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厚生労働科学研究費補助金研究報告書表紙

厚生労働科学研究費補助金

循環器疾患・糖尿病等生活習慣病対策総合研究事業

肥満を伴う高血圧症に対する防風通聖散の併用投与による，24時間自由行動下血圧及び糖脂質代謝・酸化ストレスの改善効果についての研究

（ H２２ － 循環器等（生習）－ 一般 － ００９ ）

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研究代表者　田村　功一

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厚生労働科学研究費補助金研究報告書目次

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厚生労働科学研究費補助金（循環器疾患・糖尿病等生活習慣病対策総合研究事業）
（総括）研究報告書

肥満を伴う高血圧症に対する防風通聖散の併用投与による，24時間自由行動下血圧及び糖脂質代謝・酸化ストレスの改善効果についての研究

研究代表者　田村　功一　横浜市立大学医学部循環器・腎臓内科学　准教授

研究要旨

肥満合併高血圧は動脈硬化を促進し心血管病・腎不全の根源となるため，高齢化を迎えつつある国民の健康レベルのさらなる向上のため，肥満合併高血圧への集学的治療による効率的な医療が必要である．

本研究では，肥満合併高血圧に対する集学的治療を施行する手段として，西洋学的治療介入に加えて漢方薬を用いた東洋医学的治療介入の併用による治療効果の向上を検証することを目的としている．方法としては，横浜市立大学附属病院と地域協力病院，開業医院との多施設共同研究として，試験対象選択基準を満たし文書同意が得られた肥満合併高血圧患者(目標症例数合計200症例)を無作為に2群に割り付け，防風通聖散併用投与群では，食事・運動療法及びレニン･アンジオテンシン阻害薬などを用いた西洋医学的治療介入に加えて東洋医学的治療介入手段である抗肥満漢方薬の防風通聖散を6ヶ月間併用投与する．また，通常治療継続群では，西洋医学的治療介入を同期間行う．

防風通聖散の併用投与による降圧の改善効果の評価にあたっては，全例において 24 時由行動下血圧測定を行うことにより臓器合併症予後を正確に反映する 24 時間血圧・血圧日動の解析が可能である．さらに研究代表者らが予後との関連性を明らかにした 24 時間自由下血圧測定で得られる新規指標（血圧短期変動性など）に着目した解析も行うために，防風散の併用投与による肥満合併高血圧に対する治療向上効果について独創性の高い研究成果られると考えられる．

研究分担者氏名・所属研究機関名及び所属研究機関における職名

梅村　敏・横浜市立大学・教授

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A. 研究目的

本研究では，重篤な心血管病の根源である肥満合併高血圧に対して，西洋医学的治療介入に加えて東洋医学的治療介入手段である抗肥満漢方薬の防風通聖散併用投与による治療効果向上について，24 時間自由行動下血圧測定で得られる降圧指標，糖脂質代謝，及び酸化ストレスに着目して検討する．

【研究の必要性及び特色・独創的な点】

肥満と高血圧は相互に密接な関係があり，内臓脂肪型肥満の増悪に伴って臓器合併症が増加する．よって肥満合併高血圧の治療の目的は，食事・運動療法とともに降圧目標値までの確実な降圧と内臓脂肪型肥満の効率的な改善により，心血管病や腎不全を抑制することである．また RAS の生理活性物質アンジオテンシン II(Ang II)は主要な受容体の 1 型 Ang II 受容体に作用して情報伝達系を活性化し，メタボリック症候群や心血管病の発症・進展を促進するため，メタボリック症候群合併高血圧患者に対しては高血圧治療ガイドライン(JSH2009)では RAS 阻害薬が第 1 選択薬である．しかし RAS 阻害薬単独投与では目標血圧までの確実な降圧が困難な場合が多く併用療法が必要となる．

一方漢方薬は多種生薬の混合製剤でありその薬効や作用機序は不確定な面が残り臨床試験成績も不十分なため，JSH2009 においても治療薬として推奨されるに至っていない．よって，肥満合併高血圧に対する西洋医学と東洋医学を融合させたさらなる効率的な治療を目指すために漢方薬を併用する根拠となる臨床効果の検討が必要である．

現在までに研究代表者らは 24 時間自由行動下血圧測定(ABPM)での平均血圧や血圧日内変動に加えて新規指標の基底血圧や血圧短期変動性と心血管病の合併及び降圧薬の臓器保護作用との関連性について報告している(Clin Exp Hypertens 2005,2007,2008,2009; Nephron Clin Pract 2009; Hypertens Res 2009; Atherosclerosis 2009)．本研究では防風通聖散の併用降圧効果について，全例に ABPM を施行し，従来の

24時間血圧・血圧日内変動データに加えて基底血圧・短期血圧変動性などの臓器合併症予後と関連したABPM上の新規指標を含めた多面的な検討が可能である.

B. 研究方法

本研究は，多施設共同研究として，研究代表者の田村及び研究分担者の梅村が所属する横浜市立大学附属病院が中心となって，横浜市立大学附属市民総合医療センター病院，医療連携関連の4つの地域中核病院，および横浜内科医会会長を務めABPMを用いた研究実績のある開業医が参加して行われる.

対象は，上記の各参加施設に通院中であり，試験開始前4週間以上の観察期間中に食事・運動療法とともにRAS阻害薬などによる降圧治療が行われている本態性高血圧患者(男女を問わず)とし，以下の条件を満たした者とする.

対象選択基準は，1．試験開始時の年齢が20歳以上，80歳未満，2．肥満度(BMI) 25 kg/m2以上，3．本研究の参加への文書同意，である.

除外基準は，1．20歳未満，あるいは80歳以上の患者，2．妊娠あるいは授乳している患者，妊娠している可能性のある患者，3．二次性高血圧，悪性高血圧，あるいは重症(III度)高血圧(収縮期血圧180mmHg以上，あるいは拡張期血圧110mmHg以上) の患者，4．その他，担当医師が研究対象として不適当と判断した患者，である.

本研究の参加に同意し，対象選択基準に合致した患者について，観察期間中に無作為に防風通聖散併用治療群あるいは通常治療継続群の2群への割り付けをおこなう．防風通聖散併用治療群では防風通聖散(2.5 g/日から開始し，食前又は食間に経口服用する．なお，症状により適宜増減し，最高用量は7.5 g/日)を6ヶ月間(24週間)投与し，防風通聖散による治療効果について通常降圧治療継続群を対照とした比較調査により検討する.両群ともに同様にJSH2009記載の降圧目標までの降圧を図る.

本研究は3年計画であり，平成22年度は主に対象患者の登録及び評価項目測定を行った．平成22-24年度の全研究実施期間における目標症例数は横浜市立大学附属病院80例，各参加病院・医院20例の合計200症例であり，主要評価項目は24時間自由行動下血圧測定(ABPM；試験開始時，3，6ヶ月後の計3回施行)による降圧効果(平均血圧，血圧日内変動，基底血圧，短期血圧変動性)，副次的評価項目は，BMI，腹囲，診察室血圧，糖代謝(空腹時血糖，HbA1c，HOMA-IR，血中インスリン，アディポネクチン，レジスチン濃度)，脂質代謝(TCHO, LDLC, HDLC, TG)，酸化ストレス(血中MDA-LDL, 血中ペントシジン, 尿中L-FABP濃度)，腎内RAS活性(尿中アンジオテンシノーゲン濃度)，腎機能(尿中アルブミン排泄量，尿蛋白量，推算GFR)，動脈硬化検査(血管脈波検査)，心血管病合併の有無，及び有害事象である.

本研究は，研究代表者及び研究分担者が所属しまたABPMを用いた臨床研究について実績がある横浜市立大学附属病院腎臓高血圧内科を中心とした多施設研究として遂行されているものである.

研究実施にあたっては，ABPM機器を各施設に設置することにより対象患者の登録及びABPMを含む血圧測定や検体収集は，各参加施設において行っている．しかし保険適応外の評価項目については，測定精度を上げるために各参加施設からの検体を横浜市立大学の研究室に集めて一括して（株）エスアールエルの検査室に測定を委託するとともに横浜市立大学の研究室において測定を行う．また，研究分担者の横浜市立大学附属病院長兼務の梅村が中心となって測定結果の解析を行い，同じく研究分担者の腎臓高血圧内科部長戸谷が中心となって各参加施設の腎臓高血圧内科部長との間で定期的に本研究の状況について協議するなど連絡を密にして効率的に研究を遂行している．さらに本研究実施にあたり横浜市立大学先端医科学研究センターの臨床研究支援部門の臨床・疫学研究推進室と随時連絡・協議をおこなっている.

（倫理面への配慮）

本研究は臨床研究に関する倫理指針を遵守して行われ，本研究への参加に先立ち，研究対象者である患者に対して，用いる治療薬に起こりうる副作用などを含めて十分な説明を行い，患者の自由意思による文書同意を取得する．また，患者の名前や病名等プラーバシーに関する秘密は固く守られるように細心の注意を払う.

試験薬はいずれも高血圧あるいは肥満症に対する治療薬として承認を取得しており，その承認用量範囲で使用し，また，高血圧に対する併用薬の投与は可能となっている．さらに用いる治療薬の投与禁忌や慎重投与条件を正確に把握するとともに，副作用等が認められた場合は医師が適切な治療を行う.

主要検査項目としては，2008年4月に保険適応となった携帯型自由行動下自動血圧計を用いた24時間自由行動下血圧測定(ABPM)を試験開始時，3ヶ月後，及び6ヶ月後の計3回行うことにより，非侵襲的に24時間血圧，血圧日内変動，基底血圧，及び血圧短期変動性を評価する．また，尿・血液検査及び動脈硬化検査の回数は，一般診療における検査頻度に基づき，3ヶ月(12週)間隔で合計3回行うこととしている．同様に3ヶ月(12週)間隔で合計3回行われる保険適応外の糖代謝関連指標検査(血中アディポネクチン，レジスチン濃度測定)，腎内RAS活性検査(尿中アンジオテンシノーゲン濃度測定)および酸化ストレス関連検査(血中MDA-LDL，血中ペントシジン，尿中L-FABP濃度)についての検査費用は研究費により行われている.

なお本研究計画については，横浜市立大学および各参加施設の倫理委員会に研究計画を申請して承認を得た.

C. 研究結果

本研究では，横浜市立大学附属病院と地域協力病院，開業医院との多施設共同研究として，試験対象選択基準を満たし文書同意が得られた肥満合併高血圧患者(目標症例数合計200症例)を無作為に2群に割り付け，防風通聖散併用投与群では，食事・運動療法及びレニン-アンジオテンシン系(RAS)阻害薬などを用いた西洋医学的治療介入に加えて東洋医学的治療介入手段である抗肥満漢方薬の防風通聖散を6ヶ月間併用投与する．また，通常治療継続群では，西洋医学的治療介入を同期間行う.

本研究は3年計画であり，各患者について6ヶ月間の介入試験期間をともなうものであるために，平成22年度は主に対象患者の登録及び評価項目測定を行うこととした．そこで，平成22年度は本計画の最終的な試

験計画について横浜市立大学医学部倫理委員会において承認を得た後，各参加予定施設での参加医師および倫理委員会に対して順次本研究計画について説明および申請をおこない承認を取得できたため，参加患者の登録を開始している．

近年 2 型糖尿病患者数が増加し，腎症や心血管合併症が主たる死因である．そのため 2 型糖尿病患者における腎症や心血管合併症の発症機序の解明は重要である．一方，早期腎症までの 2 型糖尿病患者に対する集約的治療が顕性腎症への移行や心血管合併症を抑制できることが報告されている．そこで，本研究のパイロット試験として，高血圧合併顕性腎症 2 型糖尿病患者に対して西洋医学的介入による血圧，糖・脂質代謝における集約的治療を 12 ヶ月間にわたって行い，薬物を含む西洋医学的集約的治療により，血圧日内変動と腎症の改善が可能かについて検討した．対象の 20 名（男 16 名，女 4 名）の高血圧を合併した顕性腎症 2 型糖尿病患者に対して ARB (valsartan)，statin (fluvastatin)投与を含む血圧，脂質・糖代謝への集約的治療を 12 ヶ月間にわたって行い，ABPM および腎機能（eGFR，UACR）に与える影響について検討した．

その結果，西洋医学的集約的治療介入開始 12 ヶ月後には開始前に比べて診察室血圧と糖・脂質代謝の有意な改善がみられた（収縮期血圧，130±2 vs 150 ±1 mmHg；拡張期血圧，76±1 vs 86±1 mmHg；空腹時血糖，117±5 vs 153±7 mg/dl；LDL-C，116±8 vs 162±5 mg/dl，P<0.0001）．また，ABPM では，介入開始 12 ヶ月後において昼間・夜間の平均血圧および血圧短期変動性の有意な改善が認められた．さらに，腎機能では，介入開始 12 ヶ月後では開始前に比較して，推算糸球体濾過量（eGFR）は不変であったが（43.1 vs 44.3 ml/min/1.73 m2，NS），尿中アルブミン排泄率（UACR）に関しては有意な減少が認められた(1228 vs 2340 mg/g-cr，P<0.05)．したがって，高血圧を合併した顕性腎症 2 型糖尿病患者に西洋医学的介入による集約的治療を行うことにより血圧日内変動の改善，eGFR の維持，および尿蛋白量の減少を期待できる可能性が明らかにされた．以上の研究成果について第 33 回日本高血圧学会総会（福岡），第 23 回国際高血圧学会（バンクーバー）において発表し，英文学術誌に掲載受理された．

D. 考察

肥満合併高血圧患者数は増加傾向にあり，合併する生活習慣病管理の面からも大きな問題となっている．また，本邦での大規模コホート研究，あるいは海外での複数のコホート研究を対象とした大規模メタ解析において，肥満による心血管病発症の促進による死亡率の増加が報告されている．したがって，肥満合併高血圧に対する包括的で効率的な治療は，心血管病の発症予防に結びつくと考えられる．高血圧における血圧管理では，診察室血圧測定のみならず家庭血圧測定や 24 時間自由行動下血圧測定（ABPM）を行うことがガイドラインにおいて推奨される．特に ABPM では血圧日内変動の詳細な評価が可能である．ABPM で評価可能な血圧日内変動関連指標のうち長時間周期の変動指標としては夜間血圧下降度（dipper，non-dipper）や早朝の血圧上昇の程度（morning surge）等があり，短時間周期の変動指標としては血圧短期変動性が挙げられる．合併症のある高血圧の病態では長時間周期の血圧変動関連指標の意義に関する研究が先行しており，例えば慢性腎臓病患者では血圧日内変動上の特徴として，夜間降圧がみられない non-dipper 型となることが多く，夜間血圧は微量アルブミン尿ー蛋白尿の程度と相関し，non-dipper 型高血圧は慢性腎臓病および心腎連関を進展させることが明らかにされている．そのため慢性腎臓病患者の血圧管理においては，末期腎不全・心腎連関の抑制のために，早朝血圧や夜間血圧も評価すべきであり，診察室血圧に加えて家庭血圧や ABPM により血圧日内変動を評価することが望ましいとされている．

一方，ABPM で評価可能な短時間周期の血圧変動関連指標である血圧短期変動性の高血圧治療における意義については未解明な部分も多く今後の詳細な検討が必要な状況である．我々は，血圧短期変動性に着目して，ABPM で測定される血圧の標準偏差(SD)あるいは変動係数(CV)を血圧短期変動性の指標とし，合併症をもつ高血圧患者における病態生理学的意義について，横断的研究，前向き研究，および介入研究による検討を行っている．例えば，慢性腎臓病合併高血圧患者を対象とした横断的研究では，慢性腎臓病合併高血圧患者において血圧短期変動性が増加していること，血中コレステロール値やノルエピネフリン濃度が血圧短期変動性と関連すること，あるいは冠動脈疾患を合併した慢性腎臓病合併高血圧患者では血圧短期変動性のさらなる増加がみられることを報告している．また，慢性腎臓病合併高血圧患者を対象とした介入研究では，降圧薬による降圧作用や長時間周期の血圧日内変動指標への作用に加えて血圧短期変動性への作用が臓器保護作用に影響を与える可能性があることを明らかにしている．これらの結果は，ABPM での血圧短期変動性が合併症をもつ高血圧における動脈硬化・臓器障害の病態を反映し合併症をもつ高血圧での血圧管理における治療標的のひとつとなりうる可能性を提唱していると考えられる．

E. 結論

本研究の結果，防風通聖散による併用治療が肥満合併高血圧患者に対して 24 時間自由行動下血圧(平均血圧，血圧日内変動，基底血圧，短期血圧変動性)改善作用，糖脂質代謝改善作用，及び酸化ストレス抑制作用を発揮することが西洋医学的に明らかになると予想される．その結果，肥満合併高血圧に対する防風通聖散を用いた東洋医学的治療介入の西洋医学的意義が確立され，肥満合併高血圧に対する集学的治療法における有力な選択肢として防風通聖散による併用療法が今後の高血圧治療ガイドラインにも採用されると期待される．

F. 健康危険情報

特になし．

G. 研究発表

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H. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
なし

2. 実用新案登録
なし

3. その他
なし

研究成果の刊行に関する一覧表

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出島　徹, 涌井広道, 増田真一朗, 田村功一, 梅村敏.	特集：腎とレニン・アンジオテンシン・アルドステロン系. 腎内アンジオテンシンⅡ受容体の分布と機能—AT1, AT2, ATRAP—.	日腎会誌	52 (2)	101-105	2010
岡野泰子, 戸谷義幸, 東　公一, 矢花眞知子, 田村功一, 梅村　敏.	腎障害を合併した高血圧患者における ARB／利尿薬合剤の有用性—基底血圧および自律神経活動と Health-Related QOL の変化について—.	日腎会誌	52 (7)	939-944	2010

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Intrarenal suppression of angiotensin II type 1 receptor binding molecule in angiotensin II-infused mice

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Wakui H, Tamura K, Matsuda M, Bai Y, Dejima T, Shigenaga A, Masuda S, Azuma K, Maeda A, Hirose T, Ishigami T, Toya Y, Yabana M, Minamisawa S, Umemura S. Intrarenal suppression of angiotensin II type 1 receptor binding molecule in angiotensin II-infused mice. *Am J Physiol Renal Physiol* 299: F991–F1003, 2010. First published August 25, 2010; doi:10.1152/ajprenal.00738.2009.—ATRAP [ANG II type 1 receptor (AT1R)-associated protein] is a molecule which directly interacts with AT1R and inhibits AT1R signaling. The aim of this study was to examine the effects of continuous ANG II infusion on the intrarenal expression and distribution of ATRAP and to determine the role of AT1R signaling in mediating these effects. C57BL/6 male mice were subjected to vehicle or ANG II infusions at doses of 200, 1,000, or 2,500 ng·kg^{-1}·min^{-1} for 14 days. ANG II infusion caused significant suppression of ATRAP expression in the kidney but did not affect ATRAP expression in the testis or liver. Although only the highest ANG II dose (2,500 ng·kg^{-1}·min^{-1}) provoked renal pathological responses, such as an increase in the mRNA expression of angiotensinogen and the α-subunit of the epithelial sodium channel, ANG II-induced decreases in ATRAP were observed even at the lowest dose (200 ng·kg^{-1}·min^{-1}), particularly in the outer medulla of the kidney, based on immunohistochemical staining and Western blot analysis. The decrease in renal ATRAP expression by ANG II infusion was prevented by treatment with the AT1R-specific blocker olmesartan. In addition, the ANG II-mediated decrease in renal ATRAP expression through AT1R signaling occurred without an ANG II-induced decrease in plasma membrane AT1R expression in the kidney. On the other hand, a transgenic model increase in renal ATRAP expression beyond baseline was accompanied by a constitutive reduction of renal plasma membrane AT1R expression and by the promotion of renal AT1R internalization as well as the decreased induction of angiotensinogen gene expression in response to ANG II. These results suggest that the plasma membrane AT1R level in the kidney is modulated by intrarenal ATRAP expression under physiological and pathophysiological conditions in vivo.

gene expression; renin-angiotensin system; angiotensin; receptor; hypertension

EVIDENCE SUGGESTS THAT THE activation of angiotensin II (ANG II) type 1 receptor (AT1R) through the tissue renin-angiotensin system plays a pivotal role in the pathogenesis and associated end-organ injury of hypertension. The carboxyl-terminal portion of AT1R is involved in the control of AT1R internalization independent of G protein coupling and plays an important role in linking receptor-mediated signal transduction to the specific pathophysiological response to ANG II (16, 41). The AT1R-associated protein (ATRAP), which is a molecule specifically interacting with the carboxyl-terminal domain of the AT1R, was cloned using a yeast-two-hybrid screening system (8, 21). The results of previous in vitro studies and ATRAP transgenic mice studies showed that ATRAP suppresses ANG II-mediated pathological responses in cardiovascular cells and tissues by promoting the constitutive internalization of AT1R (1, 7, 11, 30, 40, 44), thereby suggesting ATRAP to be an endogenous inhibitor of AT1R signaling (22, 37).

With respect to the tissue distribution and regulation of ATRAP expression in vivo, ATRAP and AT1R are broadly expressed in many tissues, including the kidney, and there is a tissue-specific regulatory balancing of the expression of ATRAP and AT1R during the development of hypertension in spontaneously hypertensive rats (35). Chronic infusion of ANG II is one of the representative models of hypertension and end-organ damage and is associated with the activation of the intrarenal renin-angiotensin system, including upregulation of renal angiotensinogen through the AT1R pathway (10, 20, 49). Furthermore, previous studies using a series of kidney cross-transplant experiments also showed that the activation of intrarenal AT1R is required for the development of ANG II-dependent hypertension and the related end-organ damage (5, 6). Thus we hypothesized that the intrarenal distribution and regulation of endogenous ATRAP expression may also be involved in the pathophysiological responses to ANG II. Accordingly, studies were performed to examine the changes in intrarenal ATRAP expression during ANG II infusion in mice and to determine the role of AT1R in mediating these responses. Furthermore, we examined whether the plasma membrane AT1R level was influenced by the ANG II-mediated decrease in the renal ATRAP level and/or by an increase in the renal ATRAP level in a transgenic model, to analyze the relationship between ATRAP and AT1R expression in the kidney.

METHODS

Materials. ANG II was purchased from Sigma. The AT1R-specific blocker olmesartan (RNH6270) was kindly supplied by Daiichi-Sankyo Pharmaceuticals (Tokyo, Japan).

Animals and ANG II infusion. Adult male C57BL/6 mice (10–12 wk of age, Oriental Yeast Kogyo) were divided into three groups (n = 6–8 mice/group) for the subcutaneous infusion of vehicle or ANG II (either 200, 1,000, or 2,500 ng·kg^{-1}·min^{-1}) via an osmotic minipump (ALZA) for 14 days. The percentage of body weight increase (% BW increase) was calculated as follows: % BW increase = [(BW at *day 14*) − (BW at baseline) × 100]/(BW at baseline). In several of the experiments, vehicle or olmesartan (10 mg·kg^{-1}·day^{-1}) in the

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drinking water was administrated for the same period. The ANG II and olmesartan dosages were determined from previous reports (10, 18, 48). Following experimental treatment, the mice were anesthetized and the tissues were removed into liquid nitrogen or fixative. The Animal Studies Committee of Yokohama City University approved all the animal experimental protocols.

Blood pressure measurements. Systolic blood pressure and heart rate were measured by the tail-cuff method (BP monitor MK-2000; Muromachi Kikai), as described previously (34, 42). BP monitor MK-2000 made it possible to measure blood pressure without preheating the animals, thus allowing the avoidance of stressful conditions (17).

Analysis of total ATRAP and AT1R protein expression. The characterization and specificity of the anti-mouse ATRAP antibody and the anti-AT1R antibody (sc-1173, Santa Cruz Biotechnology) were described previously (42). Western blot analysis was performed to examine the total protein expression of ATRAP and AT1R as described (40, 42). Briefly, whole tissue extracts were used for SDS-PAGE, and transferred membranes (Millipore) were incubated with either *1*) an anti-ATRAP antibody or *2*) an anti-AT1R antibody and subjected to enhanced chemiluminescence (Amersham Biosciences). The images were analyzed quantitatively using a Fuji LAS3000 Image Analyzer (Fujifilm) for determination of the total ATRAP and AT1R protein levels. To measure the tissue expression ratio of ATRAP to AT1R, each ATRAP protein level was divided by the corresponding total AT1R protein level obtained by reprobing, and thus was derived from the same extract.

Real-time quantitative RT-PCR analysis. Total RNA was extracted from the kidney with ISOGEN (Nippon Gene, Tokyo, Japan), and cDNA was synthesized using the SuperScript III First-Strand System (Invitrogen). Real-time quantitative RT-PCR was performed by incubating the RT product with TaqMan Universal PCR Master Mix and a designed TaqMan probe (Applied Biosystems), essentially as described previously (34). RNA quantity was expressed relative to the 18S rRNA endogenous control.

Immunohistochemistry for ATRAP and AT1R expression. Immunohistochemistry was performed as described previously (14, 42). The kidneys were perfusion-fixed with 4% paraformaldehyde, subsequently embedded in paraffin, and cut into sections of 4-μm thickness. The sections were dewaxed and rehydrated. Antigen retrieval was performed by microwave heating. The sections were treated for 60 min with 10% normal goat serum in phosphate-buffered saline and blocked for endogenous biotin activity using an Avidin/Biotin Blocking kit (Vector Laboratories). For the study of ATRAP and AT1R, the sections were incubated at 4°C overnight with either *1*) an anti-ATRAP antibody diluted at 1:100 or *2*) anti-AT1R antibody diluted at 1:100, as described previously (42). The sections were incubated for 60 min with (a) biotinylated goat anti-rabbit IgG (Nichirei), blocked for endogenous peroxidase activity by incubation with 0.3% H_2O_2 for 20 min, treated for 30 min with streptavidin and biotinylated peroxidase (DAKO), and then exposed to diaminobenzidine. The sections were counterstained with hematoxylin, dehydrated, and mounted. Immunoreactivity was semiquantitatively evaluated in a blinded manner. Briefly, 20 microscopic fields/slide were selected at random for evaluation. Examination was performed using a microscope with ×200 magnification (Olympus) and an integrated digital camera system (Olympus). Image Pro-plus computer image analysis software (Media Cybernetics, Bethesda, MD) was used to analyze the brown stain pixel density and to quantify the protein levels, as described previously (10, 15, 32, 47).

Analysis of plasma membrane AT1R expression. The plasma membrane was specifically extracted from tissues using a Plasma Membrane Extraction Kit (K268–50, Biovision) according to the manufacturer's protocol and then used for SDS-PAGE (43). Membranes (Millipore) were incubated with either *1*) anti-AT1R antibody or *2*) anti-flotillin-2 monoclonal antibody (no. 3436, Cell Signaling Technology) and subjected to enhanced chemiluminescence (Amersham Biosciences). Flotillin-2 is constitutively localized to the plasma membrane and was used as an internal control protein on the plasma membrane (36). The images were analyzed quantitatively using a Fuji LAS3000 Image Analyzer (Fujifilm) for determination of the plasma membrane AT1R protein levels.

Generation of ATRAP transgenic mice. To produce ATRAP transgenic mice, hemagglutinin (HA)-tagged mouse ATRAP cDNA was subcloned into pCAGGS expression vector, which contained a cytomegalovirus enhancer and chicken β-actin (CAG) promoter (28), and the resultant transgene construct was microinjected into the pronuclei of fertilized mouse embryos at the single-cell stage to generate transgenic mice (C57BL/6 strain). The ATRAP transgene positive (+) mice were mated with C57BL/6 wild-type mice to obtain ATRAP transgene positive (+) mice and littermate control mice for the experiments. Animal genotyping was performed as previously described. Transgenic mice were identified by PCR using 5′-TGCTT-

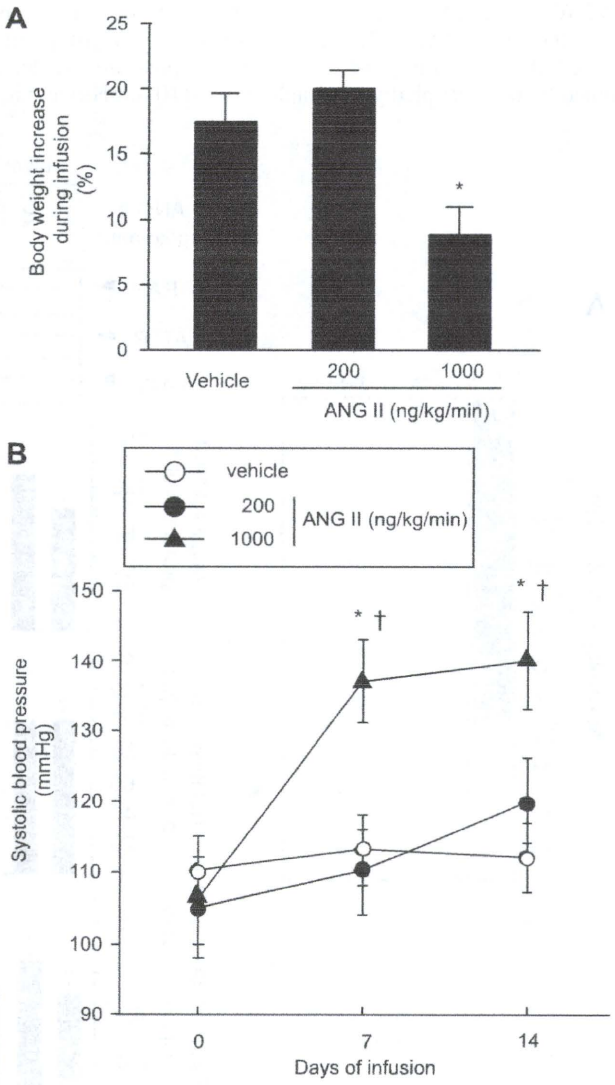

Fig. 1. Effects of continuous ANG II infusion on body weight (*A*) and systolic blood pressure (*B*) during the treatment period. Adult male C57BL/6 mice were divided into 3 groups (n = 6–8 mice/group) for the subcutaneous infusion of vehicle or ANG II (either 200 or 1,000 ng·kg^{-1}·min^{-1}) via an osmotic minipump for 14 days. The values of the percent body weight increase and systolic blood pressure are expressed as means ± SE (n = 6–8/group). *P < 0.05 vs. vehicle. †P < 0.05 vs. *day 0*.

GGGGCAACTTCACTATC-3′ as the forward primer and 5′-ACGGTGCATGTGGTAGACGAG-3′ as the reverse primer.

Statistical analysis. Values are expressed as means ± SE in the text and figures. The data were analyzed using ANOVA. If a statistically significant effect was found, a post hoc analysis with Scheffé's test was performed to detect differences between the groups. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Effects of ANG II on body weight and systolic blood pressure. Vehicle-infused mice gained BW during the study period (%BW increase, 17.2 ± 2.2%, $n = 8$) (Fig. 1*A*). Mice infused at a low dose of ANG II (200 ng·kg^{-1}·min^{-1}) displayed a similar gain in BW (%BW increase, 19.9 ± 1.4%, $n = 6$). In contrast, mice subjected to a high dose of ANG II (1,000 ng·kg^{-1}·min^{-1}) exhibited a significant inhibition of BW gain (%BW increase, 8.7 ± 2.1%, $n = 7$, $P < 0.05$ vs. vehicle and $P < 0.01$ vs. ANG II 200 ng·kg^{-1}·min^{-1}). All groups displayed the same range of systolic blood pressure, as determined by tail-cuff plethysmography (105–110 mmHg) at baseline (Fig. 1*B*). Systolic blood pressure remained stable in the vehicle-infused mice during the study period, with systolic blood pressure averaging 113 ± 6 and 112 ± 6 mmHg by *days 7* and *14*, respectively ($n = 8$). Similarly, systolic blood pressure did not exhibit any evident change in the low-dose ANG II (200 ng·kg^{-1}·min^{-1})-infused mice (110 ± 5 and 120 ± 5 mmHg by *days 7* and *14*, respectively, $n = 6$). In contrast, systolic blood pressure was significantly elevated, to 137 ± 6 and 140 ± 7 mmHg on *days 7* and *14* of ANG II infusion, respectively, in the high-dose ANG II (1,000 ng·kg^{-1}·min^{-1})-infused mice. Thus, in this study, the low dose of ANG II (200 ng·kg^{-1}·min^{-1}) corresponds to a subpressor dose, and the high dose of ANG II (1,000 ng·kg^{-1}·min^{-1}) corresponds to a pressor dose.

Suppression of ATRAP expression by ANG II in the kidney. We previously showed that ATRAP and AT1R are expressed in various mouse tissues, including the kidney, testis, and liver (42). Thus we examined whether continuous ANG II infusion would regulate ATRAP expression in a tissue-specific manner,

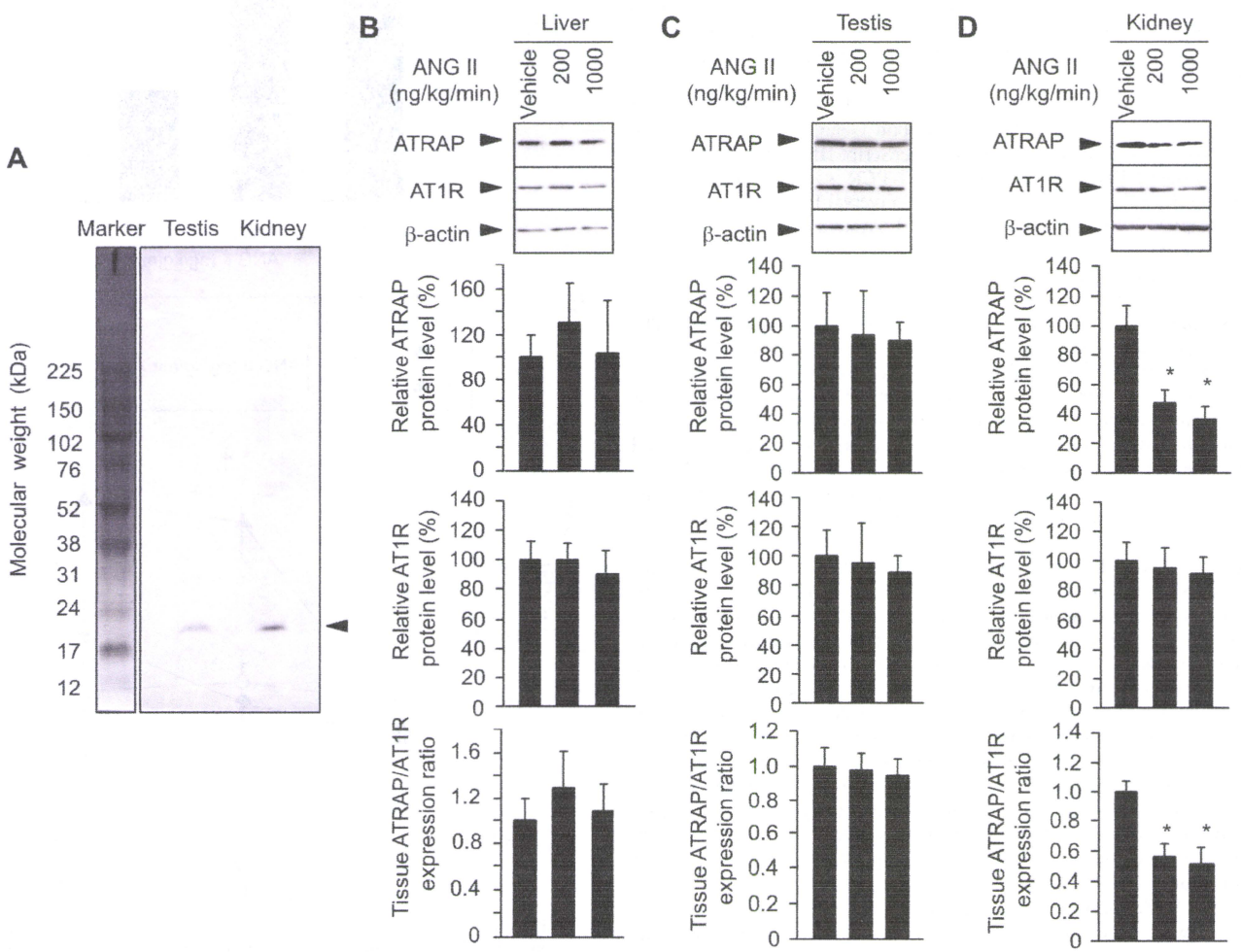

Fig. 2. Western blot showing the signal specificity of the ANG II type 1 receptor (AT1R)-associated protein (ATRAP) protein detected by the polyclonal anti-ATRAP antibody through visualization of the entire size range (*A*) and representative Western blots showing the effects of continuous ANG II infusion on the total protein expression of ATRAP and AT1R in the tissues of mice infused with vehicle or ANG II (200 or 1,000 ng·kg^{-1}·min^{-1}) for 14 days [liver (*B*); testis (*C*); kidney (*D*)]. Measurement of the ATRAP-to-AT1R ratio was performed as described in METHODS. The values were calculated relative to those obtained with extracts from mice infused with vehicle and are expressed as means ± SE ($n = 6$/group). *$P < 0.05$ vs. vehicle.

using Western blot analysis with an ATRAP-specific antibody (40, 42). Since the antibody developed against ATRAP is relatively new (42), we initially examined the signal specificity through visualization of the entire size range on Western blot analysis. Western blot analysis of tissue extracts from the testis and kidney of adult male C57BL/6 mice revealed that the polyclonal antibody for mouse ATRAP recognized a prominent band of 18 kDa, which was consistent with the predicted molecular mass of mouse ATRAP (= 18 kDa) (Fig. 2*A*).

Subsequently, we examined whether ANG II stimulation affected the expression of total ATRAP and AT1R expression using whole tissue extracts. The results of Western blot analysis showed that the hepatic and testicular protein levels of both ATRAP and AT1R were similar in the vehicle- and ANG II-infused mice, resulting in no apparent change in the relative expression ratio of ATRAP to AT1R in the liver and testis (Fig. 2, *B* and *C*). On the other hand, with respect to the renal expression of ATRAP and AT1R, although the total AT1R protein levels did not exhibit any evident change in either the vehicle-infused or ANG II-infused mice, the ATRAP protein levels at the subpressor and pressor dose in the ANG II-infused mice were significantly lower than in vehicle-infused mice after 14 days of treatment (Fig. 2*D*). As a result, the relative expression ratio of ATRAP to AT1R in the kidney was significantly suppressed at the subpressor and pressor dose in the ANG II-infused mice compared with the vehicle-infused mice (Fig. 2*D*; tissue ATRAP/AT1R expression ratio, $P < 0.05$, subpressor or pressor dose of ANG II-infused mice vs. vehicle-infused mice).

Effects of ANG II on mRNA expression of ATRAP, angiotensinogen, NADPH oxidase 4, and α-subunit of the epithelial sodium channel. We next examined the pathophysiological consequence of the observed ANG II-induced decreases in renal ATRAP expression by analyzing the mRNA expression of angiotensinogen, NADPH oxidase 4 (Nox4), and the α-subunit of the epithelial sodium channel (α-ENaC) in the kidney of the vehicle- and ANG II-infused mice. For this experiment, we also employed a higher dose of ANG II (2,500 $ng \cdot kg^{-1} \cdot min^{-1}$) for 2 wk of treatment. Systolic blood pressure was progressively elevated to 132 ± 5 and 157 ± 6 mmHg on *days 7* and *14* of ANG II infusion, respectively, from 107 ± 5 mmHg at baseline, in the higher dose ANG II (2,500 $ng \cdot kg^{-1} \cdot min^{-1}$)-infused mice.

The results of real-time quantitative RT-PCR analysis showed that ANG II infusion (200, 1,000, or 2,500 $ng \cdot kg^{-1} \cdot min^{-1}$) for 14 days led to similarly significant decreases in the renal expression of the ATRAP mRNA compared with vehicle infusion (Fig. 3*A*). With respect to the renal pathological effects of ANG II stimulation, there were significant elevations of renal angiotensinogen and α-ENaC mRNA expression by ANG II infusion (2,500 $ng \cdot kg^{-1} \cdot min^{-1}$), while the renal Nox4 mRNA expression was not affected (Fig. 3, *B–D*).

Suppression of ATRAP immunostaining by ANG II in outer medulla of the kidney. We also examined the effect of ANG II infusion on the intrarenal distribution and expression levels of ATRAP by immunohistochemical analysis. The ATRAP immunohistochemical signal was detected throughout the kidney. A relatively high level of ATRAP immunoreactivity was observed in the outer medulla, and moderate ATRAP immunostaining was also observed in the renal cortex and inner medulla in vehicle-infused mice after 14 days of treatment (Fig. 4). However, there was a significant decrease in ATRAP immunoreactivity in the outer medulla of the kidney in ANG II-infused mice. This suppression of ATRAP expression was likely to be region specific in the outer medulla, since no apparent suppression of ATRAP expression was observed in the inner medulla or cortex (Fig. 4). ANG II infusion did not affect the intrarenal distribution or the relative levels of AT1R immunoreactivity (Fig. 5).

The semiquantitative evaluation with immunohistochemical analysis revealed a region-specific reduction of ATRAP immunostaining in the outer medulla with both the subpressor (200 $ng \cdot kg^{-1} \cdot min^{-1}$) and pressor (1,000 $ng \cdot kg^{-1} \cdot min^{-1}$)

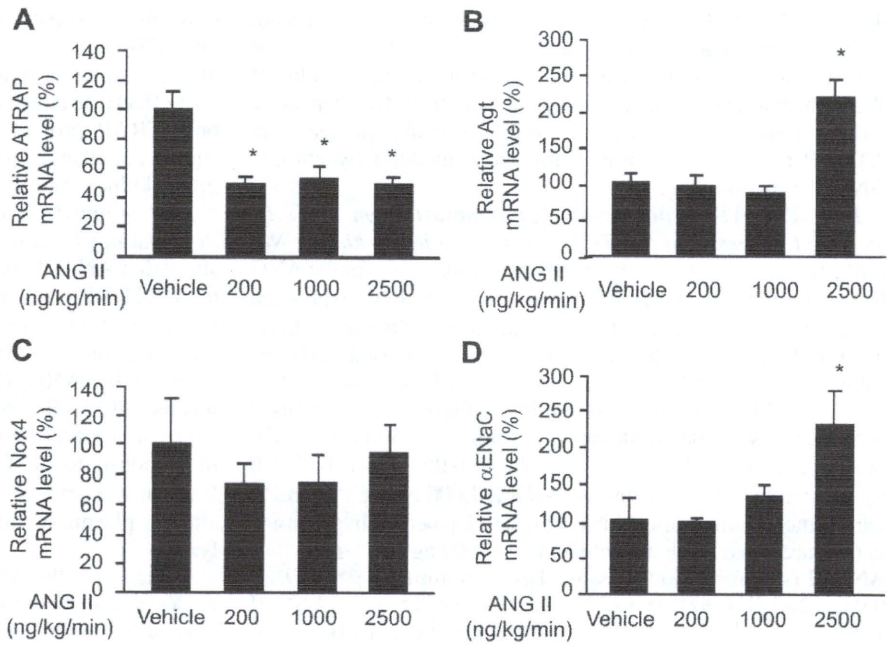

Fig. 3. Effects of continuous ANG II infusion on ATRAP (*A*), angiotensinogen (Agt; *B*), NADPH oxidase 4 (NOX4; *C*), and the α-subunit of the epithelial sodium channel (α-ENaC; *D*) mRNA expression in the mouse kidney. Real-time quantitative RT-PCR analysis shows the relative ATRAP, Agt, NOX4, and α-ENaC mRNA levels in the kidney of mice infused with vehicle or ANG II (200, 1,000, or 2,500 $ng \cdot kg^{-1} \cdot min^{-1}$) for 14 days. The values were calculated relative to those obtained with extracts from mice infused with vehicle and are expressed as means ± SE (n = 6/group). *$P < 0.05$ vs. vehicle.

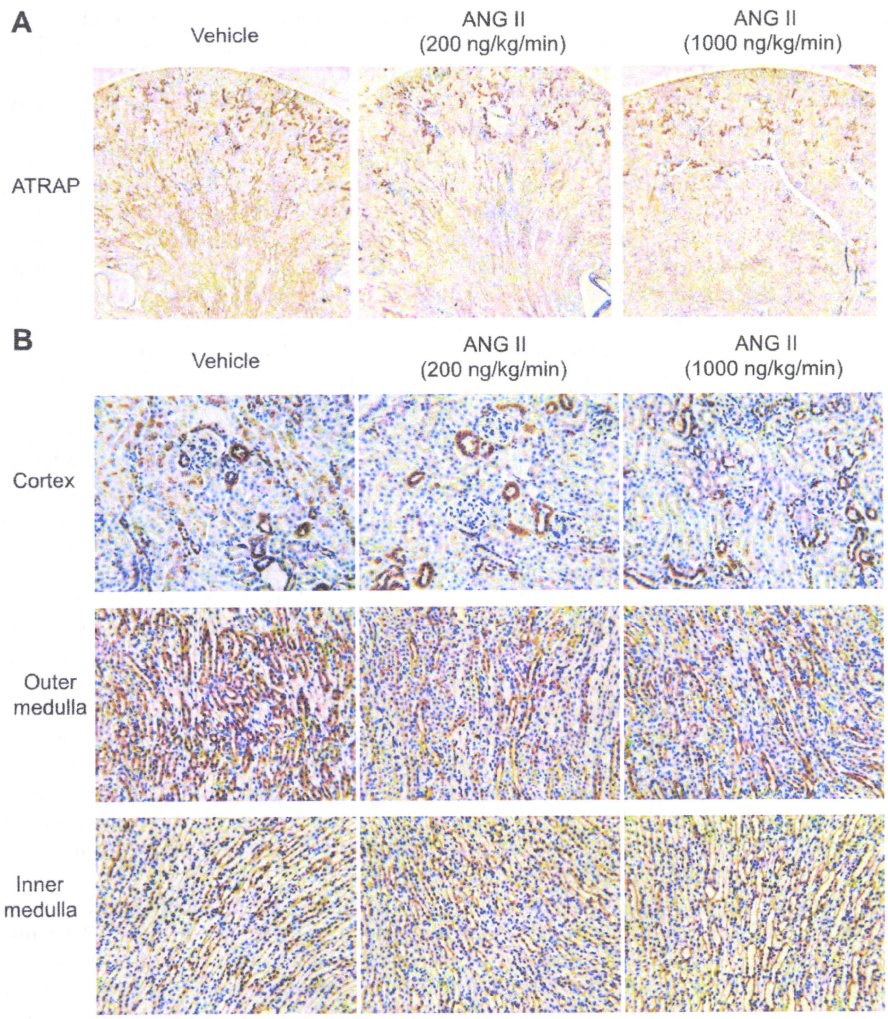

Fig. 4. Representative kidney sections showing the expression of total ATRAP protein in the kidney of mice infused with vehicle or ANG II (200 or 1,000 ng·kg^{-1}·min^{-1}) for 14 days (*A*). Positive areas for ATRAP are evident as the brown dots in the sections. Higher magnification of the kidney sections show effects of continuous ANG II infusion on the immunohistochemical localization of ATRAP expression in the renal cortex, outer medulla, and inner medulla in mice treated with vehicle or ANG II (*B*). Original magnification: ×20 (*A*); ×200 (*B*).

dose in the ANG II-infused mice, without any significant change in the pattern of intrarenal distribution or levels of AT1R immunostaining (Fig. 6). Furthermore, the results of Western blot analysis using tissue extracts from the respective kidney regions confirmed the region-specific decrease in ATRAP protein expression in the outer medulla by chronic ANG II infusion (Fig. 7).

Effects of AT1R-specific blocker olmesartan on ANG II-mediated suppression of ATRAP expression in the kidney. We further examined whether the AT1R was responsible for the ANG II infusion-mediated intrarenal suppression of ATRAP expression using the AT1R-specific blocker olmesartan. Olmesartan treatment did not affect the BW gain in mice infused with the subpressor dose (200 ng·kg^{-1}·min^{-1}, % BW increase, 20.7 ± 1.7%, n = 8), but restored normal BW gain in the mice infused with the pressor dose (1,000 ng·kg^{-1}·min^{-1}) of ANG II (% BW increase, 18.1 ± 1.6%, n = 8, $P < 0.05$, ANG II 1,000 ng·kg^{-1}·min^{-1}+olmesartan vs. ANG II 1,000 ng·kg^{-1}·min^{-1}). Olmesartan treatment also inhibited the development of hypertension in the mice treated with the pressor dose (1,000 ng·kg^{-1}·min^{-1}) of ANG II (systolic blood pressure 104 ± 6 mmHg, n = 6, $P < 0.05$, ANG II 1,000 ng·kg^{-1}·min^{-1}+olmesartan vs. ANG II 1,000 ng·kg^{-1}·min^{-1}), while olmesartan did not affect systolic blood pressure in the mice infused with the subpressor dose (200 ng·kg^{-1}·min^{-1}, 101 ± 7 mmHg, n = 6). Furthermore, olmesartan treatment completely prevented the suppressive effects of either the presser or subpressor dose of ANG II on ATRAP protein expression in the kidney (Fig. 8). No significant changes were observed in AT1R protein expression in the kidney by olmesartan treatment.

Lack of any decrease in plasma membrane AT1R expression in the kidney by chronic ANG II infusion. The results in Fig. 2 show that ANG II stimulation led to a decrease in the levels of total ATRAP protein expression in the kidney, but not other tissues, including the testis. On the other hand, the total AT1R protein expression in all of the tissues examined was unchanged by ANG II treatment (Fig. 2). Thus, to examine whether ANG II-mediated suppression of intrarenal ATRAP expression affects cell surface AT1R expression in the kidney in response to ANG II stimulation, the plasma membrane fraction was specifically extracted from the kidney and testis, and the plasma membrane AT1R protein expression was analyzed.

In the testis, the ANG II infusion at the subpressor dose (200 ng·kg^{-1}·min^{-1}) tended to decrease the expression of the plasma membrane AT1R protein, and the pressor dose (1,000

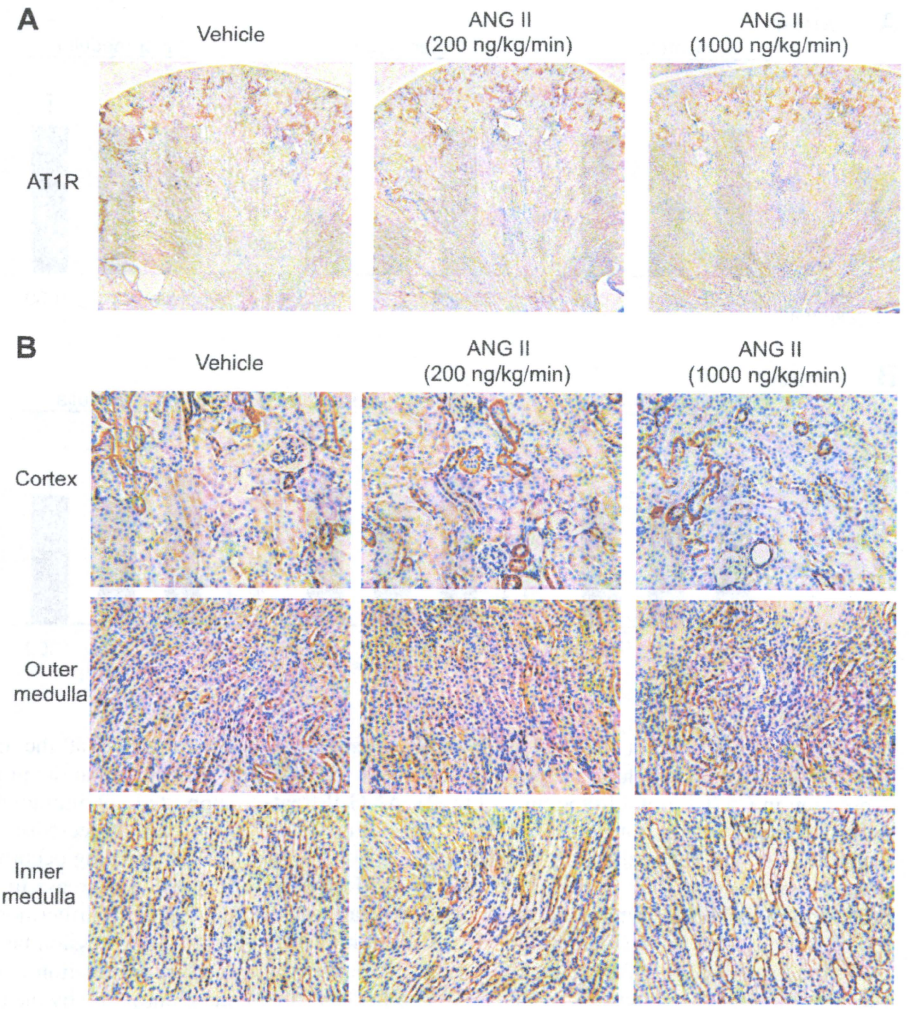

Fig. 5. Representative kidney sections showing the expression of the total AT1R protein in the kidney of mice infused with vehicle or ANG II (200 or 1,000 ng·kg^{-1}·min^{-1}) for 14 days (*A*). Positive areas for the AT1R appear as the brown dots in the sections. Higher magnification of kidney sections showing the effects of continuous ANG II infusion on immunohistochemical localization of AT1R expression in the renal cortex, outer medulla, and inner medulla in mice treated with vehicle or ANG II (*B*). Original magnification: ×20 (*A*); ×200 (*B*).

ng·kg^{-1}·min^{-1}) significantly reduced the plasma membrane AT1R protein levels (Fig. 9*A*). Since olmesartan treatment completely prevented the ANG II-induced suppressive effects on the plasma membrane AT1R protein levels in the testis (Fig. 9*A*), these results indicated that ANG II stimulation promoted AT1R internalization. In the kidney, the plasma membrane AT1R protein levels for the subpressor and pressor doses in the ANG II-infused mice were comparable to those in the vehicle-infused mice and were not affected by olmesartan treatment (Fig. 9*B*).

Decrease in plasma membrane AT1R expression in the kidney of ATRAP transgenic mice. In terms of AT1R internalization in the kidney, although ANG II stimulation decreased the ATRAP protein level and olmesartan treatment recovered it to the baseline value (Fig. 8), the plasma membrane AT1R protein level was still unaltered (Fig. 9). We hypothesized that olmesartan-mediated recovery of the downregulated ATRAP expression back to the baseline level would be insufficient to promote AT1R internalization in the kidney and that an increased expression of renal ATRAP beyond the baseline level would promote AT1R internalization and decrease plasma membrane AT1R expression. Thus, to upregulate renal ATRAP expression, we newly generated ATRAP transgenic mice using HA-tagged mouse ATRAP cDNA subcloned into the pCAGGS expression vector to test these hypotheses (Fig. 10*A*) (28).

We used these ATRAP transgenic mice for the first time to analyze a putative function of ATRAP in vivo. Western blot analysis of ATRAP expression at the protein level revealed the highest renal expression level (= 3-fold) of ATRAP (HA-ATRAP) in line 19 (Tg19), among the three lines of ATRAP transgene positive (+) mice (Fig. 10*B*), and Tg19 was therefore used for further analysis. The results of real-time quantitative RT-PCR analysis also showed a 3.7-fold increase in the baseline renal ATRAP mRNA expression over littermate control mice (Wt) in the Tg19 mice (Fig. 10*C*). While the ATRAP (HA-ATRAP) protein expression in the kidney of Tg19 mice increased compared with Wt, the total kidney AT1R protein expression in Tg19 did not differ from that in Wt (Fig. 10*D*). On the other hand, the plasma membrane AT1R protein expression in the kidney of Tg19 was significantly decreased compared with Wt at baseline (Fig. 10*E*).

Promotion of AT1R internalization and inhibition of induced expression of angiotensinogen gene in response to ANG II in the kidney of ATRAP transgenic mice. With respect to the inhibitory effect of ANG II treatment on the renal ATRAP-to-

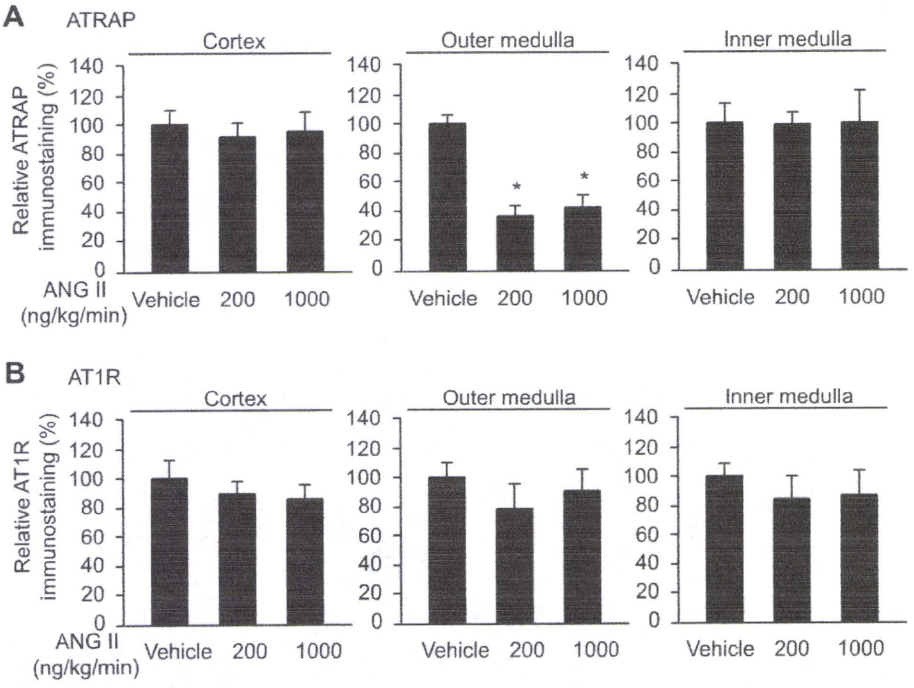

Fig. 6. Semiquantitative evaluation of the immunohistochemical analysis of ANG II-mediated effects on total ATRAP (*A*) and AT1R (*B*) protein expression in the renal cortex, outer medulla, and inner medulla in mice treated with vehicle or ANG II. The values were calculated relative to those obtained with extracts from mice infused with vehicle and are expressed as means ± SE. $*P < 0.05$ vs. vehicle.

AT1R ratio, while chronic ANG II infusion significantly decreased the ratio through a suppression of renal ATRAP expression in C57BL/6 wild-type mice (Fig. 2), ANG II treatment did not affect the ratio at all in Tg19 mice (Fig. 11*A*). Regarding AT1R internalization in the kidney, while the plasma membrane AT1R protein level was not affected by either chronic ANG II stimulation or olmesartan treatment in C57BL/6 wild-type mice (Figs. 8 and 9), it was significantly decreased by ANG II infusion in Tg19 (Fig. 11*B*), thereby indicating that enhancement of renal ATRAP expression beyond baseline promotes AT1R internalization.

Since the body size and BW of Tg19 mice were not different from the Wt at baseline (data not shown), we finally examined the physiological effects of overexpression of ATRAP in Tg19 with respect to blood pressure, response to ANG II, and target organ effects. The systolic blood pressure of Tg19 mice was comparable with that of Wt at baseline, and chronic ANG II infusion significantly and similarly increased systolic blood pressure in Tg19 and Wt (Fig. 11*C*). However, while ANG II infusion in Wt increased the angiotensinogen mRNA expression level in the kidney by 2.25-fold, the mRNA upregulation in response to ANG II infusion was significantly inhibited in Tg19 (Fig. 11*D*). These results indicate that the renal enhancement of ATRAP expression inhibits the ANG II-mediated activation of renal angiotensinogen gene expression, most likely through a promotion of AT1R internalization in response to ANG II.

DISCUSSION

The present data show that either a subpressor or pressor infusion of ANG II in mice causes a significant suppression of intrarenal ATRAP expression and that this response is dependent on the activation of AT1R. The decrease in intrarenal ATRAP expression during continuous ANG II infusion was demonstrated at the mRNA level by quantitative real-time RT-PCR, and at the protein level by Western blotting, and was supported by immunohistochemistry. In addition, the ANG II-mediated decrease in renal ATRAP expression through AT1R signaling occurred concomitantly with the lack of ANG II-induced decrease in plasma membrane AT1R expression in the kidney. Furthermore, a transgenic model increase in renal ATRAP expression beyond baseline expression was accompanied by a reduction in plasma membrane AT1R expression in the kidney, and by the promotion of renal AT1R internalization and the inhibition of an increase in renal angiotensinogen gene expression in response to ANG II.

Several previous studies have reported that activation of the intrarenal renin-angiotensin system and the AT1R pathway plays an important role in the pathogenesis of hypertension and renal injury (19, 25, 33). With respect to the mechanisms involved in ANG II-induced hypertension, the AT1R-mediated enhancement of renal angiotensinogen, collecting duct renin, intrarenal ANG II levels, medullary oxidative stress, and the failure to downregulate renal AT1R expression levels are all reported to be involved in the sustained effects of continuous ANG II elevation on eliciting hypertension (12, 13, 20, 23, 31, 50). Because the biological actions of ANG II are influenced by the AT1R expression levels, and ANG II infusion in mice specifically lacking AT1R in the kidney failed to develop hypertension (6), investigation of the renal activity of AT1R signaling in ANG II-induced hypertension is important to elucidate the mechanisms responsible for the cardiovascular and renal functional changes observed in this hypertension model.

We previously cloned ATRAP as a novel molecule which interacts with AT1R and showed that ATRAP suppressed ANG II-induced hypertrophic and proliferative responses of cardiovascular cells by inducing a constitutive internalization

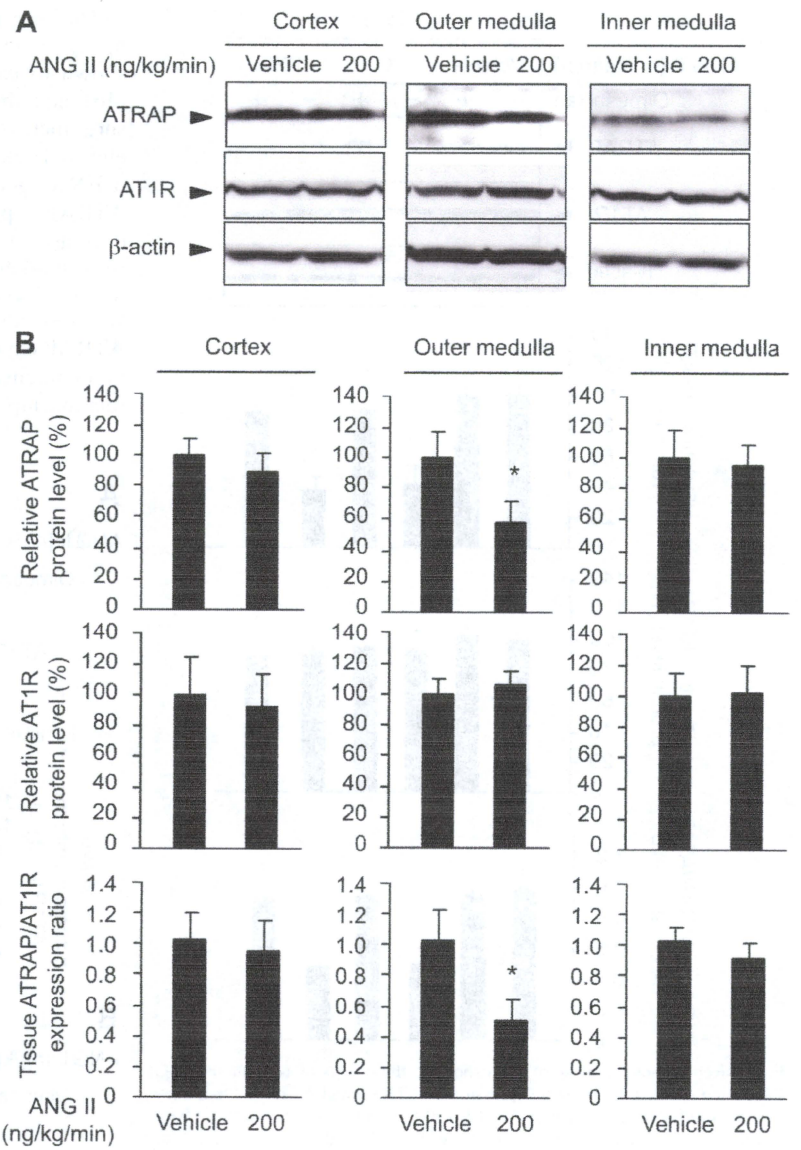

Fig. 7. Representative Western blots showing the effects of continuous ANG II infusion on the total protein expression of ATRAP and AT1R in the renal cortex, outer medulla, and inner medulla in mice infused with vehicle or ANG II ($200\ ng \cdot kg^{-1} \cdot min^{-1}$) for 14 days (*A*). Measurement of the ATRAP-to-AT1R ratio was performed as described in METHODS (*B*). The values were calculated relative to those obtained with extracts from mice infused with vehicle and are expressed as means ± SE ($n = 6$/group). *$P < 0.05$ vs. vehicle.

of AT1R (22, 37). Thus a tissue-specific regulatory balancing of ATRAP and AT1R expression may be involved in the modulation of AT1R signaling in each tissue. We previously showed that ATRAP is expressed in a variety of mouse tissues, as is the AT1R, and that dietary salt intake modulates renal ATRAP expression (42). In this study, the expression of the hepatic and testicular ATRAP protein was not affected by continuous ANG II infusion. Although activation of the tissue renin-angiotensin system is important for the pathogenesis of hypertension and is associated with organ injury, the liver and testis are not target organs of hypertensive tissue injury. Our previous studies showed that the progression of hypertension did not affect hepatic angiotensinogen gene expression in genetically hypertensive rats, which is consistent with the results in the present study (38, 39).

In terms of the regulation of the intrarenal renin-angiotensin system by ANG II stimulation, previous studies by Navar and others (9, 20, 25, 27) established that ANG II is accumulated in the kidney of rats upon infusion, a response that is prevented by AT1R-specific blockers. Further evidence from experiments using rats suggests that AT1R-specific blockers decrease intrarenal ANG II levels by preventing AT1R-mediated uptake, as well as AT1R-mediated induction of intrarenal angiotensinogen, which is a substrate of ANG II (26). We previously showed that ATRAP is abundantly expressed and widely distributed along the renal tubules from Bowman's capsule to the inner medullary collecting ducts in mice (42). In this study, while continuous ANG II infusion did not have any apparent effects on renal total AT1R protein expression in C57BL/6 wild-type mice, which is consistent with previous reports using rats (12, 13), there was a significant decrease in renal ATRAP expression in ANG II-infused mice, and thereby a marked suppression of the renal expression ratio of ATRAP to AT1R at a subpressor dose of ANG II, even without an increase in blood pressure. This suppression of the renal ATRAP expression by ANG II is AT1R dependent, as it is prevented by treatment with olmesartan.

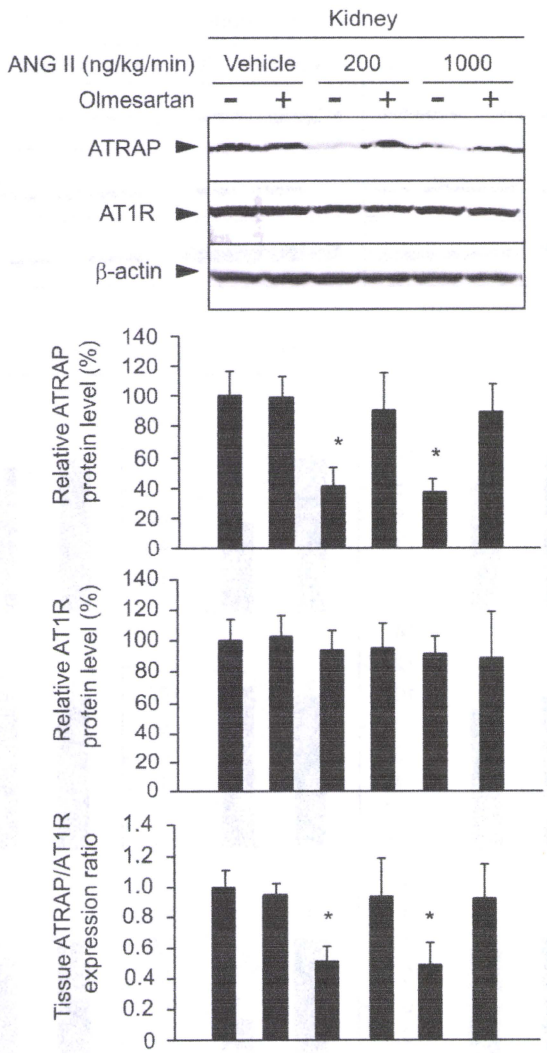

Fig. 8. Representative Western blots showing the effects of continuous ANG II infusion on the total protein expression of ATRAP and AT1R in the kidney of mice infused with vehicle or ANG II (200 or 1,000 ng·kg^{-1}·min^{-1}) with or without olmesartan treatment (10 mg·kg^{-1}·day^{-1} in the drinking water) for 14 days. Measurement of the ATRAP-to-AT1R ratio was performed as described in METHODS. The values were calculated relative to those obtained with extracts from mice infused with vehicle without olmesartan and are expressed as means ± SE (n = 6/group). *P < 0.05 vs. vehicle without olmesartan.

Previous studies also showed that chronic ANG II stimulation in rats leads to the activation of the intrarenal renin-angiotensin system, with an augmentation of renal angiotensinogen expression (20), enhancement of oxidative stress through increases in NADPH oxidase activity (3, 4), and increases in sodium retention through an upregulation of α-ENaC expression (2). On the other hand, a previous study reported that the mouse kidney is relatively resistant to ANG II, including oxidative stress, compared with the rat kidney (45). In the present study, intrarenal angiotensinogen, NADPH oxidase, and α-ENaC mRNA expression was not significantly affected by ANG II infusion of either the subpressor (200 ng·kg^{-1}·min^{-1}) or pressor dose (1,000 ng·kg^{-1}·min^{-1}) for 2 wk, despite a decrease in renal ATRAP expression.

Thus we next employed a higher dose of ANG II (2,500 ng·kg^{-1}·min^{-1}) for 2 wk of treatment, which was recently shown to cause hypertension and renal injury even in mice (46), and showed that it did provoke progressive blood pressure increases and pathological renal responses, including elevated expression levels of renal angiotensinogen and α-ENaC genes, along with a concomitant decrease in renal ATRAP expression (Fig. 3). These observations suggest that a decrease in renal ATRAP expression might be a preceding renal marker of pathological responses to ANG II stimulation in vivo. Nevertheless, because ANG II infusion of the subpressor dose already exerted a down-regulatory effect on renal ATRAP expression without increases in the renal mRNA level of angiotensinogen and α-ENaC, there was a lack of any direct relationship between ATRAP and the expression of angio-

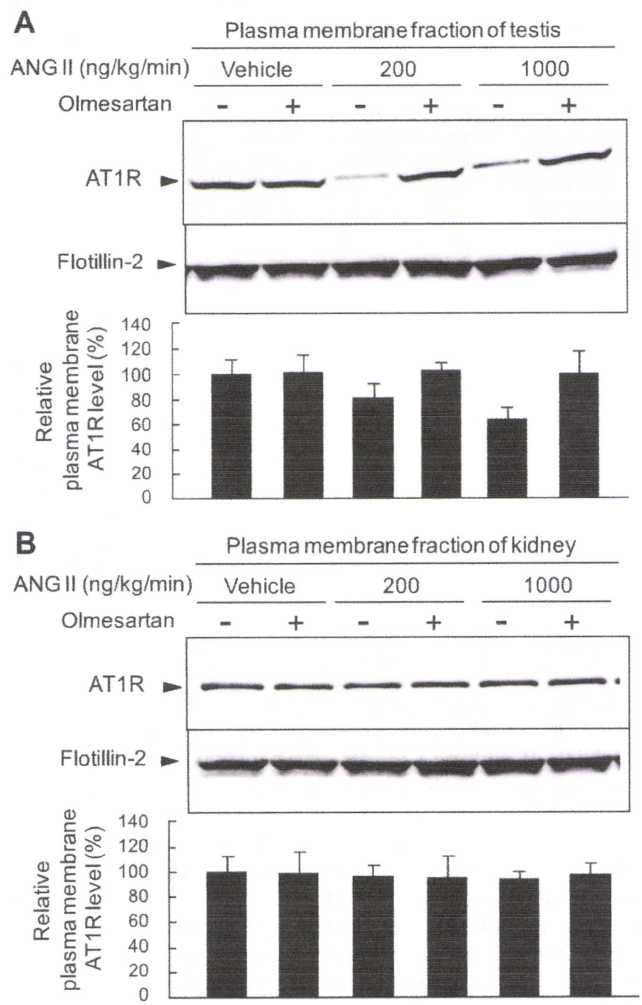

Fig. 9. Representative Western blots showing the effects of continuous ANG II infusion on the plasma membrane AT1R protein level in the tissues of mice infused with vehicle or ANG II (200 or 1,000 ng·kg^{-1}·min^{-1}) with or without olmesartan treatment (10 mg·kg^{-1}·day^{-1} in the drinking water) for 14 days [testis (*A*); kidney (*B*)]. Flotillin-2 is constitutively localized to the plasma membrane and is an internal control protein. The values were calculated relative to those using plasma membrane fractions from mice infused with vehicle without olmesartan and are expressed as means ± SE (n = 6/group). *P < 0.05 vs. vehicle without olmesartan.

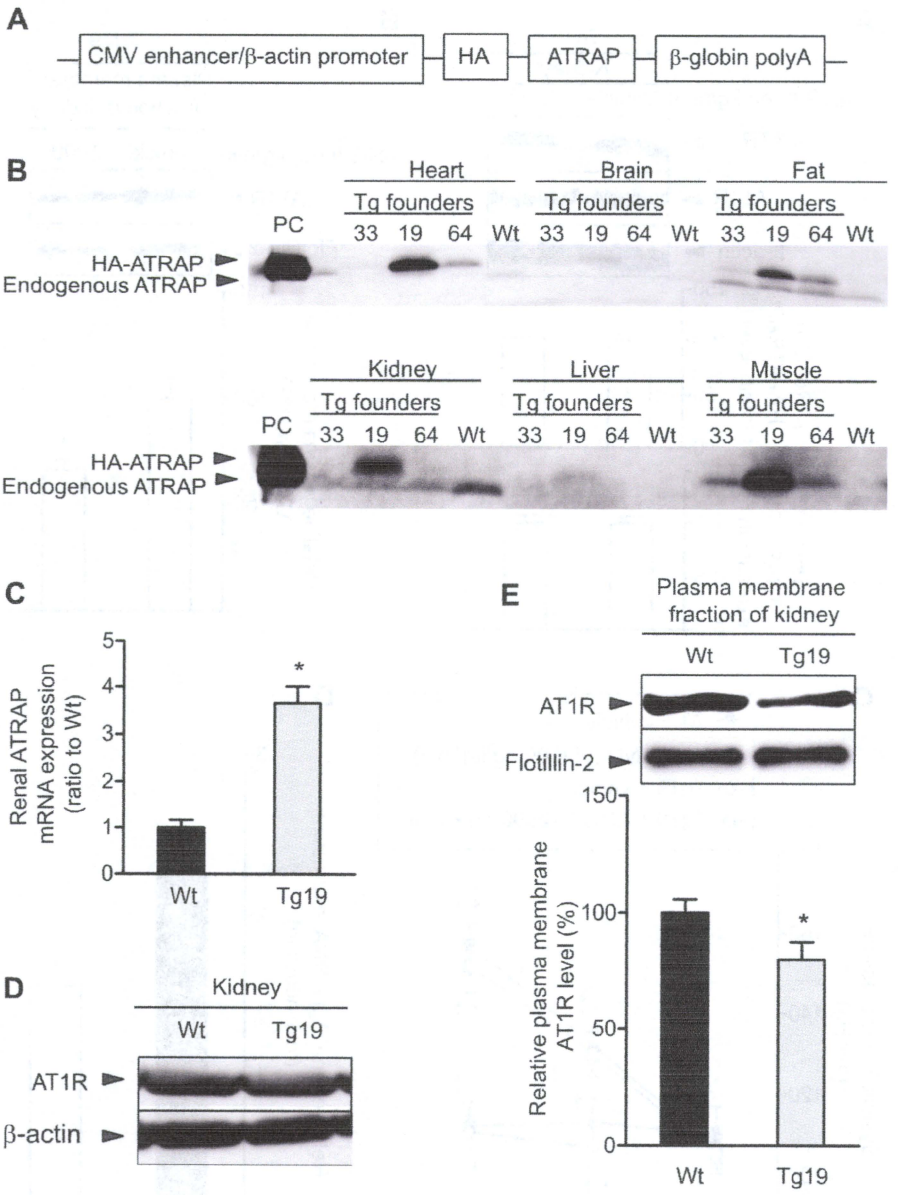

Fig. 10. Generation of ATRAP transgenic mice and decrease in the plasma membrane AT1R expression in the kidney. *A*: transgenic mice expressing ATRAP were generated on a C57BL/6J background with standard techniques. Briefly, the hemagglutinin (HA)-tagged mouse ATRAP cDNA was subcloned into the pCAGGS expression vector, which contained the cytomegalovirus (CMV) enhancer and chicken β-actin (CAG) promoter, and the resultant transgene construct was microinjected into the pronuclei of fertilized mouse embryos at the single-cell stage to generate transgenic mice (C57BL/6 strain). *B*: Western blot analysis of ATRAP expression at the protein level revealed the highest renal expression level (= 3-fold) of ATRAP (HA-ATRAP) in line 19 (Tg19), among the 3 lines of ATRAP transgene positive (+) mice obtained. Tg19 was used for further analysis in the present study. *C*: results of real-time quantitative RT-PCR analysis showed a 3.7-fold increase in the baseline renal ATRAP mRNA expression over littermate control mice (Wt) in ATRAP transgenic mice (Tg19). The values were calculated relative to those in kidneys from Wt and are expressed as means ± SE (n = 7/group). *$P < 0.05$ vs. Wt. *D*: results of Western blot analysis showed that the total kidney AT1R protein expression of Tg19 did not differ from that in Wt. *E*: results of Western blot analysis showed that the plasma membrane AT1R protein expression in the kidney of Tg19 was significantly decreased compared with Wt at baseline. The values were calculated relative to those from the plasma membrane fractions of Wt and are expressed as means ± SE (n = 9/group). *$P < 0.05$ vs. Wt.

tensinogen and α-ENaC genes in the kidney. Thus the results did not establish any causality or effect with respect to changes in renal ATRAP at this stage. Therefore, further investigation is needed to elucidate the exact molecular causal relationship between them.

The results of immunohistochemistry, including semiquantitative evaluation and Western blot analysis using the respective kidney regions, revealed a reduction of ATRAP expression in the outer medulla resulting from ANG II stimulation. Since previous studies showed that the outer medulla plays an important role in ANG II-mediated renal injury (23, 24), the suppression of renal ATRAP, particularly in the outer medullary region, may play a role in the renal pathological responses elicited by ANG II stimulation. While the intrarenal colocalization of ATRAP with AT1R suggests a functional role for ATRAP, it does not necessarily implicate ATRAP in electrolyte transport, renal injury, or hypertension, and the precise tubular function of ATRAP remains to be determined. The detailed molecular mechanism responsible for the tissue-specific AT1R-mediated suppression of ATRAP expression is still unclear. Further studies are necessary to determine the regulatory machinery of ATRAP gene transcription, including the transcription factors interacting with the promoter region of ATRAP gene and the functional effects of ATRAP on ANG II-mediated pathological responses using cultured renal tubular cells, and such studies are now underway.

Previous in vitro results suggested that ATRAP promotes AT1R internalization so as to inhibit AT1R signaling (37). In the present study, chronic ANG II infusion with either the a low or high dose caused significant suppression of endogenous ATRAP expression in the kidney, but not in the testis (Fig. 2).

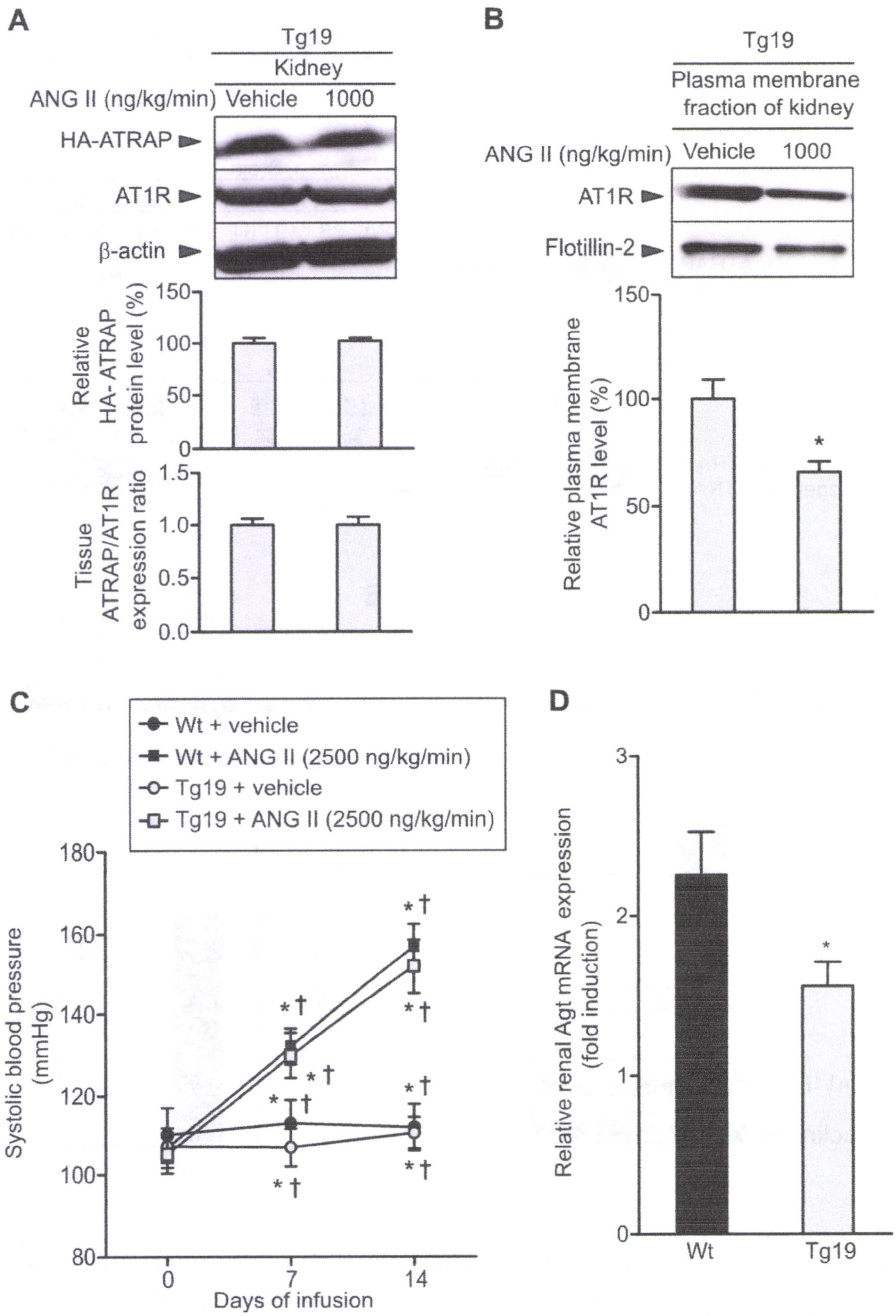

Fig. 11. Promotion of AT1R internalization and inhibition of induced expression of angiotensinogen gene in response to ANG II in the kidney of ATRAP transgenic mice. *A*: representative Western blots showing the effects of continuous ANG II infusion on the total protein expression of HA-ATRAP and AT1R in the kidney of ATRAP transgenic mice (Tg19) infused with vehicle or ANG II (1,000 $ng \cdot kg^{-1} \cdot min^{-1}$) for 14 days. Measurement of the ATRAP-to-AT1R ratio was performed as described in METHODS, and the values were calculated relative to those in extracts from Tg19 infused with vehicle and are expressed as means ± SE (n = 6/group). *B*: representative Western blots showing the effects of ANG II infusion on the plasma membrane AT1R protein level in the kidney of Tg19 infused with vehicle or ANG II (1,000 $ng \cdot kg^{-1} \cdot min^{-1}$) for 14 days. The values were calculated relative to those obtained with extracts from Tg19 infused with vehicle and are expressed as means ± SE (n = 6/group). *C*: effects of ANG II infusion on systolic blood pressure during the treatment period. Tg19 and littermate control mice (Wt) were infused with either vehicle or ANG II (2,500 $ng \cdot kg^{-1} \cdot min^{-1}$) for 14 days. The values of systolic blood pressure are expressed as means ± SE (n = 6/group). *$P < 0.05$ vs. vehicle. †$P < 0.05$ vs. *day 0*. *D*: effects of ANG II infusion on renal Agt mRNA expression in Wt and Tg19. Values are calculated as the fold-induction of those from extracts in the vehicle-infused mice and are expressed as means ± SE (n = 6/group). *$P < 0.05$ vs. Wt.

On the other hand, ANG II infusion did not alter plasma membrane AT1R expression in the kidney but significantly decreased it in the testis (Fig. 8), while total AT1R expression was not altered by ANG II in either of these tissues (Fig. 2). As a result, it is probable that testicular ATRAP promoted substantial ANG II-induced AT1R internalization, which was estimated by comparing the plasma membrane AT1R level with the total AT1R level.

In terms of AT1R internalization in the kidney, although ANG II downregulated ATRAP expression and olmesartan treatment recovered it to the baseline level (Fig. 8), the plasma membrane AT1R expression itself was not affected at all (Fig. 9), thereby still indicating the possibility that renal ATRAP exerts no effect on AT1R internalization in the kidney. We hypothesized that upregulation of renal ATRAP expression beyond baseline promotes AT1R internalization such that it is detected as a decrease in plasma membrane AT1R expression in the kidney without a change in the total AT1R protein level.

Thus, to examine the ATRAP-mediated effect on renal AT1R internalization by a different strategy in vivo, we produced ATRAP transgenic mice. The results demonstrated that enhancement of renal ATRAP expression in transgenic mice caused a decrease in the plasma membrane AT1R level, even at baseline without ANG II stimulation, irrespective of there