

Fig. 5. The effect of GW9662, a PPAR $\gamma$  antagonist, on telmisartan (Tel)-induced AT1R downregulation. VSMCs were preincubated with GW9662 (5  $\mu$ g/mL) for 30 min and incubated with or without Tel (10  $\mu$ mol/L) for 12 h. Expression of AT1R protein and  $\alpha$ -tubulin was determined by Western blot analysis. Densitometric analysis was performed as described in the legend to Fig. 2. Values (mean  $\pm$  S.E.M.) are expressed as a percent of control culture (100%) ( $n=5$ ). \* $P<0.05$  vs control. n.s. not significant.

of TZDs and a full PPAR $\gamma$  agonist, inhibited AT1R expression as previously described [16]. We examined the effect of other AT1R antagonists on AT1R expression. Can (Fig. 3) and Olm (data not shown) had no effect on AT1R expression.

### 3.2. Tel inhibits AT1R expression at the transcriptional level

Deletion mutants of AT1 promoter/luciferase fusion DNA were used to locate the response element responsible for Tel-induced AT1R suppression (Fig. 4A). The suppression was observed in all constructs from  $-980/+25$ -luc to  $-61/+25$ -luc, so we supposed that the response element may exist in the DNA segment between  $-61$  bp and  $+25$  bp. Since Sugawara et al. [16] had previously reported the crucial role of a GC-box-related sequence within the  $-58/-34$  region of the AT1R gene promoter in PPAR $\gamma$ -induced AT1R suppression, we hypothesized that the same site may also be important in Tel-induced suppression. The luciferase construct with mutation in GC box (Sp1 site) failed to respond to Tel (Fig. 4A), indicating the important role of Sp1 site in Tel-induced downregulation. In addition, Tel did not affect the degradation rate of AT1R mRNA (Fig. 4B). These data suggested that Tel inhibits AT1R gene transcription and does not affect AT1R mRNA stability.

### 3.3. Tel-induced AT1R downregulation is PPAR $\gamma$ dependent

To examine the role of PPAR $\gamma$  in Tel-induced AT1R suppression, we examined the effect of GW9662, a PPAR $\gamma$  antagonist. Although GW9662 itself did not affect AT1R expression, preincubation with GW9662 blocked AT1R suppression induced by Tel (Fig. 5).

## 4. Discussion

In the present study, we demonstrated that Tel, an AT1R antagonist, suppressed the AT1R expression through the PPAR $\gamma$ -mediated pathway. This is the first study that demonstrates the suppression of AT1R expression by AT1R antagonist in VSMCs.

The expression of AT1R was suppressed at both mRNA and protein levels. The results of the promoter assay and the mRNA stability assay suggested that the suppression occurred at the transcriptional level rather than the post-transcriptional level.

The involvement of PPAR $\gamma$  on AT1R suppression was confirmed by experiments using GW9662. GW9662 prevented the Tel-induced suppression of AT1R expression, indicating the critical role of PPAR $\gamma$  in this pathway. The ability to activate PPAR $\gamma$  is reported to be unique to Tel and irbesartan (Irb) among several AT1R antagonists [10]. In our study, though we have not examined the effect of Irb, Can (Fig. 3) and Olm (data not shown) had no effect on AT1R expression. Our results seem to be consistent with the previous report [10].

Schupp et al. reported that a subset of AT1R blockers (ARB), Tel and Irb, induced PPAR $\gamma$  activity and promoted PPAR $\gamma$ -dependent differentiation in 3T3-L1 adipocytes [9]. These ARB activated PPAR $\gamma$  by direct interaction with the ligand binding domain (LBD) of PPAR $\gamma$  [9].

The docking studies of the molecular binding model explained the difference in the ability to activate PPAR $\gamma$  among several ARB and full agonists of PPAR $\gamma$  by comparing their interaction with residues of several helices [10]. According to this model, Tel fits in the LBD of PPAR $\gamma$  surrounded by helices H3, H6, and H7. However, Tel does not interact with activation function-2 helix that is responsible for receptor activation and stabilization by full PPAR $\gamma$  agonists. Irb, Can, and Olm, so-called tetrazole-containing ARBs, made contact with helix H3 but not H7. The difference in the interaction with these helices of LBD of PPAR $\gamma$  might contribute to their potential to activate PPAR $\gamma$ .

On activation by a ligand, PPAR $\gamma$  regulates the expression of several genes involved in lipid and carbohydrate metabolism [25,26] and inflammatory responses [27]. From the molecular insight, these effects are basically due to two different transcriptional regulatory mechanisms, one is transactivation and the other is transrepression. Transactivation is dependent on PPAR $\gamma$  response element (PPRE). Upon activation, PPAR $\gamma$  forms a heterodimer with retinoid X receptor and binds to PPRE in the promoter region of the target genes such as CD36 and glucose transporter 4. In contrast, transrepression involves interference with other transcription factors such as NF- $\kappa$ B and AP-1. Because there is no consensus sequence of PPRE in the AT1R gene promoter up to  $-980$  bp, the suppression of AT1R gene transcription should have occurred through the latter mechanism.

This assumption is well substantiated by a previous study which reported that suppression of AT1R gene expression by

PPAR $\gamma$  activation was independent of PPRE [16]. The authors demonstrated that the –58/–34 region of the AT1R gene promoter, including Sp1 binding site, was essential to the PPAR $\gamma$  activator-induced AT1R suppression. They concluded that activated PPAR $\gamma$  inhibited Sp1 function by direct protein–protein interaction. Our study with Tel also demonstrated the essential role of Sp1 binding site in the suppression of AT1R expression. Therefore, Tel may inhibit Sp1 function through the activation of PPAR $\gamma$  resulting in downregulation of the AT1R expression.

Our previous studies showed that downregulation of AT1R by PPAR $\gamma$  activator attenuated cellular response to Ang II [15]. However, Ang II induced ERK activation was almost completely blocked by Tel even at 1  $\mu$ mol/L, which did not affect AT1R expression because of AT1R blocking effect (Fig. 2C). Therefore, AT1R binding effect is expected at the lower concentration of Tel and dual effect of AT1R binding and AT1R downregulation is expected at higher concentration.

TZDs, synthetic PPAR $\gamma$  activators, are reported to inhibit atherogenesis by regulating various gene expressions. Several studies have demonstrated the anti-atherogenic effects of PPAR $\gamma$  activators in both animal models and human. Rosiglitazone (Rosi), one of TZDs, was shown to have additive effects on plaque regression in the combination treatment with simvastatin in an atherosclerotic rabbit model [28]. Anti-atherogenic effect of Rosi was also reported in a diabetes–atherosclerosis mouse model [29]. AT1R antagonists are reported to suppress atherogenesis. Strawn et al. demonstrated that losartan attenuated atherogenesis in monkeys with hypercholesterolemia [30]. Based on these studies, Tel may be more efficient in suppressing atherosclerotic vascular diseases due to its properties of PPAR $\gamma$  activation and AT1R antagonism.

TZDs are also effective in improving insulin sensitivity and they are already utilized to treat patients with type 2 diabetes [31]. Although the precise mechanism for PPAR $\gamma$ -mediated insulin sensitization is not clear, TZDs reduced fasting and postprandial glucose concentration, and insulin level. Ang II also affects insulin sensitivity [32]. The inhibition of AT1R by losartan improved insulin sensitivity [33] and, in the LIFE study, losartan significantly reduced the incidence of new-onset diabetes in patients with hypertension compared with atenolol [34]. Therefore, the dual effects of Tel may synergistically improve insulin sensitivity.

The dual function of Tel, an AT1R antagonist and a partial agonist of PPAR $\gamma$ , may be quite useful for the treatment of patients with hypertension with complications such as diabetes and atherosclerosis. Considering the blockade of Ang II, the downregulation of AT1R through the activation of PPAR $\gamma$  adds further possibility of Tel. This may result in more complete inhibition of the Ang II. However, it remains to be determined whether Tel suppresses the expression of AT1R in vivo and is superior to other AT1R antagonists in terms of the inhibition of the progression of cardiovascular diseases. Further study is needed.

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## References

- [1] de Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T. International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev* 2000;52:415–72.
- [2] Brenner BM, Cooper ME, de Zeeuw D, Keane WF, Mitch WE, Parving HH, et al. Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 2001;345:861–9.
- [3] Diez J, Querejeta R, Lopez B, Gonzalez A, Larman M, Martinez Ubago JL. Losartan-dependent regression of myocardial fibrosis is associated with reduction of left ventricular chamber stiffness in hypertensive patients. *Circulation* 2002;105:2512–7.
- [4] Scheen AJ. Prevention of type 2 diabetes mellitus through inhibition of the renin–angiotensin system. *Drugs* 2004;64:2537–65.
- [5] Chiu AT, Herblin WF, McCall DE, Ardecky RJ, Carini DJ, Duncia JV, et al. Identification of angiotensin II receptor subtypes. *Biochem Biophys Res Commun* 1989;165:196–203.
- [6] Horiuchi M, Akishita M, Dzau VJ. Recent progress in angiotensin II type 2 receptor research in the cardiovascular system. *Hypertension* 1999;33:613–21.
- [7] Gillespie EL, White CM, Kardas M, Lindberg M, Coleman CI. The impact of ACE inhibitors or angiotensin II type 1 receptor blockers on the development of new-onset type 2 diabetes. *Diabetes Care* 2005;28:2261–6.
- [8] Healey JS, Baranchuk A, Crystal E, Morillo CA, Garfinkle M, Yusuf S, et al. Prevention of atrial fibrillation with angiotensin-converting enzyme inhibitors and angiotensin receptor blockers: a meta-analysis. *J Am Coll Cardiol* 2005;45:1832–9.
- [9] Schupp M, Janke J, Clasch R, Unger T, Kintscher U. Angiotensin type 1 receptor blockers induce peroxisome proliferator-activated receptor-gamma activity. *Circulation* 2004;109:2054–7.
- [10] Benson SC, Pershadsingh HA, Ho CI, Chittiboyina A, Desai P, Pravenc M, et al. Identification of telmisartan as a unique angiotensin II receptor antagonist with selective PPAR $\gamma$ -modulating activity. *Hypertension* 2004;43:993–1002.
- [11] Yki-Jarvinen H. Thiazolidinediones. *N Engl J Med* 2004;351:1106–18.
- [12] He W, Barak Y, Hevener A, Olson P, Liao D, Le J, et al. Adipose-specific peroxisome proliferator-activated receptor gamma knockout causes insulin resistance in fat and liver but not in muscle. *Proc Natl Acad Sci U S A* 2003;100:15712–7.
- [13] Marx N, Kehrle B, Kohlhammer K, Grub M, Koenig W, Hombach V, et al. PPAR activators as antiinflammatory mediators in human T lymphocytes: implications for atherosclerosis and transplantation-associated arteriosclerosis. *Circ Res* 2002;90:703–10.
- [14] Collins AR, Meehan WP, Kintscher U, Jackson S, Wakino S, Noh G, et al. Troglitazone inhibits formation of early atherosclerotic lesions in diabetic and nondiabetic low density lipoprotein receptor-deficient mice. *Arterioscler Thromb Vasc Biol* 2001;21:365–71.
- [15] Takeda K, Ichiki T, Tokunou T, Funakoshi Y, Iino N, Hirano K, et al. Peroxisome proliferator-activated receptor gamma activators downregulate angiotensin II type 1 receptor in vascular smooth muscle cells. *Circulation* 2000;102:1834–9.
- [16] Sugawara A, Takeuchi K, Urano A, Ikeda Y, Arima S, Kudo M, et al. Transcriptional suppression of type 1 angiotensin II receptor gene expression by peroxisome proliferator-activated receptor-gamma in vascular smooth muscle cells. *Endocrinology* 2001;142:3125–34.

- [17] Yang H, Lu D, Raizada MK. Angiotensin II-induced phosphorylation of the AT1 receptor from rat brain neurons. *Hypertension* 1997;30:351–7.
- [18] Gupta M, Miller BA, Ahsan N, Ulsh PJ, Zhang MY, Cheung JY, et al. Expression of angiotensin II type I receptor on erythroid progenitors of patients with post transplant erythrocytosis. *Transplantation* 2000;70:1188–94.
- [19] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 1987;162:156–9.
- [20] Takeuchi K, Alexander RW, Nakamura Y, Tsujino T, Murphy TJ. Molecular structure and transcriptional function of the rat vascular AT1a angiotensin receptor gene. *Circ Res* 1993;73:612–21.
- [21] Guo DF, Uno S, Ishihata A, Nakamura N, Inagami T. Identification of a cis-acting glucocorticoid responsive element in the rat angiotensin II type 1A promoter. *Circ Res* 1995;77:249–57.
- [22] Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning*. New York: Cold Spring Harbor Laboratory Press; 1989. p. 16.66–7.
- [23] Lin Y, Zhu X, McLntee FL, Xiao H, Zhang J, Fu M, et al. Interferon regulatory factor-1 mediates PPARgamma-induced apoptosis in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2004;24:257–63.
- [24] Back SJ, Kim JS, Nixon JB, DiAugustine RP, Eling TE. Expression of NAG-1, a transforming growth factor-beta superfamily member, by troglitazone requires the early growth response gene EGR-1. *J Biol Chem* 2004;279:6883–92.
- [25] Tontonoz P, Hu E, Devine J, Beale EG, Spiegelman BM. PPAR gamma 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. *Mol Cell Biol* 1995;15:351–7.
- [26] Singh Ahuja H, Liu S, Crombie DL, Boehm M, Leibowitz MD, Heyman RA, et al. Differential effects of rexinoids and thiazolidinediones on metabolic gene expression in diabetic rodents. *Mol Pharmacol* 2001;59:765–73.
- [27] Diep QN, El Mabrouk M, Cohn JS, Endemann D, Amiri F, Virdis A, et al. Structure, endothelial function, cell growth, and inflammation in blood vessels of angiotensin II-infused rats: role of peroxisome proliferator-activated receptor-gamma. *Circulation* 2002;105:2296–302.
- [28] Tao L, Liu HR, Gao E, Teng ZP, Lopez BL, Christopher TA, et al. Antioxidative, antinitrative, and vasculoprotective effects of a peroxisome proliferator-activated receptor-gamma agonist in hypercholesterolemia. *Circulation* 2003;108:2805–11.
- [29] Phillips JW, Barringhaus KG, Sanders JM, OYang Z, Chen M, Hesselbacher S. Rosiglitazone reduces the accelerated neointima formation after arterial injury in a mouse injury model of type 2 diabetes. *Circulation* 2003;108:1994–9.
- [30] Strawn WB, Chappell MC, Dean RH, Kivlighn S, Ferrario CM. Inhibition of early atherogenesis by losartan in monkeys with diet-induced hypercholesterolemia. *Circulation* 2000;101:1586–93.
- [31] Miyazaki Y, Mahankali A, Matsuda M, Glass L, Mahankali S, Ferrannini E. Improved glycemic control and enhanced insulin sensitivity in type 2 diabetic subjects treated with pioglitazone. *Diabetes Care* 2001;24:710–9.
- [32] Velloso LA, Folli F, Sun XJ, White MF, Saad MJ, Kahn CR. Cross-talk between the insulin and angiotensin signaling systems. *Proc Natl Acad Sci U S A* 1996;93:12490–5.
- [33] Olsen MH, Fossum E, Hoiegggen A, Wachtell K, Hjerkin E, Nesbitt SD. Long-term treatment with losartan versus atenolol improves insulin sensitivity in hypertension: ICARUS, a LIFE substudy. *J Hypertens* 2005;23:891–8.
- [34] Lindholm LH, Ibsen H, Borch-Johnsen K, Olsen MH, Wachtell K, Dahlöf B. Risk of new-onset diabetes in the losartan intervention for endpoint reduction in hypertension study. *J Hypertens* 2002;20:1879–86.

## A third-generation, long-acting, dihydropyridine calcium antagonist, azelnidipine, attenuates stent-associated neointimal formation in non-human primates

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**Background** Calcium antagonists have been shown to reduce atherogenesis and improve clinical outcomes in atherosclerotic vascular disease. No study has so far, however, addressed the effects of calcium antagonists on stent-associated neointimal formation. We therefore investigated whether a third-generation calcium antagonist, azelnidipine, attenuates in-stent neointimal formation in non-human primates.

**Method** Male cynomolgus monkeys were fed a high cholesterol diet for 4 weeks, and were randomly assigned to three groups: a vehicle group and two other groups treated with azelnidipine at 3 and 10 mg/kg per day for an additional 24 weeks ( $n = 12$  each). Multi-link stents were then implanted in the iliac artery.

**Results** Azelnidipine at the high dose reduced neointimal thickness ( $0.25 \pm 0.02$  versus  $0.19 \pm 0.02$  mm;  $P < 0.05$ ). Azelnidipine also reduced local oxidative stress and monocyte chemoattractant protein 1 (MCP-1) expression. No difference was found between the three groups in the degrees of injury score, inflammation score, plaque neovascularization, or plasma lipid levels. Azelnidipine also reduced MCP-1-induced proliferation/migration of vascular smooth muscle cells *in vitro*.

**Conclusions** This study demonstrated for the first time that azelnidipine attenuates in-stent neointimal

formation associated with the reduced expression of MCP-1 and smooth muscle proliferation/migration in the neointima. These data in non-human primates suggest potential clinical benefits of azelnidipine as a 'vasculoprotective calcium antagonist' in patients undergoing vascular interventions. *J Hypertens* 24:1881–1889 © 2006 Lippincott Williams & Wilkins.

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### Introduction

Dihydropyridine calcium antagonists have been used worldwide for patients with hypertension and atherosclerotic vascular disease. Recent clinical trials with calcium antagonists provided evidence suggesting that reducing arterial blood pressure close to normal ranges is of great importance in reducing cardiovascular events [1–4]. Notably, in the Comparison of Amlodipine versus Enalapril to Limit Occurrences of Thrombosis (CAMELOT) trial [5], compared with placebo and enalapril, the third-generation calcium antagonist amlodipine was shown to reduce hospitalization as a result of unstable angina and revascularization in patients with coronary artery disease already being treated with aspirin, beta-blockers, or statins. These latest clinical trials suggest that there might be pleiotropic actions of cal-

cium antagonists beyond blood pressure lowering. The pleiotropic actions of calcium antagonists may be distinct from pharmacological actions related to L-type calcium channel blockade, but may be attributable to their lipophilic character leading to a high affinity with membrane phospholipid of arterial wall cells such as vascular smooth muscle cells (VSMC) [6]. The vasculoprotective actions of such calcium antagonists include an improvement in endothelial function, an anti-inflammation effect, anti-oxidant function, antiproliferation of VSMC and so forth.

In animal experiments, calcium antagonists have been shown to prevent or attenuate atherosclerosis [7,8], hypertension-induced vascular remodeling [9], heart failure [10] and neointimal formation after balloon injury [11]. However, no study has so far addressed the effects of

calcium antagonists on stent-associated neointimal formation. Investigating this subject must be of clinical importance, because stent implantation is now the major revascularization technique worldwide in patients with atherosclerotic vascular disease. Although a drug-eluting stent reduces the restenosis rate in selected arterial lesions, stent-associated restenosis remains an unsolved clinical issue in high-risk lesions. The cellular mechanism of restenosis after stenting is presumed to be neointimal formation as a result of VSMC proliferation/migration, recruitment of bone marrow-derived progenitor cells or inflammation in response to injury [12–14].

Azelnidipine is a newly developed third-generation calcium antagonist and has antihypertensive action that is comparable to amlodipine [15]. Azelnidipine has strong lipophilicity and affinity to membranes of VSMC [16,17]. Therefore, the aim of this study was to investigate whether azelnidipine at a clinical dose range attenuates in-stent neointimal formation in non-human primates (cynomolgus monkeys). To gain clinical significance for the results obtained, we used a non-human primate model of stent-associated neointimal formation [12].

## Materials and methods

### Experimental animals

Thirty-six 5-year-old male cynomolgus monkeys weighing 4.2–6.6 kg were purchased. The study protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences. The animal care before and after the operation of the animal model was performed in Gaoyao Kangda Laboratory Animal Science and Technology in China. A part of this study was performed at the Station for Collaborative Research and the Morphology Core, Kyushu University Graduate School of Medical Sciences.

### Animal model of in-stent restenosis

All animals were fed a high-cholesterol diet (0.5% cholesterol and 6% corn oil) for 4 weeks before stent implantation (Fig. 1). Ticlopidine 100 mg and aspirin 81 mg were started 7 days before the initial procedure. Aspirin was to be given and continued until animals were killed at

6 months, and ticlopidine was administered for 28 days. The animals were randomly assigned to three groups as follows: (i) no treatment vehicle control group (0.5% carboxymethyl cellulose sodium salt); (ii) low-dose azelnidipine (3 mg/kg per day; donated by Sankyo Pharmaceutical Co., Tokyo, Japan) group; (iii) high-dose azelnidipine (10 mg/kg per day) group ( $n = 12$  each). Azelnidipine treatment was carried out once a day by gavage for 24 weeks. One week after grouping, all monkeys were anaesthetized with ketamine hydrochloride (10 mg/kg intramuscularly) and sodium pentobarbital (30 mg/kg intravenously), and underwent the placement of a 15 mm-long Multilink stent mounted over the 3-mm balloon implanted in the iliac artery (30 s inflation at 6 atm followed by 60 s inflation at 8 atm, resulting in a stent-to-artery ratio of 1.2 : 1.0) as described [12]. After the operation, all monkeys were fed the same high-cholesterol diet.

### Histopathology and immunohistochemistry

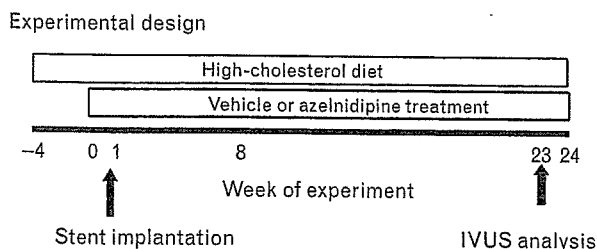
Stented arterial sections were excised and fixed for 24 h with 95% ethanol and 1% acetic acid. Each segment was divided into two parts at the centre of the stent. The proximal part was embedded in methyl methacrylate mixed with *n*-butyl methacrylate to allow for sectioning through metal stent struts. Serial sections were stained with elastica van Gieson and haematoxylin eosin. To evaluate the stent area, lumen area, medial area, and neointimal area, the neointimal thickening (neointimal area/length of internal elastica lamina; IEL) were measured. An immunohistochemical study was performed with antibodies against human smooth muscle actin (1A4; Dako, Tokyo, Japan) and von Willebrand factor (vWF; Dako). To evaluate the degree of neovascularization the number of arterioles in the intima and adventitia were counted.

The distal part was used for immunohistochemical analysis. After stent struts were gently removed with micro forceps, the tissue was dehydrated, embedded in paraffin, and cut into 5- $\mu$ m thick slices. They were subjected to immunostaining using antibodies against human monocyte chemoattractant protein 1 (MCP-1; Santa Cruz, California, USA) and C-C chemokine receptor 2 (CCR2, Santa Cruz) as described [12]. A single observer, who was blinded to the experiment protocol, performed the morphometry. All images were captured by an Olympus microscope equipped with a digital camera (HC-2500) and were analysed using Adobe Photoshop 6.0 (Adobe Systems, San Jose, California, USA) and Scion Image 1.62 (Scion, Frederick, Maryland, USA) software.

### Local oxidative stress with fluorescent dihydroethidium micrographic analysis

Frozen, enzymatically intact, sections (10- $\mu$ m thick) just at the proximal edge of stented iliac arteries were incubated with dihydroethidium (10  $\mu$ mol/l; Sigma, Tokyo,

Fig. 1



Experimental design. IVUS, Intravascular ultrasound.

Japan) in phosphate-buffered saline for 30 min at 37°C in a humidified chamber protected from light. Ethidium bromide was detected by fluorescent microscopy as described previously [18]. One representative section per artery was counted in all animals.

#### Intravascular ultrasound procedure and analysis

Twenty-three weeks after stent implantation, intravascular ultrasound imaging (IVUS) was performed. The imaging was performed using a 40 MHz ultrasonic imaging catheter (Ultra Cross; Boston Scientific, Boston, Massachusetts, USA) and an automatic pullback device, and the studies were recorded on 1/2-inch high-resolution s-VHS tapes for off-line volumetric assessment. IVUS analysis included stent area and proliferative intimal area.

#### Injury and inflammation scores

The injury and inflammatory scores were determined at each strut site, and mean values were calculated for each stented segment as previously described [19,20]. In brief, a numeric value from 0 (no injury) to 3 (most injury) was assigned: 0, endothelial denudate, IEL intact; 1, IEL lacerated, media compressed, not lacerated; 2, IEL lacerated, media lacerated, external elastica lamina compressed, not lacerated; and 3, media severely lacerated, external elastica lamina lacerated, adventitia may contain stent strut. The average injury score for each segment was calculated by dividing the sum of injury scores by the total number of struts at the examined section. The inflammation score took into consideration the extent

and density of the inflammatory infiltrate in each individual strut. With regard to the inflammatory score for each individual strut, the grading is: 0, no inflammatory cells surrounding the strut; 1, light, non-circumferential inflammatory cells infiltrate surrounding the strut; 2, localized, moderate-to-dense cellular aggregate surrounding the strut non-circumferentially; and 3, circumferential dense inflammatory cells, infiltration of the strut. The inflammatory score for each cross section was calculated in the same manner as for the injury score (sum of the individual inflammatory scores, divided by the number of struts in the examined section).

#### Biochemical measurements

Biochemical parameters listed in Tables 1 and 2 were measured. MCP-1, interleukin (IL)-8, and transforming growth factor beta 1 were measured using commercially available enzyme-linked immunosorbent assay kits for humans (Quantikine R&D Systems, Minneapolis, Minnesota, USA). 8-Iso prostaglandin F<sub>2</sub>α (isoprostanes) in the urine was measured by high-performance liquid chromatography/tandem mass spectrometry as previously reported [21]. Isoprostanes were adjusted according to the creatinine level. Urine was collected over 3 h while each animal was in a metabolic cage.

#### Proliferation and migration assay in vascular smooth muscle cells

Human coronary artery VSMC (Cambrex Bio Science Walkersville Inc., Walkersville, Tennessee, USA) were

Table 1 Systemic biochemical parameters and body weight

Parameters	Groups	Week of experiment			
		0	8	16	24
Total cholesterol (mg/dl)	Vehicle control	430 ± 51	495 ± 51	417 ± 34	646 ± 55*
	Azel 3 mg/kg	470 ± 32	555 ± 62	524 ± 70	631 ± 42*
	Azel 10 mg/kg	437 ± 33	480 ± 47	347 ± 28	568 ± 50*
HDL-cholesterol (mg/dl)	Vehicle control	20 ± 2	19 ± 4	22 ± 6	14 ± 2
	Azel 3 mg/kg	18 ± 2	14 ± 2	15 ± 2	15 ± 2
	Azel 10 mg/kg	21 ± 4	14 ± 2	17 ± 4	16 ± 2
LDL-cholesterol (mg/dl)	Vehicle control	370 ± 46	387 ± 37	315 ± 28	512 ± 39*
	Azel 3 mg/kg	381 ± 31	430 ± 46	385 ± 46	466 ± 27*
	Azel 10 mg/kg	298 ± 32	381 ± 40	267 ± 24	420 ± 29*
Triglycerides (mg/dl)	Vehicle control	16 ± 3	16 ± 4	13 ± 1	18 ± 2
	Azel 3 mg/kg	10 ± 1	16 ± 3	13 ± 2	33 ± 9
	Azel 10 mg/kg	14 ± 1	25 ± 12	12 ± 2	25 ± 4
Glucose (mg/dl)	Vehicle control	58 ± 3	67 ± 3	64 ± 2	62 ± 3
	Azel 3 mg/kg	60 ± 4	60 ± 3	63 ± 3	66 ± 3
	Azel 10 mg/kg	65 ± 4	65 ± 3	61 ± 2	59 ± 3
GOT (unit/l)	Vehicle control	52 ± 6	44 ± 6	50 ± 4	54 ± 4
	Azel 3 mg/kg	51 ± 3	43 ± 3	47 ± 4	55 ± 3
	Azel 10 mg/kg	45 ± 4	41 ± 4	50 ± 6	61 ± 6
GPT (unit/l)	Vehicle control	29 ± 7	21 ± 3	20 ± 4	23 ± 2
	Azel 3 mg/kg	25 ± 2	20 ± 2	22 ± 7	23 ± 2
	Azel 10 mg/kg	21 ± 2	17 ± 2	17 ± 5	28 ± 6
Angiotensin II (pg/ml)	Vehicle control	29 ± 8	19 ± 4	14 ± 2	43 ± 10
	Azel 3 mg/kg	12 ± 2	15 ± 3	15 ± 3	21 ± 5
	Azel 10 mg/kg	12 ± 1	11 ± 2	15 ± 3	31 ± 8
Body weight (kg)	Vehicle control	5.2 ± 0.2	5.6 ± 0.2	6.1 ± 0.2	6.1 ± 0.2
	Azel 3 mg/kg	5.2 ± 0.2	5.6 ± 0.3	5.8 ± 0.3	5.8 ± 0.3
	Azel 10 mg/kg	5.2 ± 0.2	5.6 ± 0.2	6.0 ± 0.3	5.9 ± 0.3

Azel, azelnidipine; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Data are mean ± SEM (n = 12 each). \*P < 0.05 versus 0 week.

Table 2 Systemic oxidative stress marker and cytokines

Parameters	Groups	Week of experiment			
		0	8	16	24
8-Iso-prostaglandin in urine (ng/mg creatinine)	No treatment	716 ± 86	691 ± 94	654 ± 75	603 ± 88
	Azel 3 mg/kg	714 ± 78	572 ± 92	621 ± 86	626 ± 66
	Azel 10 mg/kg	845 ± 79	674 ± 98	693 ± 92	573 ± 78*
MCP-1 in serum (pg/ml)	No treatment	44 ± 5	40 ± 11	51 ± 3	61 ± 5*
	Azel 3 mg/kg	46 ± 3	37 ± 8	45 ± 3	47 ± 5
	Azel 10 mg/kg	47 ± 4	32 ± 7	29 ± 5 <sup>†</sup> *	37 ± 4 <sup>†</sup> *
IL-8 in serum (ng/ml)	No treatment	3.0 ± 0.3	3.8 ± 0.5	4.4 ± 0.5	3.2 ± 0.5
	Azel 3 mg/kg	2.2 ± 0.3	2.9 ± 0.3	4.6 ± 0.4	3.6 ± 0.7
	Azel 10 mg/kg	2.7 ± 0.3	3.3 ± 0.4	4.2 ± 0.8	2.7 ± 0.6
TGF-β1 in serum (ng/ml)	No treatment	63 ± 6	70 ± 7	67 ± 7	47 ± 4
	Azel 3 mg/kg	55 ± 4	67 ± 3	68 ± 7	43 ± 5
	Azel 10 mg/kg	56 ± 4	73 ± 4	62 ± 4	42 ± 3

Azel, azelnidipine; IL, interleukin; MCP-1, Monocyte chemoattractant protein 1; TGF-β1, transforming growth factor beta 1. Data are mean ± SEM ( $n = 12$  each). \* $P < 0.01$  versus 0 week, <sup>†</sup> $P < 0.05$  versus no treatment group.

cultured, and placed into 48-well culture plates (Falcon 354506 Biocoat Cell Ware Human Fibronectin; Bioscience Inc., Bedford Massachusetts, USA). Proliferation was stimulated by human MCP-1 at 10 ng/ml (Sigma). Azelnidipine at 1, 10, and 100 nmol or solvent were added to the wells. Four days later, the cells were fixed with methanol and a single observer, who was blinded to the experimental protocol, counted the number of cells per plate.

The migration of rat aortic smooth muscle cells was assessed with a Boyden chamber type cell migration assay kit housing a collagen-precoated polycarbonate membrane with 8.0-μm pores (Chemicon International Inc., Temecula, California, USA), as previously described [22]. The lower chambers were filled with or without human MCP-1 at 10 ng/ml (Sigma). Then cells ( $1 \times 10^5$  cells/ml) were placed on the upper side of the membrane and allowed to migrate through the pores. After 4 h of incubation, the number of cells that migrated to the lower surface of the membrane was counted per  $\times 200$  high-power fields. Azelnidipine at 1, 10, and 100 nmol or solvent was added to the lower chamber.

The concentrations of azelnidipine at ranges of 1–10 nmol can be considered as a clinically relevant dose, because the concentrations are nearly equivalent to the concentration reported in human subjects who were orally administered a 5 or 15 mg azelnidipine tablet [23].

#### Measurements of arterial blood pressure, heart rate, and plasma azelnidipine levels

Separate preliminary experiments were performed to determine the effects of azelnidipine on arterial blood pressure and heart rate in cynomolgus monkeys under conscious conditions. On the last day of the drug treatment period, blood samples were drawn before and after drug administration, and the plasma drug concentration was determined by gas chromatography–mass spectrometry [15].

#### Statistical analysis

Data are expressed as the mean ± SE. Statistical analysis of differences was compared by analysis of variance and Dunnett's multiple comparison tests. A  $P$  value of less than 0.05 was considered to be statistically significant.

## Results

#### General status

No animals showed any adverse clinical signs (decreased spontaneous motor action, decreased food consumption, diarrhoea, limping and prone position, etc.) during the experimental period and all survived. There was no significant treatment effect on body weight among the groups (Table 1).

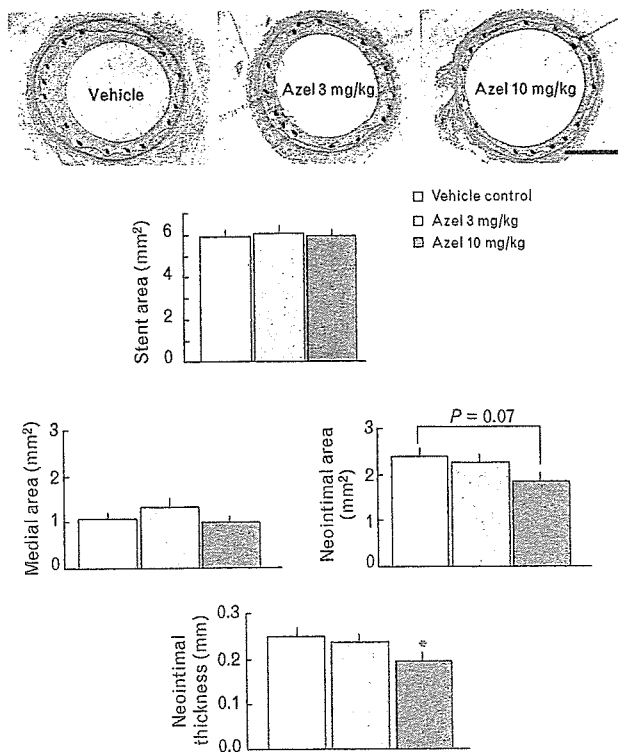
#### Histopathological and immunohistochemical analysis, and detection of local oxidative stress

There were no significant differences in stent area, IEL length, and medial area among all three groups (Fig. 2). Azelnidipine at the high dose tended to decrease the neointimal area (vehicle group,  $2.4 \pm 0.2$ ; low-dose group,  $2.3 \pm 0.2$ ; high-dose group,  $1.8 \pm 0.2$  mm<sup>2</sup>; vehicle versus high dose  $P = 0.07$ ) and significantly decreased neointimal thickening (vehicle group,  $0.25 \pm 0.02$ ; low-dose group,  $0.24 \pm 0.02$ ; high-dose group,  $0.19 \pm 0.02$ ; vehicle versus high dose  $P < 0.05$ ; Fig. 2).

Both MCP-1 and CCR2 immunoreactivity was not detected in the non-stented normal artery (data not shown). In contrast, intense MCP-1 and CCR2 immunoreactivity was evident in the neointima and media of stented arteries from the control group. The percentage of MCP-1-positive cells in the neointima decreased in the high-dose azelnidipine group (Fig. 3), whereas the percentage of CCR2-positive cells did not differ among the three groups. In contrast, there was no significant difference in the percentage of MCP-1 or CCR2-positive cells in the media among the three groups.

Local oxidative stress was measured with dihydroethidium staining. No apparent dihydroethidium fluorescence

Fig. 2



Effects of azelnidipine on stent-associated neointimal formation (histopathological analysis). (a) Representative photomicrographs of cross-sections of the stented iliac artery stained with elastic van Gieson from vehicle control group (left panel), low-dose azelnidipine (Azel) group (middle panel), and high-dose azelnidipine group (right panel) 24 weeks after stenting. Bar 1 mm. (b) Stent area (area within the stent itself), medial area (area within the external elastica lamina minus internal elastica lamina area), neointimal area (area within the internal elastica lamina minus lumen area), and neointimal thickening ( $n = 12$  each). \* $P < 0.05$  versus vehicle group. Each value represents mean  $\pm$  SEM.

was detected in the non-stented normal artery (data not shown). As shown in Fig. 4, the fluorescent signal attributable to superoxide production was markedly enhanced in the neointima and media from the control group. The intensity of dihydroethidium fluorescence in the neointima decreased in the high-dose azelnidipine group, whereas the dihydroethidium signal in the media did not differ among the three groups (Fig. 4).

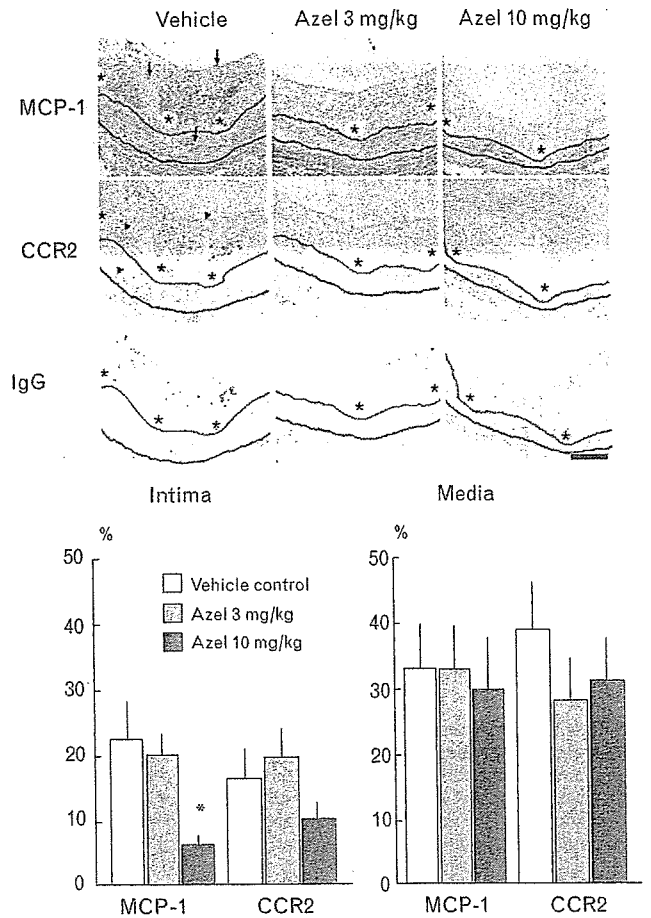
**Injury score, inflammation score, and neovascularization**

There was no significant difference in the injury score and inflammation score between the three groups (Fig. 5). Also, there were no significant differences in the degrees of neovascularization in the neointima (vWF-positive microvessels/mm<sup>2</sup>) and adventitia (vWF-positive microvessels/section; Fig. 5).

**Intravascular ultrasound imaging analysis**

The degrees of neointimal formation were also investigated by IVUS analysis. As shown in histopathological

Fig. 3



Effects of azelnidipine (Azel) on immunoreactivity for monocyte chemoattractant protein 1 (MCP-1) and C-C chemokine receptor 2 (CCR2). (a) Cross-sections of stented arteries from experimental groups stained immunohistochemically with the antibody against MCP-1 (arrows), CCR2 (arrow heads) or non-immune IgG (\* indicates stent strut sites). White and blue lines show internal and external elastica lamina, respectively. (b) The percentages of MCP-1 and CCR2-positive cells in the neointima and media ( $n = 12$  each). Bar 100  $\mu$ m. \* $P < 0.05$  versus vehicle group. Each value represents mean  $\pm$  SEM.

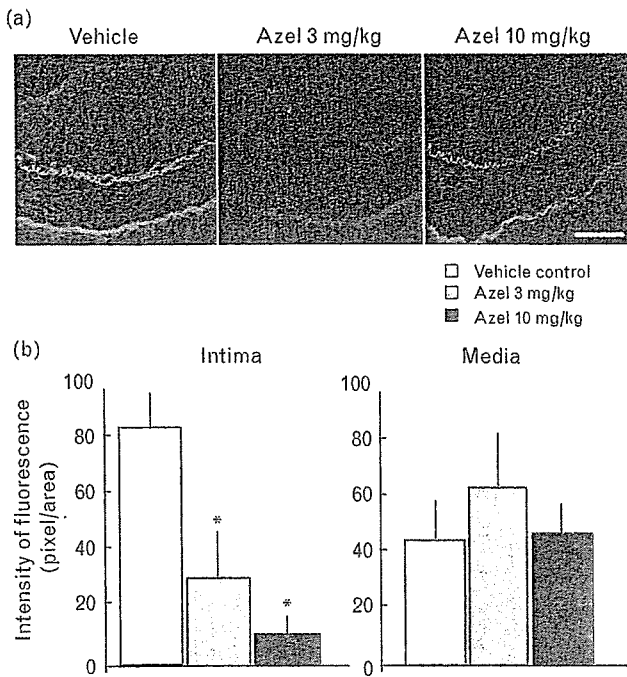
analysis, high-dose azelnidipine reduced stent-associated neointimal formation by 42% (Fig. 6).

**Systemic biochemical parameters**

Low and high doses of azelnidipine also did not affect lipid profiles, angiotensin II and liver transaminases (Table 1). Azelnidipine at the high dose decreased 8-iso-prostaglandin F2 $\alpha$  in urine at week 24 (Table 2). Serum levels of MCP-1 increased at week 24 in the vehicle group (Table 2). No increase in serum MCP-1 levels was noted in azelnidipine treatment groups. Notably, azelnidipine at the high dose reduced serum MCP-1 levels. In contrast, there were no treatment effects of azelnidipine on serum levels of IL-8 and transforming growth factor beta 1 (Table 2).



Fig. 4



Effects of azelnidipine (Azel) on local oxidative stress marker. (a) Representative superoxide detection with dihydroethidium in cross-sections of iliac arteries. Red colour expressed dihydroethidium-stained positive nuclei, and yellow or green expressed autofluorescences. Autofluorescences were agreed internal elastica lamina (upper line) and external elastica lamina (lower line). Bar 100  $\mu$ m. (b) Mean fluorescent intensity in the neointima and media ( $n = 12$  each). \* $P < 0.01$  versus vehicle group. Each value represents mean  $\pm$  SEM.

**Proliferation and migration assay of vascular smooth muscle cells**

Azelnidipine at 1, 10, and 100 nmol significantly inhibited MCP-1-induced proliferation in a dose-dependent manner (Fig. 7). Azelnidipine also reduced the MCP-1-induced migration of rat aortic smooth muscle cells (Fig. 7).

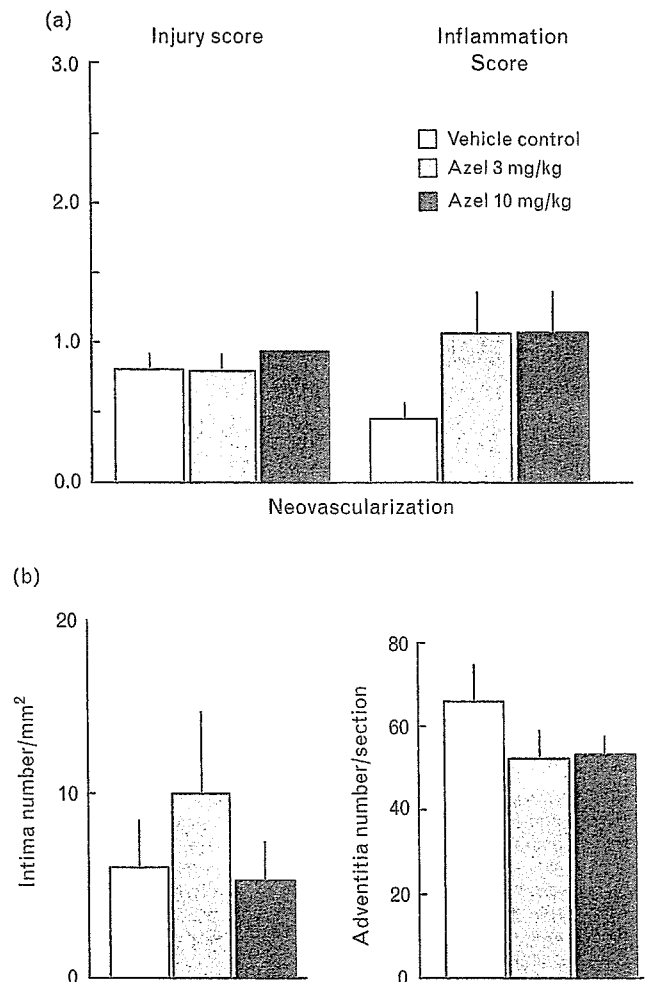
**Haemodynamic parameters and plasma azelnidipine concentrations**

Azelnidipine at the low and high doses did not affect haemodynamic parameters (Table 3). The maximum concentration ( $C_{max}$ ) of azelnidipine at 3 and 10 mg/kg per day was  $36 \pm 17$  and  $107 \pm 17$  ng/ml, respectively, in monkeys. The  $C_{max}$  after oral administration of azelnidipine at 16 mg in hypertensive individuals is reported to be  $48 \pm 19$  ng/ml [15].

**Discussion**

The present study demonstrated for the first time that a newly developed calcium antagonist, azelnidipine, at 10 mg/kg attenuated neointimal formation 6 months after stent implantation in hypercholesterolemic non-

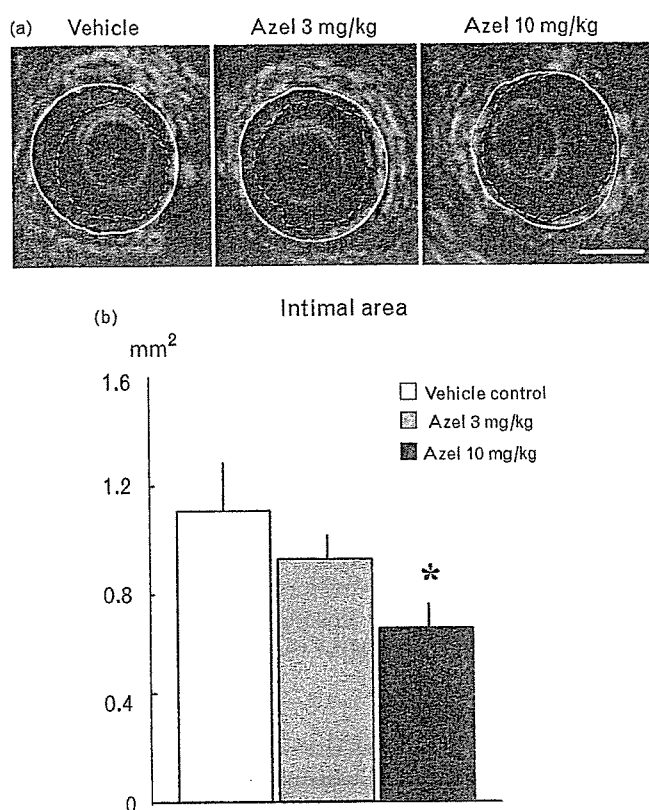
Fig. 5



Effects of azelnidipine (Azel) on injury score, inflammation score, and neovascularization. (a) Injury score and inflammation score. (b) The degrees of neovascularization in the neointima and adventitia. The neovascularization were defined as von Willebrand factor (vWF)-positive microvessels/mm<sup>2</sup> in the neointima and vWF-positive microvessels/section in adventitia ( $n = 12$  each). Each value represents mean  $\pm$  SEM.

human primates (cynomolgus monkeys). We evaluated the degrees of stent-associated neointimal formation in two ways: histopathological and IVUS analyses. Both methods demonstrated significant benefits of azelnidipine by reducing neointimal formation after stent implantation. Although an appropriate animal model for the evaluation of stent-associated neointimal formation (restenotic changes) is uncertain, the non-human primate model may gain the advantage over non-primate animal models such as rabbits and pigs, because adequate degrees of neointima develop after stenting, and vascular inflammatory and proliferative responses to injury in non-human primates are presumed to be closer to those in humans than other non-primate models. Therefore, the use of non-human primates may allow us to evaluate the efficacy of any therapies on

Fig. 6

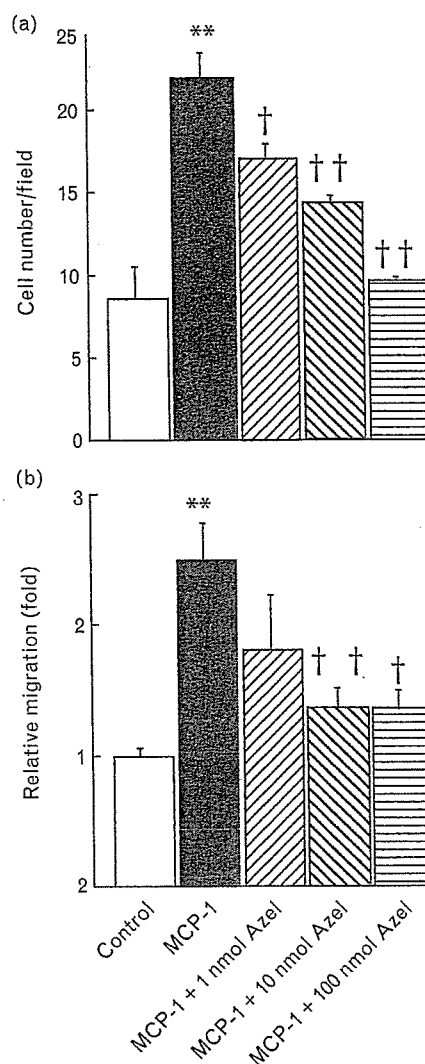


Effects of azelnidipine (Azel) on stent-associated neointimal formation by intravascular ultrasound analysis. (a) An intravascular ultrasound cross-sectional image in the treatment groups. Dot lines and solid lines show endothelial and internal elastica lamina, respectively. Bar 1 mm. (b) Effect of azelnidipine on neointimal area ( $n = 8-10$  each). \* $P < 0.05$  versus vehicle group. Each value represents mean  $\pm$  SEM.

stent-associated neointimal formation in clinically relevant conditions.

Another important point is that the both doses of azelnidipine did not significantly reduce arterial blood pressure in cynomolgus monkeys (Table 3). Furthermore, azelnidipine did not affect serum lipid levels (Table 1). Therefore, the beneficial effect of azelnidipine on stent-associated neointimal formation was independent of its effects on serum lipid or arterial blood pressure. Although the reason why the

Fig. 7



Effects of azelnidipine (Azel) on proliferation of human coronary artery smooth muscle cells (a) and on the migration of rat aortic smooth muscle cells (b). \* $P < 0.05$ , \*\* $P < 0.01$  versus control. † $P < 0.05$ , †† $P < 0.01$  versus monocyte chemoattractant protein 1 (MCP-1;  $n = 9$  each). Each value represents mean  $\pm$  SEM.

high dose of azelnidipine, which was higher than the clinical dose in respect of plasma concentrations ( $C_{\text{max}}$ ), did not reduce arterial blood pressure, it might be explained by a species difference. In addition, the fact

Table 3 Mean heart rate and blood pressure in conscious cynomolgus monkeys treated with or without azelnidipine

	Vehicle control	Azelnidipine 3 mg/kg per day	Azelnidipine 10 mg/kg per day
Heart rate (beats/min)	123 $\pm$ 11	146 $\pm$ 12	147 $\pm$ 10
Systolic blood pressure (mmHg)	101 $\pm$ 5	100 $\pm$ 4	98 $\pm$ 3
Diastolic blood pressure (mmHg)	70 $\pm$ 3	68 $\pm$ 2	64 $\pm$ 2

Data are expressed as mean  $\pm$  SEM. Cynomolgus monkeys (three males and three females) were sedated with intramuscular ketamine. A telemetry transmitter system with a catheter-type pressure manometer (TL11M2-D70-PCT; Data Sciences International, Arden Hills, Minnesota, USA) was implanted at the subcutaneous space of the abdomen, and the tip of the manometer was placed in the abdominal aorta through the femoral artery. After the animals recovered from the surgery and stabilized, arterial blood pressure and heart rate were continuously measured for at least 48 h by a telemetry receiver system (Data Sciences International) before and 7 days after the oral administration of azelnidipine at 3 or 10 mg/kg per day. Mean systolic blood pressure, diastolic blood pressure, and heart rate were examined with a computer-based analysis system equipped with a telemetry system (Primetech Inc., Tokyo, Japan).

that normotensive monkeys were used in the present study can be explored.

To investigate the potential mechanisms of the beneficial action of azelnidipine, we examined the systemic and local oxidative stress markers. Azelnidipine at 10 mg/kg slightly but significantly reduced the systemic marker (urine isoprostanes) 24 weeks after stent implantation. However, the effect of azelnidipine on systemic oxidative stress is not conclusive because of the variability of baseline values. In contrast, a representative local marker (fluorescent dihydroethidium staining) was reduced in neointimal cells (VSMC), but not in medial cells by azelnidipine at 10 mg/kg. These data support the notion that local oxidative stress in activated neointimal smooth muscle cells is important as one of the major mechanisms underlying the vasculoprotective action of azelnidipine seen in the present study.

It is now known that the oxidative stress-induced inflammatory and proliferative process is a centre stage of neointimal formation after vascular injury [24,25]. We and others have demonstrated that increased monocyte-mediated inflammation is associated with greater neointimal formation after stenting [26,27], and anti-MCP-1 gene therapy [12,14,28,29] or the administration of blocking antibody against the MCP-1 receptor [30] markedly reduced neointimal formation after vascular injury. In the present study, we therefore examined MCP-1 expression and showed a reduction in serum MCP-1 levels and MCP-1 immunoreactivity in the neointima by azelnidipine at 10 mg/kg. These data suggest that the vasculoprotective effects of azelnidipine may be attributable to the inhibition of the oxidative stress-induced upregulation of MCP-1. However, because there were no significant differences in the inflammation score, injury score, and neovascularization between the three groups, it is suggested that the beneficial effects of azelnidipine cannot be explained solely by its anti-inflammatory actions.

Emerging evidence, however, suggests that MCP-1 not only mediates monocyte-related inflammation, but also mediates the transformation, proliferation, and migration of VSMC [31–33]. We therefore hypothesized that azelnidipine inhibits the proliferation/migration of VSMC induced by MCP-1. Our present data with VSMC in culture suggest that azelnidipine might attenuate stent-associated neointimal formation at least partly by inhibiting the proliferation and migration of VSMC induced by MCP-1.

Our present data suggest that azelnidipine attenuated the proliferation and migration of VSMC in the neointima and thus decreased the production of inflammatory cytokines and growth factors after stent intervention, which in turn led to reduced neointimal formation. It has

been suggested that azelnidipine might be used for the prevention or treatment of atherosclerotic vascular disease after stenting procedures as a 'vasculoprotective calcium antagonist'. A drug-eluting stent is now becoming a major therapeutic device for patients with flow-limiting vascular stenosis. It is expected that oral azelnidipine treatment in addition to a drug-eluting stent would further decrease restenosis and clinical outcomes in patients.

In conclusion, this study provided experimental evidence suggesting that oral treatment with azelnidipine attenuates stent-associated neointimal formation in non-human primates. The beneficial effects were associated with reduced local oxidative stress, MCP-1 expression and smooth muscle cell proliferation/migration in the neointima. These data in non-human primates suggest the potential clinical benefits of azelnidipine as a 'vasculoprotective calcium antagonist' in patients undergoing vascular interventions. However, further clinical research is needed to support this notion, because the effective dose of azelnidipine was higher than the clinical dose range.

### Acknowledgments

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### References

- 1 Pitt B, Byington RP, Furberg CD, Hunninghake DB, Mancini GB, Miller ME, Riley W. Effect of amlodipine on the progression of atherosclerosis and the occurrence of clinical events. PREVENT Investigators. *Circulation* 2000; **102**:1503–1510.
- 2 The ALLHAT Officers and Coordinators for the ALLHAT Collaborative Research Group. Major outcomes in high-risk hypertensive patients randomized to angiotensin-converting enzyme inhibitor or calcium channel blocker vs diuretic: the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT). *JAMA* 2002; **288**:2981–2997.
- 3 Julius S, Kjeldsen SE, Weber M, Brunner HR, Ekman S, Hansson L, et al. Outcomes in hypertensive patients at high cardiovascular risk treated with regimens based on valsartan or amlodipine: the VALUE randomised trial. *Lancet* 2004; **363**:2022–2031.
- 4 Jorgensen B, Simonsen S, Endresen K, Forfang K, Vatne K, Hansen J, et al. Restenosis and clinical outcome in patients treated with amlodipine after angioplasty: results from the Coronary Angioplasty Amlodipine REStenosis Study (CAPARES). *J Am Coll Cardiol* 2000; **35**:592–599.
- 5 Nissen SE, Tuzcu EM, Libby P, Thompson PD, Ghali M, Garza D, et al. Effect of antihypertensive agents on cardiovascular events in patients with coronary disease and normal blood pressure: the CAMELOT study: a randomized controlled trial. *JAMA* 2004; **292**:2217–2225.
- 6 Mason RP, Marche P, Hintze TH. Novel vascular biology of third-generation L-type calcium channel antagonists: ancillary actions of amlodipine. *Arterioscler Thromb Vasc Biol* 2003; **23**:2155–2163.
- 7 Cristofori P, Lanzoni A, Quartaroli M, Pastorino AM, Zancanaro C, Cominacini L, et al. The calcium-channel blocker lacidipine reduces the development of atherosclerotic lesions in the apoE-deficient mouse. *J Hypertens* 2000; **18**:1429–1436.
- 8 Suzuki J, Iwai M, Li Z, Li JM, Min LJ, Ide A, et al. Effect of combination of calcium antagonist, azelnidipine, and AT1 receptor blocker, olmesartan, on atherosclerosis in apolipoprotein E-deficient mice. *J Hypertens* 2005; **23**:1383–1389.
- 9 Kataoka C, Egashira K, Ishibashi M, Inoue S, Ni W, Hiasa KI, et al. Novel anti-inflammatory actions of amlodipine in a rat model of arteriosclerosis induced by long-term inhibition of nitric oxide synthesis. *Am J Physiol Heart Circ Physiol* 2004; **286**:H768–H774.

- 10 Kim-Mitsuyama S, Izumi Y, Izumiya Y, Yoshida K, Yoshiyama M, Iwao H. Additive beneficial effects of the combination of a calcium channel blocker and an angiotensin blocker on a hypertensive rat-heart failure model. *Hypertens Res* 2004; **27**:771–779.
- 11 Ide S, Kondoh M, Satoh H, Karasawa A. Anti-proliferative effects of benidipine hydrochloride in porcine cultured vascular smooth muscle cells and in rats subjected to balloon catheter-induced endothelial denudation. *Biol Pharm Bull* 1994; **17**:627–631.
- 12 Ohtani K, Usui M, Nakano K, Kohjimoto Y, Kitajima S, Hirouchi Y, *et al.* Antimonocyte chemoattractant protein-1 gene therapy reduces experimental in-stent restenosis in hypercholesterolemic rabbits and monkeys. *Gene Ther* 2004; **11**:1273–1282.
- 13 Costa MA, Simon DI. Molecular basis of restenosis and drug-eluting stents. *Circulation* 2005; **111**:2257–2273.
- 14 Egashira K. Molecular mechanisms mediating inflammation in vascular disease: special reference to monocyte chemoattractant protein-1. *Hypertension* 2003; **41**:834–841.
- 15 Kuramoto K, Ichikawa S, Hirai A, Kanada S, Nakachi T, Ogihara T. Azelnidipine and amlodipine: a comparison of their pharmacokinetics and effects on ambulatory blood pressure. *Hypertens Res* 2003; **26**: 201–208.
- 16 Koike H, Kimura T, Kawasaki T, Sada T, Ikeda T, Sanbuissho A, Saito H. Azelnidipine, a long-acting calcium channel blocker with slow onset and high vascular affinity. *Annu Rep Sankyo Res Lab* 2002; **54**:1–64.
- 17 Yagil Y, Lusting A. Azelnidipine (CS-905), a novel dihydropyridine calcium channel blocker with gradual onset and prolonged duration of action. *Cardiovasc Drugs Rev* 1995; **13**:137–148.
- 18 Iwai M, Liu HW, Chen R, Ide A, Okamoto S, Hata R, *et al.* Possible inhibition of focal cerebral ischemia by angiotensin II type 2 receptor stimulation. *Circulation* 2004; **110**:843–848.
- 19 Schwartz RS, Huber KC, Murphy JG, Edwards WD, Camrud AR, Vlietstra RE, Holmes DR. Restenosis and the proportional neointimal response to coronary artery injury: results in a porcine model. *J Am Coll Cardiol* 1992; **19**:267–274.
- 20 Kornowski R, Hong MK, Tio FO, Bramwell O, Wu H, Leon MB. In-stent restenosis: contributions of inflammatory responses and arterial injury to neointimal hyperplasia. *J Am Coll Cardiol* 1998; **31**:224–230.
- 21 Ohashi N, Yoshikawa M. Rapid and sensitive quantification of 8-isoprostaglandin F<sub>2</sub>alpha in human plasma and urine by liquid chromatography-electrospray ionization mass spectrometry. *J Chromatogr B Biomed Sci Appl* 2000; **746**:17–24.
- 22 Ono H, Ichiki T, Fukuyama K, Iino N, Masuda S, Egashira K, Takeshita A. cAMP-response element-binding protein mediates tumor necrosis factor-alpha-induced vascular smooth muscle cell migration. *Arterioscler Thromb Vasc Biol* 2004; **24**:1634–1639.
- 23 Azelnidipine. CS 905, Calblock, RS 9054. *Drugs R D* 2003; **4**:122–125.
- 24 Egashira K. Clinical importance of endothelial function in arteriosclerosis and ischemic heart disease. *Circ J* 2002; **66**:529–533.
- 25 Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury. Part II: animal and human studies. *Circulation* 2003; **108**: 2034–2040.
- 26 Farb A, Weber DK, Kolodgie FD, Burke AP, Virmani R. Morphological predictors of restenosis after coronary stenting in humans. *Circulation* 2002; **105**:2974–2980.
- 27 Welt FG, Rogers C. Inflammation and restenosis in the stent era. *Arterioscler Thromb Vasc Biol* 2002; **22**:1769–1776.
- 28 Usui M, Egashira K, Ohtani K, Kataoka C, Ishibashi M, Hiasa K, *et al.* Antimonocyte chemoattractant protein-1 gene therapy inhibits restenotic changes (neointimal hyperplasia) after balloon injury in rats and monkeys. *FASEB J* 2002; **16**:1838–1840.
- 29 Egashira K, Zhao Q, Kataoka C, Ohtani K, Usui M, Charo IF, *et al.* Importance of monocyte chemoattractant protein-1 pathway in neointimal hyperplasia after periarterial injury in mice and monkeys. *Circ Res* 2002; **90**:1167–1172.
- 30 Horvath C, Welt FG, Nedelman M, Rao P, Rogers C. Targeting CCR2 or CD18 inhibits experimental in-stent restenosis in primates: inhibitory potential depends on type of injury and leukocytes targeted. *Circ Res* 2002; **90**:488–494.
- 31 Denger S, Jahn L, Wende P, Watson L, Gerber SH, Kubler W, Kreuzer J. Expression of monocyte chemoattractant protein-1 cDNA in vascular smooth muscle cells: induction of the synthetic phenotype: a possible clue to SMC differentiation in the process of atherogenesis. *Atherosclerosis* 1999; **144**:15–23.
- 32 Selzman CH, Miller SA, Zimmerman MA, Gamboni-Robertson F, Harken AH, Banerjee A. Monocyte chemotactic protein-1 directly induces human vascular smooth muscle proliferation. *Am J Physiol Heart Circ Physiol* 2002; **283**:H1455–H1461.
- 33 Viedt C, Vogel J, Athanasiou T, Shen W, Orth SR, Kubler W, Kreuzer J. Monocyte chemoattractant protein-1 induces proliferation and interleukin-6 production in human smooth muscle cells by differential activation of nuclear factor-kappaB and activator protein-1. *Arterioscler Thromb Vasc Biol* 2002; **22**:914–920.

## Overexpression of glutathione peroxidase attenuates myocardial remodeling and preserves diastolic function in diabetic heart

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**Matsushima, Shouji, Shintaro Kinugawa, Tomomi Ide, Hidenori Matsusaka, Naoki Inoue, Yukihiro Ohta, Takashi Yokota, Kenji Sunagawa, and Hiroyuki Tsutsui.** Overexpression of glutathione peroxidase attenuates myocardial remodeling and preserves diastolic function in diabetic heart. *Am J Physiol Heart Circ Physiol* 291: H2237–H2245, 2006. First published July 14, 2006; doi:10.1152/ajpheart.00427.2006.—Oxidative stress plays an important role in the structural and functional abnormalities of diabetic heart. Glutathione peroxidase (GSHPx) is a critical antioxidant enzyme that removes H<sub>2</sub>O<sub>2</sub> in both the cytosol and mitochondria. We hypothesized that the overexpression of GSHPx gene could attenuate left ventricular (LV) remodeling in diabetes mellitus (DM). We induced DM by injection of streptozotocin (160 mg/kg ip) in male GSHPx transgenic mice (TG+DM) and nontransgenic wildtype littermates (WT+DM). GSHPx activity was higher in the hearts of TG mice compared with WT mice, with no significant changes in other antioxidant enzymes. LV thiobarbituric acid-reactive substances measured in TG+DM at 8 wk were significantly lower than those in WT+DM (58 ± 3 vs. 71 ± 5 nmol/g, *P* < 0.05). Heart rate and aortic blood pressure were comparable between groups. Systolic function was preserved normal in WT+DM and TG+DM mice. In contrast, diastolic function was impaired in WT+DM and was improved in TG+DM as assessed by the deceleration time of peak velocity of transmitral diastolic flow and the time needed for relaxation of 50% maximal LV pressure to baseline value (tau; 13.5 ± 1.2 vs. 8.9 ± 0.7 ms, *P* < 0.01). The TG+DM values were comparable with those of WT+Control (tau; 7.8 ± 0.2 ms). Improvement of LV diastolic function was accompanied by the attenuation of myocyte hypertrophy, interstitial fibrosis, and apoptosis. Overexpression of GSHPx gene ameliorated LV remodeling and diastolic dysfunction in DM. Therapies designed to interfere with oxidative stress might be beneficial to prevent cardiac abnormalities in DM.

oxidative stress; diabetes mellitus; heart failure; antioxidant; hypertrophy; glutathione peroxidase

DIABETES MELLITUS OFTEN LEADS to congestive heart failure, even in the absence of any other risk factors such as coronary artery disease or hypertension, suggesting that diabetes itself causes a specific form of cardiomyopathic state, independent of vascular complications (3). Specifically, diastolic dysfunction has been regarded as a hemodynamic hallmark seen in diabetes and ultimately contributes to the development of heart failure (1, 21). Diabetes causes myocardial structural remodeling characterized by myocyte hypertrophy and apoptosis as well as interstitial fibrosis (7), which increases cardiac muscle stiffness and may contribute to impaired diastolic function. Although

the features of diabetic heart disease have been well identified, its pathogenesis and, in particular, the mechanisms underlying myocardial remodeling process have not been fully elucidated.

The excess production of reactive oxygen species (ROS), resulting in oxidative stress, is widely considered to be a cause of tissue damage associated with diabetes (2, 23). Our previous studies have demonstrated that H<sub>2</sub>O<sub>2</sub> and hydroxyl radical (·OH), generated via superoxide anion (O<sub>2</sub><sup>-</sup>·), play an important role in the development and progression of myocardial remodeling in the pacing-induced heart failure as well as in the postinfarct heart failure model (10, 28). Furthermore, in vitro studies demonstrated that ROS could directly induce hypertrophy and apoptosis in cardiac myocytes (30). Therefore, oxidative stress may contribute to the development of myocardial remodeling seen in diabetes (27). In fact, previous studies reported that antioxidants could attenuate myocardial damage in experimental diabetes (24, 36).

The first line of defense against ROS-mediated cardiac injury comprises several antioxidant enzymes including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSHPx). SOD, including mitochondrial Mn-SOD and cytosolic Cu/Zn-SOD, could provide antioxidant protection by inactivating O<sub>2</sub><sup>-</sup>·, sparing nitric oxide from destruction, and preventing O<sub>2</sub><sup>-</sup>· from forming more destructive ROS such as peroxynitrite and its reaction products, including ·OH. However, greater dismutation of O<sub>2</sub><sup>-</sup>· by SOD results in an increase of H<sub>2</sub>O<sub>2</sub>. Therefore, among these antioxidants, GSHPx is an important enzyme that performs several vital functions (28). GSHPx not only functions by removing H<sub>2</sub>O<sub>2</sub> formed after the SOD-catalyzed dismutation reaction, but also detoxifies the lipid hydroperoxides. In several in vitro studies, GSHPx alone was demonstrated to confer greater protection against oxidative damage than either SOD or catalase or the combination of SOD and catalase (31). The great efficiency of GSHPx as an antioxidant may be attributable to the fact that it is located in both the cytosol and the mitochondrial matrix and that it can utilize lipid peroxides as well as H<sub>2</sub>O<sub>2</sub> for substrates. These beneficial characteristics make GSHPx an important candidate for therapy against myocardial remodeling and failure due to increased ROS production. Our previous studies have demonstrated that GSHPx overexpression can attenuate oxidative stress as well as postinfarct cardiac remodeling and failure (28). Therefore, the purpose of this study was to determine whether overexpression of GSHPx could attenuate the structural remodeling and func-

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tional decline in the hearts with diabetes using GSHPx transgenic (TG) mice (20).

## MATERIALS AND METHODS

**TG mice.** This study was approved by our Institutional Animal Research Committee and conformed to the animal care guidelines of the American Physiological Society. We used the progeny of heterozygous breeding pairs of the C57BL/6×CBA/J hybrid mice with the overexpression of the human GSHPx1 gene (20). The coding region of the human GSHPx gene was inserted into the unique *Bam*HI site of the pHMG cassette vector. To generate TG mice, *Not*I fragments of recombinant plasmids were microinjected into the pronuclei of the C57BL/6×CBA/J hybrid. Mouse strain 23 (containing 200 copies of the human GSHPx gene) was used for further studies. To obtain TG animals and their normal littermates for experiments, TG founders (kindly provided by Drs. Oleg Mirochnitchenko and Masayori Inouye, University of Medicine and Dentistry of New Jersey) were bred with (C57BL/6 × CBA/J)<sub>F1</sub> mice. Tail clips and a PCR protocol to confirm the genotype were performed by a group of investigators.

**Induction of diabetes.** Diabetes was induced in 8-wk-old male mice, weighing 26–32 g, by injection with streptozotocin (STZ; 160 mg/kg ip). Tail vein blood glucose samples were measured 5 days after injection to insure induction of diabetes. As a control, vehicle (0.1 mol/l citrate buffer, pH 4.5) was injected in wild-type (WT) and TG mice. This assignment procedure was performed with the use of numeric codes to identify the animals.

**Echocardiographic and hemodynamic measurements.** After 8 wk of injection, echocardiographic studies were performed under light anesthesia with tribromoethanol-amylen hydrate (Avertin; 2.5% wt/vol, 8 μl/g ip) and spontaneous respiration. Two-dimensional targeted M-mode tracings were recorded at a paper speed of 50 mm/s (28).

To assess diastolic cardiac function, two-dimensional guided Doppler flow measurements of mitral inflow were obtained. Mitral inflow velocities were recorded only after extensive scanning from multiple vantage points to ensure that the maximal velocity was obtained. In most situations, this was an apical window corresponding to an “off-axis” apical window (displaced toward the parasternal window). Early and late mitral inflow velocity (E wave and A wave, respectively) and E wave deceleration time were measured from the Doppler recordings in the standard fashion, and the E-to-A ratio was calculated (17).

Under the same anesthesia with Avertin, a 1.4-Fr micromanometer-tipped catheter (Millar Instruments) was inserted into the right carotid artery and then advanced into the left ventricle (LV) to measure LV pressures. The following indexes of cardiac performance were measured and averaged from three consecutive beats; LV systolic pressure, LV end-diastolic pressure (EDP), the maximum and minimum values of the first derivative of LV pressure (LVdP/dt<sub>max</sub> and LVdP/dt<sub>min</sub>, respectively), and the time needed for relaxation of 50% maximal LV pressure to baseline value (tau). One subset of investigators, who were not informed of the experimental groups, performed *in vivo* LV function studies.

**Plasma glucose.** After completion of the cardiac function measurements performed after 2 h of fasting, blood samples were collected for the determination of plasma glucose.

**Myocardial histopathology.** After *in vivo* studies, the heart was excised and dissected into the right and left ventricles, including the septum. The LV was cut into three transverse sections: apex, middle ring, and base. From the middle ring, 5-μm sections were cut and stained with Masson's trichrome. Myocyte cross-sectional area was determined by quantitative morphometry of tissue sections from the mid-LV (28). Collagen distribution was also determined by using picrosirius red (0.1% Sirius Red F3BA in picric acid)-stained sections. Slides were left in 0.2% phosphomolybdic acid for 5 min, washed, and

left in picrosirius red for 90 min and then in 1 mmol/l HCl for 2 min and 70% ethanol for 45 s.

To detect apoptosis, tissue sections from the mid-LV were stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining. The number of TUNEL-positive cardiac myocyte nuclei was counted, and the data were normalized per 10<sup>5</sup> total nuclei identified by hematoxylin-positive staining in the same sections (28).

**Antioxidant enzyme activities and lipid peroxidation.** The enzymatic activities of GSHPx, catalase, and total SOD were measured in the LV according to the methods described previously (28).

Lipid peroxidation is a major biochemical consequence of ROS attack on biological tissue. We therefore determined the degree of lipid peroxidation in the myocardial tissue through biochemical assay of thiobarbituric acid reactive substances (TBARS) (28). In brief, LV myocardial tissue was homogenized (10% wt/vol) in 1.15% KCl solution (pH 7.4). The homogenate was mixed with 0.4% SDS, 7.5% acetic acid adjusted to pH 3.5 with NaOH, and 0.3% thiobarbituric acid. Butylated hydroxytoluene (0.01%) was added to the assay mixture to prevent autoxidation of the sample. The mixture was kept at 5°C for 60 min and was heated at 100°C for 60 min. After cooling, the mixture was extracted with distilled water and *n*-butanol-pyridine (15:1, vol/vol) and centrifuged at 1,600 g for 10 min. The absorbance of the organic phase was measured at 532 nm. The amount of TBARS was determined by absorbance with the molecular extinction coefficient of 156,000 (and expressed as μmol/g wet wt).

To further assess the lipid peroxidation by histochemical analysis, LV myocardial sections were immunolabeled with an antibody raised against 4-hydroxy-2-nonenal (HNE)-modified protein, an aldehydic byproduct of lipid peroxidation (10). In brief, paraffin-embedded tissue sections (3-μm thick) were deparaffinized with xylene and refixed with Bouin's solution for 20 min and immersed in 70, 90, and 100% ethanol to remove picric acid. To inhibit endogenous peroxidase, the sections were incubated with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min. After a rinsing in 0.01 mol/l PBS, the sections were incubated with normal goat serum (diluted to 1:10) to inhibit nonspecific binding of antibodies. The sections were further incubated with polyclonal antiserum raised against an HNE-modified histidyl peptide (Gly3-His-Gly3) conjugated with keyhole-limpet hemocyanin. After a rinsing with 0.01 mol/l PBS, the sections were incubated with biotin-labeled mouse anti-mouse IgG antiserum (diluted 1:100; Nihonyushi N213220) for 60 min and then with avidin-biotin complex (Vectastain ABC kit; 1:100) for 60 min. After a rinsing, the sections were finally incubated with 0.02% 3,3'-diaminobenzidine and 0.03% H<sub>2</sub>O<sub>2</sub> in deionized water for 6–9 min. As a negative control, the sections were also incubated with normal rabbit serum.

A morphometric analysis of HNE-positive myocardial area was performed with tissue sections stained with HNE. Each section was photographed under a microscope and magnified (final magnification, ×200). Three to four fields were randomly selected from one or two coronal sections in each animal. As a result, the HNE-positive areas were measured at approximately five to seven fields for each animal. Within each field, myocardial segments that stained positively with anti-HNE antibody were identified and were manually traced by use of a digitizing pad with a computer to calculate the traced area.

**Matrix metalloproteinases, transforming growth factor-β, and connective tissue growth factor.** We further determined the alterations of profibrotic mediators including matrix metalloproteinases (MMPs), transforming growth factor (TGF)-β, and connective tissue growth factor (CTGF) in this model.

First, the myocardial MMP levels, including MMP-2 and MMP-9, were determined in the LV using gelatin zymography (10). The LV myocardial samples were homogenized (~30-s bursts) in 1 ml of an ice-cold extraction buffer containing cacodylic acid (10 mmol/l), NaCl (0.15 mol/l), ZnCl<sub>2</sub> (20 mmol/l), NaN<sub>3</sub> (1.5 mmol/l), and 0.01% Triton X-100 (pH 5.0). The homogenate was then centrifuged (4°C, 10 min, 10,000 g), and the supernatant was decanted and saved on ice.

The pH levels of the samples were adjusted to 7.5 by use of Tris (1 mol/l). The final protein concentration of the myocardial extracts was determined using a standardized colorimetric assay. The extracted samples were then aliquoted and stored at  $-80^{\circ}\text{C}$  until the time of assay. The myocardial extracts were then directly loaded onto electrophoretic gels (SDS-PAGE) containing 1 mg/ml gelatin under non-reducing conditions. The myocardial extracts at a final protein content of 5  $\mu\text{g}$  were loaded onto the gels using a 3:1 sample buffer (10% SDS, 4% sucrose, 0.25 mol/l Tris-Cl, and 0.1% bromophenol blue, pH 6.8). The gels were run at 15 mA/gel through the stacking phase (4%) and at 20 mA/gel for the separating phase (10%), while the running buffer temperature was maintained at  $4^{\circ}\text{C}$ . After SDS-PAGE, the gels were washed twice in 2.5% Triton X-100 for 30 min each, rinsed in water, and incubated for 24 h in a substrate buffer at  $37^{\circ}\text{C}$  (50 mmol/l Tris-HCl, 5 mmol/l  $\text{CaCl}_2$ , and 0.02%  $\text{NaN}_3$ , pH 7.5). After incubation, the gels were stained with Coomassie brilliant blue R-250. The zymograms were digitized, and the size-fractionated bands, which indicated the MMP proteolytic levels, were measured by integrated optical density in a rectangular region of interest.

Next, the mRNA levels of myocardial MMPs including MMP-1, -2, -3, -8, and -9 as well as tissue inhibitors of MMPs (TIMPs) including TIMP-1, -2, -3, and -4 were determined by multiprobe ribonuclease protection assay (RPA RiboQuant, PharMingen). Each value was normalized to that of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) in each template set as an internal control, followed by calculation as a ratio to WT+Control.

The expression level of genes including the TGF- $\beta$  gene was determined by ribonuclease protection assay. The value of each hybridized probe was normalized to that of GAPDH in each template set as an internal control. CTGF protein levels were quantified by Western blot analysis using a specific antibody against recombinant mouse CTGF. To confirm the amount of loaded proteins, total proteins were also visualized by Coomassie brilliant blue staining. Within a given experiment, the densitometric values were normalized, using standards concurrently run within the same gel, and the value for each WT+diabetes mellitus (DM) or TG+DM was calculated as a ratio of WT+Control.

**Statistical analysis.** Data are expressed as means  $\pm$  SE. Between-group comparison of means was performed by one-way ANOVA, followed by *t*-tests. The Bonferroni's correction was done for multiple comparisons of means.  $P < 0.05$  was considered to be statistically significant.

## RESULTS

**Antioxidant enzymes and TBARS.** The baseline differences in antioxidant enzyme activities between WT and TG mice were determined (Table 1). In TG mice, there was a significant increase in GSHPx activities in the LV. Importantly, catalase and SOD activities were not altered in the TG hearts, indicating no effects of GSHPx overexpression on other antioxidant

enzymes. Similar results have been reported in the brain, liver, kidney, and lung obtained from the same series of mice (11, 22). The changes of antioxidant enzyme activities by the presence of diabetes were compared between WT and TG mice. GSHPx activities were comparable between WT+Control and WT+DM (Table 1). More importantly, as expected, GSHPx activities were significantly higher in TG+DM compared with WT+DM (Table 1). SOD and catalase activities were not altered in WT+DM or TG+DM compared with WT+Control.

TBARS measured in the LV obtained from WT+DM tended to be greater compared with WT+DM, which, however, did not reach statistical significance ( $P = 0.05$ ). Importantly, TBARS from TG+DM were significantly lower than those from WT+DM (Fig. 1A). In accordance with TBARS, an immunohistochemical analysis of HNE-modified protein revealed the lipid peroxides to be positively stained in myocytes from WT+DM mice, whereas only faint labeling was observed in the WT+Control, TG+Control, and TG+DM (Fig. 1B). The myocardial area stained positively with HNE was significantly smaller in TG+DM than in WT+DM.

**Body weight and plasma glucose.** There was no death in any group of mice. DM animals gained less body weight than control mice, which, however, did not differ between WT+DM and TG+DM (Table 2). At 5 days and 8 wk, DM mice had increased plasma glucose levels compared with control animals, which was not altered by GSHPx gene overexpression (Table 2).

**Echocardiography and hemodynamics.** The echocardiographic and hemodynamic data of mice at 8 wk are shown in Table 3. There was no significant difference in heart rate and aortic blood pressure among four groups of mice. Systolic function, as assessed by echocardiographic fractional shortening and  $\text{LVdP/dt}_{\text{max}}$ , was preserved as normal in both WT+DM and TG+DM mice. In contrast, diastolic function, as assessed by the deceleration time of peak velocity of transmitral diastolic flow (Dct) and the time needed for relaxation of 50% maximal LV pressure to baseline value ( $\tau$ ), was impaired in WT+DM, which was significantly attenuated in TG+DM (Table 3).  $\text{LVdP/dt}_{\text{min}}$  tended to be decreased in WT+DM compared with WT+Control ( $P = 0.05$ ) and increased in TG+DM compared with WT+DM ( $P = 0.09$ ), both of which, however, did not reach statistical significance. LVEDP was slightly, but significantly, elevated in WT+DM, which was attenuated in TG+DM.

**Myocardial histomorphometry and apoptosis.** Increased cardiac myocyte size, indicating hypertrophy, was evident in WT+DM, and this increase was significantly attenuated in TG+DM (Fig. 2). Collagen volume fraction, indicating interstitial fibrosis, in the WT+DM group was greater than in the control groups (Fig. 2). These fibrotic changes in the heart were significantly reduced in the TG+DM group.

The number of TUNEL-positive cells in the LV from the TG+DM group was significantly decreased compared with the WT+DM group and held constant relative to the control groups (Fig. 3).

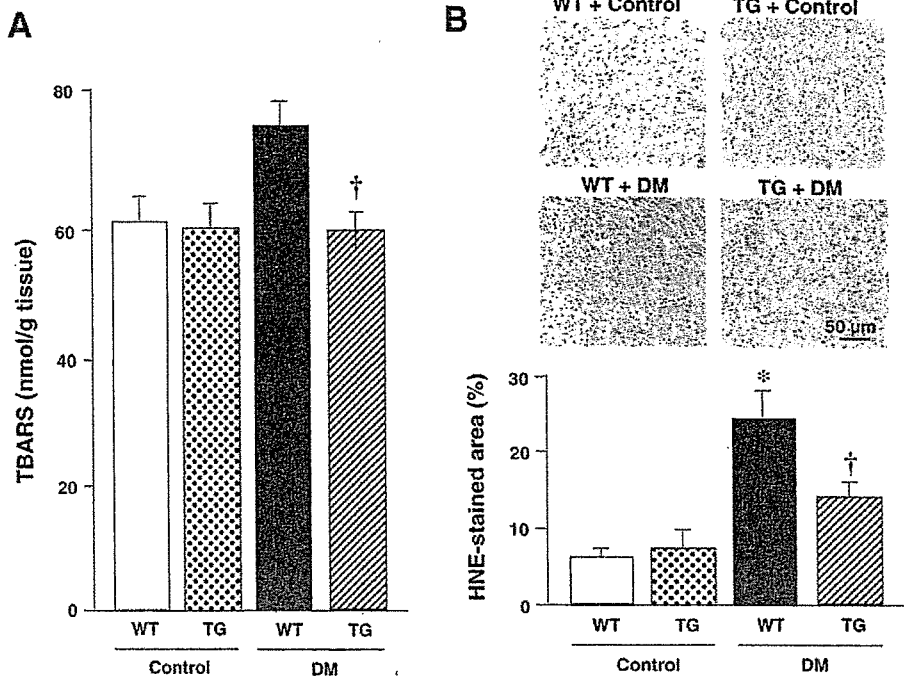
**Myocardial MMPs, TGF- $\beta$ , and CTGF.** To further assess the alterations of profibrotic mediators to be involved in interstitial fibrosis, myocardial levels of MMPs, TGF- $\beta$ , and CTGF were determined.

Table 1. Antioxidant enzymes

	WT+Control (n = 9)	TG+Control (n = 9)	WT+DM (n = 9)	TG+DM (n = 9)
GSHPx, nmol·min <sup>-1</sup> ·mg protein <sup>-1</sup>	44.9 $\pm$ 2.5	68.1 $\pm$ 5.0*†	45.6 $\pm$ 3.1	68.8 $\pm$ 8.3*†
Catalase, nmol/mg protein	67.8 $\pm$ 7.5	89.5 $\pm$ 15.4	64.4 $\pm$ 7.0	90.0 $\pm$ 10.1
Total SOD, U/mg protein	14.6 $\pm$ 1.3	15.7 $\pm$ 1.6	19.2 $\pm$ 2.4	19.0 $\pm$ 1.8

Values are means  $\pm$  SE; n is number of mice. GSHPx, glutathione peroxidase; SOD, superoxide dismutase; WT, wildtype; TG, transgenic; DM, diabetes mellitus. \* $P < 0.05$  vs. WT+Control. † $P < 0.05$  vs. WT+DM.

Fig. 1. A: lipid peroxidation as indicated by thiobarbituric acid reactive substances (TBARS) in the myocardial tissue from WT+Control, TG+Control, WT+DM, and TG+DM hearts ( $n = 14$  for each). WT, wild-type; TG, transgenic; DM, diabetes mellitus. Each assay was performed in triplicate. Values are means  $\pm$  SE.  $\dagger P < 0.05$ , difference from WT+DM value. B: representative immunohistochemical micrograph analysis and summary data for 4-hydroxy-2-nonenal (HNE)-modified histidine peptide in the myocardial tissue sections from WT+Control, TG+Control, WT+DM, and TG+DM mice ( $n = 3$  for each).  $*P < 0.05$ , difference from the WT+Control value.  $\dagger P < 0.05$ , difference from the WT+DM value.



Myocardial MMP-2 zymographic levels were increased in WT+DM, and this increase was significantly attenuated in TG+DM (Fig. 4). MMP-9 protein levels, although they were faint, were not altered in WT+DM or TG+DM. Other MMPs including MMP-1, -3, and -8 and TIMPs including TIMP-1, -2, -3, and -4 were not altered in the hearts from four groups of mice (Fig. 5).

Myocardial TGF- $\beta$  gene expression was increased in WT+DM, which was significantly attenuated in TG+DM (Fig. 6A). In parallel with TGF- $\beta$ , CTGF protein levels were increased in WT+DM, which, however, was not attenuated in TG+DM (Fig. 6B).

## DISCUSSION

The present study demonstrated that overexpression of GSHPx attenuated oxidative stress in the myocardial tissue and concurrently improved LV diastolic function as well as reduced myocyte hypertrophy, apoptosis, and interstitial fibrosis. Although the beneficial effects of various antioxidants on the diabetic heart have been demonstrated already by previous studies (6, 24, 36), the present study specifically provided direct evidence for the protective role of GSHPx against myocardial remodeling and dysfunction in this disease state.

The most effective way to evaluate the contribution of the specific antioxidant and obtain the direct evidence for a role of oxidative stress is through gene manipulation instead of the administration of antioxidants. Therefore, the novel finding of the present study from the point of view of understanding the pathophysiology and treatment of maladaptive myocardial remodeling in response to diabetes mellitus is that  $H_2O_2$  is critically involved in this disease process and may be a potential therapeutic target. Although the present study does not support the notion that the alterations in GSHPx are involved in oxidative stress in the diabetic heart, the overexpression of this gene can prevent the increase of oxidative stress as well as cardiac remodeling in diabetes mellitus.

A growing body of evidence suggests that the production of ROS is increased in the diabetic heart (14). Specifically, ROS are produced within the mitochondria of these hearts (15, 23). Furthermore, antioxidants have been shown to prevent the structural and functional alterations of the diabetes heart (6, 24, 36). Therefore, the present study not only extends the previous observation that employed antioxidants but also reveals the major role of ROS in the pathophysiology of cardiac remodeling associated with diabetes.

GSHPx is a key antioxidant that catalyzes the reduction of  $H_2O_2$  and hydroperoxides. It not only scavenges  $H_2O_2$  but also prevents the formation of other more toxic radicals such as  $\cdot OH$ . GSHPx possesses a higher affinity for  $H_2O_2$  than catalase. Furthermore, it is present in relatively high amounts within the heart, especially in the cytosolic and mitochondrial compartments (18). These lines of evidence imply the primary importance of GSHPx as a defense mechanism within the heart compared with catalase. Moreover, GSHPx is expected to exert greater protective effects against oxidative damage than SOD because greater dismutation of  $O_2^{\cdot -}$  by SOD may result in an increase of  $H_2O_2$ . Therefore, compared with SOD or catalase,

Table 2. Body weight and blood glucose data

	WT+Control ( $n = 16$ )	TG+Control ( $n = 14$ )	WT+DM ( $n = 13$ )	TG+DM ( $n = 13$ )
Body weight, g	33.5 $\pm$ 1.1	31.7 $\pm$ 1.0	27.2 $\pm$ 0.7 $\ddagger$	27.4 $\pm$ 0.7 $\ddagger$
Glucose, mg/dl (5 days after injection)	119 $\pm$ 5	114 $\pm$ 4	332 $\pm$ 24 $\ddagger$	350 $\pm$ 36 $\ddagger$
Glucose, mg/dl (8 wk after injection)	127 $\pm$ 8	125 $\pm$ 6	431 $\pm$ 36 $\ddagger$	420 $\pm$ 7 $\ddagger$

Values are means  $\pm$  SE;  $n$  is number of mice.  $\ddagger P < 0.01$  vs. WT+Control.



Table 3. Echocardiographic and hemodynamic data

	WT+Control	TG+Control	WT+DM	TG+DM
<i>Echocardiographic data</i>				
<i>n</i>	16	14	13	13
Heart rate, beats/min	466±12	448±7	417±6	420±7
LV EDD, mm	3.7±0.1	3.6±0.1	3.5±0.1	3.5±0.1
LV ESD, mm	2.4±0.1	2.3±0.1	2.3±0.1	2.2±0.1
Fractional shortening, %	35.1±0.5	35.5±0.8	34.1±0.7	36.1±0.6
IVS thickness, mm	0.71±0.02	0.70±0.02	0.68±0.02	0.68±0.02
PW thickness, mm	0.74±0.02	0.74±0.03	0.72±0.02	0.73±0.02
E/A ratio	1.49±0.06	1.60±0.12	1.28±0.06	1.31±0.07
Dct, ms	33.6±0.6	34.6±1.3	45.3±1.6‡	37.5±0.9‡
<i>Hemodynamic data</i>				
<i>n</i>	13	13	13	13
Heart rate, beats/min	438±12	439±13	413±4	431±17
Systolic aortic pressure, mmHg	98±3	101±4	102±5	101±6
Diastolic aortic pressure, mmHg	66±3	72±4	72±4	71±5
Mean aortic pressure, mmHg	76±3	82±4	83±5	82±6
LVEDP, mmHg	1.7±0.2	2.2±0.4	4.4±0.6‡	1.2±0.4‡
LVdP/dt <sub>max</sub> , mmHg/s	7,376±912	9,816±758	5,742±665	8,646±649
LVdP/dt <sub>min</sub> , mmHg/s	6,996±822	7,052±700	4,458±330	6,036±547
Tau, ms	7.8±0.2	8.2±0.4	13.5±1.2‡	8.9±0.7‡

Values are means ± SE; *n*, number of mice. LV, left ventricular; EDD, end-diastolic diameter; ESD, end-systolic diameter; IVS, interventricular septal; PW, posterior wall; E, peak velocity of early mitral flow; A, peak velocity of late mitral flow; Dct, deceleration time; EDP, end-diastolic pressure; dP/dt, change in pressure over time; tau, time needed for relaxation of 50% maximal LV pressure to baseline value. ‡*P* < 0.01 vs. WT+Control. §*P* < 0.01 vs. WT+DM.

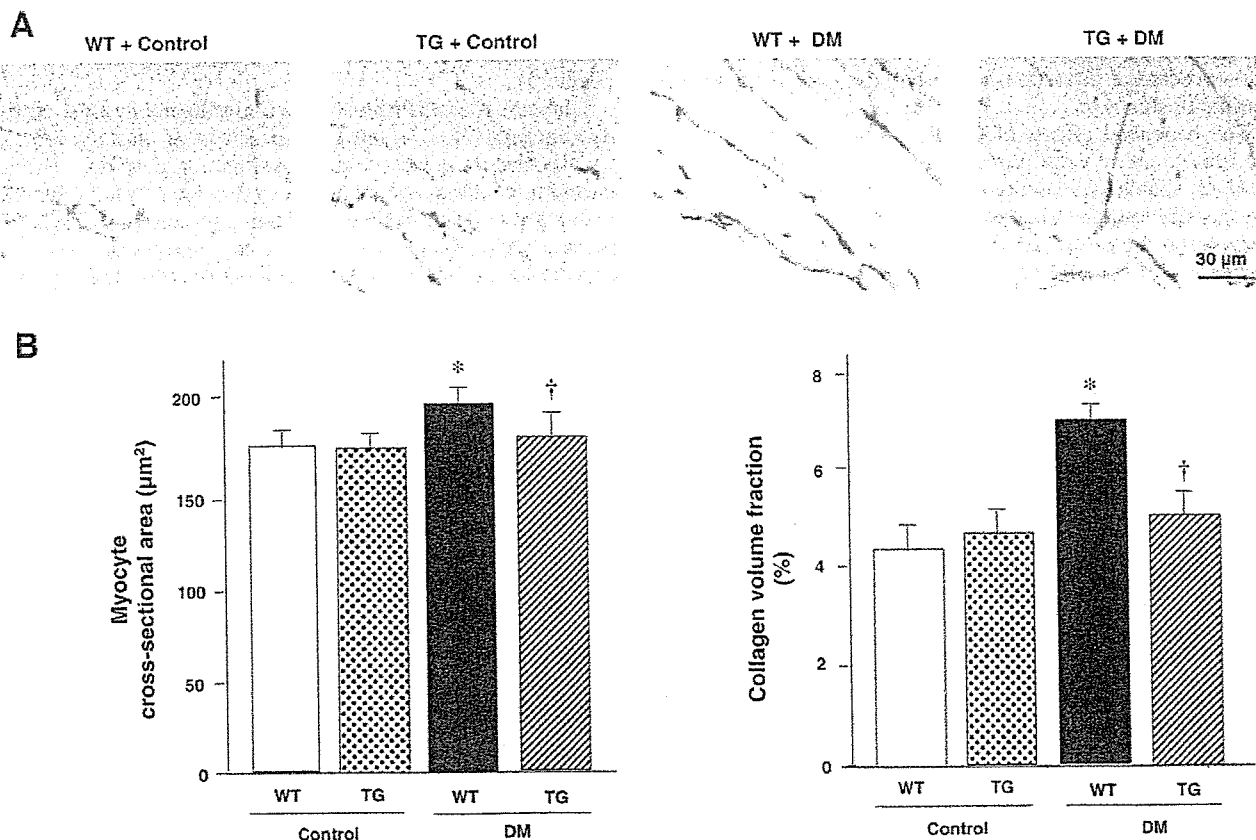
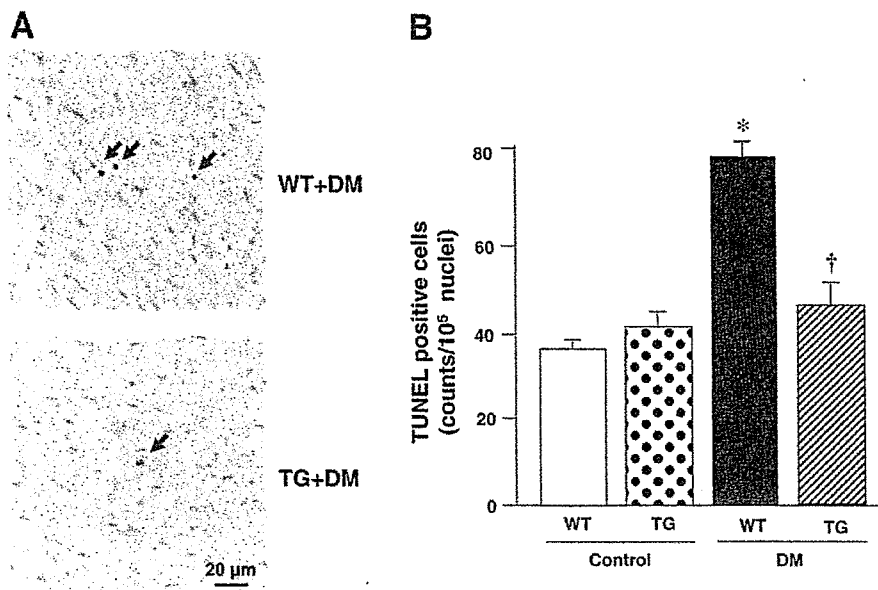


Fig. 2. A: representative photomicrographs of Masson's trichrome-stained left ventricular (LV) cross section obtained from 4 groups of mice. B: summary data for myocyte cross-sectional area and collagen volume fraction in WT+Control (*n* = 11), TG+Control (*n* = 8), WT+DM (*n* = 12), and TG+DM (*n* = 11) mice. Values are means ± SE. \**P* < 0.05, difference from the WT+Control value. †*P* < 0.05, difference from the WT+DM value.

Fig. 3. *A*: representative photomicrographs of TUNEL-stained cells (arrows) within LV cross-sections obtained from WT+DM and TG+DM mice. *B*: no. of TUNEL-positive myocytes in the LV from WT+Control, TG+Control, WT+DM, and TG+DM mice ( $n = 5$  each). Values are means  $\pm$  SE. \* $P < 0.05$ , difference from the WT+Control value. † $P < 0.05$ , difference from the WT+DM value.



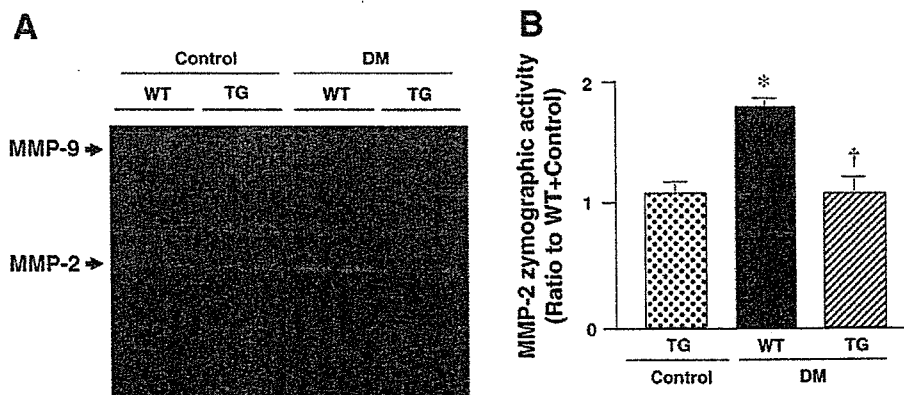
GSHPx is thought to be more effective in protecting cells, tissues, and organs against oxidative damage (5, 31). In fact, our previous studies demonstrated that the mice with GSHPx gene overexpression were more resistant to myocardial oxidative stress as well as remodeling and failure after myocardial infarction (28). Thus the present study extends the previous observation by demonstrating that they can attenuate not only post-myocardial infarction failure but also diabetes-associated cardiac dysfunction.

The beneficial effects of GSHPx overexpression shown in the present study were primarily due to its scavenging action on  $H_2O_2$  (Table 1). However, we could not completely exclude the possibility that altered expression of other antioxidant enzymes is involved. LV total SOD activities tended to be higher in WT and TG mice with diabetes; however, they did not reach statistical significance. Total SOD may be induced in the presence of DM, even though its pathophysiological significance remains uncertain. In addition, GSHPx TG mice tended to have higher levels of catalase (Table 1), which did not reach statistical significance. Nevertheless, we could not exclude the possibility that there were synergistic antioxidant effects between GSHPx and catalase against  $H_2O_2$ , because

both possess a high affinity for  $H_2O_2$ . Furthermore, the beneficial effects of GSHPx overexpression were not due to the effects on diabetes itself, because body weight and plasma glucose levels were comparable between WT+DM and TG+DM mice (Table 2). Importantly, the effects were not attributable to those of GSHPx overexpression on hemodynamics, because blood pressure and heart rate were not altered (Table 3).

Diabetes mellitus causes both diastolic and systolic cardiac dysfunction (27, 37), but an impairment of diastolic function usually occurs before systolic dysfunction develops. The impairment of diastolic function despite normal systolic function is thought to result from an increased myocardial stiffness. The present study has clearly demonstrated that the attenuation of myocardial oxidative stress by GSHPx overexpression (Fig. 1) is associated with the attenuation of diastolic dysfunction (Table 3), myocyte hypertrophy, and interstitial fibrosis (Fig. 2) in diabetes. Previous studies have demonstrated that diabetes induced by STZ leads to an increased collagen deposition (25), resulting in an increased myocardial stiffness and a decrease in LV compliance (19, 24). Although we did not directly assess the diastolic function by using the stress-

Fig. 4. *A*: representative gelatin zymography indicating myocardial matrix metalloproteinase (MMP)-2 activity in WT+Control, TG+Control, WT+DM, and TG+DM mice. *B*: summary data for myocardial MMP-2 activity in 4 groups of mice ( $n = 9$  each). Data were expressed as the ratio to WT+Control values concurrently run on the same gel. Values are means  $\pm$  SE. \* $P < 0.05$ , difference from the WT+Control value. † $P < 0.05$ , difference from the WT+DM value.



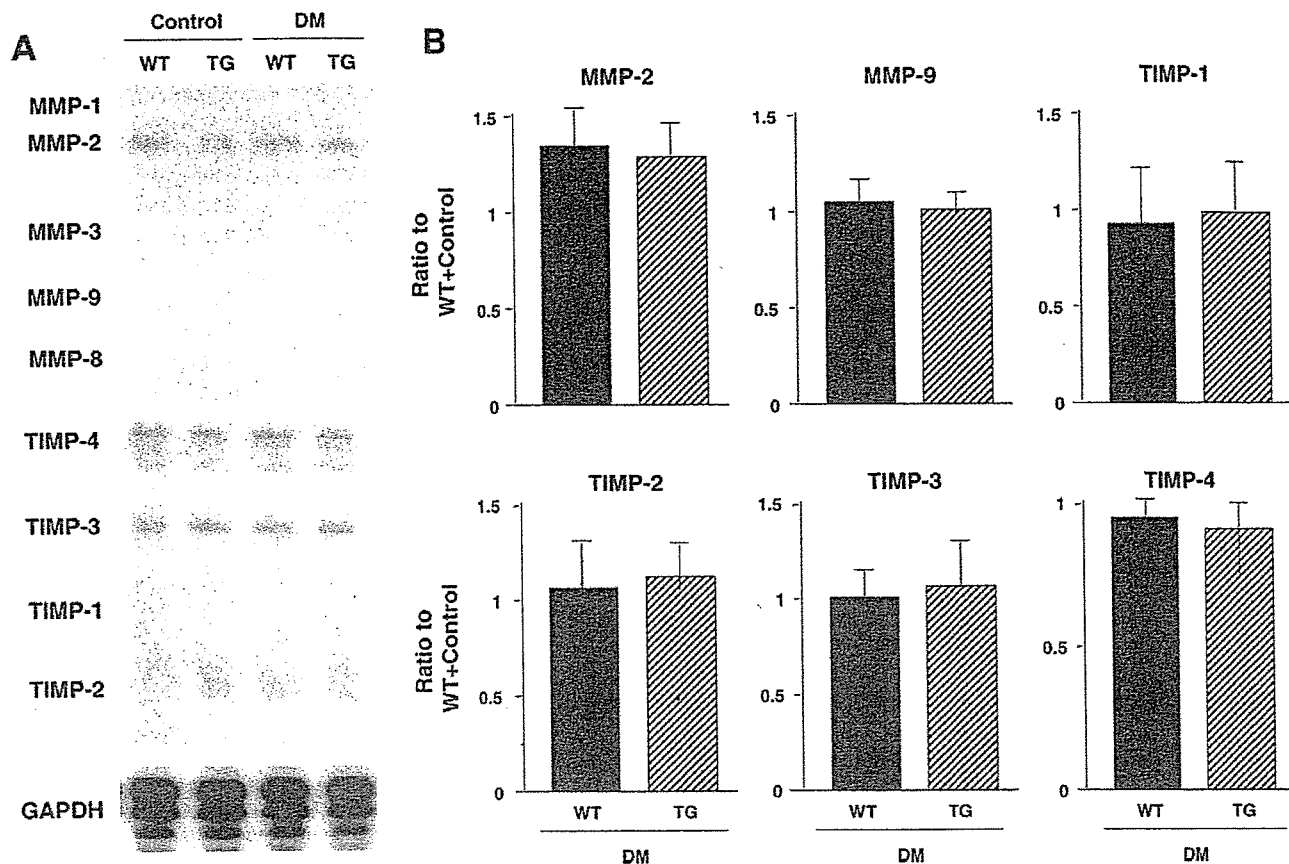


Fig. 5. *A*: representative image of myocardial gene expression of MMPs/tissue inhibitors of MMPs (TIMP). *B*: densitometric analysis of MMP and TIMP gene expression from WT+DM ( $n = 4$ ) and TG+DM mice ( $n = 4$ ). Each value was normalized to that of GAPDH in each template set as an internal control and expressed as the ratio to WT+Control ( $n = 4$ ). Values are means  $\pm$  SE.

strain relations and therefore could not comment on the contribution of myocardial stiffness to LV function in the present study, the decline in myocardial fibrosis in TG+DM mice can well be considered to contribute to the physiolog-

ical improvement of diastolic properties in these mice. In fact, prior studies have generally shown an association between increased cardiac fibrosis and diastolic chamber stiffening (35).

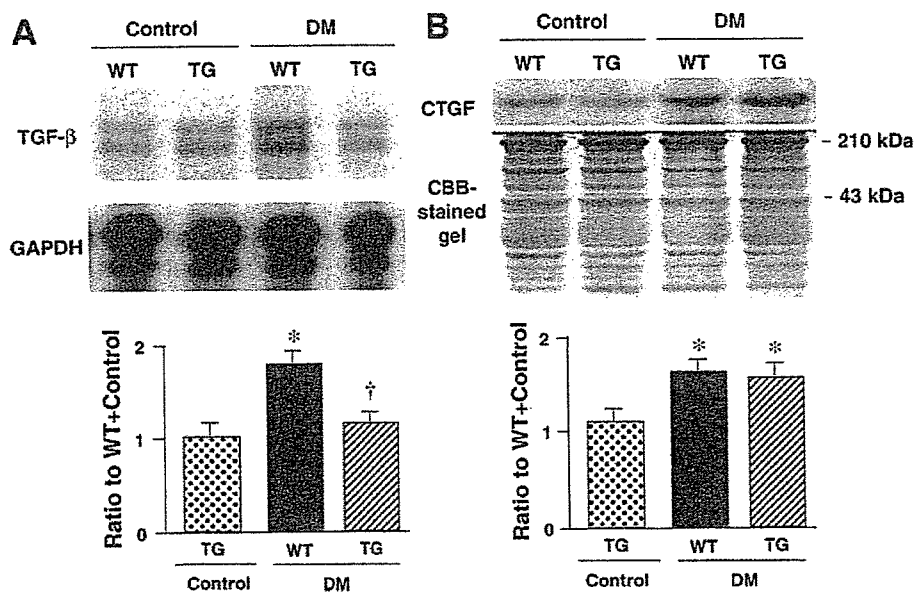


Fig. 6. Myocardial transforming growth factor (TGF)- $\beta$  (*A*) and connective tissue growth factor (CTGF; *B*) levels in WT+Control, TG+Control, WT+DM, and TG+DM mice ( $n = 6$  each). Data were expressed as the ratio to WT+Control values concurrently run on the same gel. Values are means  $\pm$  SE. \* $P < 0.05$ , difference from the WT+Control value. † $P < 0.05$ , difference from the WT+DM value.

The present results are consistent with previous studies demonstrating that ROS are involved in the structural alterations of the extracellular matrix collagens. The increase in collagens and myocardial stiffness in STZ-induced diabetic rats was prevented by treatment with aminoguanidine (24) and antioxidants (26, 34).

There may be several factors whereby oxidative stress contributes to myocardial remodeling in diabetes. First, oxidative stress is involved in cardiac myocyte hypertrophy and apoptosis (Figs. 2 and 3). It has been demonstrated that a subtle increase in ROS caused by partial inhibition of SOD results in hypertrophy and apoptosis in isolated cardiac myocytes (30), both of which are thought to contribute to diabetic myocardial damage. Specifically, recent studies show that the incidence of apoptosis is increased in the diabetic heart (4, 7), which may cause loss of contractile myocytes, compensatory hypertrophy of myocytes, and interstitial infibrosis (6). Myocyte necrosis also may be involved in the increased fibrosis from the diabetic heart. Second, oxidative stress induces the activation of MMP-2 seen in diabetes (Fig. 4). Our previous studies have demonstrated that MMP-2 activation plays an important role in the pathophysiology of cardiac remodeling (16). Moreover, MMPs have been shown to be activated by ROS in cardiac fibroblasts (29). On the basis of these findings, it is conceivable to hypothesize that increased ROS contribute to the activation of MMP and thus to the development of interstitial fibrosis in diabetes. Third, a possible role for growth factors in diabetes-related end-organ complications is increasingly being recognized. TGF- $\beta$  and CTGF can induce the production of collagen and fibronectin from cardiac fibroblasts and myocytes, and increased expression of both factors has been documented in the diabetic heart (12, 32). CTGF has a unique TGF- $\beta$  response element in its promoter region (8) and acts downstream of TGF- $\beta$ . The present study demonstrated that the attenuation of cardiac fibrosis by GSHPx overexpression was associated with the decrease in TGF- $\beta$  expression, but not with CTGF, suggesting that TGF- $\beta$  mainly contributed to cardiac fibrosis in this model. Nevertheless, we could not completely exclude the contribution of other growth factors such as VEGF, FGF, or PDGF in our model.

Approximately 20–40% of patients with heart failure have preserved systolic function and are thought to have an impairment of diastolic function as the primary mechanism leading to symptomatic heart failure (33). Diabetes is recognized as one of the major risk factors associated with diastolic heart failure (13). Despite the high prevalence of diabetes among patients with this type of heart failure, the treatment of diastolic heart failure remains empirical (9). There are currently few clinical data to support the efficacy of any particular class of drugs for diastolic heart failure. Therefore, the present study should help clarify the potential that antioxidants may play in the treatment of diastolic heart failure.

There are several limitations to be acknowledged in this study. First, the echocardiographic assessment of LV diastolic function in mice is somewhat difficult. However, intra- and interobserver variabilities of our echocardiographic measurements were small, and measurements were highly reproducible. Therefore, our technique was capable of noninvasively assessing the LV structure and function in mice. Second, the relationships between cardiac function and structural alterations were analyzed only at 8 wk after the induction of

diabetes. Longer follow-up must be performed to establish whether cardiac remodeling including myocyte hypertrophy, interstitial fibrosis, and apoptosis due to hyperglycemia may eventually lead to clinical heart failure in diabetes. Third, we employed myocyte cross-sectional area as an index of myocyte hypertrophy. Although this has been commonly used, we need to be cautious in interpreting these data, because increased myocyte cross-sectional area is not equivalent to myocyte hypertrophy, and the length of the cell is an equally important determinant of myocyte volume.

In conclusion, GSHPx overexpression inhibited the development of LV remodeling and diastolic dysfunction associated with diabetes. These beneficial effects of GSHPx were associated with the attenuation of myocyte hypertrophy, apoptosis, and interstitial fibrosis. Therapies designed to interfere with oxidative stress could be beneficial to prevent diabetic heart disease.

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#### REFERENCES

1. Abe T, Ohga Y, Tabayashi N, Kobayashi S, Sakata S, Misawa H, Tsuji T, Kohzaki H, Suga H, Taniguchi S, and Takaki M. Left ventricular diastolic dysfunction in type 2 diabetes mellitus model rats. *Am J Physiol Heart Circ Physiol* 282: H138–H148, 2002.
2. Baynes JW. Role of oxidative stress in development of complications in diabetes. *Diabetes* 40: 405–412, 1991.
3. Beckman JA, Creager MA, and Libby P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *JAMA* 287: 2570–2581, 2002.
4. Cai L, Li W, Wang G, Guo L, Jiang Y, and Kang YJ. Hyperglycemia-induced apoptosis in mouse myocardium: mitochondrial cytochrome C-mediated caspase-3 activation pathway. *Diabetes* 51: 1938–1948, 2002.
5. Cohen G and Hochstein P. Glutathione peroxidase: the primary agent for the elimination of hydrogen peroxide in erythrocytes. *Biochemistry* 2: 1420–1428, 1963.
6. Fiordaliso F, Bianchi R, Staszewsky L, Cuccovillo I, Doni M, Laragione T, Salio M, Savino C, Melucci S, Santangelo F, Scanziani E, Masson S, Ghezzi P, and Latini R. Antioxidant treatment attenuates hyperglycemia-induced cardiomyocyte death in rats. *J Mol Cell Cardiol* 37: 959–968, 2004.
7. Fiordaliso F, Li B, Latini R, Sonnenblick EH, Anversa P, Leri A, and Kajstura J. Myocyte death in streptozotocin-induced diabetes in rats in angiotensin II-dependent. *Lab Invest* 80: 513–527, 2000.
8. Grotendorst GR, Okochi H, and Hayashi N. A novel transforming growth factor beta response element controls the expression of the connective tissue growth factor gene. *Cell Growth Differ* 7: 469–480, 1996.
9. Hunt SA, Baker DW, Chin MH, Cinquegrani MP, Feldman AM, Francis GS, Ganiats TG, Goldstein S, Gregoratos G, Jessup ML, Noble RJ, Packer M, Silver MA, Stevenson LW, Gibbons RJ, Antman EM, Alpert JS, Faxon DP, Fuster V, Jacobs AK, Hiratzka LF, Russell RO, and Smith SC Jr; American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Revise the 1995 Guidelines for the Evaluation and Management of Heart Failure); International Society for Heart and Lung Transplantation; Heart Failure Society of America. ACC/AHA Guidelines for the Evaluation and Management of Chronic Heart Failure in the Adult: Executive Summary A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Commit-