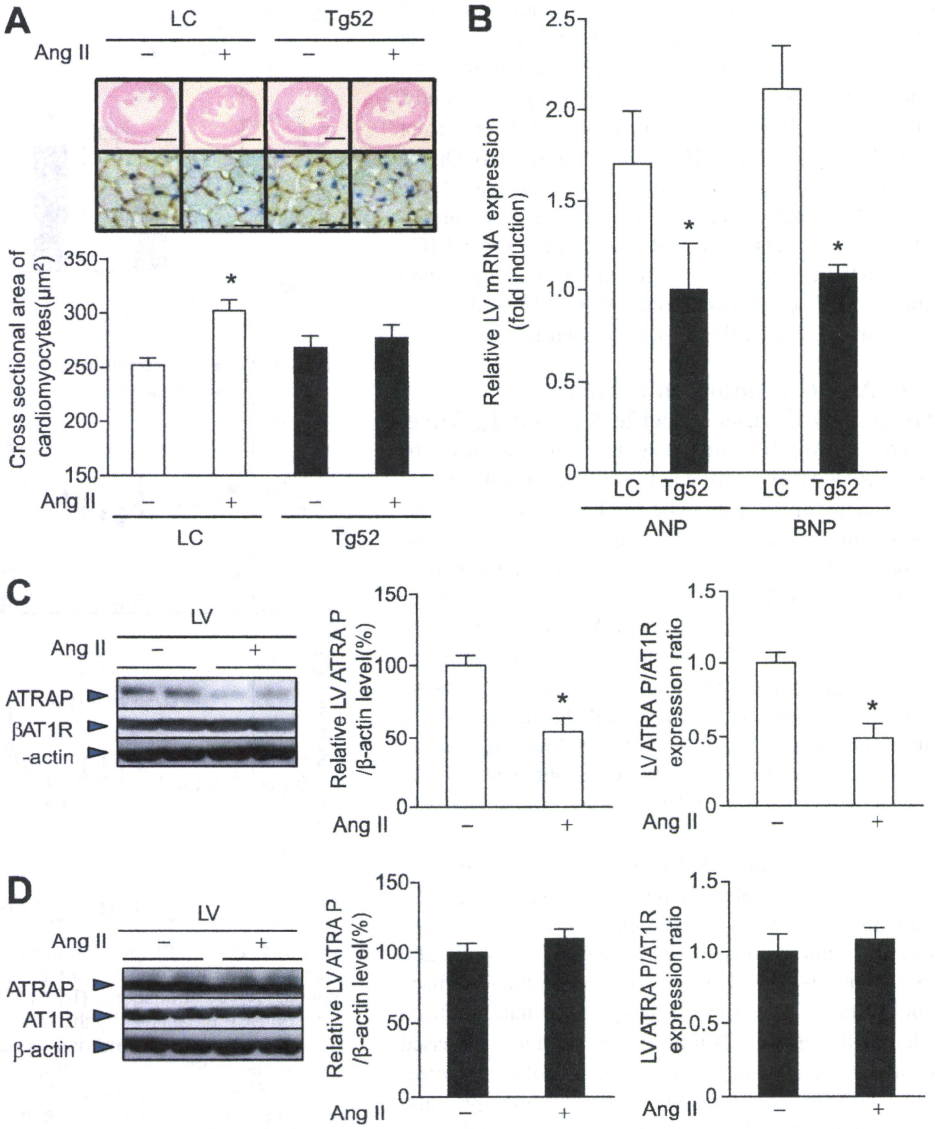

Figure 3. Cardiac hypertrophic responses by Ang II infusion into cardiac-specific ATRAP transgenic (Tg52) mice and LC mice. A, Effects of Ang II infusion on cardiac hypertrophy in Tg mice and LC mice. Representative images of hematoxylin and eosin–stained section of the hearts of all 4 groups examined (top, bar: 1 mm), representative images of antidystrophin monoclonal antibody–stained section of the LV for cell size measurement in all 4 groups (middle, magnification: ×400; bar: 20 μm), and quantitative analysis of a cardiomyocyte cross-sectional area of the LV (bottom). *$P<0.01$ vs vehicle. B, Effects of Ang II infusion on cardiac atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) mRNA expression in LC and Tg52 mice. Values are calculated as the fold induction of those achieved with extracts in the vehicle-infused group and expressed as the mean±SE (n=6 in each group). *$P<0.05$ vs LC. C, Representative Western blot and quantitative analysis of the effects of Ang II infusion on LV ATRAP and AT_1R protein expression in LC mice. Values are calculated relative to those achieved with extracts in the vehicle-infused group (Ang II) and expressed as the mean±SE (n=6 in each group). *$P<0.05$ vs vehicle. D, Representative Western blot and quantitative analysis of the effects of Ang II infusion on LV ATRAP and AT_1R protein expression in Tg52 mice. Values are calculated relative to those achieved with extracts in the vehicle-infused group (Ang II) and expressed as the mean±SE (n=6 in each group). *$P<0.05$ vs vehicle.

pletely suppressed in Tg52 mice with cardiac-specific overexpression of ATRAP.

Effects of Ang II Infusion on Hypertrophy-Related Gene Expression in Cardiac-Specific ATRAP Tg Mice

We examined whether cardiac-specific overexpression of ATRAP attenuates the cardiac hypertrophy-related gene expression induced by chronic Ang II infusion. Although Ang II infusion in LC mice increased the LV mRNA expression levels of atrial natriuretic peptide and brain natriuretic peptide by 1.7- and 2.1-fold induction, the upregulation of these mRNAs in response to Ang II infusion was completely suppressed in Tg52 mice (Figure 3B). With respect to the inhibitory effect of Ang II treatment on the cardiac ATRAP/AT_1R ratio, although Ang II infusion significantly decreased the cardiac ATRAP/AT_1R ratio through suppression of cardiac ATRAP expression in LC mice (Figure 3C), Ang II treatment did not affect the ratio at all in Tg52 mice (Figure 3D).

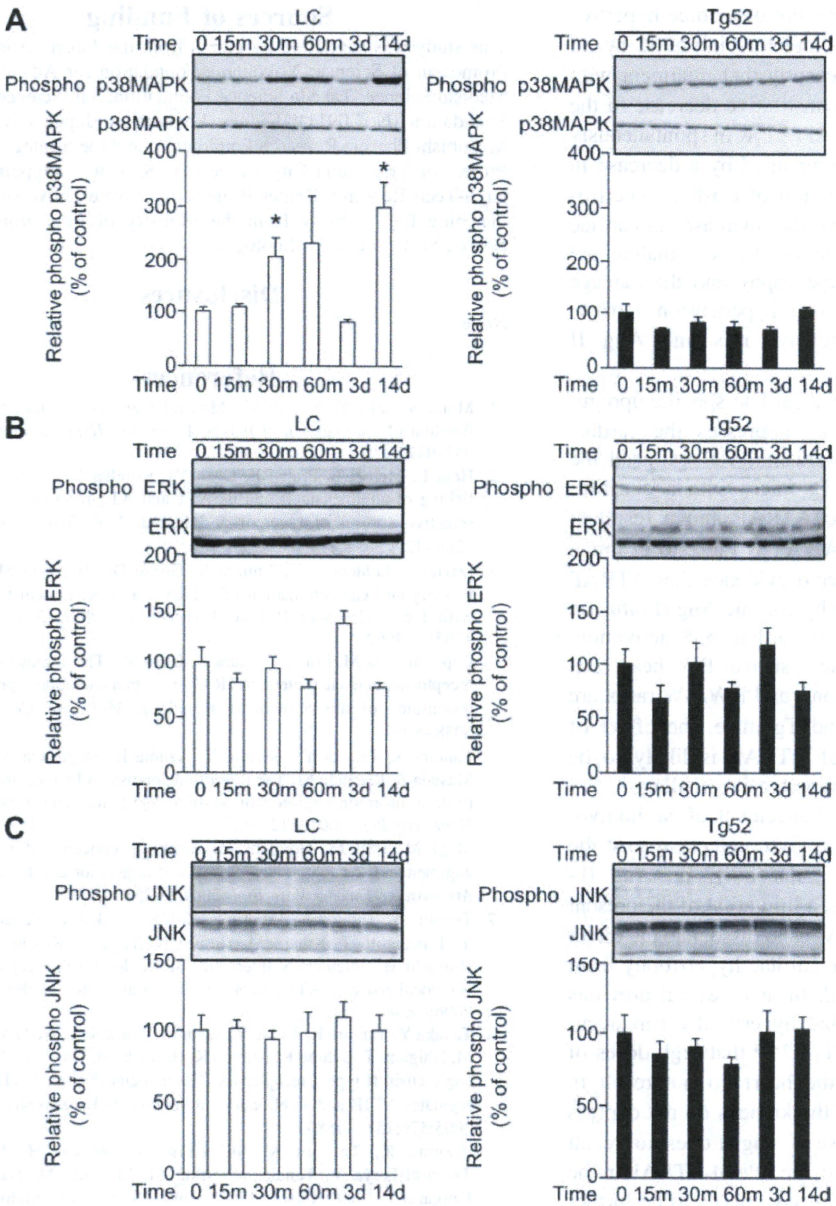

Figure 4. Effects of Ang II Infusion on MAPK activation in cardiac-specific ATRAP transgenic (Tg52) mice. Representative Western blots (top) and quantitative analysis (bottom) of the effects of Ang II infusion for 0, 15, 30, and 60 minutes and 3 and 14 days on phosphorylated and total MAPK (A, p38; B, ERK; and C, JNK) in LC mice (left, LC) and Tg52 mice (right). Values are calculated relative to those achieved with extracts in the mice at baseline (time 0) and expressed as the mean±SE (n=6 in each group). m indicates minutes; d, days. *$P<0.05$ vs baseline.

Effects of Ang II Infusion on MAPK Activation in Cardiac-Specific ATRAP Tg Mice

As the downstream effector of the AT_1R signaling pathway, MAPK plays an important role in the development of cardiac hypertrophy.[20–22] Thus, ultimately we examined the time course of LV MAPK activation in LC and Tg52 mice during Ang II treatment. LV p38 was significantly activated after 30 minutes and 14 days of Ang II infusion in LC mice (Figure 4A). However, LV p38 was not at all activated in Tg52 mice during Ang II treatment. On the other hand, LV JNK and ERK were not significantly activated by Ang II infusion in either LC or Tg52 mice (Figure 4B and 4C).

Discussion

This is the first report to our knowledge of a novel inhibitory function of cardiac ATRAP on cardiac hypertrophy in vivo. Activation of AT_1R signaling through the tissue renin-angiotensin system provokes sequential activation of signaling pathways, which leads to cardiac hypertrophy,[23–26] and chronic elevation of circulating Ang II causes sustained hypertension and associated cardiac hypertrophy.[27] The carboxyl-terminal portion of AT_1R is important for receptor internalization and activation of downstream signaling pathways.[28,29] ATRAP was cloned as a specific interacting molecule with the carboxyl-terminal domain of the AT_1 receptor by a yeast 2 hybrid screening system.[3] The results of in vitro studies showed that ATRAP suppresses Ang II–induced hypertrophic and proliferative responses by promoting a constitutive internalization of AT_1R and decreasing the p38 activity and transforming growth factor β production in cardiomyocytes and vascular smooth muscle cells, respectively, thereby suggesting that ATRAP is an endogenous inhibitor of AT_1R signaling.[8,9]

In the present study, we first showed that chronic infusion of Ang II significantly decreased the cardiac expression ratio

of ATRAP/AT_1R with the development of cardiac hypertrophy, for which the effect was completely inhibited by an AT_1R antagonist. We observed recently that treatment with an AT_1R antagonist recovered a constitutive decrease in the ratio of cardiac expression of ATRAP/AT_1R in spontaneously hypertensive rats, which was accompanied by a decrease in cardiac p38 activity and a suppression of cardiac hypertrophy.[30] Previous studies have shown that increases in cardiac p38 activity through the activation of AT_1R signaling are profoundly involved in cardiac hypertrophy and the damage incurred in genetic and experimental hypertension models, including spontaneously hypertensive rats and Ang II infusion.[31,32]

Because we had hypothesized that cardiac-specific upregulation of the ATRAP/AT_1R ratio suppresses the cardiac hypertrophy induced by Ang II infusion, we produced the cardiomyocyte-specific ATRAP Tg mice, with a constitutively high expression of cardiac ATRAP and a resultant upregulation of the cardiac ATRAP/AT_1R ratio, to examine this hypothesis. We obtained in vivo evidence that ATRAP attenuates the cardiac hypertrophy by chronic Ang II infusion concomitant with a suppression of cardiac p38 activation. Because the physiological parameters, such as BW, heart rate, radiotelemetric BP, cardiac functions, and HW/BW ratio, are comparable at baseline in LC and Tg mice, the effect of cardiac-specific overexpression of ATRAP is likely to be exerted only in the context of a hypertensive challenge.

The present study shows the enhancement of cardiomyocyte-specific ATRAP expression in vivo protects against the hypertrophic responses in mice induced by an Ang II–induced BP increase. Nevertheless, a limitation of the present study is that the results do not allow us to dissociate the direct effect of ATRAP enhancement on cardiac hypertrophy from the influence of pressure overload. In fact, several previous studies have indicated that cardiac hypertrophy can occur even in the absence of cardiac AT_1R,[33,34] that high doses of Ang II–released specifically in the heart do not result in cardiac hypertrophy,[35] and that if the kidneys do not express AT_1R, infusion of a very high dose of Ang II does not result in cardiac hypertrophy.[36] Therefore, the role of ATRAP in the regulation of cardiac hypertrophy in vivo needs to be further investigated in other models of hypertension and/or pressure or volume overload.

Perspectives

Because the results obtained in this study are essentially derived from Tg mice, it is important to exercise caution in interpreting the finding to be relevant to the pathophysiology of human cardiac hypertrophy. Nevertheless, the findings of the present study provide important information for the further investigation of the in vivo functional roles of ATRAP in the pathogenesis of cardiac hypertrophy and suggest the potential benefit of an ATRAP activation strategy. Additional studies to elucidate the molecular mechanisms of the antihypertrophic properties of cardiac ATRAP may enable a clinical application of ATRAP in the near future, such as the use of activating ligands for more efficient inhibition of AT_1R signaling in combination with inhibitors of the renin-angiotensin system.

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Disclosures

None.

References

1. Miura S, Saku K, Karnik SS. Molecular analysis of the structure and function of the angiotensin II type 1 receptor. *Hypertens Res.* 2003;26:937–943.
2. Hein L, Meinel L, Pratt RE, Dzau VJ, Kobilka BK. Intracellular trafficking of angiotensin II and its AT1 and AT2 receptors: evidence for selective sorting of receptor and ligand. *Mol Endocrinol.* 1997;11:1266–1277.
3. Daviet L, Lehtonen JY, Tamura K, Griese DP, Horiuchi M, Dzau VJ. Cloning and characterization of ATRAP, a novel protein that interacts with the angiotensin II type 1 receptor. *J Biol Chem.* 1999;274:17058–17062.
4. Lopez-Ilasaca M, Liu X, Tamura K, Dzau VJ. The angiotensin II type I receptor-associated protein, ATRAP, is a transmembrane protein and a modulator of angiotensin II signaling. *Mol Biol Cell.* 2003;14:5038–5050.
5. Tamura K, Tanaka Y, Tsurumi Y, Azuma K, Shigenaga A, Wakui H, Masuda S, Matsuda M. The role of angiotensin AT1 receptor-associated protein in renin-angiotensin system regulation and function. *Curr Hypertens Rep.* 2007;9:121–127.
6. Mogi M, Iwai M, Horiuchi M. Emerging concepts of regulation of angiotensin II receptors: new players and targets for traditional receptors. *Arterioscler Thromb Vasc Biol.* 2007;27:2532–2539.
7. Tsurumi Y, Tamura K, Tanaka Y, Koide Y, Sakai M, Yabana M, Noda Y, Hashimoto T, Kihara M, Hirawa N, Toya Y, Kiuchi Y, Iwai M, Horiuchi M, Umemura S. Interacting molecule of AT1 receptor, ATRAP, is colocalized with AT1 receptor in the mouse renal tubules. *Kidney Int.* 2006;69:488–494.
8. Tanaka Y, Tamura K, Koide Y, Sakai M, Tsurumi Y, Noda Y, Umemura M, Ishigami T, Uchino K, Kimura K, Horiuchi M, Umemura S. The novel angiotensin II type 1 receptor (AT1R)-associated protein ATRAP downregulates AT1R and ameliorates cardiomyocyte hypertrophy. *FEBS Lett.* 2005;579:1579–1586.
9. Azuma K, Tamura K, Shigenaga A, Wakui H, Masuda S, Tsurumi-Ikeya Y, Tanaka Y, Sakai M, Matsuda M, Hashimoto T, Ishigami T, Lopez-Ilasaca M, Umemura S. Novel regulatory effect of angiotensin II type 1 receptor-interacting molecule on vascular smooth muscle cells. *Hypertension.* 2007;50:926–932.
10. Yuan Z, Nimata M, Okabe TA, Shioji K, Hasegawa K, Kita T, Kishimoto C. Olmesartan, a novel AT1 antagonist, suppresses cytotoxic myocardial injury in autoimmune heart failure. *Am J Physiol Heart Circ Physiol.* 2005;289:H1147–H1152.
11. Sakai M, Tamura K, Tsurumi Y, Tanaka Y, Koide Y, Matsuda M, Ishigami T, Yabana M, Tokita Y, Hiroi Y, Komuro I, Umemura S. Expression of MAK-V/Hunk in renal distal tubules and its possible involvement in proliferative suppression. *Am J Physiol Renal Physiol.* 2007;292:F1526–F1536.
12. Shimura M, Minamisawa S, Yokoyama U, Umemura S, Ishikawa Y. Mechanical stress-dependent transcriptional regulation of sarcolipin gene in the rodent atrium. *Biochem Biophys Res Commun.* 2005;334:861–866.
13. Yoshida M, Minamisawa S, Shimura M, Komazaki S, Kume H, Zhang M, Matsumura K, Nishi M, Saito M, Saeki Y, Ishikawa Y, Yanagisawa T, Takeshima H. Impaired Ca2+ store functions in skeletal and cardiac muscle cells from sarcalumenin-deficient mice. *J Biol Chem.* 2005;280:3500–3506.
14. Mills PA, Huetteman DA, Brockway BP, Zwiers LM, Gelsema AJ, Schwartz RS, Kramer K. A new method for measurement of blood pressure, heart rate, and activity in the mouse by radiotelemetry. *J Appl Physiol.* 2000;88:1537–1544.

15. Sah VP, Minamisawa S, Tam SP, Wu TH, Dorn GW II, Ross J Jr, Chien KR, Brown JH. Cardiac-specific overexpression of RhoA results in sinus and atrioventricular nodal dysfunction and contractile failure. *J Clin Invest*. 1999;103:1627–1634.
16. Kato M, Egashira K, Takeshita A. Cardiac angiotensin II receptors are downregulated by chronic angiotensin II infusion in rats. *Jpn J Pharmacol*. 2000;84:75–77.
17. Tambascia RC, Fonseca PM, Corat PD, Moreno H Jr, Saad MJ, Franchini KG. Expression and distribution of NOS1 and NOS3 in the myocardium of angiotensin II-infused rats. *Hypertension*. 2001;37:1423–1428.
18. Hu C, Dandapat A, Sun L, Marwali MR, Inoue N, Sugawara F, Inoue K, Kawase Y, Jishage K, Suzuki H, Hermonat PL, Sawamura T, Mehta JL. Modulation of angiotensin II-mediated hypertension and cardiac remodeling by lectin-like oxidized low-density lipoprotein receptor-1 deletion. *Hypertension*. 2008;52:556–562.
19. Subramaniam A, Jones WK, Gulick J, Wert S, Neumann J, Robbins J. Tissue-specific regulation of the α-myosin heavy chain gene promoter in transgenic mice. *J Biol Chem*. 1991;266:24613–24620.
20. Hunter JJ, Chien KR. Signaling pathways for cardiac hypertrophy and failure. *N Engl J Med*. 1999;341:1276–1283.
21. Yamazaki T, Komuro I, Yazaki Y. Signalling pathways for cardiac hypertrophy. *Cell Signal*. 1998;10:693–698.
22. Izumiya Y, Kim S, Izumi Y, Yoshida K, Yoshiyama M, Matsuzawa A, Ichijo H, Iwao H. Apoptosis signal-regulating kinase 1 plays a pivotal role in angiotensin II-induced cardiac hypertrophy and remodeling. *Circ Res*. 2003;93:874–883.
23. Baker KM, Booz GW, Dostal DE. Cardiac actions of angiotensin II: role of an intracardiac renin-angiotensin system. *Annu Rev Physiol*. 1992;54:227–241.
24. Hein L, Stevens ME, Barsh GS, Pratt RE, Kobilka BK, Dzau VJ. Overexpression of angiotensin AT1 receptor transgene in the mouse myocardium produces a lethal phenotype associated with myocyte hyperplasia and heart block. *Proc Natl Acad Sci U S A*. 1997;94:6391–6396.
25. Paradis P, Dali-Youcef N, Paradis FW, Thibault G, Nemer M. Overexpression of angiotensin II type I receptor in cardiomyocytes induces cardiac hypertrophy and remodeling. *Proc Natl Acad Sci U S A*. 2000;97:931–936.
26. Zhai P, Yamamoto M, Galeotti J, Liu J, Masurekar M, Thaisz J, Irie K, Holle E, Yu X, Kupershmidt S, Roden DM, Wagner T, Yatani A, Vatner DE, Vatner SF, Sadoshima J. Cardiac-specific overexpression of AT1 receptor mutant lacking G αq/G αi coupling causes hypertrophy and bradycardia in transgenic mice. *J Clin Invest*. 2005;115:3045–3056.
27. Frank D, Kuhn C, van Eickels M, Gehring D, Hanselmann C, Lippl S, Will R, Katus HA, Frey N. Calsarcin-1 protects against angiotensin-II induced cardiac hypertrophy. *Circulation*. 2007;116:2587–2596.
28. Hunyady L, Bor M, Balla T, Catt KJ. Identification of a cytoplasmic Ser-Thr-Leu motif that determines agonist-induced internalization of the AT1 angiotensin receptor. *J Biol Chem*. 1994;269:31378–31382.
29. Tang H, Guo DF, Porter JP, Wanaka Y, Inagami T. Role of cytoplasmic tail of the type 1A angiotensin II receptor in agonist- and phorbol ester-induced desensitization. *Circ Res*. 1998;82:523–531.
30. Shigenaga A, Tamura K, Wakui H, Masuda S, Azuma K, Tsurumi-Ikeya Y, Ozawa M, Mogi M, Matsuda M, Uchino K, Kimura K, Horiuchi M, Umemura S. Effect of olmesartan on tissue expression balance between angiotensin II receptor and its inhibitory binding molecule. *Hypertension*. 2008;52:672–678.
31. Bao W, Behm DJ, Nerurkar SS, Ao Z, Bentley R, Mirabile RC, Johns DG, Woods TN, Doe CP, Coatney RW, Ohlstein JF, Douglas SA, Willette RN, Yue TL. Effects of p38 MAPK Inhibitor on angiotensin II-dependent hypertension, organ damage, and superoxide anion production. *J Cardiovasc Pharmacol*. 2007;49:362–368.
32. Park JK, Fischer R, Dechend R, Shagdarsuren E, Gapeljuk A, Wellner M, Meiners S, Gratze P, Al-Saadi N, Feldt S, Fiebeler A, Madwed JB, Schirdewan A, Haller H, Luft FC, Muller DN. p38 mitogen-activated protein kinase inhibition ameliorates angiotensin II-induced target organ damage. *Hypertension*. 2007;49:481–489.
33. Harada K, Komuro I, Shiojima I, Hayashi D, Kudoh S, Mizuno T, Kijima K, Matsubara H, Sugaya T, Murakami K, Yazaki Y. Pressure overload induces cardiac hypertrophy in angiotensin II type 1A receptor knockout mice. *Circulation*. 1998;97:1952–1959.
34. Kudoh S, Komuro I, Hiroi Y, Zou Y, Harada K, Sugaya T, Takekoshi N, Murakami K, Kadowaki T, Yazaki Y. Mechanical stretch induces hypertrophic responses in cardiac myocytes of angiotensin II type 1a receptor knockout mice. *J Biol Chem*. 1998;273:24037–24043.
35. van Kats JP, Methot D, Paradis P, Silversides DW, Reudelhuber TL. Use of a biological peptide pump to study chronic peptide hormone action in transgenic mice: direct and indirect effects of angiotensin II on the heart. *J Biol Chem*. 2001;276:44012–44017.
36. Crowley SD, Gurley SB, Herrera MJ, Ruiz P, Griffiths R, Kumar AP, Kim HS, Smithies O, Le TH, Coffman TM. Angiotensin II causes hypertension and cardiac hypertrophy through its receptors in the kidney. *Proc Natl Acad Sci U S A*. 2006;103:17985–17990.

Online Supplement

Original research article:

Cardiac-specific activation of AT1 receptor-associated protein completely suppresses cardiac hypertrophy in chronic angiotensin II-infused mice

Running title:

Cardiac ATRAP enhancement inhibits hypertrophy

Hiromichi Wakui, Kouichi Tamura, Yutaka Tanaka, Miyuki Matsuda, Yunzhe Bai, Toru Dejima, Shin-ichiro Masuda, Atsu-ichiro Shigenaga, Akinobu Maeda, Masaki Mogi, Naoaki Ichihara, Yusuke Kobayashi, Nobuhito Hirawa, Tomoaki Ishigami, Yoshiyuki Toya, Machiko Yabana, Masatsugu Horiuchi, Susumu Minamisawa, Satoshi Umemura

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Supplemental References

1. Subramaniam A, Jones WK, Gulick J, Wert S, Neumann J, Robbins J. Tissue-specific regulation of the alpha-myosin heavy chain gene promoter in transgenic mice. *J Biol Chem*. 1991;266:24613-24620.
2. Daviet L, Lehtonen JY, Tamura K, Griese DP, Horiuchi M, Dzau VJ. Cloning and characterization of ATRAP, a novel protein that interacts with the angiotensin II type 1 receptor. *J Biol Chem*. 1999;274:17058-17062.
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Table S1. Blood Pressure and Heart Rate 14 Days after Ang II Infusion in LC, Tg46, and Tg52 Mice

	LC		Tg46		Tg52	
Variable	Vehicle	Ang II	Vehicle	Ang II	Vehicle	Ang II
SBP, mmHg	112±6	121±6	109±5	120±6	105±5	112±4
HR, bpm	652±44	657±22	673±24	648±29	572±54	612±22

SBP indicates systolic blood pressure; HR, heart rate. All of the values are mean±SE (n=6 to 8).

Fig. S1

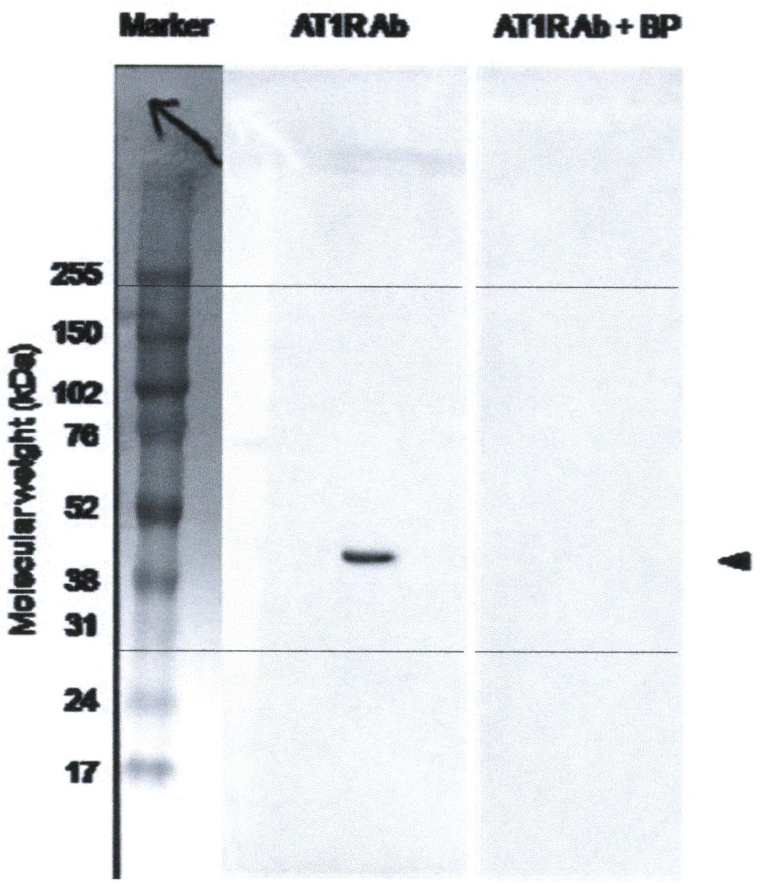

Figure S1. Western Blot Analysis of AT1R Protein in the Mouse Heart

The result of Western blot analysis showed a single protein band of approximately 42kDa in tissue extracts derived from heart of C57BL/6J wild-type mice at baseline. This single band was not observed when the antibody was preabsorbed with an AT1R-selective blocking peptide. These results demonstrate the specificity of AT1R antibody used in the present study. Ab, antibody; BP, blocking peptide.

Fig. S2

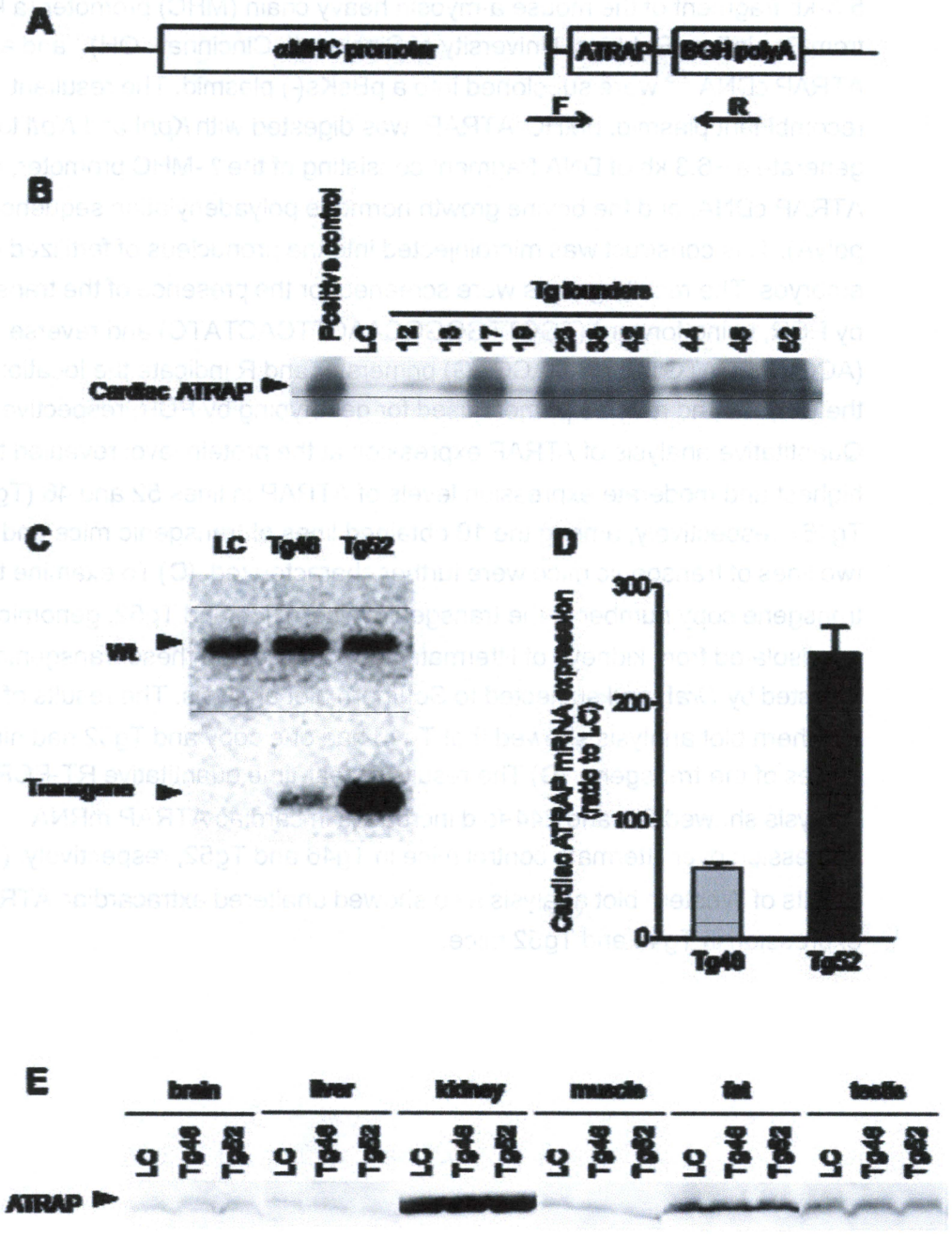

Figure S2. Generation of Cardiac-specific ATRAP Transgenic Mice

(**A**) Transgenic mice expressing ATRAP specifically in cardiomyocytes were generated on a C57BL/6J background with standard techniques. Briefly, a 5.5-kb fragment of the mouse a-myosin heavy chain (MHC) promoter (a kind gift from Dr. Jeffrey Robbins, University of Cincinnati, Cincinnati, OH) [1] and a mouse ATRAP cDNA [2,3] were subcloned into a pBsKs(-) plasmid. The resultant recombinant plasmid, pMHC-ATRAP, was digested with *Kpn*I and *Not*I to generate a ~6.3 kb of DNA fragment consisting of the ? -MHC promoter, mouse ATRAP cDNA, and the bovine growth hormone polyadenylation sequence (BGH polyA). This construct was microinjected into the pronucleus of fertilized mouse embryos. The resulting pups were screened for the presence of the transgene by PCR, using forward (TGCTTGGGGCAACTTCACTATC) and reverse (ACGGTGCATGTGGTAGACGAG) primers. F and R indicate the locations of the forward and reverse primers used for genotyping by PCR, respectively. (**B**) Quantitative analysis of ATRAP expression at the protein level revealed the highest and moderate expression levels of ATRAP in lines 52 and 46 (Tg52 and Tg46), respectively, among the 10 obtained lines of transgenic mice and these two lines of transgenic mice were further characterized. (**C**) To examine the transgene copy number in the transgenic mice, Tg46 and Tg52, genomic DNA was isolated from kidneys of littermate control mice and these transgenic mice, digested by *Dra*I, and subjected to Southern blot analysis. The results of Southern blot analysis showed that Tg46 had one copy and Tg52 had nine copies of the transgene. (**D**) The results of real-time quantitative RT-PCR analysis showed 59- and 244-fold increases in cardiac ATRAP mRNA expression over littermate control mice in Tg46 and Tg52, respectively. (**E**) The results of Western blot analysis also showed unaltered extracardiac ATRAP expression in Tg46 and Tg52 mice.

Fig. S3

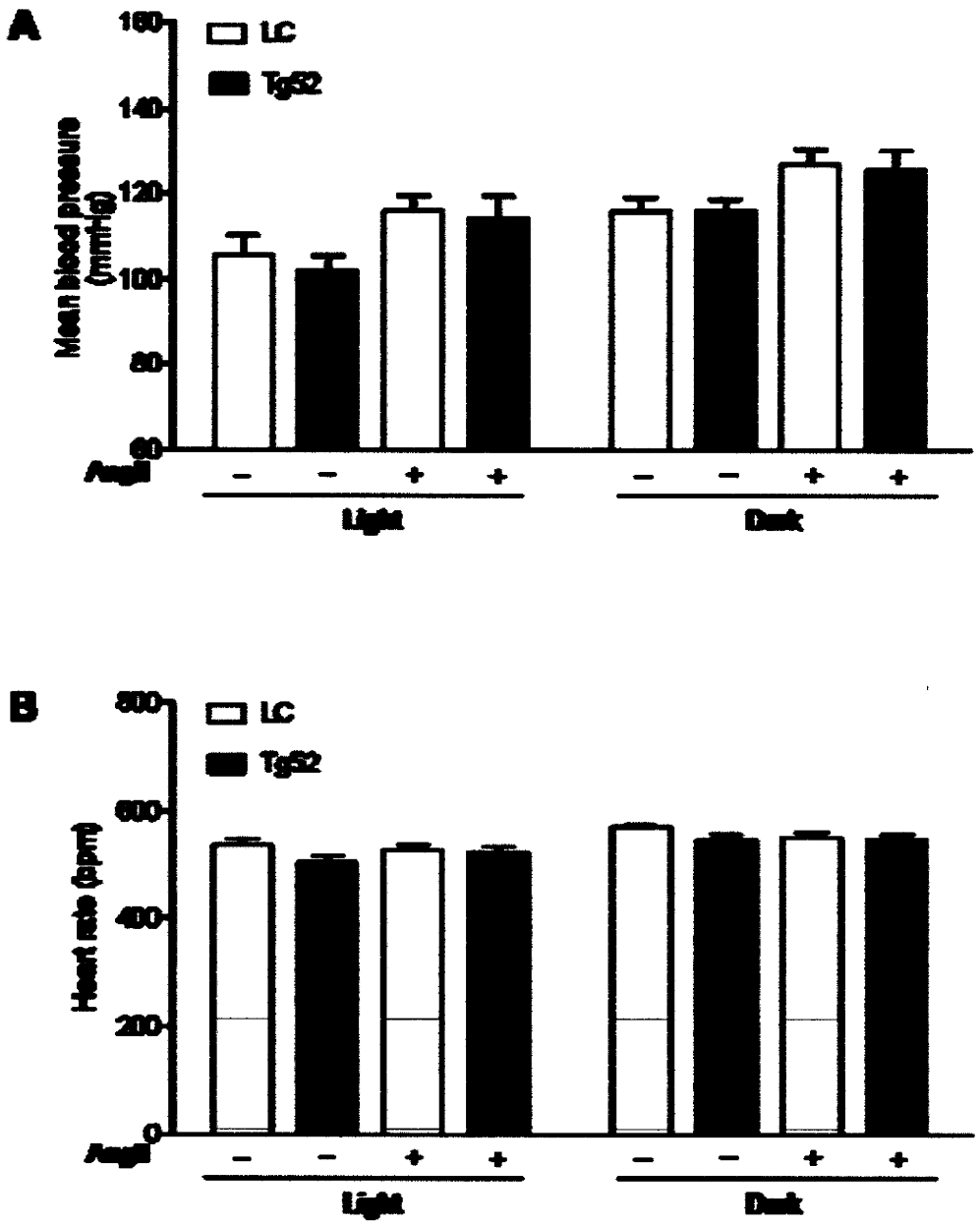

Figure S3. Direct Mean BP and HR measurement by radiotelemetric devices in LC and Tg mice

(**A**) In LC mice, Ang II infusion for 2 weeks tended to increase the mean BP (MBP) in the light period (105.7±4.6 versus 116.0±3.5 mmHg, P=0.126) and in the dark period (115.8±3.4 versus 126.9±3.8 mmHg, P=0.076), without statistical significance. Similarly in Tg52 mice, Ang II infusion tended to increase MBP in the light period (102.4±3.3 versus 114.3±5.0 mmHg, P=0.071) and in the dark period (115.9±2.8 versus 125.7±4.6 mmHg, P=0.126), also without statistical significance. (**B**) Regarding the radiotelemetric heart rate (HR), Ang II in fusion did not affect HR in LC and Tg mice in either the light period or the dark period.

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Sustained Inhibition of Oxidized Low-Density Lipoprotein Is Involved in the Long-Term Therapeutic Effects of Apheresis in Dialysis Patients

Yuko Tsurumi-Ikeya, Kouichi Tamura, Koichi Azuma, Hiroshi Mitsuhashi, Hiromichi Wakui, Ichiro Nakazawa, Teruyasu Sugano, Yasuyuki Mochida, Toshiaki Ebina, Nobuhito Hirawa, Yoshiyuki Toya, Kazuaki Uchino and Satoshi Umemura

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Sustained Inhibition of Oxidized Low-Density Lipoprotein Is Involved in the Long-Term Therapeutic Effects of Apheresis in Dialysis Patients

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Objective—Low-density lipoprotein (LDL) apheresis is a potential therapy for conventional therapy–resistant peripheral artery disease. In the present study, we examined the chronic effects of LDL apheresis on clinical parameters in vivo and endothelial cell functions in vitro in hemodialysis patients who had the complication of peripheral artery disease.

Methods and Results—Twenty-five patients were enrolled, and the responses of 19 patients to LDL apheresis were analyzed. Patients were classified into 2 groups according to change in ankle-brachial pressure index (ABI) after treatment: patients with improved ABI (responders, n=10) and patients with worsened ABI (nonresponders, n=9). In the responders, apheresis resulted in a long-term reduction of circulating levels of oxidized LDL, C-reactive protein, and fibrinogen. In human umbilical vein endothelial cells (HUVECs), the serum from the responders increased expression of activated endothelial nitric oxide synthase protein and proliferative activity. Furthermore, there was a significant correlation between ABI and activated endothelial nitric oxide synthase protein level in HUVECs treated with responder serum (R=0.427, P<0.05).

Conclusion—These results demonstrate that LDL apheresis decreases oxidized LDL and inflammation and improves endothelial cell function in the responders. This may be one of the mechanisms involved in the long-term therapeutic effect of LDL apheresis on peripheral circulation in hemodialysis patients. (***Arterioscler Thromb Vasc Biol.* 2010;30:1058-1065.)**

Key Words: atherosclerosis ■ endothelium ■ nitric oxide synthase ■ oxidized lipids ■ peripheral arterial disease ■ lipoproteins ■ oxidative stress

Cardiovascular disease is the primary cause of death in patients with end-stage renal disease. Patients on dialysis are reported to have a 10–20-fold greater risk of cardiovascular disease–associated mortality than the general population after stratification for age, gender, race, and the presence or absence of diabetes. Patients undergoing dialysis have many of the risk factors for atherosclerosis, such as hypertension, dyslipidemia, and disturbed calcium-phosphate metabolism, and, indeed, they commonly experience severe atherosclerosis, including peripheral artery disease (PAD). Low-density lipoprotein (LDL) apheresis is a potentially useful treatment for conventional therapy–resistant hypercholesterolemic patients with coronary artery disease and PAD.[1,2] Previously, it was shown that a single LDL apheresis session enhanced the peripheral microcirculation, probably by increasing the production of nitric oxide (NO) and bradykinin,[3] reducing blood viscosity and adhesion molecules,[4] and inducing endothelium-dependent vasodilatation.[5] However, the precise molecular mechanism of the long-term effects of LDL apheresis on the improvement of the peripheral circulation remains unclear and warrants further investigation.

We undertook the present study to investigate the short- and long-term effects of LDL apheresis on clinical and laboratory parameters in vivo and vascular endothelial cell function in vitro, in hemodialysis patients with PAD, and to identify factors related to the improvement of the peripheral circulation by LDL apheresis.

Methods

Patients and Study Design

The study protocol was approved by the Human Ethics Committee of Yokohama City University Hospital. A total of 25 consecutive hemodialysis patients with leg impairments and ankle-

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brachial pressure index (ABI) values less than 0.9 were recruited in Yokohama City University Hospital, and written informed consent was obtained from all participating patients. All patients were being treated with cilostazol, aspirin, icosapentate ethyl, ticlopidine, or sarpogrelate. The drug therapy was not changed during the study period. Of the 25 patients enrolled in this study, 5 patients could not complete the study because of death (n=3), amputation (n=1), or percutaneous transluminal angioplasty (n=1) during the study period, and they were excluded from the analysis.

LDL apheresis was carried out once or twice a week on nonhemodialysis days, and 10 sessions of apheresis were performed for each patient. The absolute walking distance and ABI were principally estimated on nonhemodialysis days before the 1st and 10th sessions and at 3 months after the end of treatment. ABI was measured by an oscillometric method using the Form PWV/ABI (AT Co.). Because most participating patients were unable to perform treadmill exercise because of conditions such as previous heart attack or paralysis, the absolute walking distance was evaluated by medical staff on a flat floor in the hospital. Blood samples were collected before and after the first session, at the start of the 10th session, and at 3 months after the end of treatment (before regular hemodialysis).

LDL Apheresis Technique

LDL apheresis was performed using hollow polysulfone fibers (Sulflux, Kaneka) as the plasma separator and a dextran sulfate cellulose column (Liposorber LA-15, Kaneka) as the LDL absorber. Blood flow from the arteriovenous fistula access was in the range of 80 to 100 mL/min, the plasma flow was 25 to 30 mL/min, and 3000 to 4000 mL of the plasma volume was treated per session.

Human Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Kurabo. HUVECs were maintained in HuMedia-EG2 medium (Kurabo) and were cultured as described previously.[6] Cells from the fourth to seventh passages were used for all experiments.

Determination of the Total and Activated Endothelial Nitric Oxide Synthase Protein Levels

HUVECs were cultured for 24 hours in HuMedia-EG2 medium containing 50% serum from the patients. Western blot analysis was performed essentially as described previously.[7,8] Briefly, whole cellular extracts from the HUVECs were loaded on 5% SDS-polyacrylamide gel electrophoresis gels. After protein transfer, membranes were immunoblotted with either anti-human endothelial nitric oxide synthase (eNOS) polyclonal antibody (Transduction Laboratories) or anti-human phospho-eNOS (Ser-1177) polyclonal antibody (Cell Signaling Technology). The protein levels were measured with densitometry using NIH ImageJ and were expressed relative to those achieved with HUVECs treated with the serum at baseline. Activated eNOS protein levels were normalized to the signal generated by the probe for the total eNOS protein.

Cell Proliferation Assay

HUVECs were seeded at 5×10^3 cells per well in a 96-well collagen I–coated plate (Becton Dickinson) and cultured for 48 hours in growth factor–depleted medium for preparation of the growth-arrested condition before the experiment. The growth factor–depleted medium contained HuMedia-EB2 and 2% FBS, but neither human epidermal growth factor nor human fibroblast growth factor. The HUVECs were incubated with a fresh growth factor–depleted medium containing 50% serum from patients for 24 hours. The proliferation activity assay was conducted by 5-bromo-2′-deoxyuridine (BrdUrd) incorporation assay, essentially as described previously.[9] The absorbance of samples was measured by a plate reader (ImmunoReader NJ-2100, InterMed) at 405 nm, with a reference wavelength at 490 nm. The mean absorbance was calculated for each of 10 samples.

Tube Formation Assay

Experiments on tube formation were conducted in triplicate in 24-well dishes using an angiogenesis assay kit (Kurabo) according to the manufacturer's instructions. Briefly, HUVECs cocultured with fibroblasts were cultivated in the medium containing 5% serum from patients. After 11 days, cells were fixed in 70% ethanol and then visualized with CD31 antibody (Kurabo). Vascular endothelial growth factor (VEGF) (10 ng/mL) was also tested as a positive control. Tube length was quantified using angiogenesis imaging software (Kurabo).

Statistical Analysis

Data are expressed as the mean±SE. The significance of the changes before versus after treatment was analyzed by the Wilcoxon single-rank test. Differences between responders and nonresponders were determined by the Student *t* test, Welch *t* test, or Mann-Whitney test. $P<0.05$ was deemed to be statistically significant.

Results

Effects of LDL Apheresis on Clinical Parameters

Of the 20 patients, 1 patient experienced a worsening of leg pain despite ABI improvement and was excluded from the analysis. Nineteen patient responses were investigated. The absolute walking distance improved significantly at the 10th session of LDL apheresis compared with baseline (from 171±33 m to 294±34 m, $P<0.05$) and even at 3 months after the end of the treatment (from 171±33 m to 270±42 m, $P<0.05$). Similarly, the ABI improved at the 10th session compared with baseline (from 0.59±0.04 to 0.67±0.04, $P<0.05$). Subsequently, the patients were classified into 2 groups according to the changes in the ABI at 3 months after the end of treatment. The 2 groups were patients with improved ABI (responders, n=10) and patients with worsened ABI (nonresponders, n=9). In the responders, the absolute walking distance and the ABI were significantly increased at the 10th session compared with baseline (from 118±26 m to 333±45 m, $P<0.05$; from 0.53±0.06 to 0.69±0.06, $P<0.005$; absolute walking distance and ABI, respectively) and even at 3 months after the end of treatment (from 118±26 m to 297±63 m, $P<0.05$; from 0.53±0.06 to 0.69±0.05, $P<0.005$). On the other hand, neither absolute walking distance nor ABI showed any statistically significant change during the study period in the nonresponders. The baseline characteristics in each group are shown in Table 1. There was no significant difference between the 2 groups in gender, cause of renal disease, diabetes or nondiabetes, age, body mass index, hemodialysis period, duration of PAD, grade of Fontaine classification, ABI, absolute walking distance, laboratory data, or the frequency of LDL apheresis.

Effects of LDL Apheresis on Laboratory Parameters

The short- and long-term lipid reductions by LDL apheresis in each group are shown in Table 2. Blood collection

Table 1. Comparison of Baseline Parameters Between Responders and Nonresponders

	Responders (n=10)	Nonresponders (n=9)	*P*
Gender, male/female	8/2	7/2	
Cause of renal disease			
Diabetic nephropathy	4	4	
Nephroscrelosis	2	3	
Chronic glomerulonephritis	4	2	
Diabetes/nondiabetes	4/6	4/5	
Age, years	66.3±2.8	66.1±3.5	NS
Body mass index, kg/m^2	19.8±0.7	20.8±0.9	NS
Hemodialysis period, years	7.4±1.8	7.3±2.3	NS
Duration suffering from PAD, years	6.5±3.9	6.4±4.8	NS
Fontaine classification, n	I, 1; IIa, 0; IIb, 5; III, 3; IV, 1	I, 0; IIa, 3; IIb, 4; III, 0; IV, 2	NS
ABI	0.53±0.06	0.65±0.05	NS
Absolute walking distance, m	118±26	232±58	NS
Total cholesterol, mg/dL	158±9	164±14	NS
Triglycerides, mg/dL	121±25	145±63	NS
HDL cholesterol, mg/dL	46±5	52±9	NS
LDL cholesterol, mg/dL	88±7	83±8	NS
MDA-LDL, U/L	90±10	99±20	NS
Oxidized LDL, U/L	38±3	38±5	NS
Fibrinogen, mg/dL	400±14	388±38	NS
CRP, mg/dL	0.87±0.40	0.81±0.47	NS
VEGF, pg/mL	114±14	98±24	NS
HGF, ng/mL	0.36±0.03	0.32±0.03	NS
CNP, fmol/mL	31.3±6.1	26.5±4.2	NS
Ca×P, (mg/dl)^2	50.5±4.5	52.3±6.7	NS
Intact PTH, pg/mL	299±81	232±63	NS
IL-1β, pg/mL	0.25±0.03	0.20±0.01	NS
Frequency of LDLA per week	once 6, twice 4	once 6, twice 3	NS

Parameters are shown as the mean±SE values or the number of patients. CNP indicates C-type natriuretic peptide; PTH, parathyroid hormone; IL-1β, interleukin-1β; LDLA, LDL apheresis; NS, not significant.

was performed before and after first apheresis, at the start of the 10th apheresis, and 3 months after the 10th apheresis. Therefore, lipid concentrations at the start of the 10th apheresis and 3 months after the 10th apheresis increased compared with those after the first apheresis. The serum levels of total cholesterol, LDL-cholesterol, malondialdehyde-modified (MDA)-LDL, and oxidized LDL were significantly reduced during a single session in both groups. Specifically in the responders, oxidized LDL continued to be significantly lower even at the 10th session compared with baseline. On the other hand, in the nonresponders, MDA-LDL and oxidized LDL concentrations had increased compared with the baseline levels by 3 months after the end of the treatment. These results indicate that LDL apheresis exerted a sustained lowering effect on lipid-related oxidative stress in the responders. We also measured lipid-independent oxidative stress markers, such as plasma 8-hydroxydeoxyguanosine, as well as advanced glycation end products. However, these general oxidative stress markers did not improve during the course of the study period, irrespective of the efficacy of the LDL apheresis (data not shown).

The short- and long-term changes in inflammatory markers and vasculogenerative factors induced by LDL apheresis in each group are summarized in Table 3. Although in both groups the C-reactive protein (CRP) and fibrinogen concentrations were significantly reduced during a single session, it was only in the responders that they tended to remain reduced at the 10th session compared with baseline. The plasma C-type natriuretic peptide concentration was significantly increased during a single session only in the responders. We did not find any significant short-term or long-term effects of LDL apheresis on the VEGF, hepatocyte growth factor (HGF), or interleukin-1β concentration in either group.

Effects of LDL Apheresis on Activated eNOS Expression in Vascular Endothelial Cells Exposed to Patient Serum

The total eNOS protein expression levels in HUVECs did not undergo significant change by incubation with the

Table 2. Short- and Long-Term Effects of LDL Apheresis on Lipid Concentrations

	Baseline	After 1st Apheresis	At 10th Apheresis	Three Months After 10th Apheresis	*P1*	*P2*	*P3*
Responders							
Total cholesterol, mg/dL	158±9	94±5	151±10	180±13	<0.01	NS	0.06
Triglycerides, mg/dL	121±25	101±16	110±20	107±16	NS	NS	NS
HDL cholesterol, mg/dL	46±5	41±5	51±6	60±7	NS	NS	0.06
LDL cholesterol, mg/dL	88±7	32±3	78±9	98±11	<0.01	NS	NS
MDA-LDL, U/L	90±10	44±5	87±13	95±10	<0.01	NS	NS
Oxidized LDL, U/L	38±3	20±2	32±3	38±4	<0.01	<0.05	NS
Nonresponders							
Total cholesterol, mg/dL	164±14	95±10	146±16	186±20	<0.05	NS	NS
Triglycerides, mg/dL	145±63	118±40	88±14	185±56	NS	NS	NS
HDL cholesterol, mg/dL	52±9	46±8	53±8	46±11	<0.05	NS	NS
LDL cholesterol, mg/dL	83±8	26±2	75±10	104±16	<0.05	NS	NS
MDA-LDL, U/L	99±20	40±6	90±12	118±23	<0.05	NS	<0.05
Oxidized LDL, U/L	38±5	18±2	32±4	46±6	<0.05	NS	<0.05

Parameters are shown as mean±SE values. *P1* indicates baseline vs after the 1st apheresis; *P2*, baseline vs at the 10th apheresis; *P3*, baseline vs 3 months after the 10th apheresis. NS indicates not significant.

serum collected from either group during the course of the study period (Figure 1A and 1B). However, the serum collected from the responders at the 10th session significantly increased the expression level of the activated eNOS protein in HUVECs compared with the serum after the first apheresis, although the increases in activated eNOS protein level at the 10th apheresis and at 3 months after 10th apheresis did not reach statistical significance as compared with baseline (Figure 1A). In contrast, the serum from the nonresponders did not alter the expression of the activated eNOS protein in HUVECs (Figure 1B).

Effects of LDL Apheresis on the Proliferative Activity of Vascular Endothelial Cells Exposed to Patient Serum

To examine the possible effect of LDL apheresis on the proliferative activity of HUVECs, we performed a BrdUrd incorporation assay using patient serum. The synthesis of DNA in HUVECs was significantly increased by incubation with the responder serum collected 3 months after the end of the treatment compared with the serum at baseline (Figure 2A). On the other hand, the serum from the nonresponders did not alter the synthesis of DNA in HUVECs (Figure 2B).

Table 3. Short- and Long-Term Effects of LDL Apheresis on Inflammatory Markers and Vasculogenerative Factors

	Baseline	After 1st Apheresis	At 10th Apheresis	Three Months After 10th Apheresis	*P1*	*P2*	*P3*
Responders							
Fibrinogen, mg/dL	400±14	308±18	337±32	394±35	<0.01	0.07	NS
CRP, mg/dL	0.87±0.40	0.49±0.20	0.39±0.23	0.75±0.47	<0.05	0.07	NS
VEGF, pg/mL	114±14	87±13	94±15	97±20	NS	NS	NS
HGF, ng/mL	0.36±0.03	0.31±0.03	0.31±0.03	0.36±0.04	NS	NS	NS
CNP, fmol/mL	31±6	36±7	33±6	38±7	<0.05	NS	NS
IL-1β, pg/mL	0.25±0.03	0.22±0.02	0.17±0.02	0.28±0.04	NS	NS	NS
Nonresponders							
Fibrinogen, mg/dL	388±38	264±28	340±45	427±35	<0.05	NS	NS
CRP, mg/dL	0.81±0.47	0.34±0.21	1.12±1.02	0.61±0.35	<0.05	NS	NS
VEGF, pg/mL	98±24	66±13	108±34	101±26	NS	NS	NS
HGF, ng/mL	0.32±0.03	0.31±0.04	0.38±0.05	0.40±0.06	NS	NS	NS
CNP, fmol/mL	27±4	29±3	27±4	31±4	NS	NS	NS
IL-1β, pg/mL	0.20±0.01	0.72±0.39	0.29±0.12	0.46±0.16	NS	NS	NS

Parameters are shown as the mean±SE values. *P1* indicates baseline vs after the 1st apheresis; *P2*, baseline vs at the 10th apheresis; *P3*, baseline vs 3 months after the 10th apheresis. CNP indicates C-type natriuretic peptide; IL-1β, interleukin-1β.

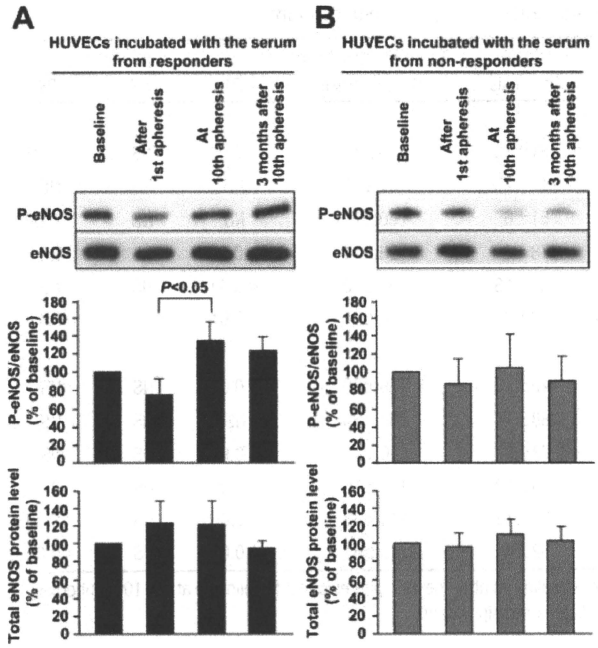

Figure 1. Representative Western blot analysis showing the expression of the activated eNOS protein, which is phosphorylated at Ser-1177 (P-eNOS) in HUVECs incubated with the serum from the responders (A) or nonresponders (B), along with the expression of the total amount of eNOS protein. Values are expressed as mean±SE.

Effects of LDL Apheresis on Tube Formation in Vascular Endothelial Cells Exposed to Patient Serum

To examine the effects of LDL apheresis on HUVEC tubular morphogenesis, we cocultured HUVECs with human fibroblasts and incubated them with patient serum. The tube length and area were evidently unchanged by incubation with the serum collected during the course of the study period in either group (Supplemental Figure IA and IB, available online at http://atvb.ahajournals.org).

Relationships Among Walking Distance, ABI, Oxidized LDL, Fibrinogen, and Activated eNOS Expression

Finally, there were statistically significant correlations between walking distance and plasma oxidized LDL ($R=-0.448$, $P<0.05$, Figure 3A) and fibrinogen ($R=-0.779$, $P<0.05$, Figure 3B) levels and activated eNOS protein level in HUVECs ($R=0.568$, $P<0.01$, Figure 3C) in responders. There was also a significant correlation between the ABI and activated eNOS protein level in HUVECs ($R=0.427$, $P<0.05$, Figure 3D) in responders. In nonresponders, there was a significant correlation between the ABI and activated eNOS protein level in HUVECs ($R=-0.470$, $P<0.05$, Supplemental Figure IIA) and a marginal correlation between walking distance and plasma oxidized LDL ($R=-0.329$, $P=0.07$, Supplemental Figure IIB). On the other hand, no significant correlation between CRP or BrdUrd incorporation and

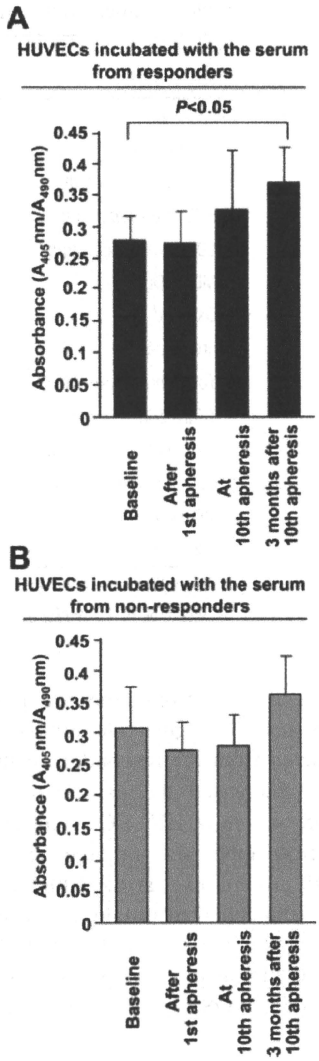

Figure 2. A 5-bromo-2′-deoxyuridine (BrdUrd) incorporation assay was performed for HUVECs incubated with the serum from the responders (A) or nonresponders (B). The absorbance of samples was measured at 405 nm, with a reference wavelength at 490 nm. Values are expressed as mean±SE.

ABI or walking distance was observed in either group (data not shown).

Discussion

It is now widely accepted that the endothelium exerts a critical role in the regulation of vascular tone, modification of lipoproteins, inflammation, thrombogenesis, and transformation of circulating monocytes into pathological foam cells. Accumulating evidence also indicates that proinflammatory and prothrombotic markers play an important role in the pathogenesis of atherosclerosis, and various factors are involved in the inflammatory and coagulatory processes that are active during atherosclerosis.[10] In particular, oxidative stress is reported to be increased in patients with end-stage renal disease[11] and has been implicated in the endothelial injury caused by oxidized LDL, a key lipid-related oxidative stress marker.[11,12]

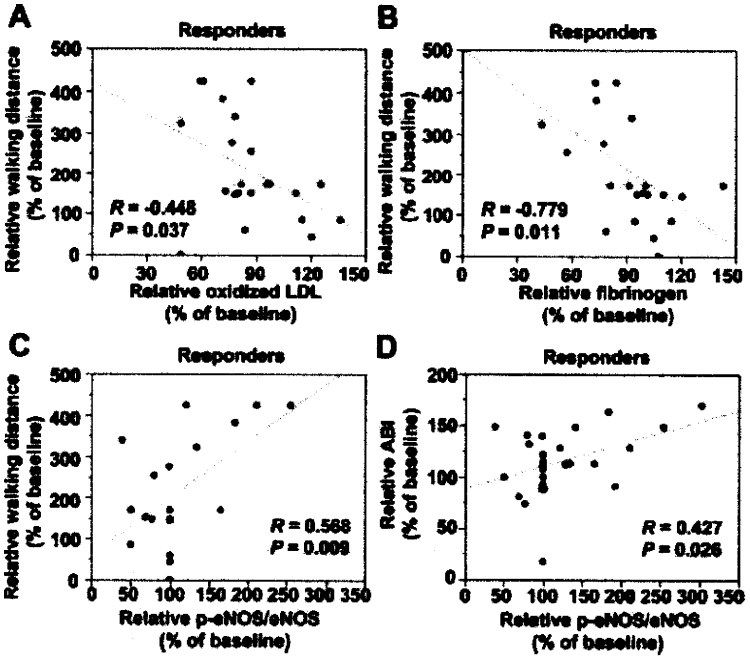

Figure 3. Relationships between the walking distance (relative walking distance) and plasma oxidized LDL (relative oxidized LDL) (A), fibrinogen (relative fibrinogen) levels (B), and activated eNOS protein level in HUVECs (p-eNOS/eNOS) (C) in the responders, and between the relative ABI and activated eNOS protein level in HUVECs (relative p-eNOS/eNOS) (D) in the responders. The respective values were calculated relative to those achieved at baseline in either the responder group or the nonresponder group.

Oxidized LDL affects endothelial cell function in various ways, eg, by inducing the expression of proinflammatory cell adhesion molecules, inhibiting endothelial cell proliferation with stimulated apoptosis, and impairing endothelial vasodilator function.[13–15] Atherosclerosis is also associated with increased concentrations of inflammatory hepatic markers, including CRP and fibrinogen.[16] CRP and fibrinogen are reported to be elevated in renal insufficiency, inducing adhesion molecule expression in endothelial cells so as to provoke an inflammatory response and the atherosclerotic process,[17] and to support the interaction of endothelial cells with matrix and inflammatory cells.[18] Both the oxidative stress and proinflammatory conditions result in a significant decrease in eNOS activity and thus impair endothelial function and promote atherosclerotic lesions.[13,15,17,19]

Previous studies demonstrated that a single LDL apheresis decreased not only the total LDL cholesterol but also the oxidized LDL, CRP, and fibrinogen concentrations as short-term effects.[5,20,21] Although previous studies reported that LDL apheresis may induce long-term reduction of the CRP and fibrinogen concentration,[21,22] there has been no report of the long-term effects of LDL apheresis on lipid-related oxidative stress. In this study, we demonstrated short-term reductions of the LDL, MDA-LDL, oxidized LDL, CRP, and fibrinogen concentrations during a single apheresis, consistent with the results of previous studies.[5,20,21] More importantly, our results disclosed a long-term reduction of oxidized LDL, CRP, and fibrinogen specifically until the 10th apheresis in the responders. Although LDL apheresis did not cause a sustained decrease in LDL cholesterol in the responders or the nonresponders, the long-term therapeutic effects of LDL apheresis were related to the relatively sustained decrease in oxidized LDL, which is a marker of lipid peroxidation.

On the other hand, LDL apheresis did not significantly affect plasma 8-hydroxydeoxyguanosine, which is a marker of DNA oxidation injury, or advanced glycation end products, which reflect protein oxidation, in the responders. A recent study also showed apheresis-mediated reduction of thiobarbituric acid reactive substances, which is a marker of lipid peroxidation, and also production of reactive oxygen species via suppression of NADPH oxidase expression in leukocytes in hemodialysis patients.[23] Therefore, these results suggest that LDL apheresis–mediated suppression of lipid peroxidation is a major contributing factor to its therapeutic effect on peripheral circulation in end-stage renal disease patients with PAD. With respect to the effects of LDL apheresis on high-density lipoprotein (HDL), because there was a short-term reduction of the HDL concentration during a single apheresis in nonresponders and a trend toward an increase 3 months after the 10th apheresis in comparison with baseline in responders (Table 2), it is possible that a differential effect on the HDL profile may have a role in its therapeutic impact.

We further examined the effects of LDL apheresis on vascular endothelial cell functions in vitro by analyzing the expression of the activated form of eNOS, which is phosphorylated at Ser-1177;[24] cellular proliferative activity; and tube formation capacity. The expression of the activated eNOS protein in HUVECs was significantly increased by incubation with the serum from the responders at the 10th session compared with the serum collected after the first apheresis. Furthermore, the proliferative activity of HUVECs was increased by the serum collected from the responders at 3 months after the end of treatment. These results suggest that a repeat of LDL apheresis is important for the responders to obtain the beneficial effect on endothelial cell function. Although the precise molec-