulation on cardiac and SR functions was assessed, with or without angiotensin-II receptor antagonist treatment recently reported to have anti-β-adrenergic activity.

Methods and Results Rats were treated with isoproterenol with (+) or without (–) candesartan (CAN) and then SR vesicles were isolated from the left ventricular muscle. Both Ca^{2+}-uptake and the amount of SR Ca^{2+}-ATPase were significantly lower in the CAN(–) group than in the shams, but those were almost normally restored in the CAN(+). Although the level of the protein kinase A (PKA)-phosphorylation of the SR Ca^{2+} release channel, known as the ryanodine receptor (RyR2), was elevated in the CAN(–), no Ca^{2+}-leak was detected. However, SIN-1 (O_2^- donor) induced Ca^{2+}-leak in the CAN(–) at a 10-fold lower dose than in the sham and CAN(+). In cardiomyocytes, SIN-1 decreased cell shortening and the peak Ca^{2+} transient and prolonged time from peak to 70% decline in CAN(–), again at 10-fold lower dose than in the sham and CAN(+).

Conclusion Chronic β-receptor-stimulation did not induce any Ca^{2+}-leak from the SR, whereas Ca^{2+}-leak was easily induced when oxidative stress was applied to the PKA-phosphorylated RyR2. Candesartan not only improved Ca^{2+}-uptake, but also prevented PKA-phosphorylation, rendering the SR less susceptible to Ca^{2+}-leak. (*Circ J* 2006; **70:** 777–786)

Key Words: Angiotensin II receptor; Calcium; Oxygen free radicals; Ryanodine receptor; Sarcoplasmic reticulum

During the development of heart failure, abnormal regulation of intracellular Ca^{2+} has been shown to contribute to the impaired cardiac contraction and relaxation[1,2] Several lines of evidence have accumulated demonstrating that decreased activity or expression of Ca^{2+}-ATPase (SERCA2a) in the sarcoplasmic reticulum (SR) may be an important cause of heart failure[3,4] In addition, abnormal Ca^{2+}-leakage through the SR Ca^{2+} release channel, known as the ryanodine receptor (RyR2), has been demonstrated as a possible trigger for the development of heart failure[5,6] In those studies of cases of heart failure, Ca^{2+}-leak was found to be induced by the dissociation of FKBP12.6 from the RyR2 as a result of the hyperphosphorylation of the RyR2. Restoration of the defective FKBP12.6-mediated stabilization of the RyR2 either by a β-blocker[7] or by a new cardioprotective agent, JTV519[8] improved cardiac function during the development of heart failure.

Angiotensin II antagonism not only prevents hypertrophy and/or interstitial fibrosis[9,10] but also attenuates the down-regulation of SERCA2a and improves intracellular Ca^{2+} handling[11,12] In a canine model of heart failure, we recently demonstrated that during the development of pacing-induced heart failure, valsartan preserved the density of β-receptors and concurrently restored SR function (increase in Ca^{2+}-uptake and prevention of Ca^{2+}-leak)[13] By acting on the presynaptic angiotensin-II receptor, valsartan may inhibit norepinephrine release and stimulate norepinephrine uptake back into the synaptic pool, in turn leading to a reduction in the adrenergic signal being transmitted into the cell. The fewer adrenergic signals may lead to a decrease in the level of RyR2-phosphorylation, and an inhibition of Ca^{2+}-leak through the receptor[13] Although valsartan did not improve resting cardiac function, it did enhance the contractility reserve, as suggested by an enhanced dobutamine response[13]

Chronic norepinephrine release from the synaptic pool in the hyperadrenergic state plays a crucial role in the pathogenesis of heart failure, regardless of the initial cause of the myocardial damage (hypertension, myocardial ischemia, cardiomyopathy etc)[14,15] However, heart failure is a complex disorder, and many other factors (oxidative stress, apoptosis etc) have been shown to concurrently influence the development of contractile dysfunction and/or the left ventricular (LV) remodeling process, together with the de-

(Received December 9, 2005; revised manuscript received March 9, 2006; accepted March 29, 2006)

Department of Medical Bioregulation, Division of Cardiovascular Medicine, Yamaguchi University School of Medicine, Ube, Japan
Mailing address: Masafumi Yano, MD, Department of Medical Bioregulation, Division of Cardiovascular Medicine, Yamaguchi University School of Medicine, 1-1-1 Minamikogushi, Ube 755-8505, Japan.
E-mail: yanoma@yamaguchi-u.ac.jp

fective regulation of intracellular Ca^{2+} homeostasis? Therefore, it remains to be elucidated whether or not angiotensin-II antagonism is indeed effective in terms of improving both hemodynamics and SR function in a model of chronic β-receptor stimulation.

We investigated the effects of continuous in vivo administration of isoproterenol (ISO) on SR function, with the co-administration of an angiotensin-II receptor antagonist, candesartan (CAN). The present results demonstrated that CAN not only accelerates Ca^{2+}-uptake but also prevents the protein kinase (PK) A-phosphorylation of the RyR2, thus rendering the SR less susceptible to oxidative-stress induced Ca^{2+}-leak.

Methods

Animals and Drug Treatments

Male Wistar rats were anesthetized with an intraperitoneal (ip) injection of pentobarbital sodium (50 mg/kg) and were implanted with ip osmotic minipumps (type ML4, Alzet). The rats were treated with 1 $mg \cdot kg^{-1} \cdot day^{-1}$ of ISO, or 0.9% NaCl (sham group). The rationale for this particular dosage of ISO was based on a previous finding, namely, that less than 2.4 $mg \cdot kg^{-1} \cdot day^{-1}$ of ISO induced a negligible hypertrophic response![6,17] The mean rate of infusion was 2.5 μl/h. On day 6, the osmotic minipumps were removed, and 2 days after the cessation of pumping, both the hemodynamics and SR function were measured. Echocardiograms were assessed in all groups before ISO infusion. The LV was catheterized with an ultraminiature catheter pressure transducer (PR 249, Millar Instruments) via the right carotid artery. During ISO infusion and 2 days after cessation of the infusion, a 7.5-MHz phased array transducer was used for analysis (Aloka 1000, Tokyo, Japan).

The angiotensin-II type I receptor antagonist, CAN (0.3 $mg \cdot kg^{-1} \cdot day^{-1}$, Takeda, Tokyo, Japan), was administered by gastric gavage for 8 days in combination with the 6-day ISO infusion and 2-day cessation of infusion. We previously demonstrated that this dose of CAN effectively improved the extent of LV remodeling in pressure-overloaded rats![8]

The investigation conformed with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The care of the animals and the protocols used were in accord with guidelines laid down by the Animal Ethics Committee of the Yamaguchi University School of Medicine.

Preparation of SR Vesicles

After the animals were killed, the atria were removed. The right and left ventricles were dissected, separated, and weighed. The tissues were snap-frozen in liquid N_2 and stored at –80°C. The rat cardiac SR was prepared as described previously, according to the method by Jeyakumar et al![9]

Beta-Receptor-Binding Assay

The membranes used for the β-receptor binding assay were prepared, and the assay was performed as described previously![13,20]

Ca^{2+}-Uptake and -Leak Assays

Ca^{2+}-uptake and Ca^{2+}-leak assays were carried out as described previously?[6–8,13]

Immunoblot Analysis

We performed immunoblot analyses of FKBP12.6 as described elsewhere?[6–8,13] Using the method of Marx et al[5] we achieved co-immunoprecipitation of FKBP12.6 from the SR using anti-RyR2 antibody (Oncogene Research Products, Uniondale, NY, USA) followed by immunoblotting with anti-FKBP12 (C-19) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The relative phosphorylation levels of the RyR2 were determined by immunoblot analysis with anti-phosphoserine?[8,9] which was kindly provided by Dr Andrew Marks (Columbia University). We carried out immunoblot analyses of SR SERCA2a and phospholamban (PLB), as described previously?[8,13] Specific antibodies against phosphoserine 16-PLB (Upstate Biotech, Waltham, MA, USA) were used, as was an epitope common to all forms of PLB (Upstate Biotech).

Preparation of Isolated Cardiomyocytes

The enzymatic isolation of rat cardiomyocytes was performed as described previously?[21] In brief, male Wistar rats were anesthetized with pentobarbital sodium (70 mg/kg ip), intubated and ventilated with ambient air. An incision in the chest was made, and the heart was quickly removed and retrogradely perfused with a collagenase-containing buffer via the aorta under constant flow. The LV myocardium was minced with scissors in fresh collagenase-containing buffer and the rod-shaped adult rat cardiomyocytes were prepared by retrograde perfusion of the hearts with 95%O_2/5%CO_2-bubbled Minimal Essential Medium (Sigma, St Louis, MO, USA) supplemented with 50 μmol/L Ca^{2+}, 0.5 mg/ml collagenase B, 0.5 mg/ml, collagenase D, and 0.02 mg/ml protease type XIV. The Ca^{2+} concentration was then gradually increased to a final concentration of 1 mmol/L by changing the incubation medium (50 μmol/L, 125 μmol/L, 300 μmol/L, and then 1 mmol/L). The isolated rat cardiomyocytes were transferred to laminin-coated glass culture dishes, and incubated for 12 h at 37°C in a 5%CO_2/95%O_2 atmosphere.

Cell Shortening and Ca^{2+} Transient Measurement

Measurements of cardiomyocyte cell shortening and intracellular Ca^{2+} were performed using fura-2 AM, as described previously?[22] Cells were stimulated by an electric field stimulator (IonOptix, MA, USA) at 0.5 Hz. A dual excitation spectrofluorometer was used to record fluorescence emissions (505 nm) elicited from excitation at 340 and 380 nm. The intracellular Ca^{2+} concentration was monitored as the ratio of the fluorescence intensities at 360 and 380 nm excitation. The myocardial cells were imaged with a CCD video camera (Myocam) attached to the microscope, and motion along a selected raster line segment was quantified by a video motion detector system (IonOptix).

Statistical Analysis

Statistical analysis was performed using analysis of variance. P values of <0.05 were accepted as statistically significant. Scheffe's protected least significant difference was used to make individual comparisons between groups. Data represent mean ± SD.

Results

Effect of CAN-Treatment on Cardiac Hypertrophy and Hemodynamics

The animal characteristics and cardiomyocyte data are summarized in Table 1. In the ISO-treated groups (ISO,

Table 1 Body Weight, Heart Weight and Cardiomyocyte Geometry

	Sham (n=12)	*ISO-treated (n=11)*	*ISO- and CAN-treated (n=11)*
Rate			
BW (g)	286±12	288±10	273±10
HW (mg)	675±30	792±48*	779±43*
HW/BW (mg/g)	2.4±0.1	2.8±0.1*	2.9±0.2*
LVW (mg)	526±26	622±35*	591±33*,#
LVW/BW (mg/g)	1.8±0.1	2.2±0.1*	2.2±0.1*
Cardiomyocyte	(n=30)	(n=30)	(n=28)
Length (μm)	122.5±4.9	128.0±6.0*	125.0±6.2*,#
Width (μm)	22.4±2.8	23.5±2.9	22.8±2.6

ISO, isoproterenol; CAN, candesartan; BW, body weight; HW, heart weight; LVW, left ventricular weight.
**p<0.05 vs Sham; #p<0.05 vs ISO-treated. n=number of rats.*

Table 2 Hemodynamic and Echocardiographic Data

	Sham (n=11)	*ISO-treated (n=12)*	*ISO- and CAN-treated (n=11)*
HR (beats/min)	423±34	374±44*	407±33
LVPSP (mmHg)	131±10	120±11	120±11
LVEDP (mmHg)	4.7±2.4	4.7±2.4	3.9±2.3
+dp/dt (mmHg/s)	10,569±1,092	6,936±983*	7,980±1,357*
−dp/dt (mmHg/s)	8,933±843	6,669±805*	7,345±1,314*
Tau	7.9±0.9 (n=9)	10.0±0.8* (n=9)	8.5±1.3# (n=9)
LVEDD (mm)	6.1±0.2	6.7±0.3*	6.5±0.4
LVESD (mm)	2.5±0.2	4.1±0.3*	3.3±0.3*,#
FS (%)	59±4	38±3*	50±3*,#

HR, heart rate; LVPSP, left ventricular (LV) peak systolic pressure; LVEDP, LV end-diastolic pressure; +dP/dt, peak +dP/dt of LV pressure; −dP/dt, peak −dP/dt of LV pressure; Tau, time constant of LV pressure decay during isovolumic relaxation period; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; FS, fractional shortening. Other abbreviations see in Table 1.
**p<0.05 vs Sham; #p<0.05 vs ISO-treated. n=number of rats.*

ISO+CAN), slight cardiac hypertrophy was observed, as evidenced by an increase in the ratio of LV weight to body weight (LVW/BW). However, no significant difference in LVW/BW was observed between the ISO-treated and ISO+CAN-treated groups, although LVW was slightly more increased in the former than in the latter group. Isolated cardiomyocytes were longer in the ISO-treated group than

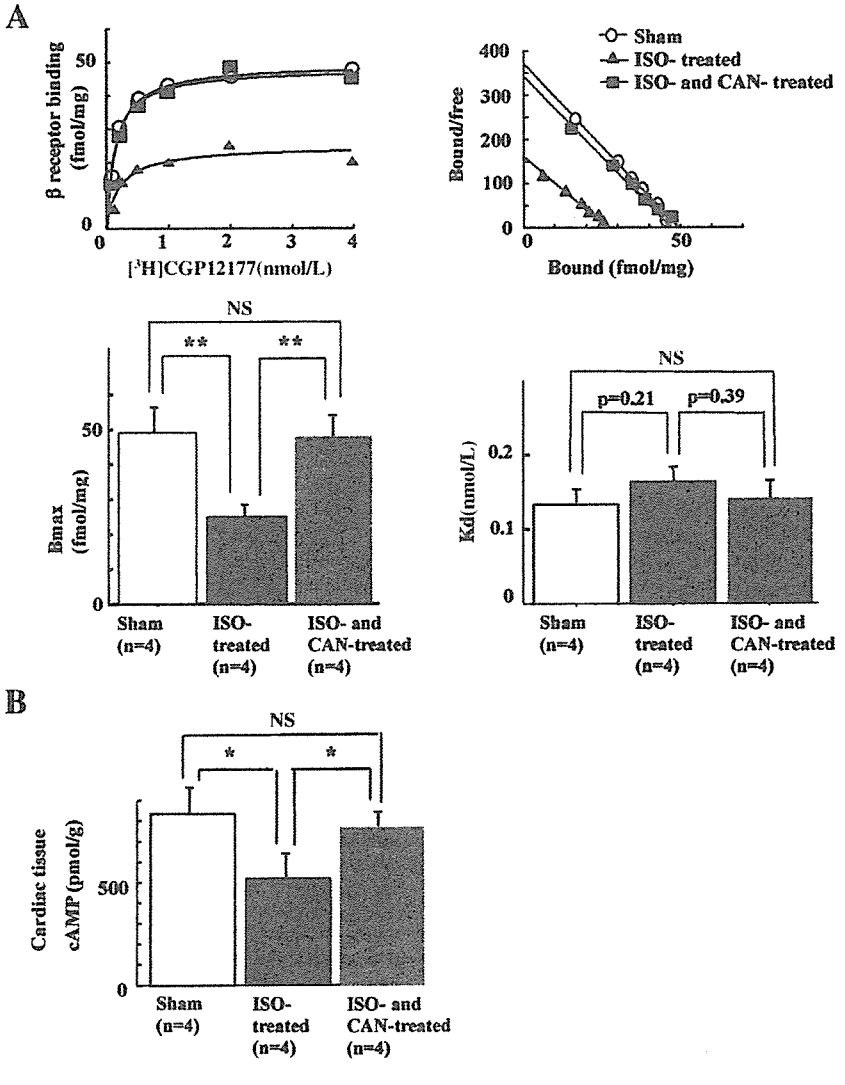

Fig 1. Effect of isoproterenol (ISO) or candesartan (CAN) treatment on the β-adrenergic system. (A: Left panel) Representative [3H]CGP12177 binding to the cardiac membrane fraction taken from the sham, ISO-treated, and ISO+CAN-treated hearts. (A: Right panel) Scatchard re-plotting of the binding data. (Lower panels) Comparison of Bmax and the Kd of [3H]CGP12177 binding. (B) Cardiac tissue cyclic AMP levels. n=number of experiments from 4 different preparations. *p<0.05; **p<0.01.

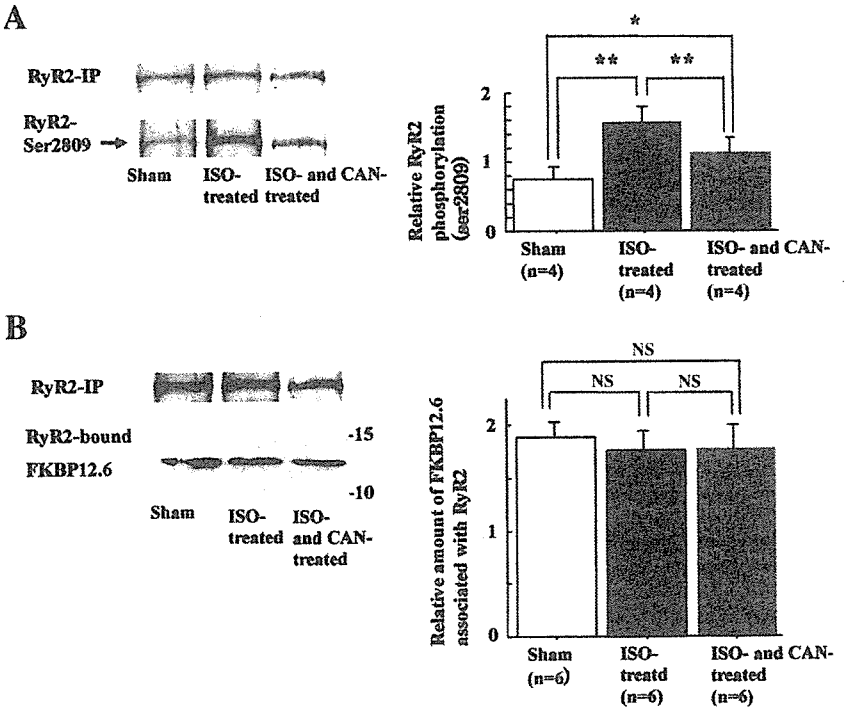

Fig 2. (A) Ptotein kinase A-mediated phosphorylation of the ryanodine receptor (RyR2) confirmed by immunoblotting with anti-2809 serine antibody. (B) Amount of RyR2-bound FKBP12.6. Each result is shown together with the densitometric analysis of the blot (Right panel). n=number of experiments from 4 different preparations. *p<0.05; **p<0.01. ISO, isoproterenol; CAN, candesartan.

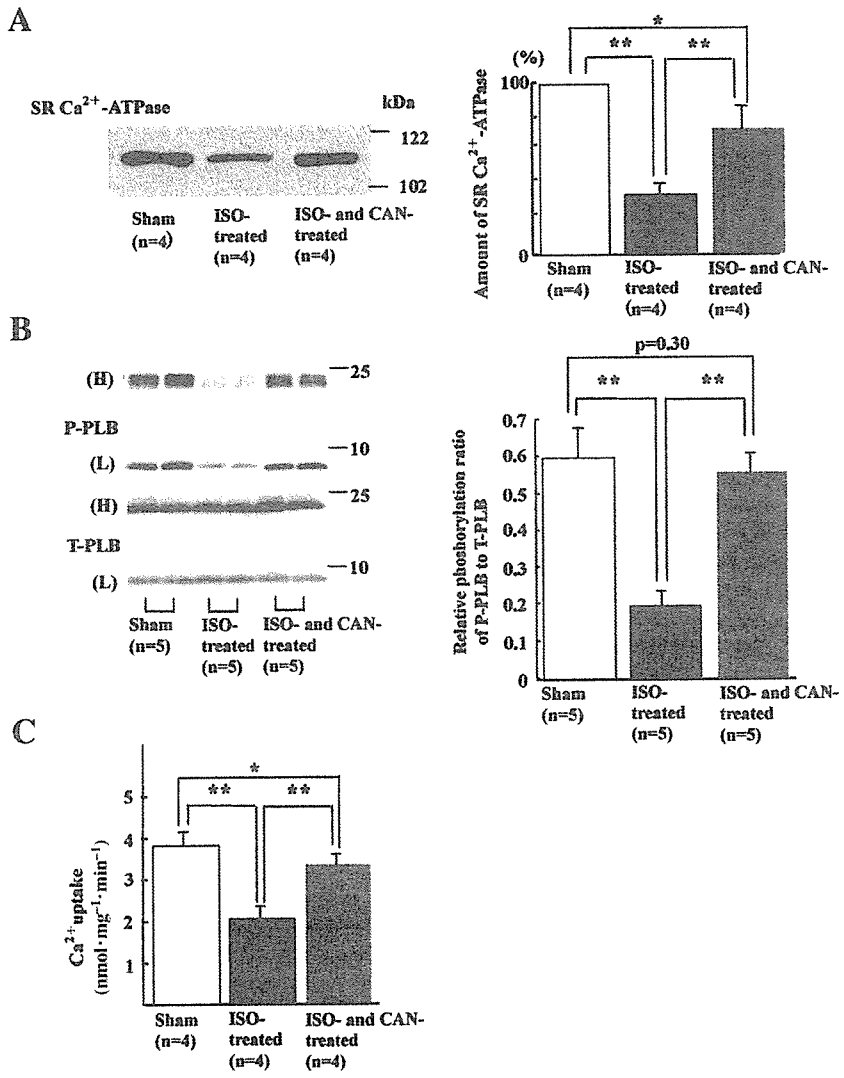

Fig 3. Amount of (A) sarcoplasmic reticulum (SR) Ca^{2+}-ATPase and (B) Ser16-phosphorylated phospholamban (p-PLB) and total phospholamban (t-PLB), each result shown together with the densitometric analysis of the blot (Right panel). The p-PLB values [sum of pentamer and monomer, in arbitrary units] were normalized with respect to the t-PLB values [sum of pentamer and monomer, in arbitrary units]. (C) Magnitude of Ca^{2+}-uptake. Ca^{2+}-uptake was induced by the addition of 0.1 mmol/L MgATP at 0.3μmol/L free [Ca^{2+}] using EGTA-Ca^{2+} buffer. n=number of experiments from 4 different preparations. *p<0.05; **p<0.01. ISO, isoproterenol; CAN, candesartan.

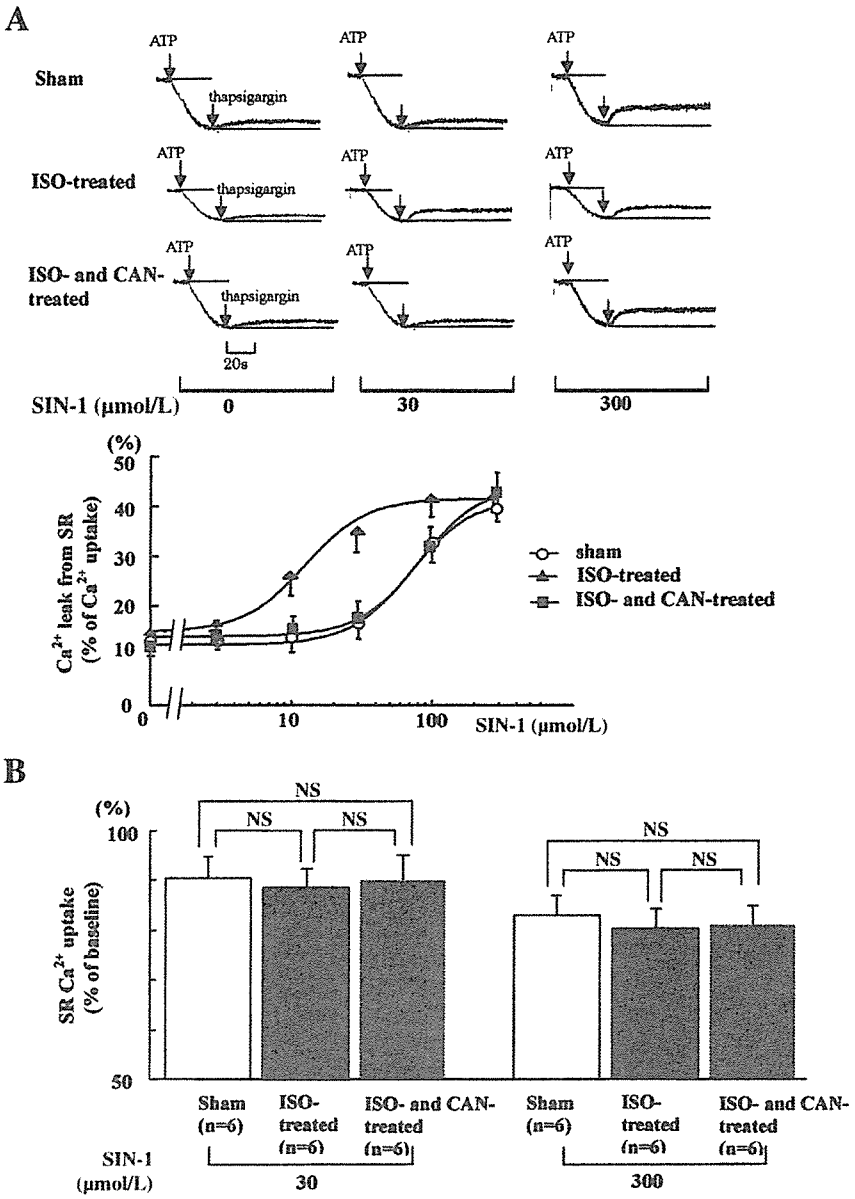

Fig 4. (A) Effect of SIN-1 on the time course of Ca^{2+}-uptake and the ensuing Ca^{2+}-leak from sarcoplasmic reticulum (SR) vesicles (Upper panel), together with the summarized data (Lower panel). *p<0.05, **p<0.01 vs sham; #p<0.05, ##p<0.01 vs isoproterenol (ISO)- and candesartan (CAN)-treated. (B) Concentration effect of SIN-1 on SR Ca^{2+}-uptake in the Sham, ISO-treated, and ISO- and CAN-treated groups. n=number of experiments from 4 different preparations.

in the other groups, but the width of these cardiomyocytes remained the same as those from the other groups. CAN treatment was found to reduce cardiomyocyte hypertrophy in terms of length, which was consistent with our previous finding that CAN reduced cardiomyocyte length, but not cardiomyocyte width, in pressure-overloaded rats[18] The hemodynamic and echocardiographic data are summarized in Table 2. The peak +dP/dt of LV pressure was lower in the ISO-treated group than in the sham group, and this peak tended to be higher in the ISO+CAN-treated group than in the ISO-treated group. The tau of LV pressure was more prolonged in the ISO-treated group than in the sham group, but it was shorter in the ISO+CAN-treated group than in the ISO-treated group.

Effects of CAN-Treatment on β-Receptor Density and Tissue cAMP Level

As shown in Fig 1A, the Bmax value for [^{3}H]CGP12177 binding (fmol/mg) was lower in the ISO-treated group than in the sham group, whereas it was restored to normal in the ISO+CAN-treated group. There was no significant difference in the Kd for [^{3}H]CGP12177 binding (nmol/L) among the 3 groups. The tissue cAMP levels were also lower in the ISO-treated group than in the sham group, whereas they were restored to normal in the ISO+CAN-treated group (Fig 1B). These results were consistent with our previous finding that angiotensin-receptor antagonist treatment restores β-adrenergic receptor signaling[13]

Effects of CAN-Treatment on Ca^{2+}-Handling Proteins in the SR

Fig 2 shows the RyR2 phosphorylation levels and the resultant changes in the RyR2-associated FKBP12.6. The levels of serine^{2809} phosphorylation were increased in the ISO-treated heart, and again were normally restored in the ISO+CAN-treated group (Fig 2A). There were no significant differences in the amount of the RyR2-associated FKBP12.6 among all groups (Fig 2B).

The protein expression of SR SERCA2a was downregulated in the ISO-treated group, but levels were restored in the ISO+CAN-treated group and approached those of the sham group (Fig 3A). Fig 3B compares the levels of Ser16-

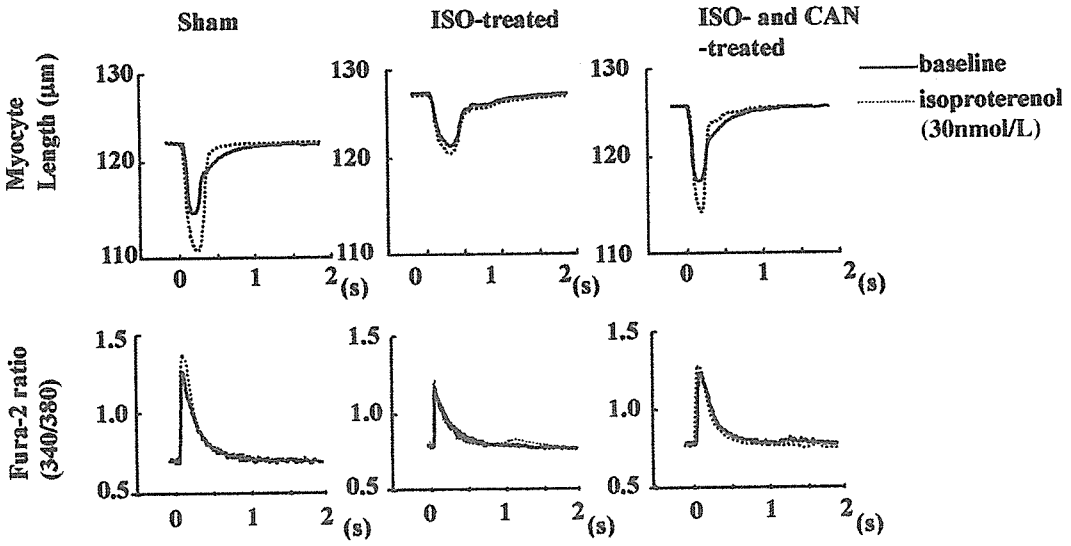

Fig 5. Representative Ca^{2+} transient and cell shortening in cardiomyocytes in the presence or the absence of isoproterenol (ISO) (30 nmol/L). CAN, candesartan.

Table 3 Effect of Isoproterenol on Cardiomyocyte Function

	Sham (n=10)	*ISO-treated (n=10)*	*ISO- and CAN-treated (n=9)*
Cardiomyocyte functions			
Percent shortening (%)			
Isoproterenol			
(−)	*7.3±0.6*	*5.6±0.9***	*7.1±1.1#*
30 nmol/L	*10.5±0.9*	*6.1±0.7***	*9.7±0.9##*
dL/dt (μm/s)			
Isoproterenol			
(−)	*119.1±8.3*	*90.6±7.3***	*109.2±12.0#*
30 nmol/L	*190.4±16.9*	*130.0±10.8***	*176.2±18.5##*
dR/dt (μm/s)			
Isoproterenol			
(−)	*102.6±7.6*	*89.1±9.3**	*98.7±8.2#*
30 nmol/L	*178.3±15.8*	*119.1±6.5**	*172.1±15.6##*
Time from peak to 70% decline (ms)			
Isoproterenol			
(−)	*220±30*	*293±19***	*215±16##*
30 nmol/L	*175±9*	*234±27***	*177±16#*
Calcium transient of cardiomyocytes			
Peak of fura-2 ratio			
Isoproterenol			
(−)	*0.54±0.13*	*0.39±0.04***	*0.46±0.04#*
30 nmol/L	*0.69±0.09*	*0.40±0.04***	*0.49±0.02#,***
Time from peak to 70% decline (ms)			
Isoproterenol			
(−)	*271±25*	*327±37***	*293±28#*
30 nmol/L	*209±15*	*272±19***	*231±19##*

Values are expressed as mean±SD.
dL/dt, longitudinal cell shortening; dR/dt, radial cell shortening. Other abbreviations see in Table 1.
**p<0.05 vs Sham, **p<0.01 vs Sham, #p<0.05 vs ISO-treated, ##p<0.01 vs ISO-treated.*
n=number of experiments from at least 4 different rat hearts.

phosphorylated PLB (p-PLB) and total PLB (t-PLB) among all groups. No difference in the levels of total PLB were observed among the groups, but there was a significant decrease in the basal level of phosphorylated PLB in the ISO-treated group. In the ISO+CAN-treated group, the level of phosphorylated PLB was restored, approaching that of the sham group. A similar result was obtained for the ratio of p-PLB to t-PLB (Right panel of Fig 3B). SR Ca^{2+}-uptake was decreased in the ISO-treated group, but was restored in the ISO+CAN-treated group, approaching that of the sham group (Fig 3C)

Effect of Oxidative Stress on SR Ca^{2+}-Uptake and Ca^{2+}-Leak

Because channel gating has been found to be modulated by a change in the redox state[23,24] we assessed the effects of an O_2^- donor SIN-1, on Ca^{2+}-leak in SR vesicles. The addition of 1 μmol/L thapsigargin produced slight Ca^{2+}-leak in the sham and ISO-treated (+ or − CAN) groups. The addition of 3–300 μmol/L SIN-1 together with 1 μmol/L thapsigargin produced a pronounced leak in all groups (Fig 4A). In the ISO-treated group, the SR Ca^{2+}-leak was induced at a much lower concentration of SIN-1 than was required to achieve the same effect in the sham group, resulting in the leftward shift of the Ca^{2+}-leak-SIN-1 relation curve (Fig 4A, Lower panel). In the ISO+CAN-treated group, the leftward shift of the SIN-1-concentration dependence of Ca^{2+}-leak was restored to approach that of the sham group (Fig 4A, Lower panel). Because Ca^{2+}-leak was induced by the destabilization of the RyR2 caused by a loss of FKBP12.6[5,6] we examined the effect of SIN-1 on FKBP12.6 dissociation from the RyR2. However, SIN-1 did not induce FKBP12.6 dissociation in the sham, ISO-treated, and ISO-and CAN-treated groups (data not shown), suggesting that the facilitation of Ca^{2+}-leak by SIN-1 is an independent event from FKBP12.6 dissociation.

Because redox agents are also known to modulate SR SERCA2a[25,26] we assessed the effects of SIN-1 on SR Ca^{2+}-uptake. When SIN-1 was added before the initiation of Ca^{2+}-uptake in the presence of 1 μmol/L JTV519 (for the prevention of Ca^{2+}-leak)[8] the magnitude of SR Ca^{2+}-uptake was slightly decreased, in a similar concentration dependent manner, in all groups (Fig 4B). There was no significant difference in SR Ca^{2+}-uptake at any concentration of SIN-1 among the 3 groups.

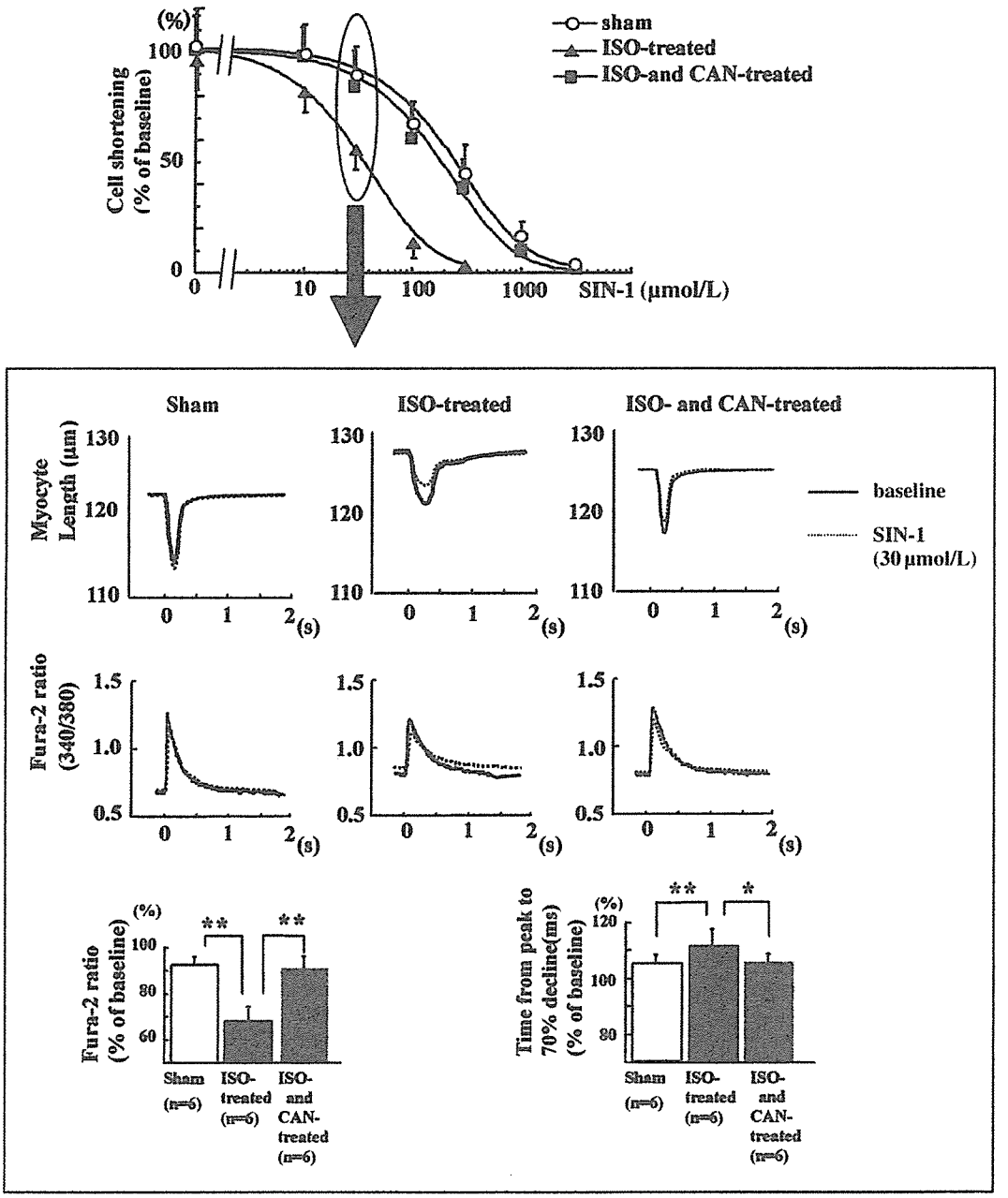

Fig 6. Concentration dependence of SIN-1 on cell shortening in cardiomyocytes (Upper panel), the representative recording of Ca^{2+} transient and cell shortening in the presence of 30μmol/L SIN1, and summarized data for peak systolic Ca^{2+} and time from peak to 70% decline (ms) at 30μmol/L SIN-1 (Lower panel). n=number of experiments from 4 different preparations. *p<0.05; **p<0.01. ISO, isoproterenol; CAN, candesartan.

Cell Shortening and Ca^{2+} Transient in Isolated Cardiomyocytes

As shown in Fig 5 and summarized in Table 3, in the ISO-treated group, cell shortening was significantly decreased compared with the sham group, an effect that was associated with decreased and prolonged Ca^{2+} transient. In contrast, in the ISO+CAN-treated group, both cell shortening and Ca^{2+} transient were restored to normal. In response to ISO treatment, less pronounced cell shortening was observed in the ISO-treated group than in the sham and ISO+CAN-treated groups. The peak of Ca^{2+} transient was also less increased in the ISO-treated group than in the sham and ISO+CAN-treated groups. The duration of Ca^{2+} transient was less abbreviated in the ISO-treated group than in the ISO+CAN-treated and sham groups. Fig 6 shows the effects of the O_2^- donor SIN-1 on cell shortening and Ca^{2+} transient. In the ISO-treated group, cell shortening deteriorated to a greater extent at a much lower concentration of SIN-1 than was observed in either the sham or ISO+CAN-treated group (Upper panel). At a lower concentration of SIN1 (30μmol/L), both cell shortening and Ca^{2+} transient were diminished in ISO-treated group (Lower panel).

Discussion

The major findings of the present study are that upon chronic stimulation of β-receptor, (1) downregulation of the β-receptor was induced, followed by decreases in tissue

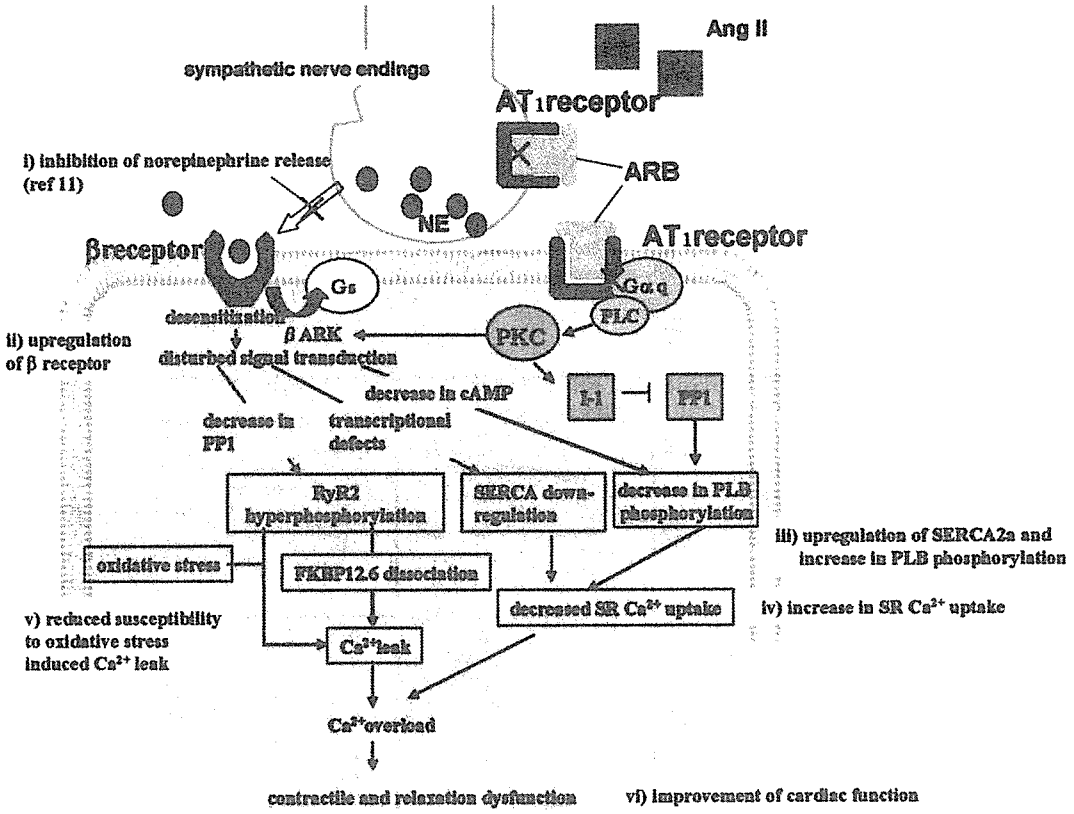

Fig 7. AngII receptor and Ca^{2+} handling in failing hearts in the hyperadrenergic state. Chronic adrenergic stimulation induces desensitization of the β-receptor (downregulation and/or uncoupling of the β-receptor) and in turn decreases the level of intracellular cAMP. The decrease in cAMP reduces the phospholamban (PLB) phosphorylation level, and in turn decreases sarcoplasmic reticulum (SR) Ca^{2+} uptake via inhibition of Ca^{2+}-ATPase (SERCA2a). Reduced SERCA2a activity is also induced by downregulation of SERCA2a mRNA expression at the transcriptional level[26] Despite the reduction in cAMP, RyR2 is hyperphosphorylated, presumably due to a decrease in the expression of membrane-bound PP1 in the ryanodine receptor (RyR2) macromolecular complex[5] Activation of the AngII receptor activates PLC, which in turn activates PKC-α. The PKC-α phosphorylates I-1, augmenting the activity of PP1 and causing hypophosphorylation of PLB[27] The PLB hypophosphorylation inhibits SERCA2a activity, thereby decreasing SR Ca^{2+} uptake. Both the Ca^{2+} leak through the RyR2 and reduced Ca^{2+} uptake lead to cardiac contractile and relaxation dysfunction. AngII receptor blocker reverses all of these abnormalities in Ca^{2+} handling by inhibiting both the presynaptic and post synaptic AT1 receptor as indicated in (i)–(vi). NE, norepinephrine; AngII, angiotensin II; AT1 receptor, AngII type 1 receptor; ARB, AngII type 1 receptor blocker; Gaq, Galpaq protein, βARC β-adrenergic receptor kinase; PLC, phospholipase C; I-1, inhibitor 1; PKC, protein kinase C.

cAMP, PLB phosphorylation, the amount of SERCA2a, and in turn Ca^{2+}-uptake in the cardiac SR, leading to impaired LV contractile and relaxation function; (2) neither Ca^{2+}-leak nor FKBP12.6-dissociation occurred, although PKA-phosphorylation of the RyR2 was observed, but O_2^- donor SIN-1 facilitated Ca^{2+}-leak at a 10-fold lower concentration of SIN-1 than that required to produce the same effect in the sham or ISO+CAN-treated groups; (3) both cell shortening and Ca^{2+} transient were impaired, again at a 10-fold lower concentration of SIN-1 than in the sham or ISO+CAN-treated rats; (4) concomitant administration of the angiotensin-II receptor antagonist CAN restored all the abnormalities induced by chronic stimulation of the β-receptor.

As shown in Fig 7, chronic β-stimulation induces downregulation of the β-receptor, which in turn decreases the tissue cAMP, leading to a reduction of the phosphorylation level of PLB and thus a decrease in SR Ca^{2+} uptake. Decreased Ca^{2+} uptake reduces the SR Ca^{2+} content, which in turn decreases cardiomyocyte shortening. The beneficial effects of CAN on LV relaxation and cardiomyocyte function are most likely attributable to the improvement in SR Ca^{2+} uptake. The mechanism by which CAN improves SR Ca^{2+} uptake may result from antagonism of both the presynaptic and postsynaptic AT1 receptors. By stimulating the presynaptic AT1 receptor, angiotensin-II enhances the sympathetic release of norepinephrine[27] Therefore, inhibition of the presynaptic AT1 receptor by CAN may reduce norepinephrine release from nerve endings and stimulate norepinephrine uptake back into the synaptic pool, in turn leading to a reduction in the adrenergic signal being transmitted into the cell. We recently found that in a pacing-induced canine heart failure model the angiotensin-II receptor antagonist valsartan indeed inhibited norepinephrine release, attenuated the downregulation of β-adrenergic receptors, and improved SERCA2a protein expression and SR Ca^{2+}-uptake activity[13] In the present study, we confirmed that in the ISO-treated rats treatment with CAN also improved SR Ca^{2+}-uptake, restored β-receptor density, and improved intracellular Ca^{2+} transient in response to ISO. Thus, the improvement in the β-receptor density and SR Ca^{2+}-uptake, which had been disturbed in the hypercatecholaminergic state, might be a class effect of angiotensin-

II antagonists. In this context, there seems to be no specific differences in the Ca^{2+} handling effects of CAN or other angiotensin-II antagonists like valsartan. Binding of angiotensin II to the postsynaptic AT1 receptor may also decrease SR Ca^{2+} uptake activity by decreasing the level of expression of SERCA2a mRNA[28] and also by increasing PKC-α activity, which in turn directly phosphorylates serine 67 in inhibitor 1, thereby augmenting the activity of protein phosphatase-1 (PP1) and causing the hypophosphorylation of PLN[29] Takeishi et al[30] also demonstrated that angiotensin-II inhibition by ramipril attenuates PKC translocation and prevents downregulation of SERCA2a and PLB protein expressions in pressure-overload hypertrophy. Collectively, angiotensin-II antagonism by CAN is likely to reverse all of the adverse effects of angiotensin II on SR Ca^{2+} uptake, and the beneficial effects of CAN on Ca^{2+} uptake leads to improved contractile and relaxation functions.

Despite the decreased level of tissue cAMP, RyR2 was phosphorylated, although the phosphorylation of PLB was increased. This discrepancy may be explained by the differential distribution of PP1 between the RyR2 macromolecular complex and the cytosolic fraction in the hyperadrenergic state in failing hearts. Marx et al[5] demonstrated that in failing hearts PKA-mediated hyperphosphorylation of the RyR2 at Ser2809 was induced mainly by a reduction of the expressions of PP1 and PP2A in the RyR2 macromolecular complex rather than by increased PKA activity.

In the current model of ISO-induced LV dysfunction, neither Ca^{2+}-leak nor FKBP12.6 dissociation from the RyR2 was observed, even though increased levels of the RyR2 phosphorylation were observed. These findings appear to conflict the previous results showing that PKA-hyperphosphorylation induces FKBP12.6 dissociation and subsequent Ca^{2+}-leak[5–8] However, the levels of PKA phosphorylation were only 1.5-fold higher in the ISO-treated group than in the sham and ISO+CAN-treated groups. In previous studies, including ours, the PKA phosphorylation levels showed greater increases (ie, ≈4-fold higher than normal)[5,7,8] Thus, the observed increase in PKA phosphorylation may not have been sufficient to exert a significant effect on FKBP12.6 dissociation and the subsequent Ca^{2+}-leak. Importantly, even though FKBP12.6 was not significantly dissociated from the RyR2 in the PKA-phosphorylated RyR2 in the basal state, the PKA-phosphorylated channel showed susceptibility to Ca^{2+}-leak upon application of oxidative stress.

There is a major controversy in the literature concerning the RyR2-FKBP12.6 interaction in hyperphosphorylated or disease conditions. Several reports have shown that hyperphosphorylation of RyR2 has no appreciable effect on the RyR2-bound FKBP12.6. Several groups reported that phosphorylation at Ser^{2808} (Ser^{2809} in rabbit) does not cause FKBP12.6 dissociation from RyR2, and that the constitutive phosphorylation of Ser^{2808} or Ser^{2809} by mutations (S2808D or S2809D) fails to disrupt the FKBP12.6-RyR2 interaction[31,32] The discrepancy may be partly ascribable to differences in experimental design. As Wehrens et al[33] suggested, the physiological ratios of FKBP12.6 to RyR2 may be important in the evaluation of FKBP12.6-dissociation from RyR. Overexpression of FKBP12.6 outside the physiological range may counteract the shift in Kd induced by PKA phosphorylation. The present study results suggest that the extent of PKA phosphorylation, which may change depending on the severity of heart failure, also appears to affect the FKBP12.6 dissociation from RyR2.

The RyR has been shown to be a target of reactive oxygen species (ROS), and the channel activity of the RyR is known to be regulated by oxidation or nitrosylation[34] It is also well established that chemical reducing agents (eg, dithiothreitol or cysteine) depress channel function[35–37] S-nitrosylation of the RyR2 has been suggested to be physiologically significant in the normal heart;[38,39] whereas excess oxidation during periods of oxidative stress can lead to deleterious loss of control[34] An important aspect of the present study is that in the purely β-receptor overstimulated heart, defective stabilization of the RyR2 could be induced by a small degree of oxidative stress, which otherwise has only a slight effect on the normal heart. That is, in the β-receptor overstimulated heart oxidative stress facilitated Ca^{2+}-leak, even under the condition of FKBP12.6 not being dissociated from RyR2. Thus, oxidative stress may change the conformational state of the RyR2, facilitating Ca^{2+}-leak under the condition of PKA phosphorylation. This notion received further support from the finding that in the ISO-treated group, SIN-1 decreased cell shortening and peak Ca^{2+} transient at a much lower concentration than was required to achieve the same effect in either the sham or ISO+CAN-treated group.

Catecholamines, including ISO, have been reported to generate ROS and form oxidation products[40] Because ROS are known causative factors in many diseases including heart failure;[41] their generation by catecholamines may also contribute to the pathogenic process. In our most recent report;[42] we demonstrated that in pacing-induced failing hearts the levels of oxidative stress are elevated, together with an increase in plasma norepinephrine, causing various abnormalities regarding Ca^{2+} regulation in failing cardiomyocytes (ie, increased intracellular level of ROS, decreased intracellular Ca^{2+} transient, oxidized RyR2, defective inter-domain interaction within RyR2, and Ca^{2+}-leak). All of these abnormalities could be reproduced by application of the NO-donor SIN-1 to normal cardiomyocyte or SR vesicles. The SIN-1-induced decrease in the peak Ca^{2+} transient was reversed by JTV519 or edaravone (free radical scavenger), but not by SEA0400 (NCX inhibitor). These findings strongly suggest that oxidative stress produces critical effects on the mechanism of channel regulation in the RyR. However, as for the cause of the decreased peak Ca^{2+} transient by SIN-1, we can not eliminate the possibility that ROS would affect the L-type Ca^{2+} channel and excitation-contraction coupling gain.

Taken together with the previous findings, both the level of intracellular ROS and oxidative stress within the RyR2 may have been increased in the ISO-treated group, and therefore channel gating of the RyR2 would be defective in ISO-treated hearts, rendering it less susceptible to Ca^{2+}-leak induced by SIN-1. Further investigation, including direct measurement of intracellular ROS and the levels of oxidative stress within the RyR2, are clearly needed.

Conclusion

In the in vivo cardiac muscle, chronic stimulation of the β-receptors was found to induce PKA phosphorylation of the RyR2 without inducing Ca^{2+}-leak, whereas oxidative stress facilitated Ca^{2+}-leak in the PKA-phosphorylated RyR2. An angiotensin-II receptor antagonist not only improved Ca^{2+}-uptake, but also prevented PKA-hyperphosphorylation, thus rendering the SR less susceptible to Ca^{2+}-leak.

Acknowledgments

This work was supported by grants-in-aid for scientific research from The Ministry of Education, Culture, Sports, Science and Technology of Japan (grant Nos. 16590689, and 16209026, 14370228 to Dr Yano and Dr Matsuzaki), Grant From Japan Cardiovascular Research Foundation (to Dr Yamamoto), and Takeda Science Foundation (to Dr Yamamoto).

References

1. Yano M, Ikeda Y, Matsuzaki M. Altered intracellular Ca^{2+} handling in heart failure. *J Clin Invest* 2005; **115:** 556–564.
2. Hasenfuss G. Alterations of calcium-regulatory proteins in heart failure. *Cardiovasc Res* 1998; **37:** 279–289.
3. Kuo TH, Tsang W, Wang KK, Carlock L. Simultaneous reduction of the sarcolemmal and SR calcium ATPase activities and gene expression in cardiomyopathic hamster. *Biochim Biophys Acta* 1992; **1138:** 343–349.
4. Cory CR, McCutcheon LJ, O'Grady M, Pang AW, Geiger JD, O'Brien PJ. Compensatory downregulation of myocardial Ca^{2+} channel in SR from dogs with heart failure. *Am J Physiol* 1993; **264:** H926–H9237.
5. Marx SO, Reiken S, Hisamatsu Y, Jayaraman T, Burkhoff D, Rosemblit N, et al. PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): Defective regulation in failing hearts. *Cell* 2000; **101:** 365–376.
6. Yano M, Ono K, Ohkusa T, Suetsugu M, Kohno M, Hisaoka T, et al. Altered stoichiometry of FKBP12.6 versus ryanodine receptor as a cause of abnormal Ca^{2+} leak through ryanodine receptor in heart failure. *Circulation* 2000; **102:** 2131–2136.
7. Doi M, Yano M, Kobayashi S, Kohno M, Tokuhisa T, Okuda S, et al. Propranolol prevents the development of heart failure by restoring FKBP12.6-mediated stabilization of ryanodine receptor. *Circulation* 2002; **105:** 1374–1379.
8. Yano M, Kobayashi S, Kohno M, Doi M, Tokuhisa T, Okuda S, et al. FKBP12.6-mediated stabilization of calcium-release channel (ryanodine receptor) as a novel therapeutic strategy against heart failure. *Circulation* 2003; **107:** 477–484.
9. Mori T, Hayashi T, Sohmiya K, Okuda N, Shimomura H, Ohkita M, et al. Mechanisms of combined treatment with celiprolol and candesartan for ventricular remodeling in experimental heart failure. *Circ J* 2005; **69:** 596–602.
10. Matsumoto R, Yoshiyama M, Omura T, Kim S, Nakamura Y, Izumi Y, et al. Effects of aldosterone receptor antagonist and angiotensin II type I receptor blocker on cardiac transcriptional factors and mRNA expression in rats with myocardial infarction. *Circ J* 2004; **68:** 376–382.
11. Bruckschlegel G, Holmer SR, Jandeleit K, Grimm D, Muders F, Kromer EP, et al. Blockade of the renin-angiotensin system in cardiac pressure-overload hypertrophy in rats. *Hypertension* 1995; **25:** 250–259.
12. Zierhut W, Studer R, Laurent D, Kastner S, Allegrini P, Whitebread S, et al. Left ventricular wall stress and sarcoplasmic reticulum $Ca^{(2+)}$-ATPase gene expression in renal hypertensive rats: Dose-dependent effects of ACE inhibition and AT1-receptor blockade. *Cardiovasc Res* 1996; **31:** 758–768.
13. Okuda S, Yano M, Doi M, Oda T, Tokuhisa T, Kohno M, et al. Valsartan restores sarcoplasmic reticulum function with no appreciable effect on resting cardiac function in pacing-induced heart failure. *Circulation* 2004; **109:** 911–919.
14. Braunwald E, Bristow MR. Congestive heart failure: Fifty years of progress. *Circulation* 2000; **102:** IV-14–IV-23.
15. Francis GS. Pathophysiology of chronic heart failure. *Am J Med* 2001; **110:** 37S–46S.
16. Boluyt MO, Long X, Eschenhagen T, Mende U, Schmitz W, Crow MT, et al. ISO infusion induces alterations in expression of hypertrophy-associated genes in rat heart. *Am J Physiol* 1995; **269:** H638–H647.
17. Stein B, Bartel S, Kirchhefer U, Kokott S, Krause EG, Neumann J, et al. Relation between contractile function and regulatory cardiac proteins in hypertrophied hearts. *Am J Physiol* 1996; **270:** H2021–H2028.
18. Obayashi M, Yano M, Kohno M, Kobayashi S, Tanigawa T, Hironaka K, et al. Dose-dependent effect of ANG II-receptor antagonist on myocyte remodeling in rat cardiac hypertrophy. *Am J Physiol* 1997; **273:** H1824–H1831.
19. Jeyakumar LH, Ballester L, Cheng DS, McIntyre JO, Chang P, Olivey HE, et al. FKBP binding characteristics of cardiac microsomes from diverse vertebrates. *Biochem Biophys Res Commun* 2001; **281:** 979–986.
20. Abrahamsson T, Ek B, Nerme V. The β_1- and β_2-adrenoceptor affinity of atenolol and metoprolol. *Biochem Pharmacol* 1988; **37:** 203–208.
21. Zhou YY, Wang SQ, Zhu WZ, Chruscinski A, Kobilka BK, Ziman B, et al. Culture and adenoviral infection of adult mouse cardiac myocytes: Methods for cellular genetic physiology. *Am J Physiol* 2000; **279:** H429–H436.
22. Hajjar RJ, Schmidt U, Kang JX, Matsui T, Rosenzweig A. Adenoviral gene transfer of phospholamban in isolated rat cardiomyocytes: Rescue effects by concomitant gene transfer of sarcoplasmic reticulum $Ca^{(2+)}$-ATPase. *Circ Res* 1997; **81:** 145–153.
23. Pessah IN, Kim KH, Feng W. Redox sensing properties of the ryanodine receptor complex. *Front Biosci* 2002; **7:** a72–a79.
24. Eu JP, Sun J, Xu L, Stamler JS, Meissner G. The skeletal muscle calcium release channel: Coupled O2 sensor and NO signaling functions. *Cell* 2000; **102:** 499–509.
25. Scherer NM, Deamer DW. Oxidative stress impairs the function of sarcoplasmic reticulum by oxidation of sulfhydryl groups in the Ca^{2+}-ATPase. *Arch Biochem Biophys* 1986; **246:** 589–601.
26. Moreau VH, Castilho RF, Ferreira ST, Carvalho-Alves PC. Oxidative damage to sarcoplasmic reticulum Ca^{2+}-ATPase at submicromolar iron concentrations: Evidence for metal-catalyzed oxidation. *Free Radic Biol Med* 1998; **25:** 554–560.
27. Lanier SM, Malik KU. Facilitation of adrenergic transmission in the canine heart by intracoronary infusion of angiotensin II: Effect of prostaglandin synthesis inhibition. *J Pharmacol Exp Ther* 1983; **227:** 676–682.
28. Koitabashi N, Arai M, Tomaru K, Takizawa T, Watanabe A, Niwano K, et al. Carvedilol effectively blocks oxidative stress-mediated downregulation of sarcoplasmic reticulum Ca^{2+}-ATPase 2 gene transcription through modification of Sp1 binding. *Biochem Biophys Res Commun* 2005; **328:** 116–124.
29. Braz JC, Gregory K, Pathak A, Zhao W, Sahin B, Klevitsky R, et al. PKC-alpha regulates cardiac contractility and propensity toward heart failure. *Nat Med* 2004; **10:** 248–254.
30. Takeishi Y, Bhagwat A, Ball NA, Kirkpatrick DL, Periasamy M, Walsh RA. Effect of angiotensin-converting enzyme inhibition on protein kinase C and SR proteins in heart failure. *Am J Physiol* 1999; **276:** H53–H62.
31. Stange M, Xu L, Balshaw D, Yamaguchi N, Meissner G. Characterization of recombinant skeletal muscle (Ser2843) and cardiac muscle (Ser2809) ryanodine receptor phosphorylation mutants. *J Biol Chem* 2003; **278:** 51693–51702.
32. Xiao B, Sutherland C, Walsh MP, Chen SR. Protein kinase A phosphorylation at serine-2808 of the cardiac Ca^{2+}-release channel (ryanodine receptor) does not dissociate 12.6-kDa FK506-binding protein (FKBP12.6). *Circ Res* 2004; **94:** 487–495.
33. Wehrens XH, Lehnart SE, Reiken SR, Deng SX, Vest JA, Cervantes D, et al. Protection from cardiac arrhythmia through ryanodine receptor-stabilizing protein calstabin2. *Science* 2004; **304:** 292–296.
34. Meissner G. Regulation of mammalian ryanodine receptors. *Front Biosci* 2002; **7:** 2072–2080.
35. Trimm JL, Salama G, Abramson JJ. Sulfhydryl oxidation induces rapid calcium release from sarcoplasmic reticulum vesicles. *J Biol Chem* 1986; **261:** 16092–16098.
36. Boraso A, Williams AJ. Modification of the gating of the cardiac sarcoplasmic reticulum Ca^{2+}-release channel by H_2O_2 and dithiothreitol. *Am J Physiol* 1994; **267:** H1010–H1016.
37. Marengo JJ, Hidalgo C, Bull R. Sulfhydryl oxidation modifies the calcium dependence of ryanodine-sensitive calcium channels of excitable cells. *Biophys J* 1998; **74:** 1263–1277.
38. Petroff MG, Kim SH, Pepe S, Dessy C, Marban E, Balligand JL, et al. Endogenous nitric oxide mechanisms mediate the stretch dependence of Ca^{2+} release in cardiomyocytes. *Nat Cell Biol* 2001; **3:** 867–873.
39. Ziolo MT, Katoh H, Bers DM. Positive and negative effects of nitric oxide on $Ca^{(2+)}$ sparks: Influence of beta-adrenergic stimulation. *Am J Physiol* 2001; **281:** H2295–H2303.
40. Zhang GX, Kimura S, Nishiyama A, Shokoji T, Rahman M, Yao L, et al. Cardiac oxidative stress in acute and chronic isoproterenol-infused rats. *Cardiovasc Res* 2005; **65:** 230–238.
41. Giordano FJ. Oxygen, oxidative stress, hypoxia, and heart failure. *J Clin Invest* 2005; **115:** 500–508.
42. Yano M, Okuda S, Oda T, Tokuhisa T, Tateishi H, Mochizuki M, et al. Correction of defective interdomain interaction within ryanodine receptor by antioxidant is a new therapeutic strategy against heart failure. *Circulation* 2005; **112:** 3633–3643.

Gender Difference in Coronary Events in Relation to Risk Factors in Japanese Hypercholesterolemic Patients Treated With Low-Dose Simvastatin

Jun Sasaki, MD; Toru Kita, MD*; Hiroshi Mabuchi, MD**; Masunori Matsuzaki, MD†; Yuji Matsuzawa, MD††; Noriaki Nakaya, MD‡; Shinichi Oikawa, MD‡‡; Yasushi Saito, MD§; Kazuaki Shimamoto, MD§§; Suminori Kono, MD¶; Hiroshige Itakura, MD¶¶,a; the J-LIT Study Group

Background Gender differences between the risk factors for coronary heart disease and coronary events were examined in the Japan Lipid Intervention Trial, a 6-year observational study.

Methods and Results Men (12,575) and women (27,013) were analyzed for risk of coronary events (acute myocardial infarction and sudden cardiac death). Simvastatin reduced serum low-density lipoprotein cholesterol (LDL-C) by 27% in both genders, and increased serum high-density lipoprotein cholesterol (HDL-C) in men (5%) and women (4%). The incidence of coronary events was lower in women (0.64/1,000 patient-years) than in men (1.57/1,000 patient-years). The risk of coronary events increased by 18% in men and 21% in women with each 10 mg/dl elevation of LDL-C, and decreased by 39% in men and 33% in women with each 10 mg/dl elevation of HDL-C. The risk increased proportionally with aging in women, but not in men. Diabetes mellitus (DM) was more strongly related to the risk of coronary events for women (relative risk 3.07) than for men (relative risk 1.58).

Conclusions The incidence of coronary events is lower in women. Serum LDL-C is related to an increased risk of coronary events to the same extent in both genders. DM seems to be a more important risk factor in women, trading off the lower risk of coronary events among them. (*Circ J* 2006; **70:** 810–814)

Key Words: Coronary events; Hyperlipidemia; Risk factors; Serum cholesterol; Sex differences

Coronary heart disease (CHD), including myocardial infarction and cardiac sudden death, is one of the leading causes of death in Japan[1] The risk of developing CHD is known to be markedly different between men and women:[2,3] CHD incidence is 2 to 5 times higher among middle-aged men than women. In the Japan Lipid Intervention Trial (J-LIT)[4–7] we previously reported that serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) concentrations were positively and serum high-density lipoprotein cholesterol (HDL-C) concentration was inversely related to CHD or cerebrovascular disease risk in patients under treatment for hypercholesterolemia. The role of coronary risk factors in the development of CHD has been studied extensively in men[8–10] but relatively few studies have investigated women[2,11]

(Received January 16, 2006; revised manuscript received March 13, 2006; accepted March 29, 2006)

*International University of Health and Welfare Graduate School of Clinical Trial Management, Fukuoka, *Kyoto University Graduate School of Medicine, Kyoto, **Kanazawa University Graduate School of Medicine, Kanazawa, †Yamaguchi University Graduate School of Medicine, Ube, ††Sumitomo Hospital, Osaka, ‡Nakaya Clinic, ‡‡Nippon Medical School, Tokyo, §Chiba University Graduate School of Medicine, Chiba, §§Sapporo Medical University School of Medicine, Sapporo, ¶Kyushu University Faculty of Medical Sciences, Fukuoka and ¶¶Ibaraki Christian University, Hitachi, Japan*

[a]Hiroshige Itakura, MD, was a Chairman of the Central Committee.

Mailing address: Jun Sasaki, MD, International University of Health and Welfare Graduate School of Clinical Trial Management, 1-3-1 Nagahama, Chuo-ku, Fukuoka 810-0072, Japan. E-mail: jsas@nifty.com

This study aimed to assess gender differences in the association of risk factors with CHD in the J-LIT data. The J-LIT is a nationwide cohort study of 52,421 hypercholesterolemic patients treated with open-labeled low-dose simvastatin (5–10 mg/day)[4,5] The J-LIT included a large number of female patients, and we were able to investigate the gender difference in the role of risk factors in the occurrence of coronary events.

Methods

Study Design

The design of the J-LIT study has been previously described[12] Briefly, study patients with serum TC concentration ≥220 mg/dl, men aged 35–70 years and postmenopausal women aged 70 years or less, were treated with 5–10 mg/day of simvastatin. Body weight, serum lipid concentrations (TC, LDL-C, HDL-C, and triglyceride (TG)) were measured at baseline, and patients were interviewed as regards family history of CHD, number of cigarettes smoked, and the amount of alcohol ingestion. Serum lipid concentrations and CHD-related events (acute myocardial infarction and cardiac sudden death) were monitored every 6 months for 6 years in all patients, including those who discontinued simvastatin. Serum lipid concentrations were determined in each study institution, and the serum LDL-C concentration was calculated using the Friedewald formula for patients with TG concentration ≤400 mg/dl[13] Study physicians recommended dietary and exercise-therapy for hyperlipidemia to all patients. Additional lipid-lowering

agents were allowed only when an adequate response in serum TC concentration was not gained by simvastatin monotherapy. Each patient was informed of the purpose and method of the study, drug efficacy and the need for long-term treatment and they gave verbal, not written, informed consent.

Subjects

Patients who had been previously treated with a lipid-lowering agent were screened for eligibility after a washout period of at least 4 weeks. For patients previously treated with probucol, the washout period was at least 12 weeks. The exclusion criteria were the occurrence of acute myocardial infarction or stroke within the past month, concurrent uncontrolled diabetes mellitus (DM), serious hepatic or renal disease, secondary hypercholesterolemia, cancer or any other illness with potentially poor survival.

Of the 52,421 patients enrolled, 5,127 were excluded because of a history of CHD, 4,934 for lack of follow-up data, and 2,772 for missing data of the covariates. Therefore, data from 39,588 patients (12,575 men, 27,013 women) were used in the present study.

Endpoints

The primary endpoints were major coronary events, defined as nonfatal and fatal myocardial infarction and sudden cardiac death. Incidence of myocardial infarction or death was counted once for each patient during the treatment, and the follow-up data thereafter were excluded from the analysis. The events were reviewed and determined by the Endpoint Classification Committee.

Statistical Analysis

The mean lipid concentrations were calculated using data available at the follow-up points in time during the treatment period. The data of lipid concentrations after the onset of events were excluded. Data during the treatment period after discontinuation of simvastatin were also included for analysis. Mean values for serum lipid concentrations and age were tested with unpaired t-test, and the prevalence of baseline characteristics were tested with the chi-square test for comparison between men and women. Patients in each sex were categorized into 5–6 groups according to the mean lipid concentrations of treatment period for TC, TG, LDL-C and HDL-C with intervals of 20, 50, 20, 10 mg/dl, respectively, and for the LDL-C/HDL-C ratio with an interval of 0.5. The reference category for the relative risk was set on the group with the lowest lipid concentrations and the lowest value of LDL-C/HDL-C ratio. Relative risks and the 95% confidence intervals (CI) were calculated using the Cox proportional hazards model with adjustment for baseline characteristics such as sex, age, hypertension, DM, body mass index (BMI), ECG abnormality, family history of CHD, alcohol ingestion and cigarette smoking. Heterogeneity between men and women was evaluated by the likelihood ratio test. Two-sided p-value <0.05 was considered statistically significant. All the statistical calculations were performed using SAS software (version 8.02, SAS Institute, Inc, Cary, NC, USA).

Results

Serum Lipids and Other Risk Factors

There were no significant difference as regards the prevalence of obesity (BMI ≥25.0 kg/m^2), hypertension, ECG abnormality, and family history of CHD between men and women (Table 1). In men, the prevalence of DM was higher (p<0.001), and cigarette smoking and alcohol ingestion were much more frequent (p<0.001).

Table 1 Baseline Characteristics of the Subjects

	Men (n=12,575)	*Women (n=27,013)*
Age (years)	*54.0 (9.1)*	*59.5 (6.5)*
Obesity (%)[a]	*36.7*	*32.2*
Hypertension (%)[b]	*45.4*	*46.3*
Diabetes mellitus (%)[c]	*20.0*	*13.9*
ECG abnormality (%)[d]	*13.4*	*12.9*
Family history of CHD (%)[e]	*5.1*	*4.8*
Cigarette smoking (%)[e]	*43.8*	*4.1*
Alcohol use (%)[e]	*73.4*	*8.7*
Lipid profiles		
Baseline (mg/dl)		
TC	*268 (41)*	*271 (31)*
LDL-C	*178 (34)*	*184 (33)*
TG	*250 (241)*	*169 (111)*
HDL-C	*49 (15)*	*55 (15)*
During the treatment (mg/dl)		
TC	*218 (31)*	*221 (29)*
LDL-C	*130 (31)*	*135 (28)*
TG	*198 (133)*	*148 (77)*
HDL-C	*51 (13)*	*57 (14)*

Figs are mean±SD unless otherwise specified.
CHD, coronary heart disease; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol.
[a] Body mass index ≥25 kg/m^2. [b] Systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥95 mmHg or medication for hypertension. [c] Fasting plasma glucose ≥140 mg/dl or medication. [d] Study physician's diagnosis. [e] Self-reported information.

Lipid profiles at baseline and during the treatment period are shown for men and women in Table 1. Men had higher concentrations of serum TG and lower concentrations of serum HDL-C at baseline and during the treatment in comparison with women. Mean percent changes in the TC, LDL-C, TG, and HDL-C concentrations from baseline to during the treatment in men were −18.8% (p<0.001), −27.2% (p<0.001), −20.9% (p<0.001), and +4.7% (p<0.001), respectively, and the corresponding values in women were −18.2% (p<0.001), −26.6% (p<0.001), −12.8% (p<0.001) and +4.4% (p<0.001), respectively.

Incidence of Coronary Events

The incidence of coronary events was greater (105/12,575) in men than in women (93/27,013) during the treatment period. Incidence rates of coronary events per 1,000 patient-years were 1.57 in men and 0.64 in women. The age-adjusted relative risk of coronary events for men vs women was 2.81 (95% CI 2.10–3.76, p<0.001).

Serum Lipid Concentrations During the Treatment Period and Risk of Coronary Events

The risk of coronary events in relation to serum lipid concentrations is shown in Table 2. Increased risk for coronary events was evident at TC ≥240 mg/dl and LDL-C ≥160 mg/dl in both men and women. An increased risk of CHD associated with elevated concentration of TG (≥250 mg/dl) was noted in women but not in men. In men, the relationship between TG and CHD risk was not measurable. A lower risk of coronary events associated with elevation in HDL-C was seen in both sexes, but the protec-

Table 2 Relative Risk of Coronary Events According to Serum Lipid Concentrations During Treatment[a)]

	Men					Women				
	N	*Event*	*RR*	*95%CI*	*p value*	*N*	*Event*	*RR*	*95%CI*	*p value*
TC (mg/dl)										
<200	*3,442*	*24*	*1.00*	*(Referent)*		*5,833*	*22*	*1.00*	*(Referent)*	
200–219	*3,643*	*23*	*0.99*	*(0.56–1.77)*	*0.984*	*8,194*	*14*	*0.52*	*(0.27–1.02)*	*0.057*
220–239	*3,029*	*25*	*1.46*	*(0.83–2.56)*	*0.192*	*7,070*	*18*	*0.88*	*(0.47–1.64)*	*0.687*
240–259	*1,431*	*15*	*2.01*	*(1.05–3.88)*	*0.036*	*3,668*	*22*	*2.19*	*(1.21–3.98)*	*0.010*
260–	*1,030*	*18*	*3.48*	*(1.86–6.52)*	*<0.001*	*2,248*	*17*	*2.82*	*(1.48–5.36)*	*0.002*
LDL-C (mg/dl)										
<120	*4,680*	*27*	*1.00*	*(Referent)*		*8,050*	*22*	*1.00*	*(Referent)*	
120–139	*3,542*	*23*	*1.24*	*(0.71–2.16)*	*0.456*	*8,418*	*17*	*0.83*	*(0.44–1.57)*	*0.566*
140–159	*2,406*	*21*	*1.84*	*(1.03–3.26)*	*0.038*	*6,185*	*19*	*1.42*	*(0.77–2.64)*	*0.263*
160–179	*1,057*	*12*	*2.60*	*(1.31–5.17)*	*0.006*	*2,673*	*17*	*3.29*	*(1.74–6.23)*	*<0.001*
180–	*648*	*17*	*6.58*	*(3.53–12.25)*	*<0.001*	*1,564*	*17*	*5.78*	*(3.03–11.00)*	*<0.001*
TG (mg/dl)										
<100	*1,521*	*11*	*1.00*	*(Referent)*		*6,337*	*18*	*1.00*	*(Referent)*	
100–149	*3,663*	*22*	*0.84*	*(0.41–1.74)*	*0.634*	*10,444*	*32*	*0.98*	*(0.55–1.76)*	*0.946*
150–199	*3,127*	*33*	*1.51*	*(0.76–3.02)*	*0.243*	*5,861*	*17*	*0.87*	*(0.44–1.71)*	*0.684*
200–249	*1,768*	*18*	*1.46*	*(0.68–3.15)*	*0.330*	*2,429*	*9*	*1.12*	*(0.50–2.53)*	*0.783*
250–	*2,494*	*21*	*1.24*	*(0.58–2.65)*	*0.572*	*1,921*	*17*	*2.62*	*(1.32–5.21)*	*0.006*
HDL-C (mg/dl)										
<40	*2,198*	*36*	*1.00*	*(Referent)*		*1,758*	*10*	*1.00*	*(Referent)*	
40–44	*2,133*	*23*	*0.64*	*(0.38–1.09)*	*0.099*	*2,794*	*17*	*1.12*	*(0.51–2.45)*	*0.776*
45–49	*2,207*	*17*	*0.44*	*(0.25–0.80)*	*0.006*	*4,101*	*24*	*1.09*	*(0.52–2.28)*	*0.819*
50–54	*1,956*	*13*	*0.39*	*(0.21–0.74)*	*0.004*	*4,440*	*13*	*0.57*	*(0.25–1.30)*	*0.179*
55–59	*1,402*	*8*	*0.33*	*(0.15–0.72)*	*0.005*	*4,053*	*13*	*0.66*	*(0.29–1.51)*	*0.324*
60–	*2,679*	*8*	*0.17*	*(0.08–0.36)*	*<0.001*	*9,867*	*16*	*0.33*	*(0.15–0.73)*	*0.006*
LDL-C/HDL-C										
<2.0	*2,851*	*11*	*1.00*	*(Referent)*		*7,426*	*11*	*1.00*	*(Referent)*	
2.0–2.4	*2,719*	*11*	*1.10*	*(0.48–2.55)*	*0.817*	*6,909*	*19*	*1.95*	*(0.92–4.10)*	*0.080*
2.5–2.9	*2,598*	*17*	*1.91*	*(0.89–4.10)*	*0.095*	*5,884*	*14*	*1.68*	*(0.76–3.72)*	*0.199*
3.0–3.4	*1,889*	*20*	*3.21*	*(1.53–6.74)*	*0.002*	*3,545*	*21*	*4.57*	*(2.19–9.54)*	*<0.001*
3.5–4.0	*1,082*	*13*	*3.87*	*(1.72–8.72)*	*0.001*	*1,728*	*12*	*5.04*	*(2.21–11.49)*	*<0.001*
4.0–	*1,194*	*28*	*8.06*	*(3.95–16.44)*	*<0.001*	*1,398*	*15*	*8.56*	*(3.88–18.88)*	*<0.001*

RR, relative risk; CI, confidence interval. Other abbreviations see in Table 1.

a) Coronary events included acute myocardial infarction and sudden cardiac death. Adjustment for age, hypertension, diabetes mellitus, body mass index, ECG abnormality, family history of CHD, cigarette smoking, and alcohol use.

Table 3 Relative Risk of Coronary Events and Baseline Characteristics[a)]

	Men					Women					Heterogeneity
	N	*Event*	*RR*	*95%CI*	*p value*	*N*	*Event*	*RR*	*95%CI*	*p value*	*p value[b)]*
Age (years)											
<55	*6,281*	*49*	*1.00*	*(Referent)*		*6,137*	*8*	*1.00*	*(Referent)*		*0.008*
55–59	*2,182*	*14*	*0.74*	*(0.41–1.34)*	*0.320*	*6,488*	*15*	*1.82*	*(0.77–4.29)*	*0.174*	
60–64	*2,164*	*17*	*0.87*	*(0.50–1.53)*	*0.627*	*7,112*	*29*	*3.02*	*(1.38–6.62)*	*0.006*	
≥65	*1,948*	*25*	*1.42*	*(0.86–2.34)*	*0.168*	*7,276*	*41*	*4.11*	*(1.92–8.82)*	*<0.001*	
Obesity[c)]	*4,621*	*40*	*0.99*	*(0.66–1.48)*	*0.956*	*8,700*	*32*	*0.91*	*(0.59–1.40)*	*0.663*	*0.676*
Hypertension[d)]	*5,705*	*68*	*2.15*	*(1.42–3.26)*	*<0.001*	*12,511*	*62*	*2.05*	*(1.32–3.18)*	*0.001*	*0.864*
Diabetes mellitus[e)]	*2,513*	*29*	*1.58*	*(1.03–2.43)*	*0.037*	*3,747*	*31*	*3.07*	*(1.99–4.74)*	*<0.001*	*0.019*
ECG abnormality[f)]	*1,681*	*26*	*1.86*	*(1.18–2.91)*	*0.007*	*3,473*	*23*	*1.67*	*(1.04–2.70)*	*0.035*	*0.972*
Family history of CHD[g)]	*637*	*10*	*2.00*	*(1.04–3.84)*	*0.038*	*1,289*	*13*	*3.34*	*(1.85–6.04)*	*<0.001*	*0.317*
Cigarette smoking[g)]	*5,506*	*52*	*1.46*	*(0.98–2.17)*	*0.063*	*1,105*	*9*	*2.94*	*(1.43–6.02)*	*0.003*	*0.148*
Alcohol use[g)]	*9,224*	*70*	*0.63*	*(0.41–0.96)*	*0.031*	*2,337*	*6*	*0.61*	*(0.26–1.45)*	*0.266*	*0.933*

Abbreviations see in Tables 1,2.

a) Coronary events included acute myocardial infarction and sudden cardiac death. Adjustment for age, hypertension, diabetes mellitus, body mass index, ECG abnormality, family history of CHD, cigarette smoking, and alcohol use. b) Heterogeneity between men and women, based on the likelihood ratio test. c) Body mass index ≥25 kg/m². d) Systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥95 mmHg or medication for hypertension. e) Fasting plasma glucose ≥140 mg/dl or medication. f) Study physician's diagnosis. g) Self-reported information.

tive association was more evident in men. The relative risk for coronary events was substantially increased in patients with LDL-C/HDL-C ≥3.0 in both men and women.

The increase in the risk of coronary events for each 10 mg/dl elevation of LDL-C concentration during the treatment period was 18% (95% CI 12–24%) in men and 21% (95% CI 15–27%) in women, and the decrease in CHD risk associated with each 10 mg/dl elevation of HDL-C concentration was 39% in men and 33% in women. The relationships of coronary events with baseline LDL-C and HDL-C concentrations were also examined, but were much weaker than those observed during the treatment period. With each 10 mg/dl elevation of LDL-C concentration at baseline, the increase in the relative risk was 7% for men

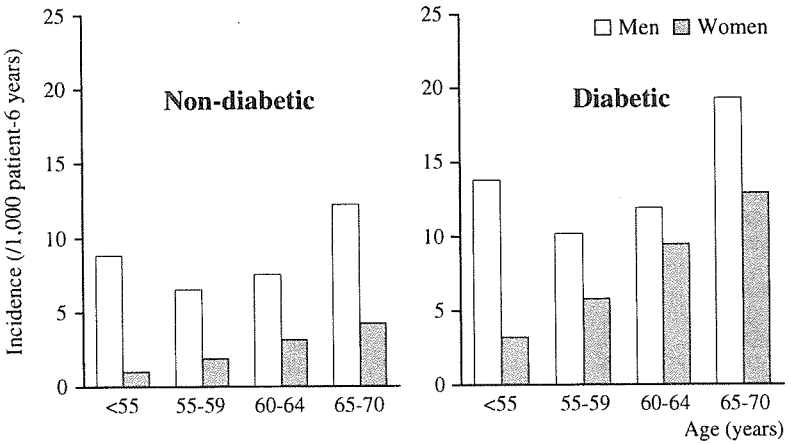

Fig 1. Estimated rates of coronary events according to age in men and women with and without diabetes mellitus (DM). Incidence rates were calculated from coronary heart disease (CHD) relative risks and the proportion of patients in each age category, for men and women separately, using Cox proportional hazards model, in which adjustment was made for age, hypertension, DM, body mass index, ECG abnormality, family history of CHD, cigarette smoking, and alcohol use.

and 9% for women and the decrease in risk with each 10 mg/dl elevation of HDL-C at baseline was 20% in both men and women.

Patient Baseline Characteristics and Risk of Coronary Events

The effect of age on the risk of coronary events was seen in women, but not in men (Table 3). Hypertension, DM, ECG abnormalities and a family history of CHD were also risk factors for coronary events in both men and women, but increased risks associated with DM and a family history of CHD were more marked for women than for men; the relative risk with DM was 1.58 in men and 3.07 in women, and the corresponding values for a family history of CHD were 2.00 in men and 3.34 in women. Obesity was unrelated to coronary events in either men or women. Although alcohol ingestion was protective in both men and women to the same extent, cigarette smoking was more strongly related to an increased risk of coronary events in women.

Discussion

This report addresses the gender differences in the relationship of serum lipid concentrations and other risk factors to CHD risk in Japanese patients under long-term treatment for hypercholesterolemia. Although serum TC and LDL-C concentrations were very similarly related to CHD risk in men and women, there was a difference between men and women in the relationship to serum TG and HDL-C concentrations. An inverse relationship of HDL-C to CHD risk was seen in men and women, but the HDL-C concentration showing a decreased risk of CHD differed by sex. The risk was significantly decreased at HDL-C ≥45 mg/dl in men and at HDL-C ≥60 mg/dl in women. The findings agree with observations published in the United States and Europe[2,3] and further indicate that the criterion of "low HDL-C" must be differential for men and women. An increased risk was observed only in women with an extremely high concentration of TG (≥250 mg/dl). Interpretation of this finding is difficult, and we do not have a clear idea about the implication of the present finding on serum TG.

In the present study, men did not show a clear increase in the risk of coronary events with increasing age, whereas there was a progressive increase in the risk with advancing age in women. The latter finding could be a reflection of the increase in serum TC and LDL-C concentrations with increasing age after menopause. The lack of an increasing trend in the association between age and coronary events in men is an unexpected finding, and may have been due to unknown characteristics of the male participants in the present study.

Whereas DM was related to increased CHD risk in both men and women, the increased risk was much greater in women, as indicated by a statistically significant interaction (p=0.019). These results did not change when further adjusted for TC or LDL-C. However, the risk difference between men and women for DM was not unique to the J-LIT patients. In a meta-analysis of 10 prospective studies, Lee et al showed that the effect of DM on the CHD risk was greater in women than in men![14] They showed that the relative risk of coronary death for DM patients vs non-DM patients was 2.58 (95% CI 2.05–3.26) in women and 1.85 (95% CI 1.47–2.33) in men (interaction p=0.045)![14] It was further noted in a later study that DM diminished the female advantage for lower CHD incidence![15] That DM is a stronger CHD risk factor in women may be related to the lower concentrations of HDL-C. Walden suggested that lower HDL-C concentrations in diabetic women as compared with men might be relevant to a stronger association between DM and CHD in women![16] In the present study, mean HDL-C concentrations in female diabetic patients were lower than those of non-diabetic patients (55.5 vs 57.5 mg/dl, p<0.001), but there was no difference in the HDL-C concentrations between the 2 groups in men (50.8 vs 51.3 mg/dl, p=0.09). The relative risk for DM was unchanged with adjustment for HDL-C. When the predicted rates of CHD incidence according to age were examined in men and women with and without DM (Fig 1), the increase in CHD incidence with aging was augmented in the presence of DM. Notably, DM diminished the women's advantage of having a lower CHD incidence in older patients.

Both cigarette smoking and family history of CHD were related to a greater increase in the risk of coronary events in women than in men. These differential increases in men and women may have been caused by random variation, as indicated by the lack of statistical significance for the interaction. As regards the effect of cigarette smoking, some studies suggest that smoking is a stronger risk factor in women than in men[2,17] but others have failed to find such a finding![18]

Finally, the present study results indicated that hypertension was an important risk factor in men and women equally, and that alcohol ingestion was protective in both sexes. These findings are in agreement with observations reported elsewhere![19–21]

In conclusion, the incidence of coronary events was 60% lower in women than in men among the J-LIT participants. Although the relationship of serum TC and LDL-C concentrations to coronary events was similar in men and women, the HDL-C concentration associated with a decreased risk of coronary events was slightly higher in women. DM was a stronger risk factor in women, and traded off the women's advantage of having a lower risk of coronary events, especially in aged patients.

Acknowledgment

This study was in part supported by a grant from Banyu Pharmaceutical Co, Ltd, Tokyo, Japan.

References

1. Ministry of Health, Labour and Welfare. Vital Statistics. Tokyo: the Ministry, 2001.
2. Njolstad I, Arnesen E, Lund-Larsen PG. Smoking, serum lipids, blood pressure, and sex differences in myocardial infarction: A 12-year follow-up of the Finnmark Study. *Circulation* 1996; **93:** 450–456.
3. Barrett-Connor E. Sex differences in coronary heart disease: Why are women so superior? The 1995 Ancel Keys lecture. *Circulation* 1997; **95:** 252–264.
4. Matsuzaki M, Kita T, Mabuchi H, Matsuzawa Y, Nakaya N, Oikawa S, et al and the J-LIT Study Group. Large scale cohort study of the relationship between serum cholesterol concentration and coronary events with low-dose simvastatin therapy in Japanese patients with hypercholesterolemia: Primary prevention cohort study of the Japan Lipid Intervention Trial (J-LIT). *Circ J* 2002; **66:** 1087–1095.
5. Mabuchi H, Kita T, Matsuzaki M, Matsuzawa Y, Nakaya N, Oikawa S, et al and the J-LIT Study Group. Large scale cohort study of the relationship between serum cholesterol concentration and coronary events with low-dose simvastatin therapy in Japanese patients with hypercholesterolemia and coronary heart disease: Secondary prevention cohort study of the Japan Lipid Intervention Trial (J-LIT). *Circ J* 2002; **66:** 1096–1100.
6. Matsuzawa Y, Kita T, Mabuchi H, Matsuzaki M, Nakaya N, Oikawa S, et al and the J-LIT Study Group. Sustained reduction of serum cholesterol in low-dose 6-year simvastatin treatment with minimum side effects in 51,321 Japanese hypercholesterolemic patients: Implication of the J-LIT study, a large scale nationwide cohort study. *Circ J* 2003; **67:** 287–294.
7. Nakaya N, Kita T, Mabuchi H, Matsuzaki M, Matsuzawa Y, Oikawa S, et al and the J-LIT Study Group. Large-scale cohort study on the relationship between serum lipid concentrations and risk of cerebrovascular disease under low-dose simvastatin in Japanese patients with hypercholesterolemia: Sub-analysis of the Japan Lipid Intervention Trial (J-LIT). *Circ J* 2005; **69:** 1016–1021.
8. Neaton JD, Wentworth D. Serum cholesterol, blood pressure, cigarette smoking, and death from coronary heart disease: Overall findings and differences by age for 316,099 white men: Multiple Risk Factor Intervention Trial Research Group. *Arch Intern Med* 1992; **152:** 56–64.
9. NIH Consensus Conference. Triglyceride, high-density lipoprotein, and coronary heart disease: NIH Consensus Development Panel on Triglyceride, High-Density Lipoprotein, and Coronary Heart Disease. *JAMA* 1993; **269:** 505–510.
10. Iwashita M, Matsushita Y, Sasaki J, Arakawa K, Kono S and the Kyushu Lipid Intervention Study (KLIS) Group. Relation of serum total cholesterol and other risk factors to risk of coronary events in middle-aged and elderly Japanese men with hypercholesterolemia: The Kyushu Lipid Intervention Study. *Circ J* 2004; **68:** 405–409.
11. Rich-Edwards JW, Manson JE, Hennekens CH, Buring JE. The primary prevention of coronary heart disease in women. *N Engl J Med* 1995; **332:** 1758–1766.
12. Matsuzawa Y, Itakura H, Kita T, Mabuchi H, Matsuzaki M, Nakaya N, et al and J-LIT Study Group. Design and baseline characteristics of a cohort study in Japanese patients with hypercholesterolemia: The Japan Lipid Intervention Trial (J-LIT). *Curr Ther Res* 2000; **61:** 219–243.
13. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972; **18:** 499–502.
14. Lee WL, Cheung AM, Cape D, Zinman B. Impact of diabetes on coronary artery disease in women and men: A meta-analysis of prospective studies. *Diabetes Care* 2000; **23:** 962–968.
15. Juutilainen A, Kortelainen S, Lehto S, Ronnemaa T, Pyorala K, Laakso M. Gender difference in the impact of type 2 diabetes on coronary heart disease risk. *Diabetes Care* 2004; **27:** 2898–2904.
16. Walden CE, Knopp RH, Wahl PW, Beach KW, Strandness E Jr. Sex differences in the effect of diabetes mellitus on lipoprotein triglyceride and cholesterol concentrations. *N Engl J Med* 1984; **311:** 953–959.
17. Asia Pacific Cohort Study Collaboration. Smoking, quitting, and the risk of cardiovascular disease among women and men in the Asia-Pacific region. *Int J Epidemiol* 2005; **24:** 1–10.
18. Ueshima H, Choudhury SR, Okayama A, Hayakawa T, Kita Y, Kadowaki T, et al. Cigarette smoking as a risk factor for stroke death in Japan: NIPPON DATA80. *Stroke* 2004; **35:** 1836–1841.
19. Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, et al and the INTERHEART Study Investigators. Effects of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): Case-control study. *Lancet* 2004; **364:** 937–952.
20. D'Agostino RB, Grundy S, Sullivan LM, Wilson P and the CHD Risk Prediction Group. Validation of the Framingham coronary heart disease prediction scores: Results of a multiple ethnic groups investigation. *JAMA* 2001; **286:** 180–187.
21. Thun MJ, Peto R, Lopez AD, Monaco JH, Henley J, Heath CW Jr, et al. Alcohol consumption and mortality among middle-aged and elderly U.S. adults. *N Engl J Med* 1997; **337:** 1705–1714.