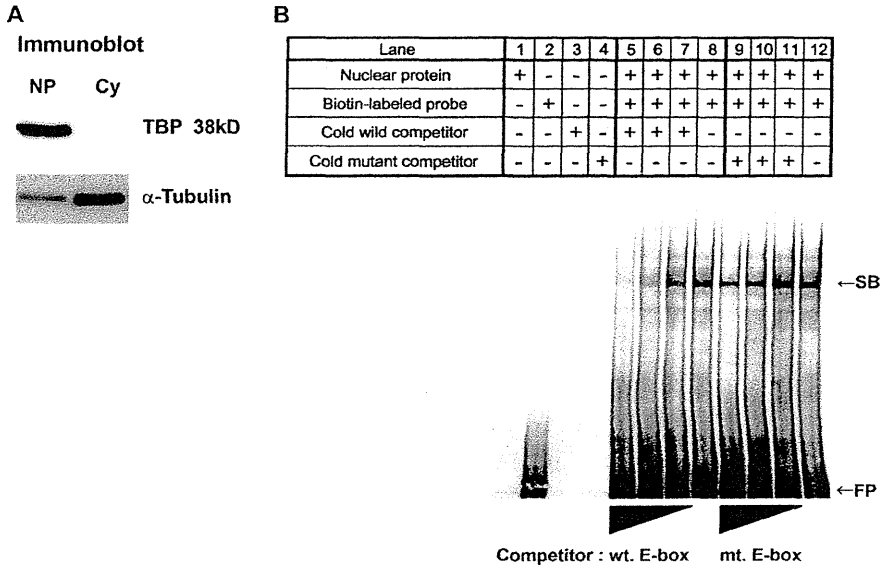

Lane	1	2	3	4	5	6	7	8	9	10	11	12
Nuclear protein	+	-	-	-	+	+	+	+	+	+	+	+
Biotin-labeled probe	-	+	-	-	+	+	+	+	+	+	+	+
Cold wild competitor	-	-	+	-	+	+	+	-	-	-	-	-
Cold mutant competitor	-	-	-	+	-	-	-	-	+	+	+	-

\Q: A FIGURE 3. **Identification of nuclear factors binding to the E-box (−72 to −43) of the mouse *Agtrap* promoter by EMSA.** *A*, immunoblot analysis shows TATA-binding protein (*TBP*) and α-tubulin in the nuclear extract (*NP*) and cytosolic extract (*Cy*), respectively. *B*, electrophoretic mobility shift and competition analyses of complexes formed by nuclear factors with the E-box (−72 to −43) of the *Agtrap* promoter. The E-box ODNs were biotin-labeled at the 3′-end and used as the labeled probe. Nuclear extracts (2 μg) from mDCT cells were incubated with the probe. In electrophoretic shift competition assay, 50, 12.5, and 2.5 pmol of unlabeled wild-type E-box (*wt*) or mutated E-box (*mt*) ODNs were added to the reaction mixture. *SB* indicates a shifted band derived from specific DNA-protein complexes. *FP*, free probes.

We next performed ChIP analysis to determine whether Usf1 and Usf2 physiologically interacted with the *Agtrap* promoter region. As shown in Fig. 4*B*, the 134-bp E-box containing the sequence from −65 to +69 of the transcriptional start site of the *Agtrap* promoter was recovered from mDCT cells after immunoprecipitation of sheared genomic DNA with an anti-USF1 antibody and anti-USF2 antibody but not after immunoprecipitation with an anti-SREBP1 antibody or anti-BMAL1 antibody. Quantitative PCR analysis confirmed that Usf1 and Usf2 are present in the *Agtrap* E-box promoter region, and the corresponding genomic DNA was enriched with both an anti-USF1 antibody (*, $p < 0.05$, *versus* IgG control) and anti-USF2 antibody (**, $p < 0.01$, *versus* IgG control) but not with an anti-SREBP1 antibody or anti-BMAL1 antibody. These data provide evidence for the occupancy by Usf1 and Usf2, but not Srebf1 or BMAL1, of the mouse *Agtrap* promoter E-box *in vivo*.

Functional Involvement of Usf1 and Usf2 in Mouse Agtrap Promoter Activity—To determine whether Usf1 and Usf2 are involved in the transcriptional regulation of the *Agtrap* gene in mDCT cells, we examined the effect of Usf1 and Usf2 siRNAs transfection on endogenous Agtrap gene expression. The 5 mRNA and protein levels of Usf1 (Fig. 5, *A* and *D*) and Usf2 (Fig. 5, *B* and *E*) were significantly decreased after transfection with their respective siRNA. In addition, although the Usf2 mRNA level was slightly increased by Usf1 knockdown (Fig. 5*B*), the Usf2 protein level was not affected (Fig. 5*E*). Intriguingly, although the siRNA reduction of Usf1 resulted in a significant increase in the levels of the Agtrap mRNA (Fig. 5*C*, $p < 0.01$, siUsf1 *versus* siCtrl) and protein (Fig. 5*F*, $p < 0.01$, siUsf1 *versus* siCtrl), Usf2 knockdown significantly decreased the Agtrap mRNA (Fig. 5*C*, $p < 0.01$, siUsf2 *versus* siCtrl) and protein (Fig. 5*F*, $p < 0.01$, siUsf1 *versus* siCtrl). These results show that Usf1 and Usf2 exert negative and positive regulatory effects on Agtrap gene expression, respectively.

Pathophysiological Relevance of Usf1 and Usf2 in Mouse Agtrap Gene Expression in the Kidney—To understand the pathophysiological roles of Agtrap in target organ injury, it is necessary to investigate the regulation of the expression of the *Agtrap* gene in response to pathological stimuli. UUO is a well established experimental model of progressive tubulo-interstitial fibrosis. UUO leads to changes in renal hemodynamics, inflammatory responses in the kidney, tubular hypertrophy, and interstitial fibrosis of the affected kidney by stimulating the renin-angiotensin system (39). Since we previously showed that the Agtrap mRNA level was suppressed in the affected kidney by UUO (20), we examined whether the change in *Agtrap* gene expression is accompanied by any modulation of the *Usf1* or *Usf2* gene expression in the UUO kidney. According to the results of quantitative RT-PCR analysis, while the Usf1 mRNA expression was significantly up-regulated in the affected kidney after 7 days of UUO (Fig. 6*B*), the Usf2 mRNA expression was F6 significantly down-regulated in the affected kidney by UUO (Fig. 6*C*), with a concomitant decrease in the Agtrap mRNA expression (Fig. 6*A*). These results *in vivo* are consistent with the notion that Usf1 and Usf2 are inhibitory and stimulatory transcription factors for the *Agtrap* gene, respectively.

Functional Involvement of the Two Adjacently Located E-box Motifs in Proximal Human AGTRAP Promoter Activity—To evaluate the evolutionary and functional conservation of the regulation of *AGTRAP* gene expression by the E-box, we examined the activity of the *AGTRAP* proximal promoter with or without an E-box mutation using luciferase reporter assay. Because the promoter of the human isologous gene *AGTRAP* has two adjacently located E-box motifs (Fig. 2*A*), we analyzed both of them. As shown in Fig. 7*A*, the 575-bp human *AGTRAP* F7 proximal promoter fragments (−480 to +95 of the putative transcriptional start site) exhibited substantial luciferase activity in human kidney-derived HEK293 cells. In addition, muta-

TABLE 1
Primer sequences used in the study

Primers		Primer sequences
Construction of wild-type and mutated *Agtrap* promoter-containing plasmids		
−4950 to −353	Forward	5′GCCATTCCCTGAGCTGTTGAGGGCCCTTCACTGAAAGGCTTCTTGGT3′
	Reverse	5′CTTTGTGAGGATCAGTGAATGAATTCATGTCCATAAAGATAAAAAGTGA3′
−2871 to 353	Forward	5′CTAGAGAAGGTACCCAAGGAGCTAAACGGATCTGCAACCCTATAG3′
	Reverse	5′CTTTGTGAGGATCAGTGAATGAATTCATGTCCATAAAGATAAAAAGTGA3′
−2018 to −353	Forward	5′GCTATGTGGTTAAAGGCACTTGCCACACCAGCCTGTCGACTGGCC3′
	Reverse	5′CTTTGTGAGGATCAGTGAATGAATTCATGTCCATAAAGATAAAAAGTGA3′
−1200 to +72	Forward	5′ggggtaCCAACTTTTGCTATGTTGGGCAAGTGGACTCCA3′
	Reverse	5′cgggatccGAACTCGGGAACAAACTTCCT3′
−900 to +72	Forward	5′ggggtaCCCCTTTCTTGACTTCAGGTCCTGTCTCCCTTTCC3′
	Reverse	5′cgggatccGAACTCGGGAACAAACTTCCT3′
−541 to +72	Forward	5′gcggtACCTGCCTGTGTGTGTATATGCACTT3′
	Reverse	5′cgggatccGAACTCGGGAACAAACTTCCT3′
−381 to +72	Forward	5′gcggtACCCTCACTTTTTATCTTTATGG3′
	Reverse	5′cgggatccGAACTCGGGAACAAACTTCCT3′
−302 to +72	Forward	5′ggggtacCTTGTGCAAGGGAAGTAAGA3′
	Reverse	5′cgggatccGAACTCGGGAACAAACTTCCT3′
−150 to +72	Forward	5′gcggtaCCCTAGGCTGCAGAAATCCC3′
	Reverse	5′cgggatccGAACTCGGGAACAAACTTCCT3′
Construction of mutated *Agtrap* promoter-containing plasmids		
X-box-mt	Forward	5′CTGCCTGACGCCGTCTCCTATCATACGGCTGCAGTCACGTGGCCG3′
	Reverse	5′CGGCCACGTGACTGCAGCCGTATGATAGGAGACGGCGTCAGGCA3′
E-box-mt	Forward	5′TCCTAGCAACCGGCTGCATTCAAATGGCCGCGCGAGTTGGCCT3′
	Reverse	5′AGGCCAACTCGCGCGGCCATTTGAATGCAGCCGGTTGCTAGGA3′
GC-box-mt	Forward	5′GAACTGGCGCAACGCGACGGTTGGCGCCAGGAAGTTTGTTCCCGA3′
	Reverse	5′TCGGGAACAAACTTCCTGGCGCCAACCGTCGCGTTGCGCCAGTTC3′
X/E-box-mt	Forward	5′TCCTATCATACGGCTGCATTCAAATGGCCGCGCGAGTTGGCCT3′
	Reverse	5′AGGCCAACTCGCGCGGCCATTTGAATGCAGCCGTATGATAGGA3′
Electrophoretic mobility shift assay (EMSA) and oligonucleotide precipitation assay		
E-box ODN		5′CCGGCTGCAGTCACGTGGCCGCGCGAGTTG3′ and 5′CAACTCGCGCGGCCACGTGACTGCAGCCGG3′
E-box-mt ODN		5′CCGGCTGCATTCAAATGGCCGCGCGAGTTG3′ and 5′CAACTCGCGCGGCCATTTGAATGCAGCCGG3′
X-box ODN		5′CTGCCTGACGCCGTCTCCTAGCAACCGGCTG3′ and 5′CAGCCGGTTGCTAGGAGACGGCGTCAGGCAG3′
Chromatin immunoprecipitation (ChIP) assay		
Mouse *Agtrap* promoter	Forward	5′CCTAGCAGCAAGAGCAGCT3′
	Reverse	5′GAACTCGGGAACAAACTTCCT3′
Human *AGTRAP* promoter	Forward	5′ACAGTCCGCTTCCTGGAATA3′
	Reverse	5′GCCGCTTGGTTGCTAGGAGACGGCGTCGGCAGC3′
Human *AGTRAP* exon 3	Forward	5′GGCTGCATTGTATTCTCAGG3′
	Reverse	5′CTTATGGCGTCGATGGAGTC3′

tions of either of the two adjacently located E-box motifs significantly decreased the luciferase activity (Fig. 7*A*). Mutations of both E-box motifs further reduced the luciferase activity (Fig. 7*A*). These results indicate that the two adjacently located E-box motifs are important for the basal transcriptional activity directed by the *AGTRAP* promoter.

USF1 and USF2 Bind the AGTRAP Promoter Region—We further performed ChIP analysis to examine whether USF1, USF2, or BMAL1 physiologically interacts with the *AGTRAP* promoter region. For ChIP analysis, we prepared primer sets for the *AGTRAP* promoter region and internal exon 3 (Fig. 7*B*). A 161-bp fragment of the proximal upstream region of the two adjacently located E-box motifs in the *AGTRAP* promoter was recovered after immunoprecipitation of sheared genomic DNA from HEK293 cells with an anti-USF1 antibody and anti-USF2 antibody but not after immunoprecipitation with an anti-SREBP1 antibody or anti-BMAL1 antibody. Quantitative PCR analysis showed that USF1 and USF2 are present at the *AGTRAP* E-box promoter region, and the corresponding genomic DNA was enriched with an anti-USF1 antibody (**, $p < 0.01$, *versus* IgG control) and anti-USF2 antibody (**, $p < 0.01$, *versus* IgG control), respectively, but not with an anti-SREBP1 antibody or anti-BMAL1 antibody (Fig. 7*B*). Among these factors USF1 and USF2, but not BMAL1 and SREBP1, proteins were also detected in the co-immunoprecipitates from HEK293 cells on immunoblot analyses (Fig. 7*C*). However, USF1, USF2, SREBP1, and BMAL1 did not interact with the *AGTRAP* exon 3 region, which is a negative control region, without the E-box (Fig. 7*B*). These data indicate the occupancy by USF1 and USF2, but not SREBP1 or BMAL1, of the human *AGTRAP* promoter region.

DISCUSSION

Despite the accumulating evidence supporting the involvement of an altered expression of *Agtrap* gene at local tissue sites in the pathogenesis of hypertension and related kidney injury, little is known about the transcriptional regulation of *Agtrap* expression. In this study, we showed that the promoter region from −150 to +72 of the mouse *Agtrap* 5′-flanking sequence, which is considered to contain important regulatory elements, directs *Agtrap* gene transcription in normal culture.

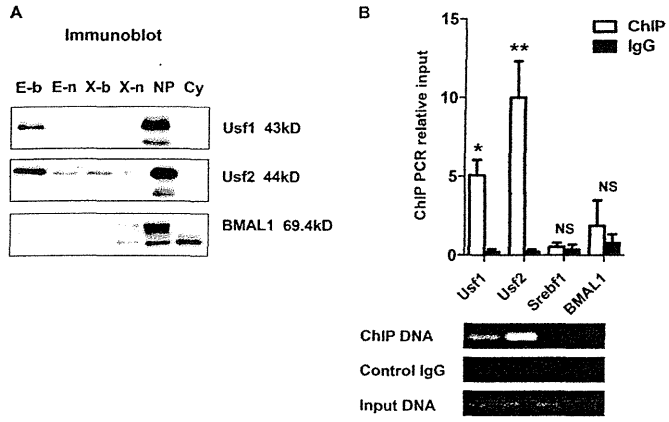

FIGURE 4. **Identification of Usf1 and Usf2 interaction with the E-box (−72 to −43) of the mouse *Agtrap* promoter by streptavidin-biotin complex assay and ChIP assay.** *A*, streptavidin-biotin complex assay. Nuclear extracts from mDCT cells were incubated with streptavidin immobilized on agarose beads. The streptavidin-biotin-DNA complex was eluted with SDS buffer and visualized by immunoblot analysis. *E-b*, E-box biotin-labeled probe; *E-n*, E-box nonlabeled probe; *X-b*, X-box biotin-labeled probe; *X-n*, X-box nonlabeled probe; *NP*, nuclear extracts; *Cy*, cytosolic extracts. *B*, ChIP assay. The mDCT cells were treated with an anti-USF1 antibody, anti-USF2 antibody, anti-SREBP1 antibody, anti-BMAL1 antibody, or control IgG (rabbit anti-HA antibody). Co-immunoprecipitated DNA was purified and estimated by quantitative PCR. In the *upper panel* the relative amount of DNA fragment detected per antibody is shown. In the *lower panel*, the quantitative PCR products, which were loaded on 3% agarose gels and visualized by ethidium bromide staining, are shown. Experiments were independently repeated at least three times, and data are expressed as the means ± S.E. *, $p < 0.05$; **, $p < 0.01$, *versus* control IgG. *NS*, not significant.

We analyzed the region from −381 to +72 based on the results showing maximum promoter activity. The results of luciferase assay using deletion mutants revealed the minimally required proximal promoter region from −150 to +72 that contains the X-box, E-box, and GC-box consensus motifs is able to direct substantial transcription of the *Agtrap* gene. Among these binding motifs, we confirmed that the E-box specifically binds Usf1 and Usf2 by employing EMSA, streptavidin-biotin complex assay, and ChIP. Such E-box-Usf1/Usf2 binding is functionally important in activating *Agtrap* expression for the following reasons: 1) mutation of the E-box to prevent Usf1/Usf2 binding reduces *Agtrap* promoter activity (Fig. 2); 2) transfection of siRNA for Usf1 increases and Usf2 decreases endogenous Agtrap mRNA and protein expression (Fig. 5), and 3) the decrease in Agtrap mRNA expression in the affected UUO kidney is accompanied by changes in Usf1 and Usf2 mRNA (Fig. 6). Taken together, these data indicate that Usf1 and Usf2 negatively and positively regulate *Agtrap* gene transcription, respectively. Because Usf1 and Usf2 bind to DNA with the same E-box sequence specificity, they most likely regulate *Agtrap* gene expression in a competitive manner.

Recently, the E-boxes in the promoter regions of renin and angiotensinogen were shown to be direct targets of Usf1 and Usf2 and suggested to be involved in the pathogenesis of both hypertension and renal injury (33, 56–58). In this study, it is

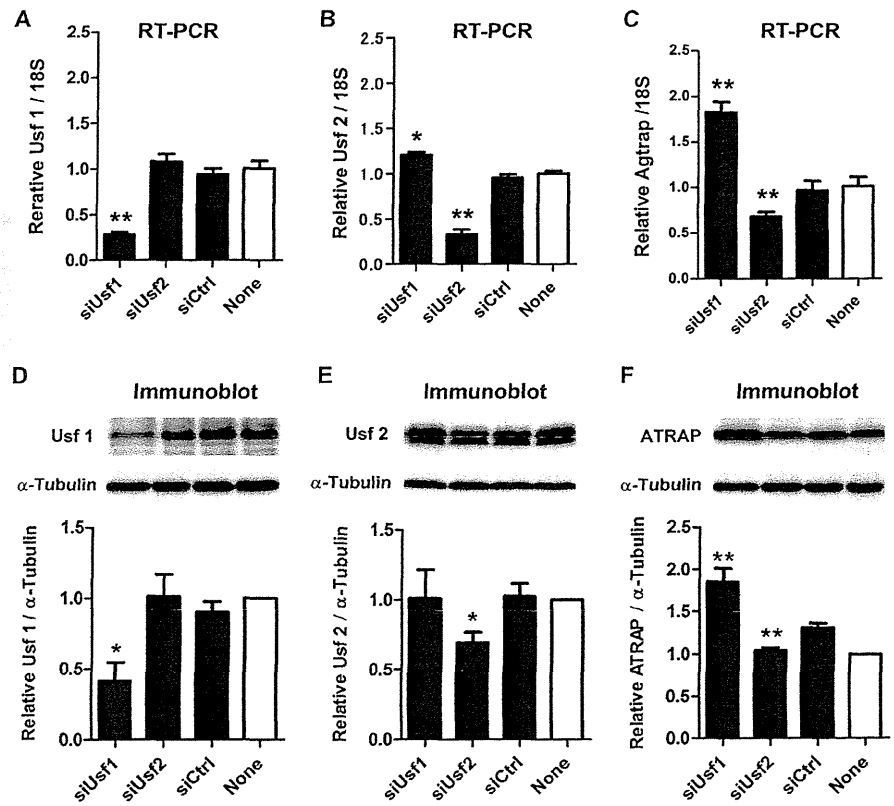

FIGURE 5. **Effects of specific knockdown of Usf1 and Usf2 by small interference (si)RNA on endogenous *Agtrap* gene expression in mDCT cells.** *A–C*, quantitative RT-PCR analysis showing the effects of respective siRNA transfection on the relative Usf1 (*A*), Usf2 (*B*), and Agtrap (*C*) mRNA levels. RNA quantity was normalized to the signal generated by constitutively expressed 18 S rRNA and is expressed relative to that achieved with extracts derived from nontreated mDCT cells (none). Experiments were independently repeated at least three times, and the data are expressed as the means ± S.E. *, $p < 0.05$; **, $p < 0.01$, *versus* control siCtrl. *D–F*, immunoblot analysis showing the effects of the respective siRNA transfection on the relative Usf1 (*D*), Usf2 (*E*), and Agtrap (*F*) protein levels. Representative immunoblots are shown, and protein expression levels are expressed relative to those achieved with extracts derived from nontreated mDCT cells (*none*). Experiments were independently repeated at least three times, and data are expressed as the means ± S.E. *, $p < 0.05$; **, $p < 0.01$, *versus* control siCtrl.

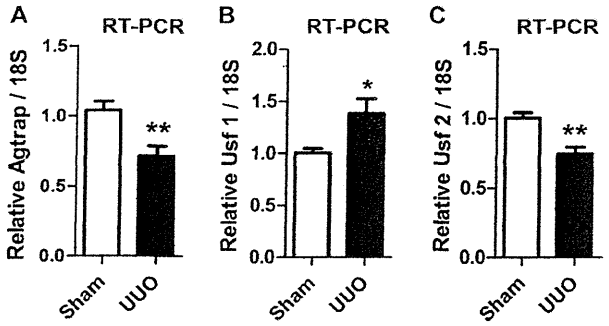

FIGURE 6. **Regulation of Usf1, Usf2, and *Agtrap* mRNA in the affected kidney by UUO.** *A–C*, quantitative RT-PCR analysis showing the effects of UUO on the relative Agtrap (*A*), Usf1 (*B*), and Usf2 (*C*) mRNA levels. RNA quantity was normalized to the signal generated by the constitutively expressed 18 S rRNA and is expressed relative to the level achieved with extracts derived from sham-operated kidney ($n = 6$). *, $p < 0.05$; **, $p < 0.01$, *versus* sham. Data are expressed as the means ± S.E.

shown that Agtrap, an emerging modulator of the renin-angiotensin system, is another target gene of Usf1 and Usf2, and that *Agtrap* gene expression is activated through the binding of Usf2 and inhibited through the binding of Usf1 to the same canonical E-box sequence in the *Agtrap* proximal promoter region.

Both Usf1 and Usf2 are reportedly activators of gene transcription via homodimerization or heterodimerization, with similar trans-activating capacities (59, 60), and they have also been proposed to function as repressors of a number of target genes (61). However, the results of this study show that Usf1 and Usf2 exert opposing regulatory effects on the expression of the same gene. Consistent with this notion, similarly opposing effects of Usf1 and Usf2 on the E-box of plasminogen activator inhibitor-1 gene, a key regulator of the fibrinolytic system, have been reported (62, 63). With respect to an interaction between Usf and other transcription factors, a previous study reported a contrasting functional and physical interaction between Usf and Sp1, a GC-box binding transcription factor, in the transcriptional regulation of the deoxycytidine kinase gene in liver-derived HepG2 cells (64). In the regulation of the deoxycytidine kinase promoter, the combination of Usf1 and Sp1 exhibited additive trans-activation at lower concentrations of Sp1, although Sp1 was inhibitory at higher levels, whereas trans-activation by Usf2 and Sp1 was synergistic in HepG2 cells (64). In this study, although the E-box and GC-box were found to be adjacently located in the *Agtrap* promoter, the results of luciferase assay showed a positive and independent stimulatory effect of these binding motifs in kidney-derived mDCT cells (Fig. 2), possibly because of a difference in the network of transcription factors in the liver and kidney. However, it is still possible that a functional interplay of Usf1 and Usf2 with putative transcription factors other than Sp1 is involved in the opposing regulatory effect exerted by Usf1 and Usf2 on *Agtrap* gene expression (Fig. 5). Further studies are needed to elucidate the molecular mechanisms, including kinase cascades, such as PI3K (28), which are involved in the differential regulatory functional effect of Usf1 and Usf2 on *Agtrap* gene expression. Studies are also needed to examine the possible role of E-box modulation by methylation at the core CpG in the Usf1/Usf2 recognition site (5′-CACpGTG-3′) in the regulation of the *Agtrap* promoter (64).

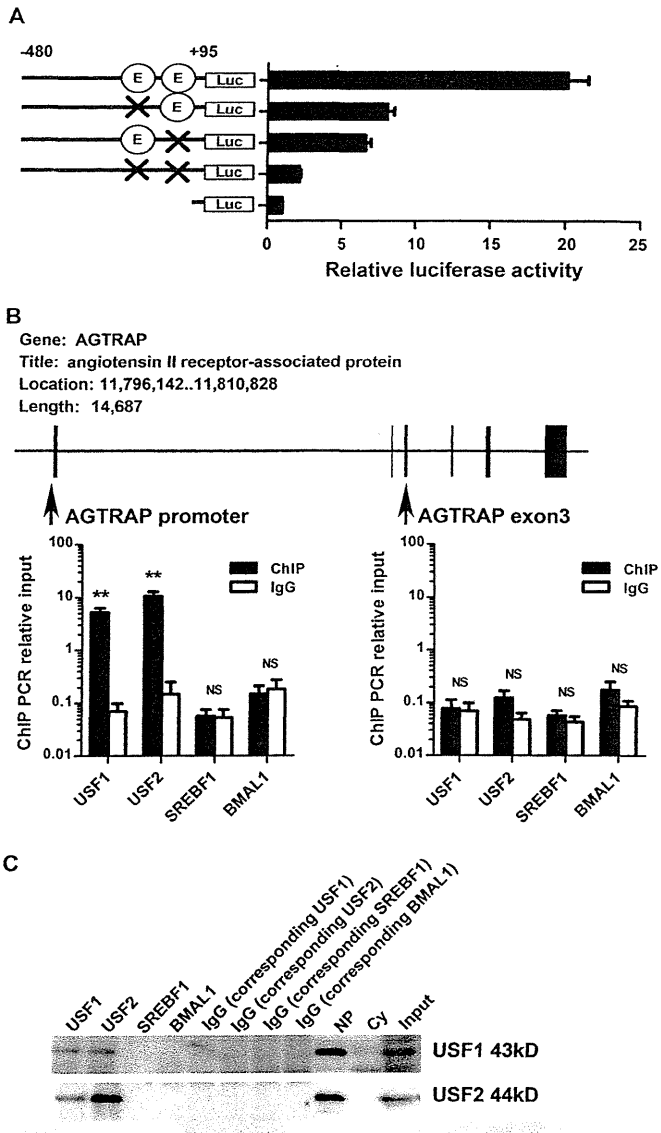

FIGURE 7. **Involvement of the E-box motifs in the regulation of the human *AGTRAP* promoter.** *A*, involvement of the two E-box motifs in the transcriptional activation of the *AGTRAP* promoter in HEK293 cells. The effects of mutations in the two E-box motifs on the transcriptional activity of the *AGTRAP* promoter-luciferase hybrid gene in HEK293 cells are shown. The relative luciferase activities were calculated relative to those achieved with the promoterless control plasmid. Data are expressed as the means ± S.E. ($n = 4$). *B*, identification of USF1 and USF2 interaction with the *AGTRAP* proximal promoter region by ChIP analysis. Schematic representation of the *AGTRAP* gene structure and the approximate genomic positions for the enrichment of the *AGTRAP* promoter and exon3 region by ChIP assay are shown (*upper panel*). The results of ChIP assay with an anti-USF1 antibody, anti-USF2 antibody, anti-SREBP1 antibody, or anti-BMAL1 antibody are shown (*lower panel*). Data are expressed as the means ± S.E. ($n = 4$). **, $p < 0.01$, *versus* control IgG (rabbit anti-HA antibody). *NS*, not significant. *C*, identification of USF1 and USF2 in the co-immunoprecipitates from the AGTRAP proximal promoter region in ChIP analysis. Co-immunoprecipitated proteins with respective specific antibodies or their corresponding control IgG in ChIP assay were subjected to immunoblot analysis and were visualized by TrueBlot (Affymetrix). *NP*, nuclear extracts; *Cy*, cytosolic extracts; *Input*, input reference.

Cardiovascular and renal diseases are closely related to circadian rhythms, which are under the control of an internal biological clock mechanism. The binding of the transcription factors BMAL1 and CLOCK to multiple extra- and intragenic E-boxes is reported to play an important role in the circadian

rhythm-related regulation of certain genes in peripheral, cardiovascular, and renal tissues (65–67). However, the present results do not indicate any significant interaction of BMAL1 with the E-box of the mouse *Agtrap* promoter (Fig. 4). This may be because the BMAL1-CLOCK heterodimer binds to multiple E-boxes of target genes despite there being a single E-box in the mouse *Agtrap* proximal promoter (66). The human *AGTRAP* promoter contains two adjacently located E-box motifs (Fig. 2). However, we did not obtain any evidence to indicate the interaction of BMAL1 with these two adjacently located E-box motifs in the *AGTRAP* promoter, at least in human kidney-derived cells (Fig. 7). Further studies are needed to examine the potential interaction of the BMAL1-CLOCK heterodimer with the adjacently located two E-box motifs in the *AGTRAP* promoter in other cells or tissues such as fat or liver, so as to exert cell type- or tissue-specific function. However, the results of the promoter assay and ChIP analysis clearly indicate the functional interactions of USF1/USF2 and the adjacently located two E-box motifs are involved in the regulation of the human *AGTRAP* promoter.

In summary, the results of this study show that Usf1 and Usf2 regulate *Agtrap* gene transcription through their interaction with the E-box in the mouse *Agtrap* promoter. Furthermore, the *in vitro* and *in vivo* results of siRNA transfection in mDCT cells and UUO in mice, respectively, suggest that Usf1 decreases and Usf2 increases *Agtrap* gene expression through the binding of Usf1/Usf2 to the E-box. We also demonstrated functional E-box-USF1/USF2 binding in the human *AGTRAP* promoter, thereby suggesting that a strategy of modulating the E-box-USF1/USF2 binding may have novel therapeutic potential.

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Therapeutic Apheresis and Dialysis

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Therapeutic Potential of Low-Density Lipoprotein Apheresis in the Management of Peripheral Artery Disease in Patients With Chronic Kidney Disease

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Abstract: Cardiovascular disease (CVD) is a major cause of death in patients with chronic kidney disease (CKD). Patients with CKD are reported to have a significant greater risk of CVD-associated mortality than that of the general population after stratification for age, gender, race, and the presence or absence of diabetes. CKD itself is also an independent risk factor for the development of atherosclerosis, and in particular, patients undergoing dialysis typically bear many of the risk factors for atherosclerosis, such as hypertension, dyslipidemia and disturbed calcium-phosphate metabolism, and commonly suffer from severe atherosclerosis, including peripheral arterial disease (PAD). Low-density lipoprotein (LDL) apheresis is a potentially valuable treatment applied to conventional therapy-resistant hypercholesterolemic patients with coronary artery disease and PAD. Although previous and recent studies have suggested that LDL apheresis exerts beneficial effects on the peripheral circulation in dialysis patients suffering from PAD, probably through a reduction of not only serum lipids but also of inflammatory or coagulatory factors and oxidative stress, the precise molecular mechanisms underlying the long-term effects of LDL apheresis on the improvement of the peripheral circulation remains unclear and warrants further investigation. **Key Words:** Chronic kidney disease, Hemodialysis patients, Low-density lipoprotein apheresis, Oxidative stress, Peripheral artery disease.

Cardiovascular disease (CVD) has a major effect on the prognosis of patients with chronic kidney disease (CKD), particularly for those patients on dialysis, and atherosclerotic vascular changes play a critical role. Renal deterioration in CKD promotes hypertension, dyslipidemia, insulin resistance, disturbed calcium-phosphate metabolism and renal anemia, and these are all risk factors for atherosclerosis. In addition, chronic inflammation, oxidative stress and variability in blood pressure and circulating blood volume also promote atherosclerotic vascular changes in dialysis CKD patients. Among the systemic atherosclerotic vascular diseases, peripheral arterial disease (PAD) is prevalent in dialysis CKD patients. In dialysis CKD patients, the PAD lesions are prone to being distributed in the arteries of the lower limbs and exhibit a severely stenotic lumen

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with vascular calcification without the development of any collateral circulation, and patients with CKD and PAD have higher mortality rates than those with either of these conditions alone (1).

Percutaneous transluminal angioplasty (PTA) for the treatment of PAD is reported to suffer from the problem of an increased rate of restenosis in dialysis CKD patients (2). PAD in HD patients is often therapy resistant and closely associated with an increased risk of cardiovascular mortality, morbidity, and hospitalization as well as a reduced Health-Related Quality of Life (HRQOL), even after bypass surgery and leg amputation (3,4). In certain countries, including most of the countries in Europe, LDL apheresis is the treatment of choice in patients with homozygous familial hypercholesterolemia (FH), particularly those refractory to statins, as described in a recent review (5). LDL apheresis has been shown to exert beneficial effects on aortic and coronary atherosclerosis and to reduce the risk of coronary artery disease in patients with homozygous FH (6,7). Recently LDL apheresis has become the adjuvant treatment of choice for dialysis CKD patients with PAD, particularly those refractory to statins and on whom it is difficult to perform PTA or bypass surgery (8,9). In this review we briefly summarize the clinical applications of LDL apheresis in Japan and mechanistic basis for its benefit in PAD in dialysis patients.

PRESENT INDICATION OF LDL APHERESIS FOR THERAPY OF PAD IN JAPAN

In Japan, indicated usage of LDL apheresis for the treatment of PAD under the government system of insurance coverage, includes the following. (i) clinical signs of poor peripheral circulation, such as cold, decolored or ulcerated extremities, or intermittent claudication consistent with a Fontaine classification of Grade II or more; (ii) refractoriness to conventional medical or surgical treatment; (iii) an excessively high LDL cholesterol (LDL-C) or total cholesterol (TC) levels (LDL-C >140 mg/dL or TC >220 mg/dL) in spite of drug treatment (10). Japanese government insurance coverage permits 10 sessions of LDL apheresis for each patient to be carried out during a 3-month period.

THE PATHOGENESIS OF LIPOPROTEIN ABNORMALITIES IN CKD

Although hypercholesterolemia is a major requisite for the governmental insurance-covered application of LDL apheresis for the treatment of PAD in Japan, the regulatory system of lipid metabolism is reported to be highly disturbed in CKD patients, owing to alterations in apolipoproteins, lipid transfer proteins, lipolytic enzymes and lipoprotein receptors (11,12). Previous studies demonstrated that LDL particles are heterogeneous with respect to their size, density and lipid composition (13,14). Among the LDL particles, the smaller and denser LDL particles (small dense low-density LDL particles) are more atherogenic (13), and the small dense low-density LDL phenotype is strongly associated with the development of coronary heart disease (15).

In CKD patients, triglyceride concentrations increase while HDL cholesterol (HDL-C) concentrations decline, and there is a progressive accumulation of the more atherogenic, small dense low-density LDL particles, in spite of low-to-normal TC and LDL-C levels (11). In addition, in dialysis CKD patients, dyslipidemia is typified by a marked increase in triglyceride-rich apo B-containing particles, a decreased HDL concentration and a predominance of small dense LDL particles, with a normal LDL-C level but an increased lipoprotein(a) (Lp[a]) concentration. Also, there is reportedly a persistent disturbance in the apolipoprotein profile, with reduced apo AI and apo AII concentrations and significant increases in the apoB, apoCIII and apoE concentrations (11,16).

LDL APHERESIS FOR THE TREATMENT OF PAD

There are presently several systems of LDL apheresis in use, including cascade and lipid filtration, immunoadsorption, heparin-induced LDL precipitation, dextran sulfate LDL adsorption, and the LDL hemoperfusion (17,18). In Japan, LDL apheresis therapy using the strategy of LDL absorption with dextran sulfate (Liposorber LA-15, Kaneka, Japan) is most commonly performed (19,20). Low-molecular dextran sulfate (MW 4500) selectively absorbs all substances containing apoB. The binding mechanism is the direct interaction between the dextran sulfate and the positively charged surface of apoB-containing lipoproteins (LDL-C, very low density lipoprotein-cholesterol [VLDL-C], and Lp[a]). Dextran sulfate has a structure similar to that of the LDL receptor and seems to act as a type of pseudoreceptor. Approximately 2.5 g LDL-C can be bound per column. After primary separation, the plasma is perfundated through the columns, where all material containing apoB such as cholesterol, LDL-C, VLDL-C, and triglycerides is absorbed, but without any absorption of HDL-C, which does not contain apoB (18).

In our hospital, LDL apheresis is performed using hollow polysulfone fibers (Sulflux, Kaneka, Osaka, Japan) as the plasma separator and a dextran sulfate cellulose column (Liposorber LA-15, Kaneka) as the LDL absorber. Blood flow from the A-V fistula access in the case of dialysis CKD patients is typically in the range of 80–100 mL/min, the plasma flow is 25–30 mL/min, and 3000–4000 mL of the plasma volume is treated per session. Heparin or nafamostat mesilate is given as an anticoagulant for extracorporeal circulation. To maintain good adsorption efficiency with an increasing quantity of plasma treated, two columns arranged in a row are used in turn automatically, so that one column can be washed with the specific liquid and regenerated after the treatment of the plasma while the other is in operation (MA-03 system, Kaneka) (21,22). LDL apheresis is carried out once or twice a week on non-HD days in the case of dialysis CKD patients, and 10 sessions of apheresis are performed in each patient.

THERAPEUTIC EFFECTS OF LDL APHERESIS IN DIALYSIS CKD PATIENTS WITH PAD

Although dialysis CKD patients with PAD often exhibit low-to-normal TC and LDL-C levels along with increased triglycerides and reduced HDL-C levels, previous studies showed that LDL apheresis by the dextran sulfate cellulose column (Liposorber LA-15, Kaneka) is clinically effective even in PAD patients undergoing HD (23–28). Since PAD patients undergoing HD tend to be resistant to any treatment and are at high risk for lower-extremity amputation, LDL apheresis is suggested to be a useful strategy in the multidisciplinary approach for therapy of PAD (Fig. 1).

In a recent clinical study we conducted, 25 dialysis CKD patients with PAD were enrolled, and the therapeutic effects of LDL apheresis in 19 patients were ultimately analyzed (22). 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 HD). The absolute walking distance and ankle-brachial pressure index (ABI) were principally estimated on non-HD days prior to the 1st and 10th sessions and at 3 months after the end of treatment, and the long-term periods in this study were defined as the time from the 1st session to the 10th session and the time from the 1st session up to the third month after the end of the 10th sessions.

Because most of these patients were unable to perform treadmill exercise because of conditions such as a previous heart attack or paralysis, the absolute walking distance was evaluated by medical staff on a flat floor in the hospital. Of the 25 patients enrolled, five patients could not complete the study because of death ($N = 3$), amputation ($N = 1$) or PTA ($N = 1$) during the study period, and they were excluded from the analysis. On the whole, the absolute walking distance improved significantly by the 10th session of LDL apheresis compared with baseline and was still improved even at 3 months after the end of the treatment (Table 1) (22). Similarly, the ABI was improved by the 10th session compared with baseline (Table 1). Subsequently, the patients were classified into two groups according to the changes in the ABI at 3 months after the end of

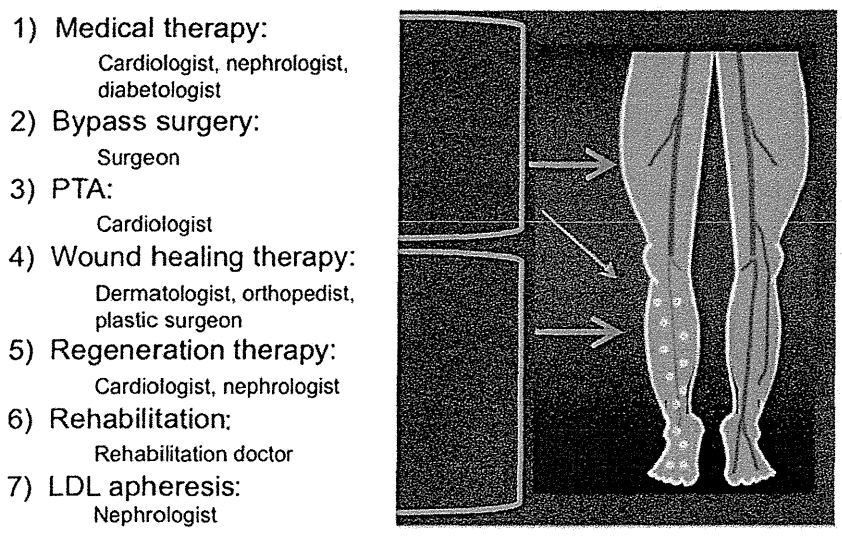

FIG. 1. Multidisciplinary therapeutic approach to peripheral arterial disease (PAD) in Japan.

TABLE 1. *Therapeutic effects of low density lipoprotein (LDL) apheresis in dialysis chronic kidney disease (CKD) patients with peripheral arterial disease (PAD)*

Clinical parameters	Baseline	After 1st apheresis	At 10th apheresis	3 months after 10th apheresis	*P1*	*P2*	*P3*
Total patients ($N = 19$):							
Walking distance (m)	171 ± 33	N/A	294 ± 34	270 ± 42	N/A	<0.05	<0.05
ABI	0.59 ± 0.04	N/A	0.67 ± 0.04	0.64 ± 0.04	N/A	<0.05	NS
ABI responders ($N = 10$):							
Walking distance (m)	118 ± 26	N/A	333 ± 45	297 ± 63	N/A	<0.05	<0.05
ABI	0.53 ± 0.06	N/A	0.69 ± 0.06	0.69 ± 0.05	N/A	<0.005	<0.005
LDL cholesterol (mg/dL)	88 ± 7	32 ± 3	78 ± 9	98 ± 11	<0.01	NS	NS
Oxidized LDL (U/L)	38 ± 3	20 ± 2	32 ± 3	38 ± 4	<0.01	<0.05	NS
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
ABI non-responders ($N = 9$):							
Walking distance (m)	232 ± 58	N/A	254 ± 49	238 ± 59	N/A	NS	NS
ABI	0.65 ± 0.05	N/A	0.63 ± 0.05	0.59 ± 0.06	N/A	NS	NS
LDL cholesterol (mg/dL)	83 ± 8	26 ± 2	75 ± 10	104 ± 16	<0.05	NS	NS
Oxidized LDL (U/L)	38 ± 5	18 ± 2	32 ± 4	46 ± 6	<0.05	NS	<0.05
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

Parameters are shown as the mean ± standard error. *P1* indicates the baseline vs. after the 1st apheresis; *P2*, the baseline vs. at the 10th apheresis; *P3*, the baseline vs. 3 months after the 10th apheresis (Modified from Tsurumi-Ikeya Y et al. *Arterioscler Thromb Vasc Biol* 2010;30:1058–1065). ABI, ankle-brachial pressure index; CRP, C-reactive protein; N/A, not applicable; NS, not significant.

treatment. The two groups were patients with an improved ABI (ABI responders, $N = 10$) and patients with a worsened ABI (ABI non-responders, $N = 9$). The serum levels of LDL-C and oxidized LDL, along with the C-reactive protein (CRP) and fibrinogen concentrations were significantly reduced after a single session in both groups. However, in the responders, LDL apheresis showed a trend toward a long-term reduction of the circulating levels of oxidized LDL, CRP, and fibrinogen (Table 1), in addition to a short-term dramatic decrease in the TC and LDL-C levels after each LDL apheresis session (Table 1).

When the baseline parameters of the ABI responders and non-responders were compared in order to analyze factors involved in the therapeutic effects of LDL apheresis, the walking distance as well as ABI tended to be lower in the ABI responders than in the ABI non-responders (walking distance, 118 ± 26 vs. 232 ± 58 m, $P = 0.08$; ABI, 0.53 ± 0.06 vs. 0.65 ± 0.05, $P = 0.12$), thereby suggesting that dialysis CKD patients with severe symptoms of PAD may be afforded a long-term therapeutic benefit by LDL apheresis. However, although the absolute walking distance and ABI still remained significantly improved in the responders 3 months after the tenth apheresis compared to these parameters at baseline, the LDL apheresis-mediated decrease in the oxidized LDL, CRP and fibrinogen concentrations lasted until just after the tenth apheresis, but not at 3 months after the tenth apheresis. Thus, there is a discrepancy between the long-term therapeutic effects of LDL apheresis on the clinical parameters of the walking distance and ABI, and the improvements in the laboratory parameters, including oxidized LDL, CRP and fibrinogen.

Thus, in order to examine the mechanism by which the absolute walking distance and ABI improved, even though the LDL apheresis did not result in a decrease in either oxidized LDL or inflammation at 3 months after the tenth apheresis, additional statistical correlation analyses were performed (Fig. 2) (22). As a result, there were statistically significant correlations between the walking distance and the plasma oxidized LDL ($R = -0.448$, $P < 0.05$, Fig. 2A) and fibrinogen ($R = -0.779$, $P < 0.05$, Fig. 2B) levels in the responders. In the non-responders, there was only a marginal correlation between the walking distance and the plasma oxidized LDL ($R = -0.329$, $P = 0.07$, Fig. 2C). Therefore, the therapeutic effects of LDL apheresis appear to be related to a chronic reduction of oxidized LDL and fibrinogen, as revealed by the significant negative relationships between the walking distance and the laboratory parameters.

Nevertheless, one of the obvious limitations of this study is the small number of enrolled patients. In addition, most of the patients were unable to perform treadmill exercise because of conditions such as previous heart attack or paralysis, but these are two distinctly different pathologies. Thus, although there should be at least four groups, that is, responders and non-responders in patients with heart attack and those with paralysis, respectively, to be strictly correct for the purposes of analysis, this was not possible due

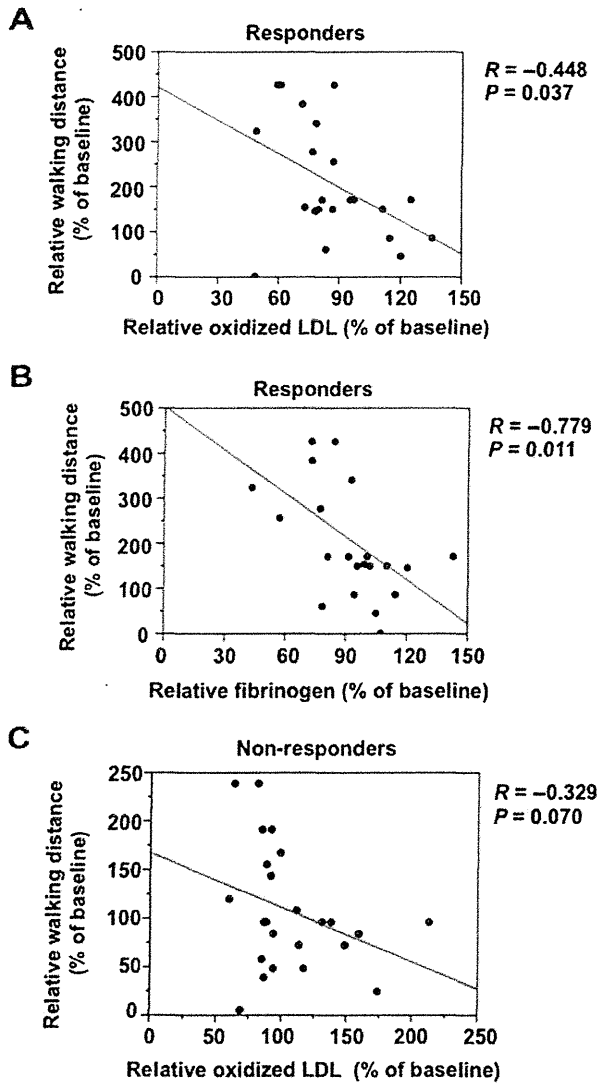

FIG. 2. Relationships between the walking distance (relative walking distance) and plasma oxidized low density lipoprotein (LDL) (relative oxidized LDL) (A), fibrinogen (relative fibrinogen) levels (B) in the responders, and between the walking distance (relative walking distance) and plasma oxidized LDL (relative oxidized LDL) (C) in the non-responders. The respective values were calculated relative to those achieved at baseline in either the responder group or the non-responder group (Modified from Tsurumi-Ikeya Y et al. *Arterioscler Thromb Vasc Biol* 2010; 30: 1058–65).

to the limited number and type of patients available. Furthermore, there is a possibility that other factor(s), which have not been identified in these studies, play a critical role in mediating the long-term therapeutic effects of LDL apheresis in CKD patients with PAD. Also, the components of the non-responders' serum responsible for the insufficient clinical improvement remain to be determined. Therefore, further studies, such as investigations using mass spectrometry, are needed (29).

MECHANISMS INVOLVED IN THE THERAPEUTIC EFFECTS OF LDL APHERESIS IN DIALYSIS CKD PATIENTS WITH PAD

Low density lipoprotein apheresis not only improves clinical symptoms rapidly, but also results in sustained improvement, although the mechanism has not been fully elucidated. Recently, LDL-C crystals in the phagosome of vascular macrophages were shown to directly activate Nod-like receptor family, pyrin domain–containing 3 (NLRP3) inflammasomes and thus to trigger atherogenesis in the early phase (30,31), and previous studies demonstrated that a single LDL apheresis decreased not only the TC and LDL-C concentrations, but also the oxidized LDL-C, CRP and fibrinogen concentrations in the short term (18). Dialysis CKD patients are reportedly characterized by higher levels of oxidative and inflammation than healthy subjects (11,32–34). Oxidative stress and inflammation are correlated strongly with tryglycerides, VLDL-C, apoC-III and apoC-III bound to apoB-containing lipoproteins, but not with either TC or LDL-C (35).

With respect to the long-term effects of LDL apheresis on lipid-related oxidative stress, a previous study has shown both acute and chronic effects of LDL apheresis in lowering the susceptibility of LDL to oxidation in non-CKD patients with severe, genetically determined hypercholesterolemia (36). In our previous study, the therapeutic effects of LDL apheresis were related to the relatively sustained decrease in oxidized LDL and fibrinogen, which are markers of lipid peroxidation and blood coagulation, respectively (Table 1, Fig. 2) (22). Other studies also showed LDL apheresis-mediated reduction of thiobarbituric acid-reactive substances, thiobarbituric acid being a marker of lipid peroxidation, and also a production of reactive oxygen species via the suppression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expression in leukocytes in HD patients (28). Therefore, these results suggest that LDL apheresis-mediated suppression of lipid peroxidation is one of the contributing factors to its therapeutic effect on the peripheral circulation in end-stage renal disease patients with PAD. Other potential mechanisms have also been proposed to play a role in the therapeutic effects of LDL apheresis on atherosclerotic vascular lesions (Table 2).

The therapeutic effects of LDL apheresis on the inflammatory profile have also been reported. Stefanutti et al. showed that LDL apheresis resulted in an anti-inflammatory and anti-atherogenic cytokine profile in the plasma of non-CKD patients with

TABLE 2. *Proposed effects of low density lipoprotein (LDL) apheresis*

- Reduction of whole blood and plasma viscosity
- Improvement of red blood cells (deformability)
- Increase of vasodilation factors (bradykinin, NO, PGI_2)
- Reduction of coagulation factors (fibrinogen etc.)
- Reduction of cell adhesion factors (ICAM-1, ELAM-1etc.)
- Reduction of CRP and MMP-9
- Inhibition of platelet activation
- Increase of HGF and endothelial progenitor cells

CRP, C-reactive protein; ELAM-1, endothelial leukocyte adhesion molecule-1 (E-selectin); HGF, hepatocyte growth factor; ICAM-1, intercellular adhesion molecule-1; MMP-9, matrix metalloproteinase-9; NO, nitric oxide; PGI_2, prostacyclin.

severe dyslipidemia and pre-existing angiographically demonstrated atherosclerotic lesions, that is, those patients at the highest level of individual cardiovascular risk (37). In another previous study, several cytokines and complement activation products, which are important for the progression of vascular atherosclerosis as well as plaque instability, were differently affected by the three apheresis columns DL-75 (whole blood adsorption), LA-15 (plasma adsorption), and EC-50W (plasma filtration) (38). This was true even in cases in which the LDL-C was reduced equally by all of them, and the adsorption columns displayed an apparently more beneficial inflammatory profile than the filtration device (38).

Vascular endothelial cells play important preventive roles against the development of atherosclerotic vascular disease (39). Previous studies showed that a single LDL apheresis session enhanced the peripheral microcirculation, probably by increasing the production of nitric oxide (NO) and bradykinin (40), reducing blood viscosity and adhesion molecules (41), and inducing endothelium-dependent vasodilatation (42). Another study demonstrated that endothelium-dependent vasodilation was significantly increased even 4 weeks after the final LDL apheresis in dialysis CKD patients with PAD (26). Thus, to investigate the molecular mechanism involved in the long-term therapeutic effects of LDL apheresis on endothelial cells, we examined the effects of LDL apheresis on vascular endothelial cell functions in vitro by analyzing the expression of the activated form of endothelial nitric oxide synthase (eNOS), which is phosphorylated at Ser-1177 (43), and cellular proliferative activity (22). The expression of the activated eNOS protein in human umbilical vein endothelial cells (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 (22). Furthermore, the proliferative activity of HUVECs was increased by the serum collected from the responders at 3 months after the end of treatment (22). Collectively, these results suggest that the therapeutic effects of LDL apheresis on CKD patients with PAD are at least partly dependent on the sustained reduction of oxidized LDL-C and fibrinogen, along with the activated eNOS-mediated improvement of endothelial cell function (Fig. 3). Because the

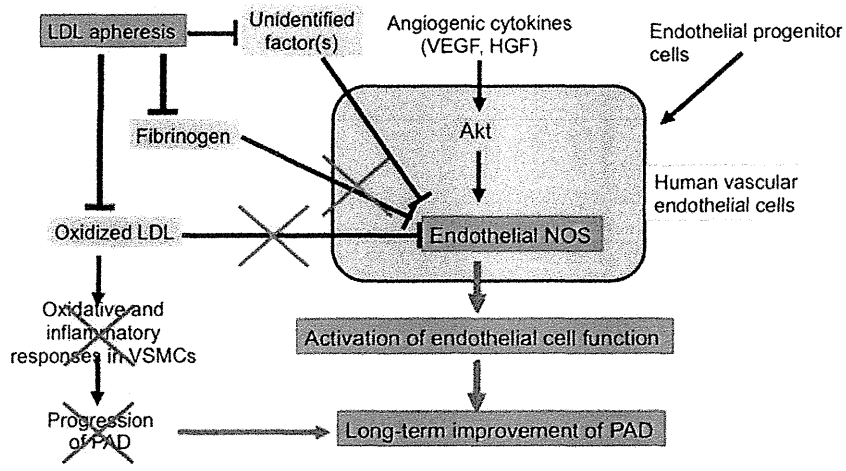

FIG. 3. Low density lipoprotein (LDL) apheresis exerts long-term therapeutic effects on peripheral arterial disease (PAD) in chronic kidney disease (CKD) patients, at least partly via a sustained reduction of oxidized LDL and fibrinogen, along with an activated endothelial nitric oxide synthase (eNOS)-mediated improvement of endothelial cell function. There is a possibility that other factor(s), which have not been identified yet, may play a critical role in mediating the therapeutic effects of LDL apheresis on endothelial cellular function. Further efforts, such as microarray analysis, are needed to identify the precise molecular mechanism of the LDL apheresis-mediated effects on endothelial cells and to improve the therapeutic efficacy of LDL apheresis. HGF, hepatocyte growth factor; PAD, peripheral arterial disease; VEGF, vascular endothelial growth factor; VSMCs, vascular smooth muscle cells.

activation of endothelial cells is an important strategy for the amelioration of the atherosclerotic vascular process (39) and there is a possibility that other factor(s), which have not been identified yet, play a critical role in mediating the therapeutic effects of LDL apheresis on endothelial cellular function, further investigative efforts, such as microarray analysis, should be used to identify the precise molecular mechanism of the LDL apheresis-mediated effects on endothelial cells and to improve the therapeutic efficacy of LDL apheresis (44).

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Enhanced Angiotensin Receptor-Associated Protein in Renal Tubule Suppresses Angiotensin-Dependent HypertensionNovelty and Significance

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Enhanced Angiotensin Receptor-Associated Protein in Renal Tubule Suppresses Angiotensin-Dependent Hypertension

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See Editorial Commentary, pp 1150–1152

Abstract—We have previously shown that angiotensin II type 1 receptor-associated protein (ATRAP/*Agtrap*) interacts with the angiotensin II type 1 receptor and promotes constitutive internalization of the receptor so as to inhibit the pathological activation of its downstream signaling but preserve baseline physiological signaling activity. The present study was designed to investigate the role of renal ATRAP in angiotensin II–dependent hypertension. We generated transgenic mice dominantly expressing ATRAP in the renal tubules, including renal distal tubules. The renal ATRAP transgenic mice exhibited no significant change in blood pressure at baseline on normal salt diet. However, in the renal ATRAP transgenic mice compared with wild-type mice, the following took place: (1) the development of high blood pressure in response to angiotensin II infusion was significantly suppressed based on radiotelemetry, (2) the extent of daily positive sodium balance was significantly reduced during angiotensin II infusion in metabolic cage analysis, and (3) the renal Na^+-Cl^- cotransporter activation and α-subunit of the epithelial sodium channel induction by angiotensin II infusion were inhibited. Furthermore, adenoviral overexpression of ATRAP suppressed the angiotensin II–mediated increase in the expression of α-subunit of the epithelial sodium channel in mouse distal convoluted tubule cells. These results indicate that renal tubule–dominant ATRAP activation provokes no evident effects on blood pressure at baseline but exerts an inhibitory effect on the pathological elevation of blood pressure in response to angiotensin II stimulation, thereby suggesting that ATRAP is a potential target of interest in blood pressure modulation under pathological conditions. (***Hypertension.* 2013;61:1203-1210.**) • Online Data Supplement

Key Words: angiotensin II ■ angiotensin receptors ■ basic science ■ gene expression/regulation ■ hypertension (kidney) ■ membrane transport/ion channels ■ receptors

Activation of angiotensin II (Ang II) type 1 receptor (AT_1R) through the tissue renin–angiotensin system plays a pivotal role in the pathogenesis of hypertension and associated end-organ injury. In addition, the activation of renal AT_1R signaling plays a key role in the altered renal sodium handling, which occurs in angiotensin-dependent hypertension.[1–3] This is consistent with Guyton's hypothesis that defective handling of sodium by the kidney with a consequent dysregulation of body fluid volume is the requisite final common pathway in the pathogenesis of hypertension.[4] The carboxyl (C)-terminal domain of AT_1R is involved in the control of AT_1R internalization independent of G protein coupling.[5,6] It plays an important role in linking receptor-mediated signal transduction with the specific biological response to Ang II. The AT_1R-associated protein (ATRAP/*Agtrap*) has been identified as the specific binding protein of the C-terminal domain of AT_1R.[7,8] ATRAP is expressed in many tissues, including the kidney, as is AT_1R. Our preceding studies suggest that ATRAP selectively suppresses Ang II–mediated pathological activation of AT_1R signaling in cardiovascular cells, and that cardiac ATRAP enhancement ameliorates cardiac hypertrophy in chronic Ang II–infused mice without affecting baseline cardiovascular function including blood pressure (BP).[9–13]

With respect to the intrarenal distribution of ATRAP, its protein was found to be widely expressed along the renal tubules,

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with a weak level in the vascular smooth muscle cells of the vasculature, including the interlobular arteries, Bowman capsule, podocytes, and mesangial cells in the glomerulus.[13,14] However, despite there being abundant kidney ATRAP expression and that various pathological stimuli, including Ang II, are reported to downregulate renal ATRAP expression, little is known about actual function of renal ATRAP.[15,16] The present study was designed to obtain in vivo evidence of renal ATRAP, with a special focus on Ang II–dependent hypertension by using transgenic (Tg) mice with a pattern of kidney-dominant ATRAP overexpression.

Materials and Methods

This study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal studies were reviewed and approved by the Animal Studies Committee of Yokohama City University. Methods are described in detail in the online-only Data Supplement.

Results

Generation of Renal ATRAP Tg Mice

We generated Tg mice with a pattern of kidney-dominant overexpression of ATRAP (Figure S1B and S1C in the online-only Data Supplement). One of 6 lines of ATRAP Tg mice exhibited renal overexpression of the transgene hemagglutinin-tagged mouse ATRAP (HA-ATRAP) in comparison with wild-type (Wt) littermate mice (Figure 1B and Figure S1C) with scant levels of the transgene HA-ATRAP protein in the other tissues examined (Figure 1A). As shown in Figure 1B, the level of renal total ATRAP protein expression detected by the anti-ATRAP antibody was ≈10-fold higher in Tg mice (endogenous ATRAP and transgene HA-ATRAP) than in Wt mice (endogenous ATRAP).

To determine the expression and distribution of the ATRAP protein in the kidney of Tg mice, we performed an immunohistochemical examination using anti-HA antibody, anti-ATRAP antibody, and antibodies to specific nephron markers (Figure 1C). Although the renal expression of the HA-ATRAP protein prevailed over the endogenous ATRAP protein, histological analysis revealed a similar intrarenal distribution of HA immunostaining (transgene HA-ATRAP protein detected by the anti-HA antibody) and ATRAP immunostaining (transgene HA-ATRAP and endogenous ATRAP proteins detected by the anti-ATRAP antibody), mainly in the cortex.

We next stained consecutive sections with markers specific to the tubular segments. We used a polyclonal antibody against aquaporin-2, which is specifically expressed in the collecting ducts; a monoclonal antibody against calbindin-D, a calcium-binding protein expressed primarily in the distal convoluted tubules (DCT) and connecting tubules; and a monoclonal antibody against megalin, which is specifically expressed in the proximal convoluted tubules; and found that a high level of ATRAP immunostaining was predominantly detected along the renal distal tubules from the DCT to connecting tubules in the renal cortex. As shown in Figure 1D, the distal tubule–dominant expression of HA-ATRAP transgene was quantified by a laser capture microdissection method. The ATRAP mRNA expression in the distal tubules of renal cortex was ≈33.7-fold higher in Tg mice than in Wt mice. However, the ATRAP mRNA expression in the proximal tubules of renal cortex was only 3.5-fold higher in Tg mice than in Wt mice.

Suppression of Ang II–Dependent Hypertension in Renal ATRAP Tg Mice

The baseline 24-hour mean systolic BP (SBP), measured by a radiotelemetry method, was comparable between Wt and Tg mice (male, 14–18 weeks of age; 126±2 versus 122±1 mm Hg, unpaired t test; P=0.12; Figure 2A and 2C). However, the SBP elevation by Ang II infusion (1000 ng/kg per min) was significantly suppressed in Tg mice compared with Wt mice (Figure 2A; 2-way repeated measures ANOVA F=7.476; P=0.0257; Figure 2B; unpaired t test; P=0.0023). We also examined the effect of a higher dose of Ang II infusion (2000 ng/kg per min) on the BP of Wt and Tg mice, and the difference in the Ang II-induced SBP elevation between Wt and Tg mice was more prominent at the higher dose (2000 ng/kg per min) of Ang II (Figure 2C; 2-way repeated measures ANOVA F=9.035; P=0.0012) (Figure 2D; unpaired t test; P=0.0017).

Increase in Urinary Sodium Excretion in Renal ATRAP Tg Mice

We hypothesized that renal enhancement of ATRAP might suppress angiotensin-dependent hypertension by influencing the handling of renal sodium and performed metabolic cage analysis (Figure S2A–S2D). Because urinary sodium excretion was significantly increased in Tg mice compared with Wt mice during the infusion period (Figure S2D; 2-way repeated measures ANOVA F=12.91; P=0.0029), we analyzed daily sodium balance during Ang II infusion and cumulative sodium balance during the early phase (day 1–6) of Ang II infusion to more exactly compare the status of renal sodium handling between Tg and Wt mice.

As shown in Figure 3A, although sodium balance was comparable in Tg and Wt mice at baseline, the extent of daily positive sodium balance was significantly reduced in Tg mice compared with Wt mice during Ang II infusion (2-way repeated measures ANOVA F=11.37; P=0.0046). Furthermore, the extent of cumulative positive sodium balance during the early phase (day 1–6) was also significantly decreased in Tg mice compared with Wt mice (Figure 3B; 2-way repeated measures ANOVA F=7.04; P=0.043) consistently with facilitated natriuresis as a mechanism for the resistance to hypertension in Tg mice.

With respect to the role of increased natriuresis during the later phase (day 7–9) in the lower BP in Tg mice (Figure 3A and Figure S2D), the difference in SBP between Tg and Wt mice became larger from day 8 to day 11 (Figure 2C; the SBP difference between Tg and Wt mice, 17 mm Hg on day 8 and 31 mm Hg on day 11), which also is consistent with facilitated natriuresis as the mechanism for the resistance to hypertension in Tg mice. However, body weight changes tended to be larger in Tg mice than Wt mice, but the differences did not reach statistical significance (Figure S2E). Accordingly, these results indicate that renal distal tubule–dominant overexpression of ATRAP suppressed Ang II–dependent hypertension, probably via a suppression of sodium reabsorption in vivo.

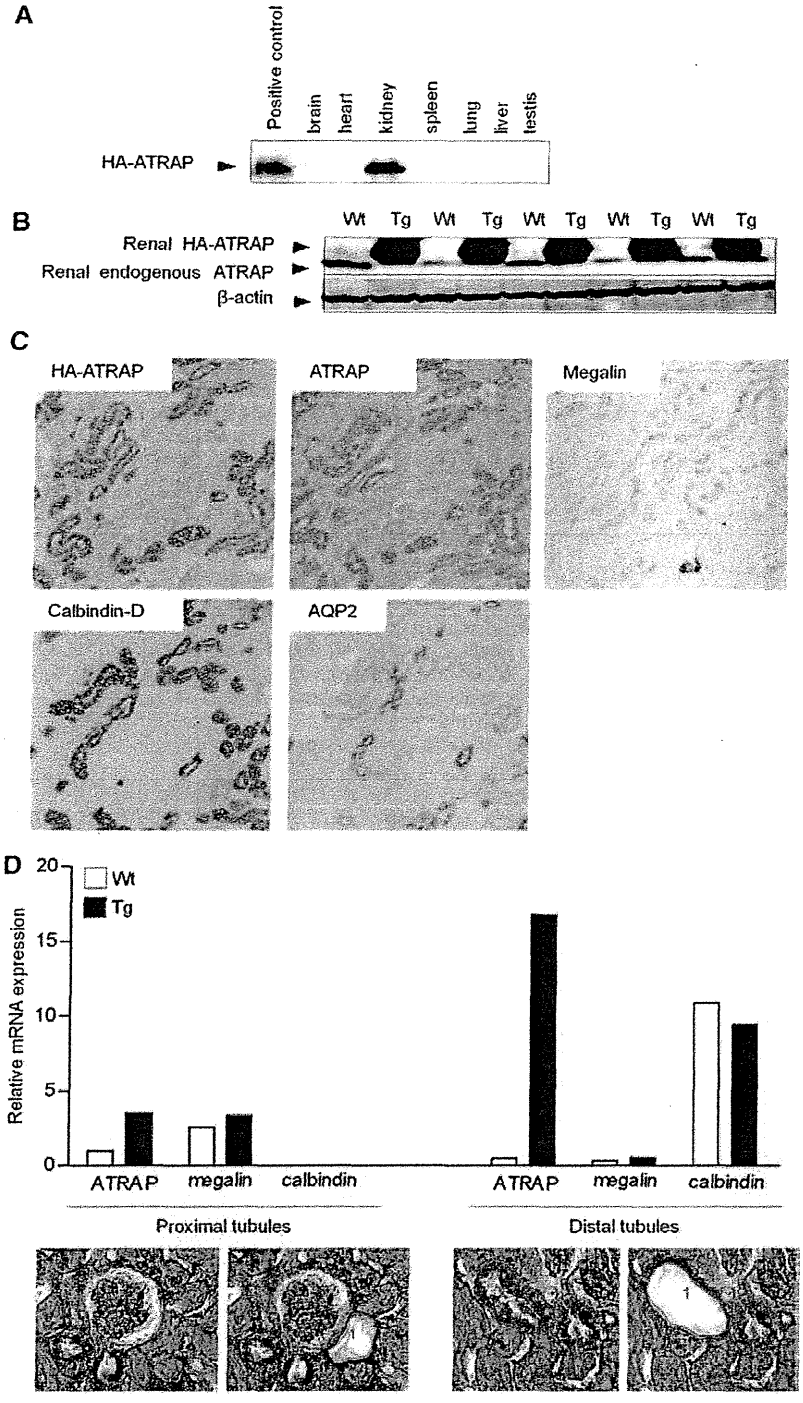

Figure 1. Expression and localization of the hemagglutinin angiotensin II type 1 receptor-associated protein (HA-ATRAP) transgene in renal ATRAP transgenic (Tg) mice. **A**, Representative Western blot analysis of the HA-ATRAP transgene with polyclonal anti-HA antibody. **B**, Representative Western blot analysis of the total ATRAP protein expression in the kidney of wild-type (Wt) and Tg mice. **C**, Renal cortical section showing expression of the HA-ATRAP transgene in renal tubules detected by anti-HA antibody (**top left**). Consecutive sections showing total ATRAP protein expression (transgene HA-ATRAP and endogenous ATRAP) detected by anti-ATRAP antibody (**top center**). Consecutive sections were also stained with a monoclonal antibody against megalin (**top right**), a specific marker of proximal tubules, a monoclonal antibody against calbindin-D (**bottom left**), a specific marker of distal convoluted tubules (DCT) and connecting tubules (CNT), and a polyclonal antibody against aquaporin-2 (AQP2; **bottom right**), a specific marker of collecting ducts. Original magnification, ×100. **D**, Representative image of a hematoxylin/eosin-stained section of the proximal (**bottom left**) and distal (**bottom right**) tubules in the renal cortex before and after laser microdissection. Original magnification, ×400. Quantitative analysis (**top**) of mRNA expression in the proximal and distal tubules of the renal cortex. Values are calculated relative to those obtained for ATRAP mRNA expression in extracts from proximal tubules of Wt mice and are expressed as the mean (n=4 in each group).

Suppression of Phosphorylated Na^+–Cl^- Cotransporter and α-Subunit of the Epithelial Sodium Channel Expression in the Kidneys of Tg Mice

To examine mechanisms involved in the suppression of sodium reabsorption in response to Ang II in Tg mice, we compared renal mRNA expression of the major sodium transporters (sodium-proton antiporter 3, NHE3; sodium-potassium-two-chloride cotransporter, NKCC2; Na^+–Cl^- cotransporter, NCC; and epithelial sodium channel, ENaC subunits). Age-matched Wt and Tg mice were divided into 4 groups: (1) vehicle-infused Wt mice, (2) Ang II–infused Wt mice, (3) vehicle-infused Tg mice, and (4) Ang II–infused Tg mice. The results of quantitative real time–polymerase chain reaction analysis showed that Ang II infusion for 11 days significantly increased the renal mRNA levels of αENaC by 2.3-fold, and the βENaC and γENaC mRNA levels also tended to increase in response to Ang II infusion, but without statistical significance in Wt mice (Figure S3). On the contrary, the Ang II–mediated upregulation of αENaC mRNA was significantly suppressed in Tg mice.

With respect to protein expression of sodium transporters, the renal NHE3 protein levels were similar in Tg and Wt mice at baseline and decreased to a similar degree after Ang II infusion (Figure 4A). The phosphorylated NKCC2 levels were similar

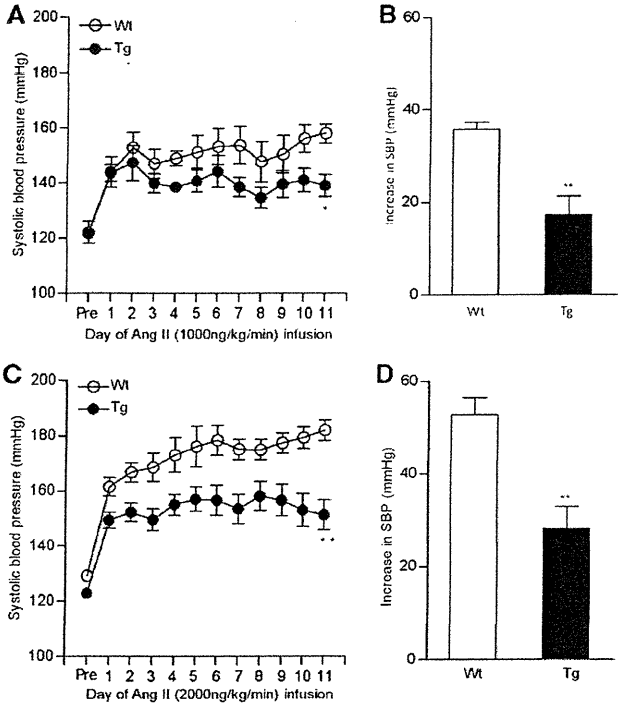

Figure 2. Effects of angiotensin II (Ang II) infusion on blood pressure (BP) analyzed by the radiotelemetric method in wild-type (Wt) and renal angiotensin II type 1 receptor-associated protein transgenic (Tg) mice. **A**, Daily and 24-hour systolic BP (SBP) in Wt and Tg mice before (pre) and during 11 days of Ang II (1000 ng/kg per min) infusion. Values are expressed as the mean±SE (n=5 in each group), **P*<0.05 vs Wt mice. **B**, The increase in SBP during Ang II (1000 ng/kg per min) infusion was significantly less in Tg mice (17±4 mm Hg) compared with Wt mice (36±2 mm Hg). Values are expressed as the mean±SE (n=5 in each group), ***P*<0.01 vs Wt mice. **C**, Daily and 24-hour SBP in Wt and Tg mice before (pre) and during 11 days of Ang II (2000 ng/kg per min) infusion. Values are expressed as the mean±SE (n=6–7 in each group), ***P*<0.01 vs Wt mice. **D**, The increase in SBP during the Ang II (2000 ng/kg per min) infusion was significantly less in Tg mice (28±5 mm Hg) compared with Wt mice (53±4 mm Hg). Values are expressed as the mean±SE (n=6–7 in each group), ***P*<0.01 vs Wt mice.

in Tg and Wt mice at baseline and decreased in both groups by Ang II with a tendency to be lower in Tg mice than in Wt mice but without statistical significance (30±6 versus 46±6%, *P*=0.086; Figure 4B). However, although expression of phosphorylated NCC, which is the activated form of NCC and plays an important role in sodium reabsorption, was increased by Ang II infusion by 2.2-fold in Wt mice, the Ang II–mediated induction of phosphorylated NCC was significantly suppressed in Tg mice (Figure 4C). Furthermore, the Ang II–mediated increase in the renal αENaC protein expression, which was observed in Wt mice (1.9-fold), was abolished in Tg mice (Figure 4D).

To further examine whether the cellular localization of αENaC at the apical membrane is altered in Tg mice, confocal microscopy analysis using anti-αENaC antibody was performed. Under baseline conditions, both Wt and Tg mice exhibited a similar αENaC immunostaining pattern in renal cortex, with a denser staining at the apical membrane of distal tubule cells (Figure 5A). Ang II infusion did not obviously affect the cellular distribution of αENaC immunostaining in either Wt or Tg mice but strongly enhanced αENaC immunostaining intensity only in Wt mice. These findings suggest that the inhibitory effect of distal tubule ATRAP on sodium reabsorption in response to Ang II is not caused by suppressed localization of αENaC to the apical membrane but rather is mediated through downregulation of αENaC expression.

ENaC is activated by aldosterone through its binding to the mineralocorticoid receptor. Therefore, to analyze the direct effect of ATRAP on αENaC, we examined whether overexpression of ATRAP would suppress the Ang II–mediated ENaC subunit expression in mouse DCT cells by performing adenoviral transfer of recombinant ATRAP. Although Ang II (10^{-6} mol/L) treatment of mouse DCT cells infected with control bacterial β-galactosidase cDNA (Ad. LacZ) increased the αENaC mRNA expression, mouse DCT cells infected with adenoviral vector containing ATRAP cDNA (Ad.HA-ATRAP) exhibited an inhibition of the Ang II-induced enhancement (Figure 5B), thereby indicating that ATRAP directly suppressed the Ang II–mediated activation of αENaC expression, independent of the aldosterone-mineralocorticoid receptor pathway.

Discussion

This is the first report, to the best of our knowledge, of an inhibitory function of renal tubular ATRAP in angiotensin-dependent hypertension without an influence on baseline BP. In this study, chronic Ang II infusion was performed at 1000 and 2000 ng/kg per min to examine the effects of distal tubule–dominant overexpression of ATRAP on the Ang II–mediated BP increase. Although the higher dose of Ang II (2000 ng/kg per min) is reported to provoke a reduction in food intake and to cause Ang II–induced wasting and skeletal muscle atrophy,[17] the lower dose of Ang II (1000 ng//kg per min) has been used in many previously performed experiments in mice,[18,19] and suppression of the Ang II–induced BP increase by the distal tubule–dominant overexpression of ATRAP was observed with both the lower and higher doses of Ang II in the present study.

In the present study, the BP at baseline was not affected by renal ATRAP overexpression. On the contrary, the genetic inactivation of other renin–angiotensin system components, such as angiotensinogen, renin, and AT_1R, was reported to result in significant decreases in BP, as well as an alteration in renal morphology and function compared with Wt mice even under baseline conditions.[20,21] Thus, ATRAP would be expected to act as a minor player among the renin–angiotensin system components, at least in terms of BP regulation and renal morphological development under physiological conditions. However, the results of present study seem to be consistent with those of our previous studies, which showed that ATRAP is not a general inhibitor of the AT_1R signaling as are the clinically available AT_1R-specific blockers, but rather specifically inhibits the pathological activation of its downstream signaling with preservation of baseline physiological signaling activity.[9–13]

With regard to the regulatory role of renal tubule AT_1R in renal sodium handling, a previous study reported that Ang II did not affect proximal tubule fluid reabsorption or sodium delivery to distal nephron segments, but sodium reabsorption in distal nephron segments was increased in Ang II–infused