特集　高齢者循環器疾患の診断と治療—update—

高齢者心不全の診断と治療*

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高齢者心不全診断（急性および慢性心不全）

1．臨床症状

左心不全による心拍出量低下から，運動耐容能が低下し，自覚症状として全身倦怠感や食欲不振が起こる．他覚的には活動低下，臥床傾向，認知症の増悪，意識障害が起こる．左心不全が進行するに従って，肺うっ血による低酸素血症をきたす．自覚症状は労作時呼吸困難，咳，痰，喘鳴である．さらに進行すれば安静時呼吸困難から起座呼吸，チアノーゼを起こす．肺うっ血時，聴診ではcrackles(wheezingあるいはpiping)を聴取する．右心不全症状としての頸静脈怒張，浮腫，肝腫大，胸水，腹水も認める．

2．心不全誘発因子

心臓基礎疾患を有する高齢患者の心不全誘発因子としては，呼吸器感染症，心筋虚血，貧血，不整脈(頻脈あるいは徐脈)，過労，利尿薬自己中断，水分摂取過剰がある．

3．心不全診断法

胸部X線・心電図・動脈血ガス・血液検査・心臓超音波検査を行う．重症度を判定し，基礎疾患を鑑別する．基礎疾患は急性心筋梗塞，陳旧性心筋梗塞，弁膜症，拡張型心筋症，高血圧症の順に多い．なお，単なる頻脈型心房細動によるうっ血性心不全も少なからずみられる．正確な重症度診断と治療方針の決定のためには，スワンガンツカテーテル法による心血行動態評価が有効である．しかし，認知症，あるいは病院の設備の面から施行が難しい場合は中心静脈圧測定のみでも有効である．血中BNP値上昇は左室壁に伸展負荷がかかっていることの鋭敏な示標である．治療効果により正常域まで低下することも多い．最近，心不全の病態として左室収縮能が低下したものと，拡張能が低下したもの，そして両者の合併したものが存在することが明らかになってきた．超音波検査で，収縮能低下は駆出率低下として判定し，拡張能低下は，洞調律例についてはドップラー左室流入血流波形から求めたE/Aの減少(加齢とともに出現)，逆に偽正常化(病的拡張能低下)，さらには超正常化(拘束型)により判定する．拡張能低下型心不全は高齢者にとくに多いといわれているが，実態は定かではない．以下に述べる心不全治療法と薬剤選択は主に収縮能低下型心不全に対して培われてきたもので，拡張能低下型に対してはエビデンスが確立されておらず，今後の課題である．

* Diagnosis and management of heart failure in elderly.

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急性心不全治療のすすめ方

1．心原性ショック

急性心不全患者で，緊急処置を要するものは，循環・呼吸が維持しえない心原性ショックの場合である．酸素飽和度モニターを設置，気道確保，酸素投与し，動脈血ガスを採取し，心電図モニター，Aラインによる血圧持続モニターを装着する．静脈路を確保し，開始液を点滴する．房室ブロックや洞停止などによる高度徐脈のとき，経皮経静脈的一時ペーシングを考慮する．心室頻拍のときは直流放電も考慮する．低血圧に対しドーパミン3～5 μg/kg/min持続点滴を開始する．それでも自発呼吸が微弱あるいは停止すれば，気管内挿管を考慮する．急性心筋梗塞による心原性ショックと診断すれば，心臓カテーテル検査室において経皮的大動脈バルーンパンピングを行い，経皮的冠動脈形成術施行を前提とした緊急冠動脈造影検査を行う．この際，高齢者であることから，もともとのADL，心疾患の既往・他疾患の合併など関連情報を早急に収集し，家人に対し病態を説明し，どこまで治療を施すかを十分相談した上で施行する．点滴による保存的薬物療法までが治療の許容範囲と家人から制限されることも多い．

2．バイタルが安定した急性心不全

半座位安静にし，絶食とする．動脈血酸素飽和度96％以上を目標にして酸素投与する．胸部X線所見から肺うっ血の程度，胸水量を把握する．カテーテルにより，中心静脈圧あるいは肺動脈楔入圧の値をモニターする．基礎点滴量を乳酸加リンゲル液20cc/時とする．尿道バルーンカテーテル挿入，フロセミドを10～20mg静注し，反応尿量を測定していく．酸素飽和度改善，フロセミドに反応する尿量，中心静脈圧の低下状況に合わせ，次の日から増減する．毎日の水分出入バランスを十分負に維持し，胸部X線で肺うっ血や胸水を減少させるようにもっていく．中心静脈圧の至適目標値は3～5 cmである．一般の収縮期心不全の場合は10cm以上がうっ血の危険域である．うっ血が解除されて低酸素血症が軽快してくれば，フロセミドを経口に変え，食事を開始する．食事量および摂取水分量(300～500cc/日に制限する)と利尿薬投与量の兼ね合いを考慮し，水分バランスを負に維持する．初診時血圧が100mmHg以上あれば，心不全の病態の悪循環を断ち切って予後を改善することが証明されている．アンジオテンシン変換酵素阻害薬(angiotensin converting enzyme inhibitor：ACEI)をできるだけ早期から継続的に経口内服させる(たとえばエナラプリル2.5～5 mg)．

急性期に患者が高血圧であったときには，左室後負荷を軽減させるため，速やかな降圧が必要である．ニトログリセリン舌下と引き続いてのニトログリセリン，ニカルジピンあるいはジルチアゼムの持続静注，またはACEIのカプトプリルを噛み砕いて舌下内服させることが有効である．

定期的に血液検査(Na, K, BUN, Cr, Ht, 血清浸透圧)をチェックし，利尿による電解質異常や脱水が起こらないようにする．低カリウム血症発生に留意し，スピノロラクトン12.5mgやカリウム製剤を併用する．しかし，高齢者には潜在性腎機能低下(血清クレアチニン値が正常でも)があり，血清カリウムが低下しないこともしばしば見受けられる．高齢では低栄養のため著しい低アルブミン血症により血清膠質浸透圧が低下し，間質うっ血があるにもかかわらず血管内脱水を生じている場合も多い．このとき，利尿薬の効果が低減し血圧低下も起こりやすい．呼吸困難が改善されれば，中心静脈圧が上昇しないように(10cm以上)留意して，アルブミン製剤点滴(4～5 g/時)を考慮する．

頻脈性心房細動あるいは洞性頻脈でも，ジゴキシン0.25mgを静注する．至適血中濃度到達に時間がかかるので，はじめに多く，のち減量して投与する(たとえば第1日と第2日目に0.25mg，第3日目以降は0.125mg)．以降腎機能を考慮しながら投与し続け，血中濃度を0.5～2.0ng/m*l*に維持する．

フォレスターIV型，これが測定できなくても，心臓超音波で明らかに中等度以上の左室収縮率低下のある場合，あるいはI, II型でも血管内脱水や腎機能障害合併によってフロセミドのみで十分な尿量が得られないとき，ドーパミン製剤を併用する．専用静脈路からの持続点滴で2～3

μg/kg/minから開始，反応が得られるまで増量する(最大10μg/kg/min)．それでも利尿が得られず，うっ血が解除できないときは，ヒト心房性Na利尿ペプチド製剤(0.05～0.1μg/kg/min)併用，あるいはホスホジエステラーゼ阻害薬ミルリノン(0.25～0.5μg/kg/min)への変更を試みるが，血圧低下に注意する．

基礎疾患が虚血性心疾患であるとき，冠動脈造影検査を施行し，経皮的冠動脈形成術あるいは冠動脈バイパス術を行う．人工心肺を使用せず(off pump)，心拍動下に冠動脈バイパス術を行う侵襲の少ないMIDCABも広まりつつある．

いったんうっ血が解除されたあとも，尿量や血圧の維持にドーパミンが必要で離脱困難な場合，ピモベンダン内服に変更し，退院にもっていける場合も多い．ピモベンダン2.5mg(分2)がドーパミン3～5μg/kg/minに相当する．

慢性心不全の治療

慢性心不全は，重症度確定(NYHA分類)と基礎疾患鑑別を行う．心臓超音波検査が簡便かつ正確である．うっ血(肺あるいは体)が存在する症例は入院させる．入院が不可能なときには外来で経口利尿薬を処方するが，頻回に通院させ，脱水などの副作用には十分注意する．うっ血が存在する間の治療は急性心不全治療に準ずる．うっ血が軽快したあとは精査を行う．虚血性心疾患における冠動脈病変の確認とインターベンション治療の適応決定，および弁膜症の重症度評価と手術適応決定のための心臓カテーテル検査は，患者の予後を判定する重要な検査である．

大規模臨床治験から，虚血性心疾患および拡張型心筋症による慢性心不全治療において，心機能を改善させ生命予後を延長させる薬物は，ACEI，β遮断薬，アンジオテンシンII受容体拮抗薬(angiotensin II receptor blocker：ARB)，アルドステロン拮抗薬であることが明らかにされている．

1．ACE阻害薬(ACEI)

CONSENSUS試験[1]では，平均70歳のNYHA IV度心不全に対しエナラプリルを投与し，心機能改善，心不全悪化抑制，生命予後改善が得られた．

SOLVED予防試験[2]では，駆出率35％未満の無症候性心不全の無投薬例に対しエナラプリルを投与し，心不全発症率減少，生命予後改善が得られ，SOLVED治療試験[3]では，駆出率35％未満のNYHA II～III度心不全でジギタリス，利尿薬などほかの心不全治療薬投与中の例に対し，エナラプリル投与によりうっ血性心不全発症率減少，生命予後改善が得られた．X-SOLVED試験[4]は，上記2試験の12年間にわたる長期フォロー結果であり，エナラプリルは12年後も累積生存率を改善させていた．とくに，最初の4年間投与したあとに中止した症例でも，その後長期にわたり薬物効果を維持できたことは注目に値する．なお，ACEIは基礎疾患が虚血性心疾患でもそれ以外でも効果を発揮する．これらの試験でACEIの心不全に対する有効性が確立された．さらに高齢者心不全で高頻度に合併している誤嚥性肺炎(不顕性誤嚥を含む)に対してACEIの咳反射亢進作用が予防効果を発揮する．投与において注意すべき副作用は，血圧低下，腎機能悪化，血清K値上昇である．

2．β遮断薬

カルベジロールスタディ[5]ではカルベジロール，CIBIS II試験[6]ではビソプロロール，MERIT-HF試験[7]ではメトプロロールが，基礎疾患の種類にかかわらず収縮能の低下した(駆出率35～40％未満)心不全患者での心不全悪化を防止し，生命予後を改善させた．これはACEIの効果とは独立していた．COPERNICUS試験[8]は，NYHA IV度の重症心不全患者を対象として施行され，ここでもカルベジロールは予後改善に働いた．実際の投与法は，急性期のうっ血が消失したあとの心機能低下症例において，血圧が100mmHg以上あれば低容量から開始(メトプロロールは5mg/日，カルベジロールは2.5mg/日)，血圧や心拍数の著しい低下がないことをみつつ，1週間ごとの心臓超音波検査で心機能低下を認めないこと，うっ血性心不全症状の発生がないことを確認して，2.5～5mgずつ増量する．カルベジロールでは10～20mgに増量するが，心機能改善は数か月以降になることもありえる．本邦ではじめての大規模臨床試験であるMUCHA試験[9]では，駆出率40％以下の拡張型心筋症あるいは虚血性心疾患患

者で，カルベジロールを2.5mgから10mgまで増量していくと，容量依存性に駆出率が改善し，心不全入院率が減少することを証明した．すなわち，少量であっても，予後改善に対し効果があることが示され，高齢者ではとくに希望がもてる．

基礎疾患が虚血性心疾患でも拡張型心筋症でも重篤な心室性不整脈による突然死があり得るが，β遮断薬は難治性心室期外収縮の発生を抑制し，突然死予防に働く面からもできるかぎり投与するのが望ましい．

3．アンジオテンシンII受容体拮抗薬(ARB)

ELITE II 試験[10]では，平均70歳の心不全例に対するACEIのカプトプリルとARBロサルタンとの効果の比較を行った結果，生命予後改善について有意差は認めなかった．CHARM試験[11]では，ACEIを使用せず，かつ駆出率40％未満の心不全例に対し，カンデサルタンにより，心不全悪化抑制，生命予後改善が得られた．この効果は年齢別に分類しても同様であった．以上，ARBはACEIと同等の効果をもつと考えられている．しかし，いまだACEIより勝るというエビデンスは出ていない．なお，CHARM added試験[12]では，駆出率の低下した虚血性心疾患でカンデサルタンをACEIに併用すると，ACEIのみに比べ心不全再発を抑制し，生命予後を改善させた．現在のところ，ARBはACEIと同等に扱ってよく，ACEIが咳などの副作用で投与できない患者にはとくに推奨できる．注意すべき副作用はACEIと同様である．

4．ジギタリス

DIGトライアル[13](1997年)では，低濃度(0.8ng/ml)投与により洞調律の心不全に効果があり，心不全入院頻度を低下させえたが，生命予後改善のエビデンスは出ていない．なお，心房細動例では心拍数コントロールとして使用され有効である．高齢者では排泄が遅延するので，血中濃度を測定しつつ，徐脈，房室ブロックやその他のジギタリス中毒の発現に十分注意する．

5．アルドステロン拮抗薬

以前から心不全治療のとき，ループ利尿薬の副作用である血清K低下作用を防止するため併用されていた抗アルドステロン薬のスピノロラクトンが心不全の生命予後を改善した(RALES試験[14])．NYHA III～IV度で左室駆出率35％以下の患者に25mg/日を3年間投与すると，死亡率を30％低下させえた．高齢者において副作用としての血清K値上昇には注意する．

以上の大規模臨床試験の結果に基づいて，ACC/AHAから慢性心不全の重症度に応じた治療の実践法ガイドラインが発表されている[15]．

〔Stage A〕 糖尿病，高血圧，冠動脈疾患などを有する心不全ハイリスク例には心機能が正常のときからACEIまたはARBを投与する．

〔Stage B〕 陳旧性心筋梗塞や弁膜症などを有し，左室駆出率が低下した無症候例には，ACEIまたはARBに加え，可能なかぎりβ遮断薬を併用し心機能改善を図る．

〔Stage C〕 主に労作時心不全症状を有する例では，Stage Bで使用した薬剤にさらにジギタリスと利尿薬およびアルドステロン拮抗薬を併用し，うっ血の治療と予防を図る．

〔Stage D〕 十分な投薬にもかかわらず，安静時から呼吸困難があり，入退院を繰り返す例では，強心薬の持続静注か，機械的サポート，心臓移植，あるいは逆に終末期ケアが必要である．

高齢患者の場合，Stage Dは終末期ケア(緩和医療)が主になると考えられる．

高齢者心不全診療における注意点

1．病歴把握が困難であること

高齢者では多かれ少なかれ認知症があることが多いため，易疲労感や呼吸困難などの自覚症状を正確に訴えられない．さらに家人を含め周囲が放置していると重症化しやすい．対策は普段から家人がよく注意して世話をすることであり，家人のみでは不十分なときは介護認定に基づくケアを積極的に受けさせる．

2．入院加療・侵襲的検査が困難であること

認知症が原因で不穏を起こすため，入院してベッド上安静，酸素マスク，尿道バルーン，末梢あるいは中心静脈点滴路留置など治療に必須な手段を確保しにくい．対策は，家人つきそいで個室に入れること，点滴期間をできるだけ短くし早期に経口薬に切り替えて早期離床を図ること，一歩下がって入院させずに在宅・病診連

携を利用した外来診療を行うことである．

3．至適体液量域がせまいこと

輸液過剰や脱水を起こしやすい．対策は治療効果をきめ細かに判定することである．

4．薬物安全域がせまいこと

腎機能低下が潜在し，ジギタリスなどの薬物血中濃度上昇や利尿薬による脱水，降圧薬による低血圧が起こりやすい．対策は，高齢者では薬を通常の半分量から開始することである．

5．栄養状態が悪いこと

慢性的胸水貯留があり，血清膠質浸透圧が低下し，利尿薬の効果が不十分である．対策はアルブミン製剤の使用や，高カロリー中心静脈栄養あるいは経口，経管栄養を行うことである．

6．服薬コンプライアンスが悪いこと

対策は家人に管理させることである．

7．ADLが低下しやすいこと

入院により筋力低下，認知症進行などADL低下が現れやすく，退院・家庭療養に移行し難い．対策はリハビリテーションである．

8．治療の限界について

心不全はあらゆる心疾患の終焉像であり，高齢患者では心不全のせいでADLが低下してゆき，やがて寿命がつきる経過をとることも多い．したがって，軽快する見込みのないときは，強心剤など点滴薬による延命治療はできるだけ避けるよう考慮すべきである．

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Attenuation of Inflammatory Vascular Remodeling by Angiotensin II Type 1 Receptor–Associated Protein

Akira Oshita, Masaru Iwai, Rui Chen, Ayumi Ide, Midori Okumura, Shiori Fukunaga, Toyofumi Yoshii, Masaki Mogi, Jitsuo Higaki, Masatsugu Horiuchi

Abstract—To explore the role of angiotensin II Type 1 receptor–associated protein (ATRAP) in vascular remodeling, we developed transgenic mice for mouse ATRAP cDNA and examined remodeling after inflammatory vascular injury induced by polyethylene cuff placement. In ATRAP transgenic (ATRAP-Tg) mice, ATRAP mRNA was increased 3- to 4-fold in the heart, aorta, and femoral artery. ATRAP-Tg mice showed no significant change in body weight, systolic blood pressure, heart rate, and heart/body weight ratio. However, cell proliferation and neointimal formation in the injured artery were attenuated in ATRAP-Tg mice. The increase in NADPH oxidase activity and the expression of $p22^{phox}$, a reduced nicotinamide-adenine dinucleotide/reduced nicotinamide-adenine dinucleotide phosphate oxidase subunit, after cuff placement was also attenuated in ATRAP-Tg mice. Moreover, activation of extracellular signal–regulated kinase, signal transducer and activator of transcription 1, and signal transducer and activator of transcription 3 after cuff placement was significantly reduced in ATRAP-Tg mice. Pressor response and cardiac hypertrophy induced by angiotensin II infusion and pressure overload were also attenuated in ATRAP-Tg mice. These results suggest that ATRAP plays an important role in vascular remodeling as a negative regulator. (***Hypertension*. 2006;48:671-676.**)

Key Words: receptors, angiotensin II ■ signal transduction ■ vascular diseases ■ muscle, smooth, vascular

The cardiovascular actions of angiotensin II (Ang II) are mainly mediated by the Ang II type 1 (AT_1) receptor. Previous reports indicate that the intracellular carboxyl-terminal tail of the receptor plays an important role in activation of receptor-coupled G protein and internalization of the AT_1 receptor.[1–6] We cloned a novel AT_1 receptor-associated protein (ATRAP) using a yeast 2-hybrid screening system.[7] ATRAP has 3 transmembrane domains and interacts with the intracellular carboxyl-terminal domain of the AT_1 receptor, but it does not interact with the AT_2 receptor, m_3 muscarinic receptor, bradykinin B_2 receptor, endothelin ETB receptor, or β_2-adrenergic receptor. It is reported that ATRAP modulates AT_1 receptor function in COS-7 cells, human embryonic kidney 293 cells, and cultured mouse cardiomyocytes. Overexpression of ATRAP significantly decreases the number of AT_1 receptors on the cell surface and also decreases the degree of p38 mitogen-activated protein kinase phosphorylation, activity of the c-fos promoter, and protein synthesis on Ang II treatment.[7–9] We also reported that overexpression of ATRAP in cultured vascular smooth muscle cells (VSMCs) enhanced internalization of the AT_1 receptor and attenuated DNA synthesis and activation of extracellular signal-regulated kinase (ERK), Akt, and signal transducer and activator of transcription (STAT) induced by Ang II.[10]

Polyethylene cuff placement around the femoral artery induces inflammatory vascular injury and remodeling responses accompanied by an increase in AT_1 and AT_2 receptor expression. VSMC proliferation, neointimal formation, inflammatory response, and oxidative stress in vascular injury were significantly attenuated in AT_1 a receptor-deficient mice.[11–13] Moreover, administration of an AT_1 receptor blocker, valsartan, also decreased vascular remodeling. These results strongly suggest that ATRAP may act as an important regulator of VSMC proliferation and vascular remodeling. In the present study, we prepared transgenic mice for ATRAP and examined its role in inflammatory vascular injury induced by polyethylene cuff placement around the femoral artery.

Methods

Transgene Constructs and Screening of Transgenic Mice

The hybrid cytomegalovirus enhancer/chicken β-actin (CAG) promoter and a mouse ATRAP cDNA were subcloned into the plasmid pCAG-GS. The plasmid containing the CAG promoter and ATRAP cDNA was microinjected into the pronuclei of fertilized mouse embryos at the single-cell stage to generate transgenic mice (C57BL/6 strain). Transgenic mice were identified by PCR using 5′-ATGGAGCTGCCTGCCGTGAA-3′ as the forward primer and 5′-GTTCACGGTGCATGTGGTAG-3′ as the reverse primer.

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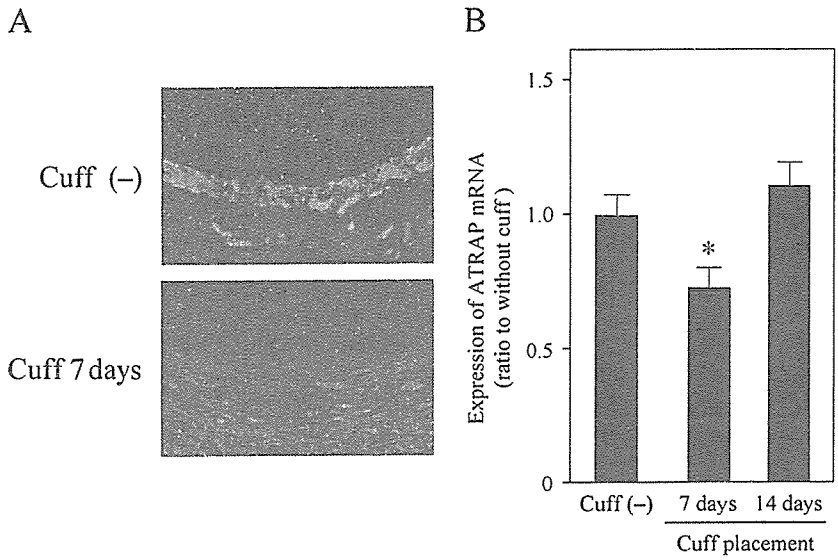

Figure 1. Expression of ATRAP in injured femoral artery after cuff placement in WT mice. Cuff placement around the femoral artery was performed as described in the Methods section, and artery samples were obtained after cuff placement. Immunostaining and real-time quantitative RT-PCR analysis for ATRAP were performed as described in the Methods section. A, Representative result of immunohistochemical staining for ATRAP in femoral artery. Top, without cuff placement; bottom, 7 days after cuff placement. B, Change in ATRAP mRNA expression in femoral artery. Values are standardized against GAPDH mRNA level. Values are mean±SE (n=6 to 8 for each group). *$P<0.05$ vs without cuff.

Cuff Placement

Adult male ATRAP transgenic (ATRAP-Tg) mice (10 to 12 weeks of age) and wild-type mice (C57BL/6J) were used in this study. The mice were housed in a room in which lighting was controlled (12 hours on and 12 hours off), and room temperature was kept at 25°C. They were given a standard diet (MF, Oriental Yeast Co, Ltd) and water ad libitum. The experimental protocol was approved by the Animal Studies Committee of Ehime University. Inflammatory vascular injury was induced by polyethylene cuff placement around the femoral artery under anesthesia with intraperitoneal injection of ketamine (70 mg/kg) and xylazine (4 mg/kg) according to methods described previously,[11–14] and morphometric analysis to measure neointimal area was performed as described previously.[11–13] The heart/body weight ratio was calculated as whole heart weight (milligrams) divided by body weight (grams). Blood pressure and heart rate were measured under consciousness by the indirect tail-cuff method with a blood pressure monitor (MK-1030, Muromachi Kikai Co, Ltd). The mice were held in a case where temperature was kept at 37°C.

Infusion of Ang II and Aortic Banding

Ang II was infused intraperitoneally at a dose of 1 μg/kg per minute for 2 weeks using an osmotic minipump. The aortic banding was performed under anesthesia with intraperitoneal injection of ketamine (70 mg/kg) and xylazine (4 mg/kg) according to the method described previously.[15,16] After 4 weeks, the hearts were excised, weighed, and heart/body weight ratio was expressed as heart weight (milligrams) versus body weight (grams) as described previously.[15,16]

Immunohistochemical Staining

Rabbit polyclonal antibody against the epitope mapped at the C-terminus of ATRAP (CPFASLENKGQAAPRG) was prepared by the Peptide Institute, Inc. Anti-$p22^{phox}$ antibody was purchased from Santa Cruz Biotechnology Inc.[13] Formalin-fixed, paraffin-embedded sections were prepared using femoral artery at 7 days after cuff placement. Proliferating cell nuclear antigen (PCNA) was stained with anti-PCNA antibody (Novocastra Laboratories, Ltd.) using an M.O.M. immunodetection kit (Vector Laboratories, Inc).[11,13] ATRAP and $p22^{phox}$ were stained using biotin-labeled secondary antibodies and Cy3-labeled streptavidin as described previously.[11,12] Paraffin-embedded sections were incubated with anti-$p22^{phox}$ and ATRAP antibody, washed, and incubated with biotin-labeled secondary antibodies, then incubated with Cy3-labeled streptavidin. Serial sections treated with secondary antibodies alone did not show specific staining. Samples were examined with a Zeiss Axioskop microscope equipped with a computer-based imaging system.[13]

Western Blot Analysis

Total proteins were prepared from pooled arteries (n=3, each pool contained 4 arteries from 4 mice) at 7 days after cuff placement. Phosphorylation of ERK, STAT1, and STAT3 was detected by Western blot using specific antibodies as described previously.[13,17]

NADPH Oxidase Activity

A tissue protein sample was prepared from the femoral artery at 7 days after cuff placement by homogenization in 500 μL of ice-cold Tris-sucrose buffer. Reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase activity was quantified by cytochrome c method from the absorbance with or without superoxide dismutase, as described previously.[18,19]

Real-Time RT-PCR

Total RNA was extracted from the femoral arteries (n=3, each pool contained 4 arteries from 4 mice). Real-time quantitative RT-PCR was performed with Premix Ex *Taq* (Takara Bio Inc). PCR primers for ATRAP were the same as for genotyping; for the AT_1 receptor they were 5′-GTTCCTGCTCACGTGTCTCA-3′ (forward) and 5′-CATCAGCCAGATGATGATGC-3′ (reverse); for the AT_2 receptor they were 5′-CCTGCATGAGTGTCGATAGGT-3′ (forward) and 5′-CCAGCAGACCACTGAGCATA-3′ (reverse); and for GAPDH they were 5′-ATGTAGGCCATGAGGTCCAC-3′ (forward) and 5′-TGCGACTTCAACAGCAACTC-3′ (reverse).

Statistical Analysis

The data were analyzed by 1-way ANOVA. If a statistically significant effect was found, post hoc analysis by Bonferroni's test was performed to detect the difference between the groups. A value of $P<0.05$ was considered statistically significant.

Results

Expression of ATRAP in Injured Artery After Cuff Placement in Wild-Type Mice

Expression of ATRAP in the femoral artery was examined by real-time RT-PCR and immunohistochemical staining. Figure

Systolic Blood Pressure, Heart Rate, and Heart Weight/Body Weight Ratio

Strain	SBP, mm Hg	HR, bpm	HW/BW, mg/g
WT	99.5±2.0	603±9	4.37±0.09
ATRAP-Tg	99.0±9.3	597±10	4.47±0.14

SBP indicates systolic blood pressure; HR, heart rate; HW/BW, heart/body weight ratio. Values are mean±SE. n=5 for each group.

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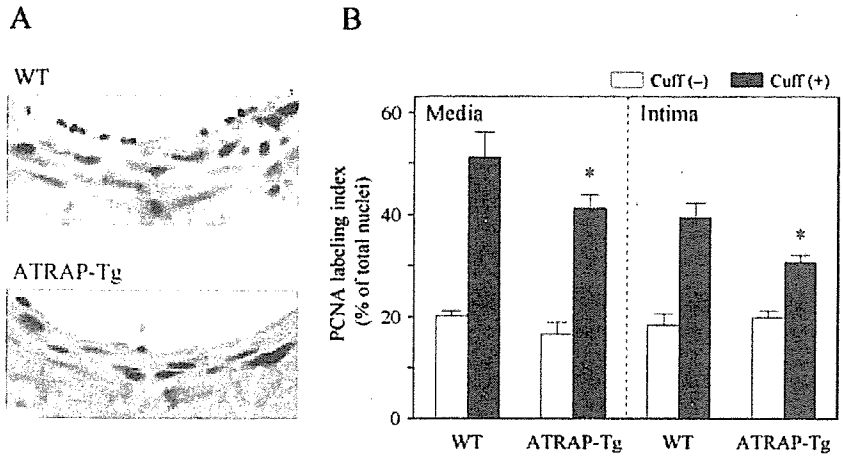

Figure 2. Cell proliferation in injured femoral artery after cuff placement in ATRAP-Tg mice. Cuff placement around the femoral artery was performed, and PCNA was detected as described in the Methods section. Cell proliferation was measured as the ratio of PCNA-positive nuclei to total nuclei in the femoral artery at 7 days after cuff placement. Values are mean±SE (n=6 to 8 for each group). *$P<0.05$ vs WT.

1A shows immunostaining of ATRAP in the femoral artery at 7 days after cuff placement or sham operation. ATRAP was observed mainly in the media of the intact artery. ATRAP was transiently decreased in the media at 7 days after cuff placement. This decrease in ATRAP after cuff placement was accompanied by a decrease in mRNA (Figure 1B). The decrease in ATRAP protein and mRNA had recovered by 14 days after cuff placement.

Blood Pressure, Heart Rate, Heart/Body Ratio, and ATRAP Expression in ATRAP-Tg Mice

Three founder lines that transmitted the transgene were established by Southern blot (ATRAP-Tg 10, 11, and 15). Because ATRAP-Tg 15 showed the highest expression of ATRAP mRNA (Figure I, available online at http://hyper.ahajournals.org), heterozygous animals of this line were used in the present study. Body size, body weight, and behavior of ATRAP-Tg mice were not different from those of C57BL/6J (wild-type [WT]) mice. As shown in the Table, systolic blood pressure, heart rate, and heart/body weight ratio in ATRAP-Tg mice were not significantly different from those in WT mice. ATRAP expression increased ≈3- to 4-fold in the heart, aorta, and femoral artery of ATRAP-Tg mice (Figure II). Media/lumen ratio of the intact femoral artery in ATRAP-Tg mice also did not differ from that in WT mice (Figure IIIA). Expression of the AT_1 receptor in the heart, aorta, and femoral artery was not significantly different between WT and ATRAP-Tg mice (Figure IIIB).

Effect of ATRAP on VSMC Proliferation and Neointimal Formation After Cuff Placement in ATRAP-Tg Mice

Cuff placement induces proliferation of VSMC and neointimal formation in the femoral artery.[12,14,20] Figure 2 shows PCNA labeling index in the media and intima at 7 days after cuff placement in WT and ATRAP-Tg mice. The control (noninjured) levels of PCNA index were not significantly different between WT and ATRAP-Tg groups. However, the increase in PCNA index in the injured artery was suppressed in ATRAP-Tg mice. Related to the change in PCNA index, neointimal formation at 14 days after cuff placement was also attenuated in ATRAP-Tg mice (Figure 3).

Inhibition of Oxidative Stress After Cuff Placement in ATRAP-Tg Mice

The increase in in situ superoxide production in the injured artery at 7 days after cuff placement was significantly attenuated in ATRAP-Tg mice. It is reported that superoxide

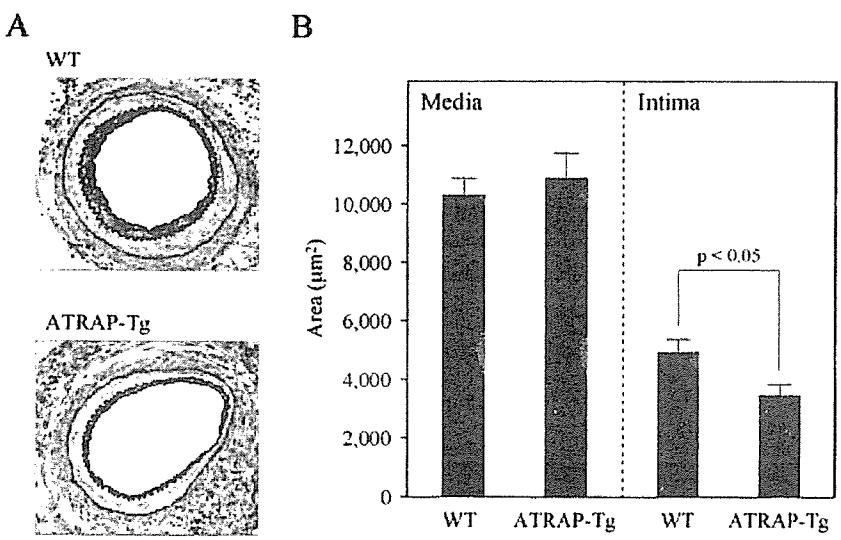

Figure 3. Neointimal formation in injured femoral artery after cuff placement in ATRAP-Tg mice. Cuff placement around the femoral artery and morphometric measurement were performed as described in the Methods section. Areas of media and neointima in the femoral artery were measured at 14 days after cuff placement in cross-sections after elastica van Gieson staining. Values are mean±SE (n=6 to 8 for each group).

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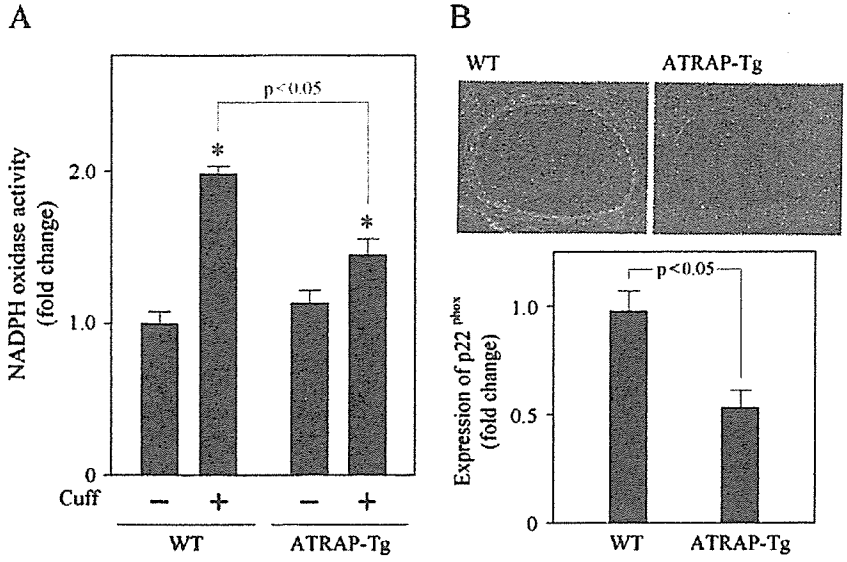

Figure 4. NADPH oxidase activity and expression of $p22^{phox}$ in injured artery after cuff placement in ATRAP-Tg mice. Cuff placement around the femoral artery was performed, and protein samples were prepared at 7 days after cuff placement as described in the Methods section. NADPH oxidase activity was measured, and immunostaining was performed as described in the Methods section. A, NADPH oxidase activity in femoral arteries after cuff placement. B, Representative results of immunohistochemical staining of $p22^{phox}$ from 5 independent experiments (top) and measurement of fluorescence (bottom). Values are mean±SE (n=6 to 8 for each group). *$P<0.05$ vs without cuff.

production is mainly mediated by NADPH oxidase.[13,21,22] NADPH oxidase activity in the injured artery was lowered in ATRAP-Tg mice (Figure 4A). Moreover, expression of $p22^{phox}$, a membrane-associated reduced nicotinamide-adenine dinucleotide/NADPH oxidase subunit, was also attenuated in ATRAP-Tg mice (Figure 4B).

Change in Intracellular Signaling in the Injured Artery of ATRAP-Tg Mice

As reported previously, cuff placement increases phosphorylation of ERK, STAT1, and STAT3 via AT_1 receptor stimulation.[17,23] The phosphorylation level of these markers was not significantly different between WT and ATRAP-Tg mice (Figure 5). However, the increase in phosphorylation of these markers after cuff placement was suppressed in ATRAP-Tg mice.

Effect of ATRAP on Cardiac Hypertrophy

The degree of cardiomyocyte hypertrophy was evaluated by calculating the ratio of heart weight/body weight. This parameter did not differ between WT and ATRAP-Tg mice before aortic banding (Figure 6). Heart-to-body weight ratio was increased 4 weeks after aortic banding in both mice strains, whereas these parameters were smaller in ATRAP-Tg mice (Figure 6A). Administration of Ang II, at 1 μg/kg per minute for 14 days, increased heart/body weight ratio with the increase in systolic blood pressure, whereas increases in these parameters were less in ATRAP-Tg mice (Figure 6B and 6C).

Discussion

In the present study, we demonstrated that VSMC proliferation, neointimal formation, and inflammatory response in the injured artery after cuff placement were inhibited in ATRAP-Tg mice compared with those in WT mice. The AT_1 receptor is a 7 *trans*-membrane receptor distributed in most adult tissues and mediates the major action of Ang II. The intracellular carboxy-terminal of the AT_1 receptor is capable of binding with intracellular signaling molecules like PLC-γ1, SHP-2, and Jak2.[3,4] ATRAP is a novel protein associated with the intracellular carboxy-terminal domain.[7] Previous reports suggest that ATRAP accelerates internalization of the AT_1 receptor and attenuates the AT_1 receptor-mediated response.[8–10] Functional analysis of the effects of ATRAP on

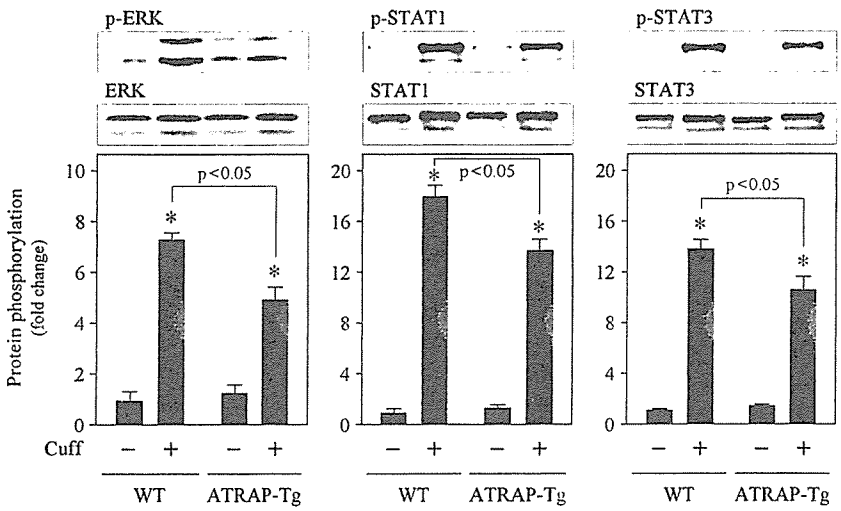

Figure 5. Phosphorylation of ERK, STAT1, and STAT3 in injured femoral artery after cuff placement in ATRAP-Tg mice. Tissue samples were prepared from arteries 7 days after cuff placement, and signaling molecules were detected as described in the Methods section. Top, Representative results of Western blot for phosphorylated and total ERK, STAT1, and STAT3 using 3 different pooled samples. Bottom, Densitometric analysis of Western blots. Values are mean±SE. *$P<0.05$ vs without cuff. p indicates phospho.

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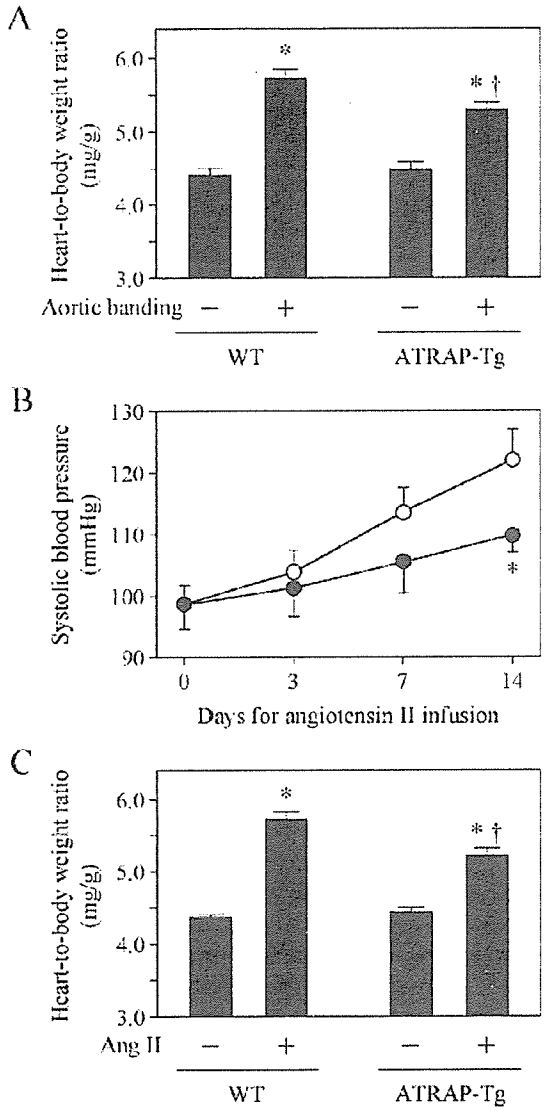

Figure 6. Effect of ATRAP overexpression on the response to Ang II infusion and aortic banding. Ang II (1 μg/kg per minute) was administered intraperitoneally for 14 days using osmotic minipump. Aortic banding was performed as described in the Methods section. Measurement of blood pressure and heart/body weight ratio were performed as described in the Methods section. A, Heart/body weight ratio 4 weeks after aortic banding. B, Systolic blood pressure induced by intraperitoneal infusion of Ang II. ○, WT mice; ●, ATRAP-Tg mice. C, Heart/body weight ratio after Ang II administration for 14 days. Values are mean±SE (n=5 to 8 for each group). *$P<0.05$ vs without aortic banding or Ang II. †$P<0.05$ vs WT without aortic banding (A) or Ang II infusion (C).

Ang II-induced AT_1 receptor signaling revealed decreases in the generation of inositol lipids, Ang II–stimulated transcriptional activity of the c-*fos* promoter and STAT, and cell proliferation. A recent study showed that ATRAP interacts with calcium-modulating cyclophilin ligand and decreases Ang II– or calcium-modulating cyclophilin ligand-induced nuclear factor of activated T cell transcriptional activation.[24] We reported that overexpression of ATRAP increased internalization of the AT_1 receptor in cultured VSMCs. In these cells, an increase in thymidine incorporation and in ERK phosphorylation induced by Ang II was attenuated. These results indicate that ATRAP suppresses AT_1 receptor–mediated signaling by stimulating internalization of the receptor. On the other hand, Ang II is involved in vascular remodeling through stimulation of the AT_1 receptor. Using an animal model of inflammatory vascular injury induced by polyethylene-cuff placement around the femoral artery, we demonstrated that VSMC proliferation, neointimal formation, and inflammatory response were markedly suppressed by blockade of AT_1 receptor–mediated signaling with an ARB or AT_1 receptor gene knockout.[11,12,17,23] Because the overexpression of ATRAP suppresses AT_1 receptor–mediated signaling, it is suggested that the increased ATRAP activity may inhibit vascular remodeling. In fact, ATRAP expression was decreased in the injured artery at 7 days after cuff placement in WT mice, when neointimal formation and inflammation were not yet obvious (Figure 1A and 1B). Therefore, it is possible that change in ATRAP level may affect remodeling of the injured artery.

To examine the function of ATRAP in an in vivo injury model, we developed ATRAP-Tg mice. Expression of ATRAP mRNA in the heart, aorta, and femoral artery in these mice was ≈3- to 4-fold higher than in WT mice (Figure II). However, ATRAP-Tg mice showed no significant change in growth, body weight, heart rate, blood pressure, and heart/body weight ratio (Table). These results indicate that overexpression of ATRAP did not affect basal physiological markers. Expression of AT_1 receptor mRNA was not significantly changed in the heart, aorta, and femoral artery of ATRAP-Tg mice. The effect of ATRAP on internalization of the AT_1 receptor may appear only when the AT_1 receptor is stimulated by Ang II.

ATRAP-Tg mice showed a decrease in vascular remodeling induced by cuff placement (Figure 1A and 1B). Because AT_1 receptor stimulation increases inflammation, VSMC proliferation, and oxidative stress,[21,25] the results in ATRAP-Tg mice suggest that overexpression of ATRAP inhibits AT_1 receptor–mediated responses in vascular injury. We have performed additional studies to examine whether the overexpression of ATRAP could also reduce cardiac hypertrophy related to Ang II and observed that cardiac hypertrophy induced by pressure overload or Ang II infusion and pressor response induced by Ang II infusion were attenuated in ATRAP-Tg mice. These results suggest that overexpression of ATRAP attenuates the cardiac hypertrophy mediated mainly by AT_1 receptor stimulation, although it is also possible that the inhibition of cardiac hypertrophy in ATRAP-Tg mice may also be caused by the reduced pressor response, because myocardial hypertrophy can be because of elevated blood pressure, and overexpression of ATRAP attenuated the pressor response to Ang II infusion in our study.

In our vascular injury model, oxidative stress, such as superoxide production and NADPH oxidase activity, was increased after cuff placement.[13] This increase in oxidative stress was also suppressed in ATRAP-Tg mice (Figure 4). Moreover, phosphorylation of intracellular signaling molecules like ERK, STAT1, and STAT3, which are stimulated through the AT_1 receptor, was attenuated in the injured artery of ATRAP-Tg mice (Figure 5).

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Perspectives

Our results suggest that an increase in ATRAP expression in vivo attenuates AT_1 receptor–mediated signaling and thereby reduces vascular remodeling. There could be the possibility that increase in expression of ATRAP could be having effects in the intact animal other than AT_1 receptor inhibition. However, the detailed mechanisms of the function of ATRAP, for example, the regulation of ATRAP expression, possible ligands for ATRAP, and the regulatory mechanism of the action of ATRAP on phosphorylation and/or dephosphorylation of signaling molecules need to be clarified. The results of the present study suggest that ATRAP may be a novel drug target for the treatment of pathological vascular remodeling.

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Disclosures

None.

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Original Article

Regression of Atherosclerosis by Amlodipine *via* Anti-Inflammatory and Anti-Oxidative Stress Actions

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We examined whether amlodipine, an L-type calcium channel blocker (CCB), has an inhibitory effect on oxidative stress and inflammatory response, and thereby atherosclerosis, in apolipoprotein E–deficient (ApoEKO) mice. Adult male ApoEKO mice (6 weeks of age) were fed a high-cholesterol diet (HCD) for 8 or 10 weeks with or without oral administration of amlodipine (3 mg/kg/day) for 10 weeks or for only the last 2 weeks of the HCD. After HCD feeding, atherosclerotic lesion formation, *in situ* superoxide production and nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase activity were evaluated in the proximal aorta. The expressions of NADPH oxidase subunits ($p47^{phox}$ and rac-1), monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) were determined with immunohistochemistry and quantitative real-time reverse-transcription polymerase chain reaction. After 8 to 10 weeks of HCD administration to ApoEKO mice, marked atherosclerotic lesion formation was observed in the proximal aorta. In the atherosclerotic lesion, superoxide production, the expression of NADPH oxidase subunits, and NADPH oxidase activity were enhanced, and the expressions of MCP-1, ICAM-1, and VCAM-1 were increased. These changes were suppressed in mice that were treated with amlodipine for 10 weeks concomitant with HCD administration, with no significant change in blood pressure and plasma cholesterol level. We also observed that treatment with amlodipine for only the last 2 weeks regressed the atherosclerotic lesions with a decrease in oxidative stress and vascular inflammation. Inhibition of the atherosclerotic lesion area and lipid area in the proximal aorta by amlodipine was correlated with its inhibitory actions on oxidative stress, inflammation and the production of adhesive molecules. These results suggest that amlodipine not only inhibits atherosclerotic lesion formation, but also regresses atherosclerosis, and that these effects are at least partly due to inhibition of oxidative stress and inflammatory response. (*Hypertens Res* 2006; 29: 457–466)

Key Words: **atherosclerosis, calcium channel blocker, inflammation, oxidative stress**

Introduction

L-type calcium channel blockers (CCBs) are widely used in the treatment of both hypertension and coronary heart disease (*1*, *2*). In the Prospective Randomized Evaluation of the Vascular Effects of Norvasc Trial (PREVENT), the CCB amlodipine reduced the rate of cardiac events and inhibited the progression of carotid artery atherosclerosis (*3*). Such vasoprotective effects of CCBs are mediated, at least in part, by an

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improvement of endothelial dysfunction, including the restoration of nitric oxide production. Zhang and Hintze reported that amlodipine increased nitric oxide generation in canine coronary vessels (*4*). However, the detailed mechanism of the inhibitory effects of CCBs on atherosclerosis formation is not entirely clear. Moreover, the question of whether CCBs might actually retard atherosclerosis has not been adequately investigated.

The production of reactive oxygen species (ROS; *e.g.*, superoxides, peroxynitrite, *etc.*) together with inflammatory factors such as chemokines, cytokines, and adhesion molecules has been shown to be increased in atherosclerotic lesions (*5, 6*). Nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase consists of plasma membrane subunits and cytosolic subunits and plays an important role in superoxide production. Rac-1 is a small guanosine triphosphate–binding protein of the Ras superfamily that is necessary, along with other components (*e.g.*, $p47^{phox}$, $p22^{phox}$, nox-1, nox-4, *etc.*) for activation of NADPH oxidase (*7, 8*). Moreover, rac-1 has been shown to mediate the shear-induced tyrosine phosphorylation of mitogen-activated protein kinase *via* regulation of the flow-dependent redox changes in endothelial cells under pathological conditions, such as endothelial dysfunction associated with atherosclerosis (*9*). Some CCBs decrease inflammation (*10*) and oxidative stress (*11*) in atherosclerosis. However, the anti-atherosclerotic action of amlodipine has not yet been well examined. Apolipoprotein E–deficient (ApoEKO) mice are commonly used to study the pathogenesis of human atherosclerosis (*12*). In this study, we used these mice to examine the effects of amlodipine on the progression and possible regression of atherosclerosis, with particular focus on the anti-inflammatory and anti-oxidative stress actions.

Methods

Animals and Treatment

Adult male ApoEKO mice (6 weeks of age; Jackson Laboratory, Bar Harbor, USA) received a standard normal diet (ND) (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) or high-cholesterol diet (HCD; 1.25% cholesterol, 10% coconut oil in MF) for 8 or 10 weeks and water ad libitum (*13*). Amlodipine (UK-48340; donated by Pfizer Inc., New York, USA) was administered daily by gavage at a dose of 3 mg/kg/day for 10 weeks. In some mice, amlodipine was administered for only the last 2 weeks of HCD administration. There were thus 5 groups: ND; HCD for 8 weeks; HCD for 10 weeks; HCD for 10 weeks + amlodipine for 2 weeks; and HCD for 10 weeks + amlodipine for 10 weeks. The plasma cholesterol concentration and systolic blood pressure (SBP) were measured as described previously (*13*). The Animal Studies Committee of Ehime University approved the experimental protocol of this study.

Table 1. Systolic Blood Pressure and Plasma Cholesterol Level in ApoEKO Mice

	SBP (mmHg)	Cholesterol (mg/dl)
ND	99.5±0.7	619.3±19.6
HCD for 8 weeks	100.4±1.5	1,153.3±47.5*
HCD for 10 weeks	100.5±1.4	1,147.1±42.0*
HCD for 10 weeks + Aml for 2 weeks	99.8±1.1	1,194.6±65.9*
HCD for 10 weeks + Aml for 10 weeks	98.7±1.0	1,216.0±49.8*

ApoEKO mice were treated with HCD and amlodipine was given as described in Methods. ApoEKO mice, apolipoprotein E–deficient mice; SBP, systolic blood pressure; ND, normal standard diet; HCD, high-cholesterol diet; Aml, amlodipine at 3 mg/kg/day. Values are the mean±SEM. $n=7$ for each group. *$p<0.05$ *vs.* the ND group.

Atherosclerotic Lesion Size

The area of atherosclerotic lesions in the proximal aorta and the lipid area in the aortic wall and the atherosclerotic lesions were determined by taking intermittent cross sections throughout a 2–3 mm length piece of the ascending aorta, followed by oil-red O staining and counterstaining with hematoxylin (*14*). Quantitative analysis was performed with Densitograph imaging software (ATTO Corporation, Tokyo, Japan). The mean value of 5 sections was taken as the value for each animal.

In Situ Detection of Superoxide Production

Superoxide production was detected in the proximal aorta using freshly frozen sections stained with dihydroethidium (10 μmol/l), and the intensity of fluorescence was measured as previously described (*15*).

NADPH Oxidase Activity

A tissue protein sample was prepared from the aorta after homogenization in 500 μl ice-cold Tris-sucrose buffer (*16*). NADPH oxidase activity was quantified by the cytochrome c method from the absorbance with or without superoxide dismutase (SOD), as previously described (*16*).

Real-Time Reverse-Transcription Polymerase Chain Reaction

Quantitative real-time reverse-transcription polymerase chain reaction (PCR) was performed using Premix Ex Taq™ (Takara Bio Inc., Shiga, Japan). The PCR primers for monocyte chemoattractant protein-1 (MCP-1) were 5′-TTAACG CCCCACTCACCTGCTG-3′ (forward) and 5′-GCT

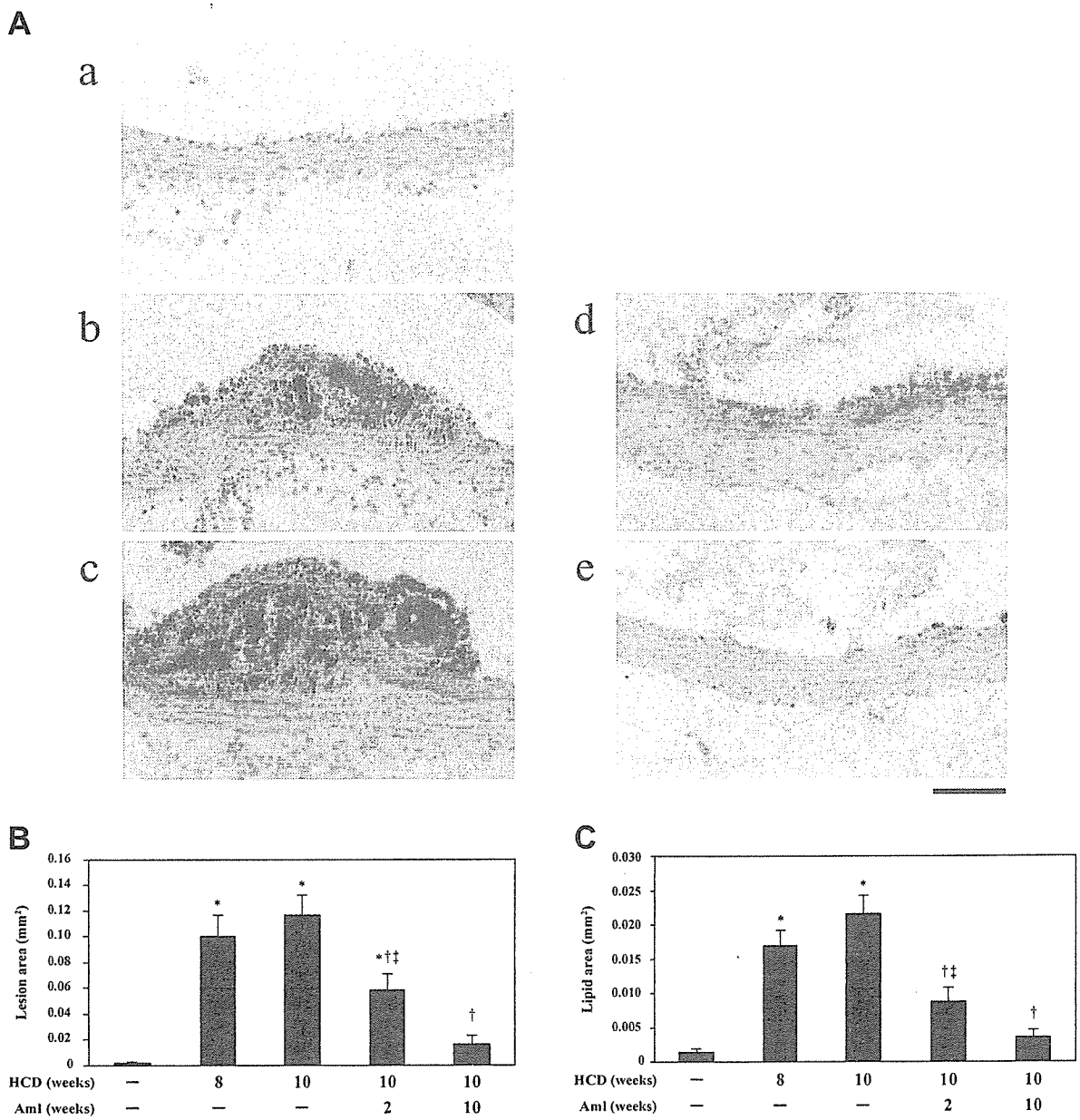

Fig. 1. *Atherosclerotic lesions of the proximal aorta from apolipoprotein E–deficient (ApoEKO) mice fed a high-cholesterol diet (HCD). A: Representative oil red O–stained cross-section of the proximal aorta of ApoEKO mice showing lipid deposition. a: Normal standard diet (ND); b: HCD for 8 weeks; c: HCD for 10 weeks; d: HCD for 10 weeks + 3 mg/kg/day amlodipine for the last 2 weeks; e: HCD for 10 weeks + 3 mg/kg/day amlodipine for 10 weeks. Magnification: ×200. The scale bar represents 100 μm. B: Morphometry of atherosclerotic area in cross-sections of the proximal aorta. C: Morphometry of lipid accumulation in cross-sections of the proximal aorta. The mean value of 5 sections was taken as the value for each animal. Aml, amlodipine (3 mg/kg/day). Values are the mean±SEM* (n=*7 or 8 per group).* *$p < 0.05$ vs. *ND,* †$p < 0.05$ vs. *HCD for 10 weeks,* ‡$p < 0.05$ vs. *HCD for 8 weeks.*

TCTTTGGGACACCTGCTGC-3′ (reverse); those for rac-1 were 5′-CCAGTGAATCTGGGCCTATG-3′ (forward) and 5′-ACAGTGGTGTCGCACTTCAG-3′ (reverse); those for $p47^{phox}$ were 5′-GTCCCTGCATCCTATCTGGA-3′ (forward) and 5′-GGGACATCTCGTCCTCTTCA-3′ (reverse); those for intercellular adhesion molecule-1 (ICAM-1) were

5′-GGCACCCAGCAGAAGTTGTT-3′ (forward) and 5′-CCTCAGTCACCTCTACCAAG-3′ (reverse); those for vascular cell adhesion molecule-1 (VCAM-1) were 5′-TCT CTCAGGAAATGCCACCC-3′ (forward) and 5′-CACAGC CAATAGCAGCACAC-3′ (reverse); and those for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were 5′-TGCGACTTCAACAGCAACTC-3′ (forward) and 5′-ATG TAGGCCATGAGGTCCAC -3′ (reverse).

Immunofluorescent Staining of p47^{phox} in Atherosclerotic Lesions

Immunofluorescence was assessed using freshly frozen sections that were incubated with anti-p47^{phox} antibody, washed and incubated with biotin-labeled secondary antibodies, and then incubated with Cy3-labeled streptavidin. Serial sections treated with secondary antibodies alone did not show specific staining. Samples were examined with a Zeiss Axioskop microscope equipped with a computer-based imaging system (*13*).

Data Analysis

All values are expressed as the mean±SEM. The data were analyzed using one-way ANOVA. If a statistically significant difference was found, Newman-Keuls' test was performed for post-hoc analysis to detect the differences among groups. Pearson's correlation coefficient was used to assess the correlation between the atherosclerotic lesion area or lipid area and each of the markers for oxidative stress, inflammation and adhesive molecules. Values of $p<0.05$ were considered statistically significant.

Results

Inhibitory Effect of Amlodipine on Atherosclerotic Lesion Formation in ApoEKO Mice Fed a High-Cholesterol Diet

In the preliminary experiments, we administered amlodipine at a dose of 0.5 or 3.0 mg/kg/day for 10 weeks. Neither dose affected the SBP in ApoEKO mice. However, a dose of 3.0 mg/kg/day but not 0.5 mg/kg/day inhibited atherosclerotic lesion formation. Therefore, in the following experiments, we used amlodipine at 3.0 mg/kg/day. Plasma cholesterol levels in ApoEKO mice were markedly increased after administration of the high-cholesterol diet ($p<0.05$ *vs.* the ND group; Table 1). After 8 or 10 weeks of the HCD diet, atherosclerotic lesion formation with lipid accumulation was observed in the proximal aortas of ApoEKO mice (Fig. 1). Administration of amlodipine (3 mg/kg/day) concomitant with HCD for 10 weeks markedly inhibited atherosclerotic lesion formation in ApoEKO mice (Fig. 1), but did not affect the SBP or plasma cholesterol level (Table 1). Interestingly, even when amlodipine was administered for only the last 2 weeks of the 10-week HCD, the atherosclerotic lesions that had already formed were significantly reduced ($p<0.05$ *vs.* HCD alone for 8 or 10 weeks, respectively; Fig. 1).

Effect of Amlodipine on Oxidative Stress and Inflammatory Response

Figure 2A shows the results of the *in situ* detection of superoxide production by dihydroethidium in the proximal aorta. The measured fluorescence intensities are shown in Fig. 2B. Superoxide production was increased by HCD treatment for 8 or 10 weeks (Fig. 2A, B). We also observed an increase in NADPH oxidase activity in atherosclerotic lesions by HCD feeding (Fig. 2C). Expression of p47^{phox}, a subunit of NADPH oxidase, detected by immunofluorescence was also increased in atherosclerotic lesions and the aortic wall in the proximal aorta after HCD treatment (Fig. 3). The expressions of the mRNAs of p47^{phox} and rac-1 were also increased in the atherosclerotic aorta (Fig. 4A, B). Treatment with amlodipine for 10 weeks inhibited the increase in superoxide production, NADPH oxidase activity, and expression of p47^{phox} and rac-1 in atherosclerotic lesions. Moreover, treatment with amlodipine for only the last 2 weeks of HCD feeding also decreased superoxide production, NADPH oxidase activity, and expression of p47^{phox} and rac-1 in atherosclerotic lesions.

In addition, the expressions of MCP-1, ICAM-1, and VCAM-1 were increased by HCD treatment (Fig. 4C–E). Administration of amlodipine for 10 weeks inhibited these changes. Amlodipine also decreased the increased expression of MCP-1, ICAM-1, and VCAM-1 when it was administered for only the last 2 weeks of the 10-week HCD feeding (Fig. 4C–E). Moreover, as shown in Table 2, three of the groups, *i.e.*, the ND, 10-week HCD, and 10-week HCD + 10-week amlodipine groups, showed a significant correlation between both the atherosclerotic lesion area and the lipid area in the proximal aorta and each of superoxide production and p47^{phox}, rac-1, MCP-1, ICAM-1 and VCAM-1 expression.

Discussion

We have demonstrated that simultaneous administration of amlodipine with HCD feeding for 10 weeks inhibited atherosclerotic lesion formation in ApoEKO mice, without affecting the SBP or plasma cholesterol level. Moreover, even when amlodipine was administered for only the last 2 weeks of the 10-week HCD, the size of the atherosclerotic lesions was significantly reduced. These inhibitory actions of amlodipine were accompanied by inhibitions of oxidative stress and the inflammatory response. It appears that this type of inhibitory action on atherosclerosis is not specific to amlodipine, since similar effects have been observed in experimental studies with the CCBs azelnidipine (*11*) and lacinidipine (*17*, *18*), as well as in a clinical study with these agents (*19*).

Previous papers reported that atherosclerotic lesions were formed in ApoEKO mice given a regular (normal) diet for a

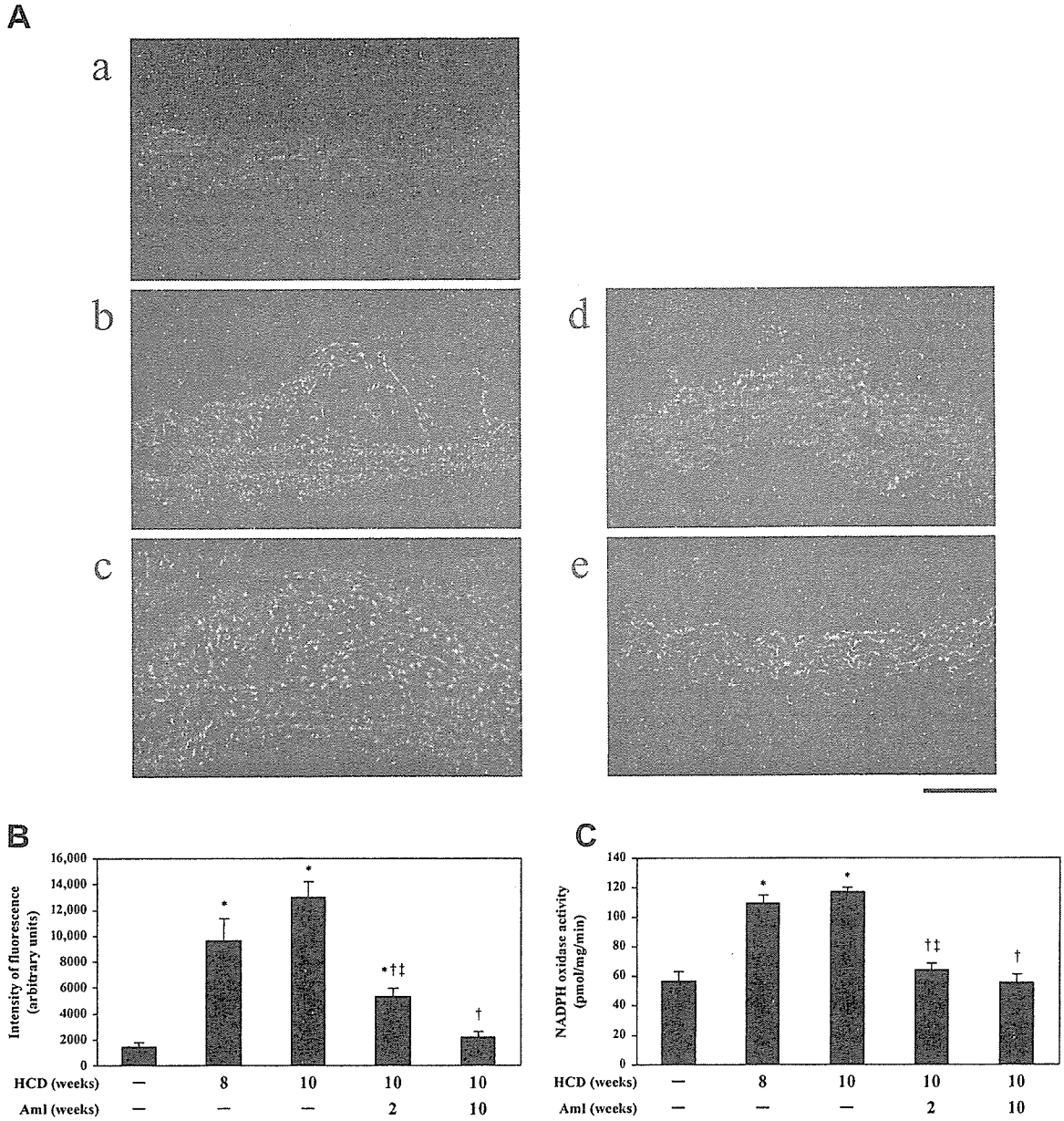

Fig. 2. In situ *detection of superoxide production in the proximal aorta of apolipoprotein E–deficient (ApoEKO) mice treated with high-cholesterol diet (HCD). Animals were treated and aortic samples were taken as described in Fig. 1. A: Representative detection of superoxide production with dihydroethidium staining in cross-sections of the proximal aorta of ApoEKO mice. a: Normal standard diet (ND); b: HCD for 8 weeks; c: HCD for 10 weeks; d: HCD for 10 weeks + 3 mg/kg/day amlodipine for the last 2 weeks; e: HCD for 10 weeks + 3 mg/kg/day amlodipine for 10 weeks. Magnification: ×200. The scale bar represents 100 μm. B: Measurement of fluorescence intensity for superoxide production. The mean value of 3 sections was taken as the value for each animal. Values are the mean±SEM for measurement of intensity (n=6 per group). C: NADPH oxidase activity in the atherosclerotic aorta of ApoEKO mice treated with HCD. Aml, amlodipine (3 mg/kg/day); NADPH, nicotinamide-adenine dinucleotide phosphate. Values are the mean±SEM (n=6 or 7 per group).* $^{*}p<0.05$ *vs. ND,* $^{\dagger}p<0.05$ *vs. HCD for 10 weeks,* $^{\ddagger}p<0.05$ *vs. HCD for 8 weeks.*

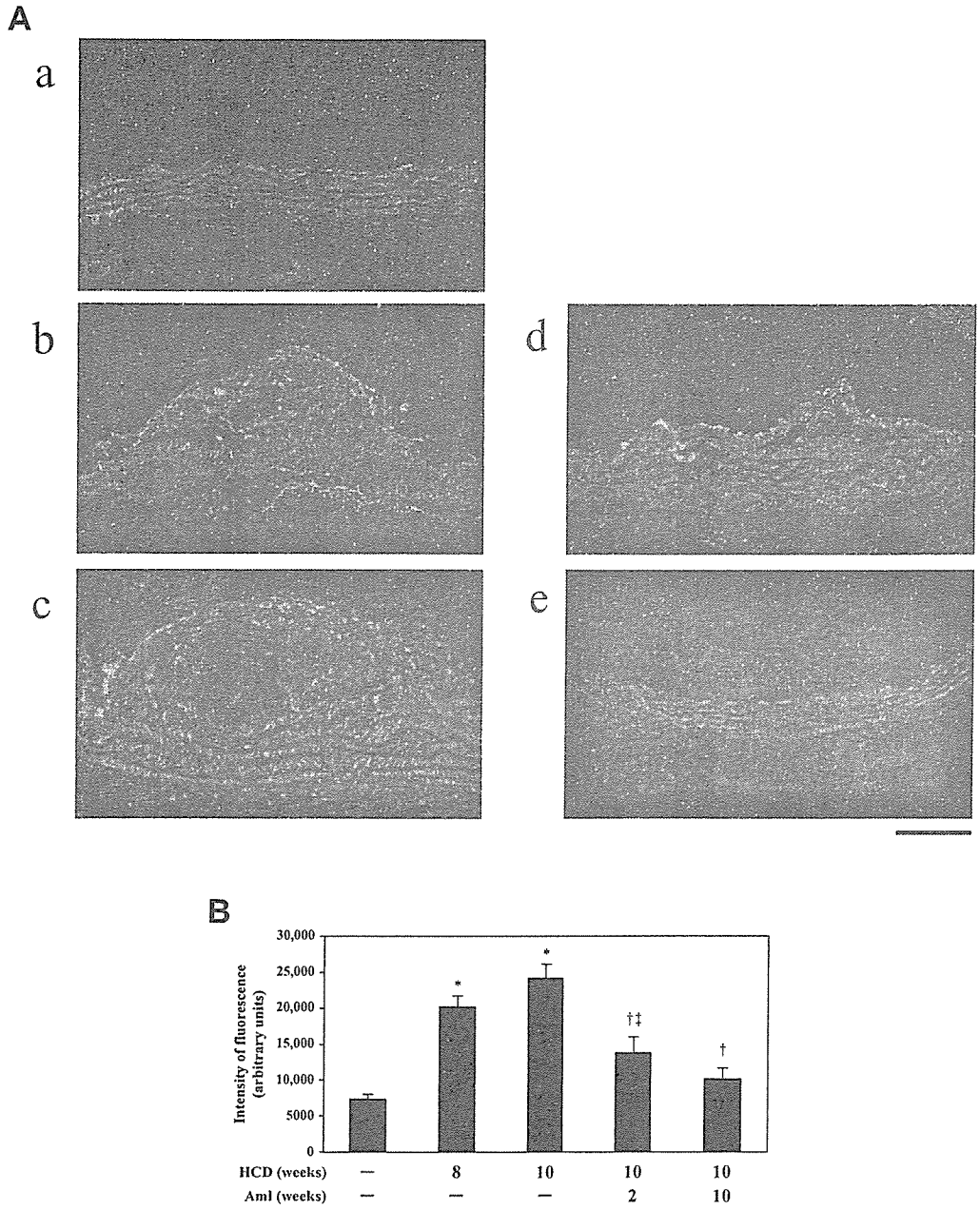

Fig. 3. *Immunohistochemical staining of p47^{phox} in the proximal aorta of apolipoprotein E–deficient (ApoEKO) mice treated with high-cholesterol diet (HCD). Animals were treated and aortic samples were taken as described in Fig. 1. A: Representative immunostaining of p47^{phox} in cross-sections of the proximal aorta of ApoEKO mice. a: Normal standard diet (ND); b: HCD for 8 weeks; c: HCD for 10 weeks; d: HCD for 10 weeks + 3 mg/kg/day amlodipine for the last 2 weeks; e: HCD for 10 weeks + 3 mg/kg/day amlodipine for 10 weeks. Magnification: ×200. The scale bar represents 100 μm. B: Measurement of fluorescence intensity for p47^{phox}. The mean value of 3 sections was taken as the value for each animal. Aml, amlodipine (3 mg/kg/day). Values are the mean±SEM for measurement of intensity (n=6 per group).* *$^{*}p<0.05$ vs. ND, $^{\dagger}p<0.05$ vs. HCD for 10 weeks, $^{\ddagger}p<0.05$ vs. HCD for 8 weeks.*

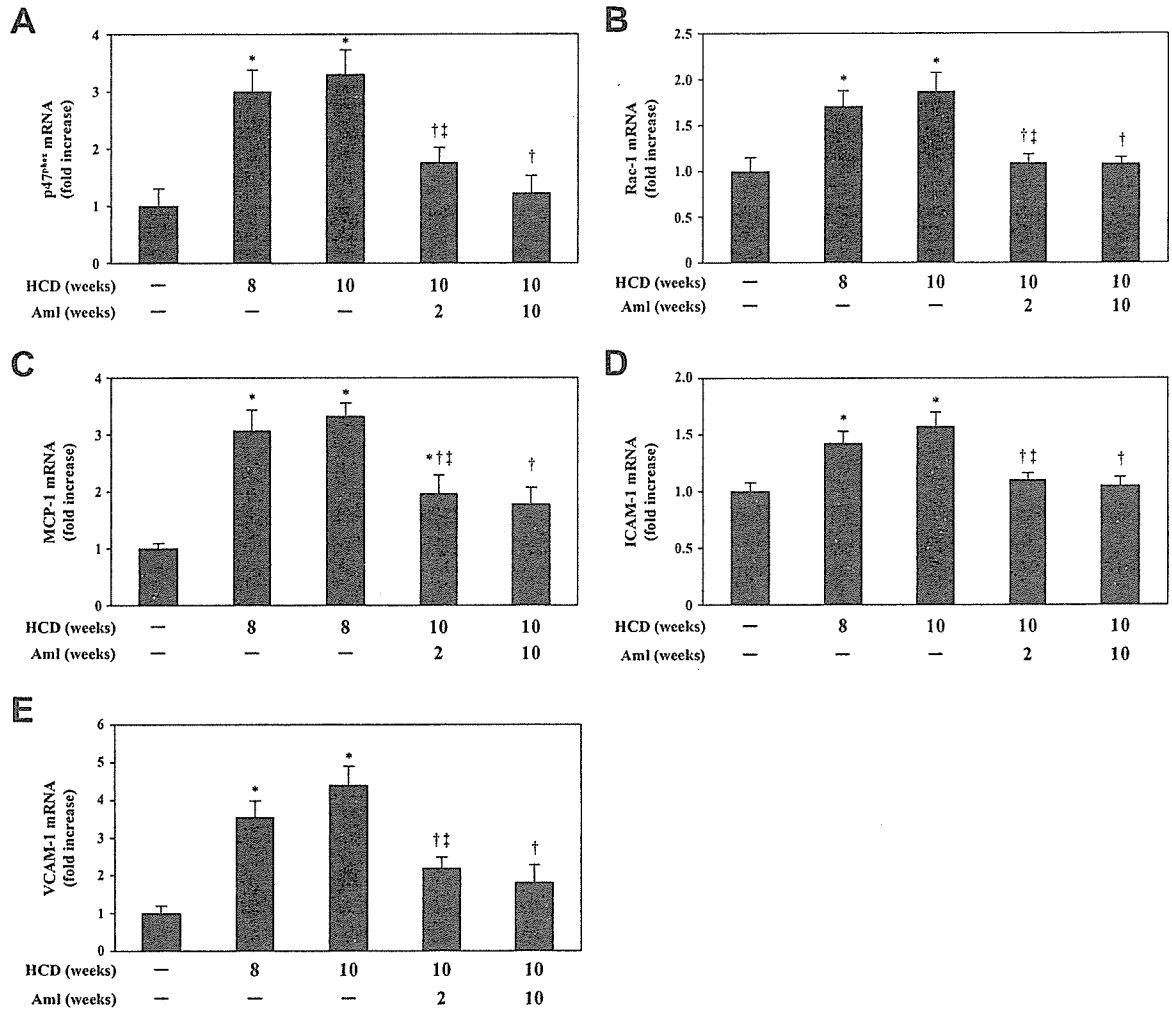

Fig. 4. *Expression of p47^{phox}, rac-1, MCP-1, ICAM-1 and VCAM-1 in the atherosclerotic aorta of apolipoprotein E–deficient (ApoEKO) mice treated with high-cholesterol diet (HCD). Aortic samples were taken from ApoEKO mice as described in the Methods section. Levels of mRNA for p47^{phox} (A), rac-1 (B), MCP-1 (C), ICAM-1 (D) and VCAM-1 (E) were assayed by quantitative real-time reverse-transcription polymerase chain reaction as described in the Methods section. Values are the mean±SEM (n=6 to 8 per group).* *$^{*}p<0.05$ vs. ND, $^{\dagger}p<0.05$ vs. HCD for 10 weeks, $^{\ddagger}p<0.05$ vs. HCD for 8 weeks. ND, normal standard diet; Aml, amlodipine (3 mg/kg/day); MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.*

longer period, like 20 weeks (*20*) or 30 weeks (*21*). However, in our study, the atherosclerotic lesions were not significantly formed in ApoEKO mice given a regular diet for 10 weeks, and it was not examined whether similar inhibitory changes by amlodipine are observed in ApoEKO mice treated with regular diet for a longer feeding period. Other research groups also used high-cholesterol diet or Western-style diet to ApoEKO mice to make atherosclerotic lesions, and they observed no apparent atherosclerosis with regular diet in ApoEKO mice (*22*). The reason for such a discrepancy of the atherosclerotic lesion formation in ApoEKO mice by regular diet is not yet clear, however, the discrepancy may depend on 1) the difference of nutritional component of regular (normal) diet from different commercial sources, and/or 2) the different feeding period of regular diet in ApoEKO mice.

There are controversial findings in regard to the effect of amlodipine on atherosclerosis. Candido *et al.* (*20*) reported that amlodipine did not significantly reduce atherosclerosis even at a hypotensive dose (6 mg/kg/day for 20 weeks) in diabetic ApoEKO mice. They reported that in the ApoEKO mice treated with streptozotocin, the vascular renin-angiotensin system plays a critical role in mediating acceleration of ath-

Table 2. Correlation between Atherosclerotic Lesion Area, Lipid Area and the Markers for Oxidative Stress and Inflammation and Adhesive Molecules

	Correlation coefficient	
	Lesion area	Lipid area
Superoxide production	0.972‡	0.913‡
$p47^{phox}$ mRNA expression	0.715†	0.623*
Rac-1 mRNA expression	0.877‡	0.871‡
MCP-1 mRNA expression	0.818‡	0.732†
ICAM-1 mRNA expression	0.886‡	0.874‡
VCAM-1 mRNA expression	0.867‡	0.868‡

MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1. *$p<0.01$,†$p< 0.001$,‡$p<0.0001$.

erosclerosis, and this may explain why irbesartan was superior to amlodipine in reducing the diabetes-associated acceleration of atherosclerosis. Takai *et al.* (*23*) reported that amlodipine tended to decrease the atherosclerotic area in monkeys fed a high-cholesterol diet, although this effect was not statistically significant. In contrast, others have reported that amlodipine inhibited atherosclerosis in non-diabetic animal models of atherosclerosis (*24*, *25*). These apparently conflicting results may be partly related to the different animal models (diabetic or non-diabetic ApoEKO mice) or different species (mice or monkeys) used.

The results of a clinical trial, the CAMELOT study, showed that amlodipine tended to inhibit the progression of plaque formation in the coronary arteries (*26*). This result, like some of those described above, suggests that amlodipine has an inhibitory effect on atherosclerosis. Moreover, our results suggest that amlodipine not only inhibits atherosclerosis, but also induces regression of atherosclerosis. These inhibitory actions of amlodipine seem to be associated with inhibition of inflammatory responses and oxidative stress. Amlodipine has also been shown to be effective in improving left ventricular hypertrophy by controlling blood pressure (*27*). Inflammation plays an important role in atherosclerosis formation (*5*, *28*). In our study, the expressions of MCP-1, ICAM-1, and VCAM-1 were increased by HCD treatment (Fig. 4C–E). Oxidative stress is also an important factor in inflammatory responses (*7*, *29*). Previous reports indicated that markers of oxidative stress, such as NADPH oxidase activity, were increased in atherosclerotic lesions (*8*, *21*). NADPH oxidase consists of subunits of membrane components (Nox-1, Nox-4, and $p22^{phox}$) and cytosolic components ($p47^{phox}$ and rac-1, *etc.*). In our study, superoxide production, NADPH oxidase activity, and the expression of the cytosolic components $p47^{phox}$ and rac-1 were markedly increased in atherosclerotic lesions (Figs. 2, 3, and 4A, B). Li *et al.* reported that both calcium influx through receptor-regulated channels and mobilization of intracellular calcium were crucial events for oxidation of lipids in monocytes, a superoxide anion–dependent event (*30*). In our study, amlodipine inhibited the expression of MCP-1, ICAM-1 and VCAM-1, and oxidative stress, such as that related to superoxide production and NADPH oxidase activation, when administered for only the last 2 weeks as well as for all 10 weeks of HCD feeding (Figs. 2, 4C–E).

There was no significant difference in NADPH oxidase activity and the expressions of $p47^{phox}$ fluorescence, rac-1, MCP-1, ICAM-1, or VCAM-1 between the 2-week amlodipine treatment group and 10-week amlodipine treatment group (Figs. 3B, 4B–E). These results seem to be discrepant from those that the inhibition of atherosclerotic lesions, lipid deposition, and superoxide production by amlodipine were stronger in the 10-week amlodipine group than in the 2-week amlodipine group (Figs. 1B, C and 2B). In a previous report, amlodipine was found to preserve SOD activity and reduce the oxidizability of low density lipoproteins (*31*). It has also been reported that amlodipine reduces oxidative stress by restoring copper/zinc-containing SOD activity in the heart in hypertensive rats (*32*). Moreover, amlodipine has been shown to increase nitric oxide (NO) production *via* stimulation of the production of endothelial nitric oxide synthase (NOS) (*33*, *34*) and to inhibit the infiltration of monocytes/macrophages into atherosclerotic lesions (*24*) and the expression of collagens type I, III, and IV (*35*). In the present study, these anti-atherosclerotic actions of amlodipine may have played a role in the changes in atherosclerotic lesions, lipid deposition and superoxide production in the groups administered amlodipine for 2 weeks or 10 weeks.

The mechanism by which amlodipine regresses atherosclerotic lesions is not yet clear. In the present study, administration of amlodipine for the last 2 weeks of the 10-week HCD period reduced the expression of ICAM-1 and VCAM-1, suggesting that amlodipine inhibited the infiltration of monocytes/macrophages into the atherosclerotic lesions. It has been reported that amlodipine decreased the DNA synthesis induced by basic fibroblast growth factor through inhibition of extracellular signal regulated kinase (ERK) activity in human vascular smooth muscle cells (VSMCs) (*36*). It has recently been reported that macrophage apoptosis is associated with diminished lesion cellularity and decreased lesion progression (*37*), and that amlodipine regressed aortic wall hypertrophy in spontaneous hypertensive rats through enhanced apoptosis (*38*), suggesting that apoptosis is involved in the regulation of atherosclerosis. These results suggest that both the inhibition of VSMC proliferation and the enhancement of apoptosis might be involved in the regression of atherosclerotic lesions by amlodipine.

Moreover, a significant correlation was observed between both the atherosclerotic lesion area and the lipid area in the proximal aorta and each of superoxide production and $p47^{phox}$, rac-1, MCP-1, ICAM-1 and VCAM-1 expression in three experimental groups, *i.e.*, the ND, 10-week HCD, and 10-week HCD + 10-week amlodipine (Table 2). These results also suggest that the inhibitory actions of amlodipine on oxidative stress, inflammation and the production of adhesive