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ヒトES細胞を用いたin vitro血管神経細胞分化システムによる「虚血脳再生ホルモン」の探索とホルモン補償による新規認知症治療法の開発に関する研究

平成18～20年度　総合研究報告書

研究代表者 慶應義塾大学医学部腎臓内分泌代謝内科 伊藤 裕

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総合研究報告書

[ヒトES細胞を用いたin vitro血管神経細胞分化ｼｽﾃﾑによる「虚血脳再生ホルモン」の探索とホルモン補償による新規認知症治療法の開発]

研究代表者　伊藤 裕　慶應義塾大学 医学部 腎臓内分泌代謝内科 教授

研究要旨　認知症はきわめて深刻な医学的問題であるが、これまで根治的治療法は開発されておらず、最先端医学の結集による新規治療法の開発に期待が寄せられている。そこで本研究ではホルモン補償による認知症治療法の開発と、ヒトES細胞由来血管-神経前駆細胞の虚血脳再生治療への応用に向けた検討を推進した。その結果、我々が虚血脳再生作用を見出した血管ホルモンであるアドレノメデュリンが、血管再生ならびに神経再生を促進する多彩な機構が明らかとなり、また、ヒト血管性認知症を模倣する新規マウスモデルにおいても、アドレノメデュリンが有効であることが示された。ヒトES細胞由来血管前駆細胞の同定ならびにヒトES細胞から神経系各系統への分化誘導法の開発に成功するとともに、神経前駆細胞移植による虚血脳治療の可能性を明らかにした。また、血管前駆細胞の減少が心血管系疾患発症と相関することを見出した。これらの知見は、内分泌学，神経内科学，再生医学の知識と手法を集学的に結集することで得られた、われわれ独自の発想に基づく成果である。

A. 研究目的

認知症は極めて深刻な医学的問題であるが、これまで根治的治療法は開発されておらず、最先端医学の結集による新規治療法の開発に期待が寄せられている。

我々はこれまで血管ホルモンの臨床的意義について研究を続け、血管拡張ホルモンであるナトリウム利尿ペプチド(NP)やアドレノメデュリン(AM)が血管トーヌスを制御するのみならず、血管再生を促進することを明らかにした。また脳梗塞後に発現が著増する血管拡張ペプチドであるAMの病態生理学的意義について検討を続け、AMが虚血脳において血管再生作用と神経再生作用を発揮することを明らかにし、AMを虚血脳保護再生ホルモンと位置付けた。また胚性幹細胞(ES細胞)の再生医療への応用を目指し、血管内皮細胞と血管平滑筋細胞に分化しうるサルES細胞由来"血管前駆細胞(VPC)"を同定した。

これらの経験を踏まえ本研究では、内分泌学，神経内科学，再生医学の知識を集学的に結集し、ホルモン補償による新規認知症治療法の開発、ヒトES細胞から血管，神経細胞への分化誘導法の開発およびその血管，神経細胞分化システムにおける分化誘導能を薬効の指標とした新規虚血脳再生ホルモンの探索、血管神経前駆細胞の心血管系疾患発症ならびに虚血脳再生への関与の解明を目指した研究を推進した。

B. 研究方法

研究代表者伊藤は、虚血脳再生ホルモンの認知症患者への治療応用に関する研究を行い、AMが虚血脳を再生する機構を多面的に検討した。スピロノラクトンを用いてAMの下流エフェクターとしての意義が注目されているアルドステロンを阻害することによりマウスに20分間中大脳動脈閉塞術を施行して、アルドステロン抑制を介したAMの作用を検討した。また、ヒトES細胞由来血管前駆細胞(VPC)の同定と、分化動態の検討を行い、VPCから分化誘導させた血管細胞の各成分（内皮細胞、血管平滑筋細胞）をそれぞれ単独ないしは混合して、大腿動脈結紮閉塞性動脈硬化症モデルマウスに移植し、移植細胞の生着ならびに血管再生効果を検討した。マウスES細胞由来血管前駆細胞(VPC)から内皮細胞への誘導過程でAMを添加し、分化挙動を解析した。

分担研究者高橋は、ヒト血管性認知症を模倣する新規脳虚血モデルの開発を試み、虚血脳再生ホルモン投与による、神経機能，認知機能改善効果の検証を行った。両側頚動脈狭窄術(bilateral carotid artery stenosis: BCAS)を施行した慢性脳低灌流モデルマウスにおける白質病変進展の検討を行い、認知能の検討ならびにレーザードップラー血流測定、組織学的・行動学的評価を行った。BCASを施行したマウスにおいてアドレノメデュリンならびにマトリックスメタロプロテアーゼ(MMP)阻害薬を投与し、白質保護効果ならびに認知症発症への関与を検討した。

分担研究者吉政は、メタボリックシンドローム患者における認知機能障害の解析と血中血管神経前駆細胞動態の解析を行った。メタボリックシンドローム患者において末梢血中CD34陽性血管内皮前駆細胞数を測定して、心血管障害に関連するパラメーターとの相関を検討した。

分担研究者山原は、血管ホルモン投与による虚血性疾患治療法の開発と血管前駆細胞の治療応用に関する検討を行った。肺気腫モデルマウスや、局所循環障害を病態基盤とするリンパ浮腫モデルマウスを用いてAMの組織再生作用を多面的に検討した。

分担研究者近藤は、ヒトES細胞由来神経前駆細胞の同定ならびに神経系各系統への誘導法の開発とin vitro血管神経細胞分化誘導系を用いた新規虚血脳再生薬剤の探索、さらには得られた神経前駆細胞のサル大脳局所への移植法の開発を行った。

C. 研究結果

研究代表者伊藤は、スピロノラクトン投与マウスに20分間大脳動脈閉塞術を施行すると、虚血脳再生因

子である bFGF や VEGF の発現が増加して、神経再生および血管再生が促進し、梗塞域が縮小することを見出した(図 1)。このことより、AM によるアルドステロン産生抑制が、AM の虚血脳保護再生機構の一つであることを明らかにした。ヒト ES 細胞由来 VEGF-R2 陽性 TRA-1 陰性細胞がヒト ES 細胞由来 VPC であることを示し、また大腿動脈結紮下肢虚血マウスへの局所細胞移植において、ヒト ES 細胞由来 VPC を内皮細胞、壁細胞へ分化させ両者を混合移植すると、それらを単独で移植するよりも良好な血管再生効果を得ることを見出した(図 2)。さらには ES 細胞由来 VPC に AM を添加することで動脈内皮へ効率的に分化することを明らかにした。これらの知見より、ヒト ES 細胞由血管前駆細胞の細胞移植治療への応用可能性と、血管再生治療における AM の有用性が示された。

分担研究者高橋は、皮質下血管性認知症患者の脳組織を非神経疾患、アルツハイマー病患者と病理学的に比較し、血管性認知症患者における白質血管構築の異常を見出した。またマウスにコイルを用いて両側頚動脈狭窄術(BCAS)を施行すると、術後一ヶ月で血管性認知症患者と類似した白質病変が生じて、短期記憶が選択的に傷害されることを明らかにした(図 3)。MMP-2 遺伝子欠損ノックアウトマウスや MMP 阻害薬投与マウスを BCAS により慢性脳低還流状態にしたところ、血液脳関門障害と白質病変の改善が認められた。したがって血管性認知症患者における白質病変の形成に、MMP による血液脳関門障害が関与する可能性が考えられた。AM 過剰発現トランスジェニックマウスに、BCAS を施行すると、大脳局所で早期の血流回復とグリオーシスの有意な抑制を認めた。このことより、慢性脳低灌流によって生ずる白質病変に対して AM が保護再生効果を発揮する可能性が示された。

分担研究者吉政は、メタボリックシンドローム(MS)患者において、末梢血中 CD34 陽性血管前駆細胞(EPC)数が内臓脂肪面積と負の相関を示して減少し、慢性腎臓病発症に関与する可能性を示した。また糖尿病患者において末梢血中 EPC 数を測定し、心血管障害に関与するパラメーターとの関連を調べたところ、EPC 減少と相関して左室肥大が増悪することを見出した。これらの知見より、末梢血中 CD34 陽性血管前駆細胞数の減少が、心血管系疾患発症に関与する可能性が明らかにされた。

分担研究者山原は、AM の組織再生能を多面的に検討し、肺気腫モデルマウスへの AM 投与により骨髄から Sca-1 陽性細胞が動員され、内皮あるいは肺上皮細胞マーカー陽性細胞に分化し、肺気腫を修復することを明らかにした。褥瘡モデルマウスへの AM 投与により、AM が血管再生作用を発揮して創傷治癒を促進することを見出した。また AM がリンパ管内皮細胞において炎症惹起性細胞接着分子発現を抑制することで抗炎症作用を発揮し、リンパ浮腫を軽減することを見出した。

分担研究者近藤は、マウス線維芽細胞上で維持培養されたサル ES 細胞を bFGF, EGF 添加条件下で接着培養を行うと神経幹細胞が得られることを見出し、ニューロン、アストロサイト、オリゴデンドロサイト各細胞系への分化法を確立した。また、サル ES 細胞由来神経幹細胞を脳線条体へ局所移植すると移植した細胞が生着し、神経系各細胞へ分化することを明らかにした(図 4)。さらには、ヒト ES 細胞からも同様の手法で神経幹細胞の同定ならびに神経幹細胞増殖継代法の開発に成功した。また、ヒト ES 細胞由来神経幹細胞から神経系各細胞への分化誘導が可能であることを明らかにし、薬剤の薬効ならびに副作用を簡便かつ短時間でスクリーニングに応用可能なヒト ES 細胞由来神経細胞分化システムを確立した。

D. 考察

AM が虚血脳再生を促進する多彩な機構を明らかにした。ヒト血管性認知症を模倣する新規マウス認知症モデル BCAS を開発し、中大脳動脈閉塞による急性梗塞巣のみならず、大脳慢性低還流による白質病変においても AM が神経保護作用を発揮することを見出した。ヒト ES 細胞から神経系各系統への分化誘導法の開発に成功するとともに、血管前駆細胞ないしは神経前駆細胞移植による虚血脳治療の可能性を明らかにした。また、血管前駆細胞の減少が心血管系疾患発症と相関することを見出した。

E. 結論

内分泌学, 神経内科学, 再生医学の知識ならびに手法を有機的に組み合わせることで、独自の発想による新規認知症治療法開発を推進することができた。今後とも AM を用いた虚血脳再生療法の臨床応用に向けた活動ならびに、ES 細胞から神経細胞への in vitro 分化システムを用いた虚血脳再生ホルモンの探索を継続する。

F. 研究発表

1. 論文発表

研究成果の刊行に関する一覧表 参照

G. 知的所有権の取得状況

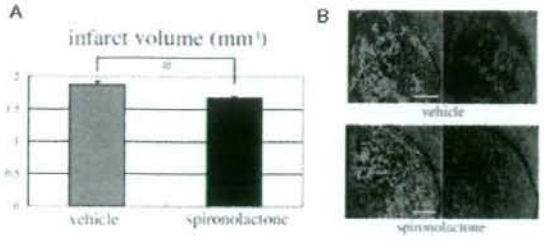
1. 特許取得

[高橋]

近藤科江、田中正太郎、藤田祐之、猪原匡史、冨本秀和、平岡眞寛. 発明名称「生体光イメージングプローブ」、特願 2008-251351 号 (出願日 : H20. 9. 29)、整理番号 A8864 (国際特許)

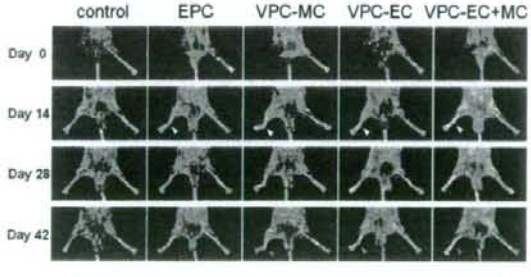
(図 1) スピロノラクトン投与マウスにおける梗塞域の縮小

スピロノラクトンでアルドステロン作用を阻害したマウスに中大脳動脈 20 分閉塞脳梗塞モデルを施行したところ、梗塞域の縮小を認めた。A. 梗塞域の大きさ　B. 虚血線状体免疫染色 青:ニューロンマーカー NeuN 緑:グリアマーカーGFAP



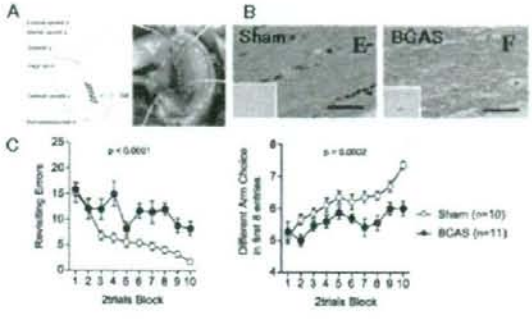
(図 2) ヒト ES 細胞由来内皮細胞と壁細胞の混合移植による良好な血管再生効果

大腿動脈結紮下肢虚血マウスへの局所細胞移植において、ヒト ES 細胞由来血管前駆細胞 (VPC) を内皮細胞 (VPC-EC)、壁細胞 (VPC-MC) へ分化させ両者を混合移植すると、それらを単独で移植するよりも良好な血管再生効果を得られた。EPC:末梢血由来内皮前駆細胞



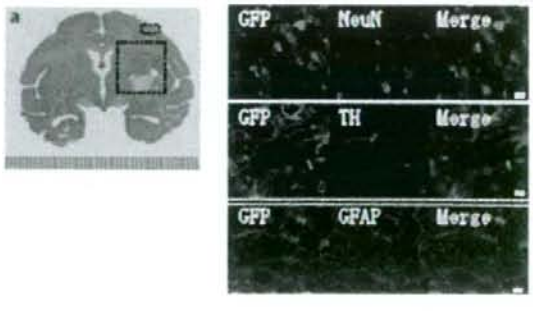
(図 3) マイクロコイルを用いた両側頸動脈狭窄術 (BCAS) による短期記憶の選択的障害

マウスにコイルを用いて BCAS を施行すると、術後一ヶ月で血管性認知症患者と類似した白質病変が生じて、短期記憶が選択的に傷害された。　A. BCAS の方法　B. BCAS 後の白質細胞の脱落　C. BCAS 後の短期記憶障害



(図 4) カニクイザル ES 細胞由来神経幹細胞のサル大脳局所での生着と神経 3 系統への分化

サル ES 細胞を bFGF, EGF 添加条件下で接着培養を行うと神経幹細胞が得られることを見出した。その神経幹細胞を脳線条体へ局所移植すると、移植した細胞が生着し、神経系各細胞へと分化した。NeuN: ニューロンマーカー　TH: ドーパミン産生細胞マーカー　GFAP: グリアマーカー



研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Mitsuishi M, Miyashita K, Muraki A, Itoh H.	Angiotensin II Reduces Mitochondrial Content in Skeletal Muscle and Affects Glycemic Control.	Diabetes.	58	710-717	2009
Ishiguro K, Hayashi K, Sasamura H, Sakamaki Y, Itoh H.	"Pulse" treatment with high-dose angiotensin blocker reverses renal arteriolar hypertrophy and regresses hypertension.	Hypertension.	53	83-89	2009
Iigaya K, Kumagai H, Nabika T, Harada Y, Onimaru H, Oshima N, Takimoto C, Kamayachi T, Saruta T, Itoh H.	Relation of blood pressure quantitative trait locus on rat chromosome 1 to hyperactivity of rostral ventrolateral medulla.	Hypertension.	53	42-48	2009
Oyamada N, Sone M, Miyashita K, Park K, Taura D, Inuzuka M, Sonoyama T, Tsujimoto H, Fukunaga Y, Tamura N, Itoh H, Nakao K.	The role of mineralocorticoid receptor expression in brain remodeling after cerebral ischemia.	Endocrinology.	149	3764-3777	2008
Mitsuishi M, Miyashita K, Itoh H.	cGMP rescues mitochondrial dysfunction induced by glucose and insulin in myocytes.	Biochem Biophys Res Commun	367	840-845	2008
Park K, Itoh H, Yamahara K, Sone M, Miyashita K, Oyamada N, Sawada N, Taura D, Inuzuka M, Sonoyama T, Tsujimoto H, Fukunaga Y, Tamura N, Nakao K.	Therapeutic potential of atrial natriuretic peptide administration on peripheral arterial diseases.	Endocrinology	149	483-491	2008
Yamahara K, Sone M, H. Itoh, Yamashita J, Yurugi-Kobayashi T, Homma K, Ting-Hsing Chao, Miyashita K, Kwijun Park, Oyamada N, Sawada N, Taura D, Fukunaga Y, Tamura N, Nakao K.	Augmentarion of Nevascularization in Hindlimb Ischemia by Combined Transplantation of Human Embryonic Stem Cells-derived Endothelial and Mural Cells.	PLoS ONE	3	e1666	2008
Sone M, H. Itoh, Yamahara K, Yamashita J, Yurugi-Kobayashi T, Nonoguchi A, Suzuki Y, Ting-Hsing Chao, Sawada N, Fukunaga Y, Miyashita K, Kwijun Park, Oyamada N, Sawada N, Taura D, Tamura N, Kondo Y, Nito S, Suemori H, Nakatsuji N, Nishikawa S, Nakao K.	Pathway for Differentiation of Human Embryionic Stem Cells to Vascular Cell Components and Their Potential for Vascular Regeneration.	Arterioscler.Thromb.Vasc.Biol.	27	2127-2134	2007
Hasegawa K, Wakino S, Tatematsu S, Yoshioka K, Homma K, Sugano N, Kimoto M, Hayashi K, H. Itoh	Role of Asymmetric Dimethylarginine in Vascular Injury in Transgenic Mice Overexpressing Dimethylarginie, Dimethylaminohydrolase 2.	Circ.Res.	101	e2-e10	2007
Sakoda M, Ichihara A, Kaneshiro Y, Takemitsu T, Nakazato Y, Nabi AH, Nakagawa T, Suzuki F, Inagami T, Itoh H.	(Pro)renin receptor-mediated activation of mitogen-activated protein kinases in human vascular smooth muscle cells.	Hypertens Res.	30	1139-1146	2007
Yurugi-Kobayashi T, Itoh H, Schroeder T, Nakano A, Narazaki G, Kita F, Yanagi K, Hiraoka-Kanie M, Inoue E, Ara T, Nagasawa T, Just U, Nakao K, Nishikawa S, Yamashita JK.	Adrenomedullin/cyclic AMP pathway induces Notch activation and differentiation of arterial endothelial cells from vascular progenitors.	Arterioscler Thromb Vasc Biol.	26	1977-1984	2007

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
K. Miyashita, H. Itoh, H. Arai, T. Suganami, N. Sawada, Y. Fukunaga, M. Sone, K. Yamahara, T. Yurugi-Kobayashi, K. Park, N. Oyamada, N. Sawada, D. Taura, H .Tsujimoto, T-H. Chao, N. Tamura, M. Mukoyama, K. Nakao	The neuroprotective and vasculo-neuro- regenerative roles of adrenomedullin in ischemic brain and its therapeutic potential.	Endocrinology	147	1642-1653	2006
Yamamoto Y, Ihara M, Oakley AE, Roger L, Slade JY, Polvikoski TM, Moss T, Kalaria RN.	Neuropathological Correlates of Temporal Pole White Matter Hyperintensities in CADASIL	Stroke		in Press	2009
Tomimoto H, Ihara M, Takahashi R, Fukuyama H.	Functional imaging in Binswanger's disease	Rinsho Shinkeigaku.	48	947-950	2008
Shibata M, Yamasaki N, Miyakawa T, Ohtani R, Ihara M, Takahashi R and Tomimoto H	Selective impairment of working memory in a mouse model of chronic cerebral hypoperfusion.	Stroke	38	2826-2832	2007
Ihara M, Tomimoto H, Ishizu K, Yoshida H, Sawamoto N, Hashikawa K and Fukuyama H.	Association of vascular parkinsonism with impaired neuronal integrity in the striatum	J Neural Transm	114	277-584	2007
Tomimoto H, Lin Jin-Xi, Ihara M, Ohtani R, Matsuo A and Miki Y.	Subinsular vascular lesions; an analysis of 119 consecutive autopsied brains	Eur J Neurol	14	95-101	2007
Nakaji K, Ihara M, Takahashi C, Itohara S, Noda M, Takahashi R, Tomimoto H	Matrix metalloproteinase-2 plays a critical role in the pathogenesis of white matter lesions after chronic cerebral hypoperfusion in rodents.	Stroke	37	2816-2823	2006
Okada S, Makino H, Nagumo A, Sugisawa T, Fujimoto M, Kishimoto I, Miyamoto Y, Kikuchi-Taura Akie, Soma T, Taguchi A, Yoshimasa Y	Circulating CD34-Positive Cell Number Is Associated With Brain Natriuretic Peptide levrl in Type 2 Diabetic Patients	Diabetetes Care	31	157-158	2008
Kokubo Y, Okamura T, Yoshimasa Y, Miyamoto Y, Kawanishi K, Kotani Y, Okayama A, Tomoike H:	Impact of Metabolic Syndrome Components on the Incedence of Cardiovascular Disease in a General Urban Japanese Population: The Suita Study.	Hypertens Res	31	2027-2035	2008
Okamura T, Kokubo Y, Watanabe M, Higashiyama A, Miyamoto Y, Yoshimasa Y, Okayama A.	Low-density lipoprotein cholesterol and non-high-density lipoprotein cholesterol and the incidence of cardiovascular disease in an urban Japanese cohort study: The Suita study	Atherosclerosis		in press	2008
Makino H, Okada S, Nagumo A, Sugisawa T, Miyamoto Y, Kishimoto I, Akie TK, Soma T, Taguchi A, Yoshimasa Y	Pioglitazone treatment stimulates circulating CD34-positive cells in type 2 diabetes patients.	Diabetes Res Clin Pract	81	327-330	2008
Makino H, Doi K, Hiuge A, Nagumo A, Okada S, Miyamoto Y, Suzuki M, Yoshimasa Y.	Impaired flow-mediated vasodilatation and insulin resistance in type 2 diabetic patients with albuminuria.	Diabetes Res Clin Pract.	79	177	2008
Suzuki M, Takamisawa I, Yoshimasa Y, Harano Y	Association between insulin resistance and endothelial dysfunction in type 2 diabetes and the effects of pioglitazone.	Diabetes Res Clin Pract	76	12-17	2007

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Jin D, Harada K, Ohnishi S, Yamahara K, Kangawa K, Nagaya N.	Adrenomedullin induces lymph-angiogenesis and ameliorates secondary lymphoedema.	Cardiovasc Res.	80	339-345	2008
Ohnishi S, Sumiyoshi H, Kitamura S, Nagaya N.	Mesenchymal stem cells attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions.	FEBS Lett.	581	3961-3966	2007
Yanagawa B, Kataoka M, Ohnishi S, Kodama M, Tanaka K, Miyahara Y, Ishibashi-Ueda H, Aizawa Y, Kangawa K, Nagaya N.	Infusion of adrenomedullin improves acute myocarditis via attenuation of myocardial inflammation and edema..	Cardiovasc Res.	76	110-118	2007
Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H, Ishino K, Ishida H, Shimizu T, Kangawa K, Sano S, Okano T, Kitamura S, Mori H.	Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction.	Nat Med.	12	459-465	2006
K. Saeki, Y. Yogihashi, M. Nakahara, S.Matsuyama, A. Koyanagi, H. Yagita, M. Koyanagi, Y. Kondo, A. You.	Highly efficient and feeder-free production of subculturable vascular endothelial cell from primate embryonic Stem cells	J. Cell Physiol.	217	261-280	2008
H. Michibata, T. Okuno, N. Konishi, K. Wakimoto, K. Kyono, K. Aoki, Y. Kondo, K. Takata, Y. Kitamura, T. Taniguchi	Inhibition of mouse GPM6A expression leads to decreased differentiation of neurons derived from mouse embryonic stem cells	Stem Cell Development	4	641-651	2008
M. Yamamoto, N. Tase, T. Okuno, Y. Kondo, S. Akiba, N. Shimozawa, K. Terao	Monitoring of gene expression in differentiation ob embryoid bodies from cynomolgus monkey embryonic stem cells in the presence of bisphenola,	Journal of Toxicological Sciences	32	301-310	2007
M. Nakahara, K. Saeki, Y. Yogiashi, A. Kimura, A. Horiuchi, N. Nakamura, A. Yoneda, K. Saeki, S. Matsuyama, M. Nakamura, T. Toda, Y. Kondo, Y. Kaburagi, A. Yuo.	The protein expression profile of cynomolgus embryonic stem cells in two-dimensional gel electrophoresis: a successful identification of multiple proteins ausing human databases.	Journal of Electrophoresis	51	1-8	2007

Angiotensin II Reduces Mitochondrial Content in Skeletal Muscle and Affects Glycemic Control

Masanori Mitsuishi, Kazutoshi Miyashita, Ayako Muraki, and Hiroshi Itoh

OBJECTIVE—Blockade of angiotensin (Ang) II has been shown to prevent new-onset type 2 diabetes. We focused on the effects of AngII on muscle mitochondria, especially on their biogenesis, as an underlining mechanism of type 2 diabetes.

RESEARCH DESIGN AND METHODS—C2C12 cells and C57bl/6 mice were used to examine roles for AngII in the regulation of muscle mitochondria and to explore whether the effect was mediated by type 1 AngII receptor (AT1R) or type 2 receptor (AT2R).

RESULTS—C2C12 cells treated with 10^{-8}–10^{-6} mol/l AngII reduced the mitochondrial content associated with downregulation of the genes involved in mitochondrial biogenesis. The action of AngII was diminished by blockade of AT2R but not AT1R, whereas overexpression of AT2R augmented the effect. AngII increased mitochondrial ROS and decreased mitochondrial membrane potential, and these effects of AngII were significantly suppressed by blockade of either AT1R or AT2R. Chronic AngII infusion in mice also reduced muscle mitochondrial content in association with increased intramuscular triglyceride and deteriorated glycemic control. The AngII-induced reduction in muscle mitochondria in mice was partially, but significantly, reversed by blockade of either AT1R or AT2R, associated with increased fat oxidation, decreased muscle triglyceride, and improved glucose tolerance. Genes involved in mitochondrial biogenesis were decreased via AT2R but not AT1R under these in vivo conditions.

CONCLUSIONS—Taken together, these findings imply the novel roles for AngII in the regulation of muscle mitochondria and lipid metabolism. AngII reduces mitochondrial content possibly through AT1R-dependent augmentation of their degradation and AT2R-dependent direct suppression of their biogenesis. ***Diabetes* 58:710–717, 2009**

Recent studies have shown that mitochondrial content and function are significantly reduced in the skeletal muscle of patients with type 2 diabetes (1,2). Percutaneous biopsy of vastus lateralis muscle has revealed that subsarcolemmal mitochondria, which are believed to be crucial for glucose transport and fatty acid oxidation, were decreased in type 2 diabetic patients, compared with body weight–matched nondiabetic patients (2). Moreover, reduced mitochondrial content and function in muscle have been also observed in pre-diabetic subjects with a family history of type 2 diabetes (3). Recent microarray analyses have revealed that expression of genes involved in mitochondrial biogenesis and oxidative phosphorylation is coordinately decreased in the skeletal muscle of patients with type 2 diabetes (4,5), for example, peroxisome proliferator–activator receptor γ co-activator 1α (PGC1α), a representative transcriptional cofactor for the determination of mitochondrial content and function, and nuclear respiratory factor 1 (NRF1). Furthermore, it has been demonstrated that mitochondrial function evaluated by the rate of ATP synthesis is diminished in the skeletal muscle of diabetic patients and family history–positive pre-diabetic patients (6,7). These findings imply that reduced mitochondrial content in the skeletal muscle is likely to contribute to the development of insulin resistance and type 2 diabetes (8,9).

Angiotensin (Ang) II, which is composed of eight amino acids, is one of the most important molecules in the renin-angiotensin system. It provokes sodium reabsorption, vasoconstriction, and elevation of blood pressure and also plays a critical role in the physiological regulation of electrolytes and water homeostasis. However, an excess of AngII may lead to tissue damage, such as atherosclerosis, cardiomegaly, and heart and renal failure. AngII is known to exert its biological effects via two functional receptors, type 1 and type 2 angiotensin II receptors (AT1R and AT2R, respectively). To date, most of the known cardiovascular effects of AngII are believed to be attributable to AT1R (10). Recent large-scale clinical trials, including HOPE (Heart Outcomes Prevention Evaluation), LIFE (Losartan Intervention for Endpoint), CHARM (Candesartan in Heart Failure—Assessment of Mortality and Morbidity), and VALUE (Valsartan Antihypertensive Long-term Use Evaluation), have demonstrated that ACE inhibitors or angiotensin II receptor blockers (ARB) prevent new onset of type 2 diabetes via their ability to attenuate AngII signaling (11). As a result of these findings, the significance of AngII for the development of insulin resistance and regulation of energy metabolism has been attracting considerable attention (12–14).

It has been demonstrated that AngII provokes insulin resistance in the skeletal muscle through multiple mechanisms. AngII treatment was found to augment reactive oxygen species (ROS) production by stimulating NADPH oxidase in cultured skeletal muscle cells, thus activating multiple redox-sensitive signaling including nuclear factor-κB (NF-κB) and increasing proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), which impair insulin action (15,16). Inhibition of insulin signaling by AngII at multiple levels including insulin receptor, insulin receptor substrate 1, and phosphatidylinositol 3-kinase has been demonstrated in aortic smooth muscle cells (17). Stimulation of primary cultured human preadipocytes by AngII was found to inhibit differentiation to mature adipocytes, suggesting that the effect of AngII on adipose tissue

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may influence glycemic control (18). Furthermore, AngII is known to affect other hormones that are related to the regulation of blood pressure, for example, by promoting the release of aldosterone and vasopressin. It has been suggested that changes in catecholamine production by genetic disruption of AT1R in mice may affect glycemic control (19). Diminished tissue perfusion and lowered blood potassium concentration caused by AngII may also be involved in the development of insulin resistance. These findings indicate that various mechanisms are likely to be involved in AngII-induced insulin resistance. However, the effect of AngII on muscle mitochondrial content and subsequent influence on glycemic control has not yet been elucidated.

In a previous study, we showed that cGMP was involved in the regulation of mitochondrial content and function of cultured C2C12 myotubular cells by altering the expressions of the genes related to mitochondrial biogenesis and the antioxidant system (20). Interestingly, cGMP is an intracellular second messenger of the vasodilating substances, natriuretic peptides and nitric oxide, both of which exert antagonistic effects to AngII actions. In the current study, we therefore focused on the effects of AngII on muscle mitochondria, especially on the regulation of their biogenesis, and their relationship with the pathogenesis of glucose intolerance.

RESEARCH DESIGN AND METHODS

Cell culture. C2C12 cells (RIKEN BioResource Center, Tsukuba, Japan) were grown in a low-glucose (100 mg/dl) medium, as described previously (20). Cells were fully differentiated, grown to overconfluence, and treated in a 24-well dish with or without 10^{-6}–10^{-8} mol/l AngII (Sigma, St. Louis, MO); an AT1R blocker, RNH6270 (Olmesartan, 10^{-5} mol/l, a gift from Daiichi-Sankyo, Tokyo, Japan); or an AT2R blocker, PD123319 (10^{-5} mol/l; Sigma). Unless indicated otherwise, total DNA, RNA, and proteins were extracted from the cells after 48 h of treatment.

Transient overexpression of angiotensin receptors in vitro and RNA interference. We constructed angiotensin receptor overexpressing vectors by fusing the chicken β-actin promoter-driven vector, pCAGGS (21) (a gift from Dr. J. Miyazaki, Osaka University, Japan) with cDNA of mouse AT1R (GenBank accession no. NM_177322) or AT2R (NM_007429). C2C12 cells were transiently transfected with these vectors (0.1 μg/well) using the Lipofectamine LTX Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. We then generated small interfering RNAs (siRNAs) for genetic blockade of AT1R and AT2R. C2C12 cells were transfected with these siRNAs, or scrambled RNA as negative control (Stealth RNAi Negative Control, Invitrogen), by means of a Lipofectamine RNAi Max Reagent (Invitrogen) according to the manufacturer's instructions.

Quantification of mitochondrial DNA copy number and gene expressions by real-time PCR. Quantitative PCR analysis was performed by standard methods. Details of the methods used are provided in the supplemental materials (available in an online-only appendix at (http://diabetes.diabetesjournals.org/cgi/content/full/db08-0949.

Quantification of mitochondrial mass, ROS production, and membrane potential and ATP content. Mitochondrial mass, mitochondrial ROS production, and membrane potential of the C2C12 cells were determined, respectively, with the aid of the fluorescent dyes: MitoTracker Green FM, MitoSOX Red, and JC-1 (Molecular Probes, Eugene, OR), following the same procedures as described previously (20). The fluorescent intensity was estimated by a multi-plate reader (Wallac ARVO SX; Perkin Elmer, Wellesley, MA). ATP content of the cells was determined with the chemiluminescence method (ATP bioluminescence Assay Kit HS II; Roche Diagnostics, Basel, Switzerland). For microscopic analysis, C2C12 cells were stained with the fluorescent probes and observed with a confocal microscope (LSM 510; Carl Zeiss, Oberkochen, Germany).

Quantification of protein levels by Western blotting. Western blotting was performed by standard methods. Details of the methods used are provided in the supplemental materials.

Animal experiments. All animal experiments were performed in accordance with the animal care and use guidelines of Keio University. Male C57bl/6 mice (Nihon Clea, Tokyo, Japan) were maintained under specific pathogen-free conditions, and exogenous AngII was infused at a dose of 0.1 μg/g body wt/day, which is recognized as a subpressor dose that does not influence blood pressure (22). The infusion was carried out via an osmotic mini-pump (Alzet model 1002; Durect, Cupertino, CA) subcutaneously implanted when the mice were 8 weeks old. The AngII-infused mice were treated with or without the AT1R blocker CS-866 (Olmesartan medoxomil, a precursor of olmesartan with a longer effect in vivo than olmesartan, and a gift from Daiichi-Sanko) or the AT2R blocker PD-123319, four times per week by oral administration (10 mg/kg body wt) with a stomach sonde. On day 10, the mice were killed for tissue harvest.

Glucose tolerance test, indirect calorimetry, and quantification of serum insulin concentration, muscle triglyceride, and enzyme activity of mitochondria. For the glucose tolerance test, the mice were fasted for 8 h and intraperitoneally injected with glucose at 2.0 g/kg body weight. Blood samples were collected from the tail vein, and the blood glucose level was promptly determined with the glucose dehydrogenase method (ACCU-CHEK Aviva, Roche Diagnostics). Oxygen consumption and fat oxidation in mice were determined by means of indirect calorimetry, which was performed for 24 h from 2000 until the next day (ARCO-2000; Arco Systems, Kashiwa, Japan). Serum insulin concentration was determined with an enzyme-linked immunosorbent assay kit (Ultra-Sensitive Mouse Insulin ELISA kit, 200710; Morinaga, Yokohama, Japan). Lipids in quadriceps were extracted with the Folch method (23), and the triglyceride level was determined with a commercially available kit (Triglyceride E-test; Wako, Osaka, Japan). We performed Oil-red-O staining of the muscle, and the method is provided in the supplemental materials. Enzyme activity of the mitochondrial proteins, cytochrome C oxidase (COX) and β-hydroxyacyl-CoA dehydrogenase (β-HAD), was determined in the skeletal muscle by a commercially available assay kit (Mitochondria Activity Assay Kit, Bio Chain, Hayward, CA) and a standard method (24), respectively.

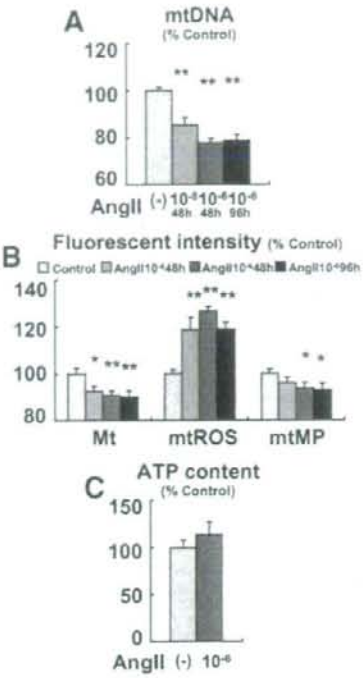

FIG. 1. AngII reduces mitochondrial content in association with an increase in mitochondrial ROS production in C2C12 cells. C2C12 cells were incubated with or without AngII (at the concentrations indicated: 10^{-8} or 10^{-6} mol/l) for 48 or 96 h. *A*: Mitochondrial DNA copy number (mtDNA) estimated by quantitative PCR analysis (n = 12 per group). *B*: Mitochondrial density (Mt), mitochondrial ROS production (mtROS), and mitochondrial membrane potential (mtMP) estimated with the aid of fluorescent probes (n = 12). *C*: ATP content determined with the chemiluminescence method (n = 12). The values were standardized to those for the control (no AngII treatment). *P < 0.05, **P < 0.01 vs. control.

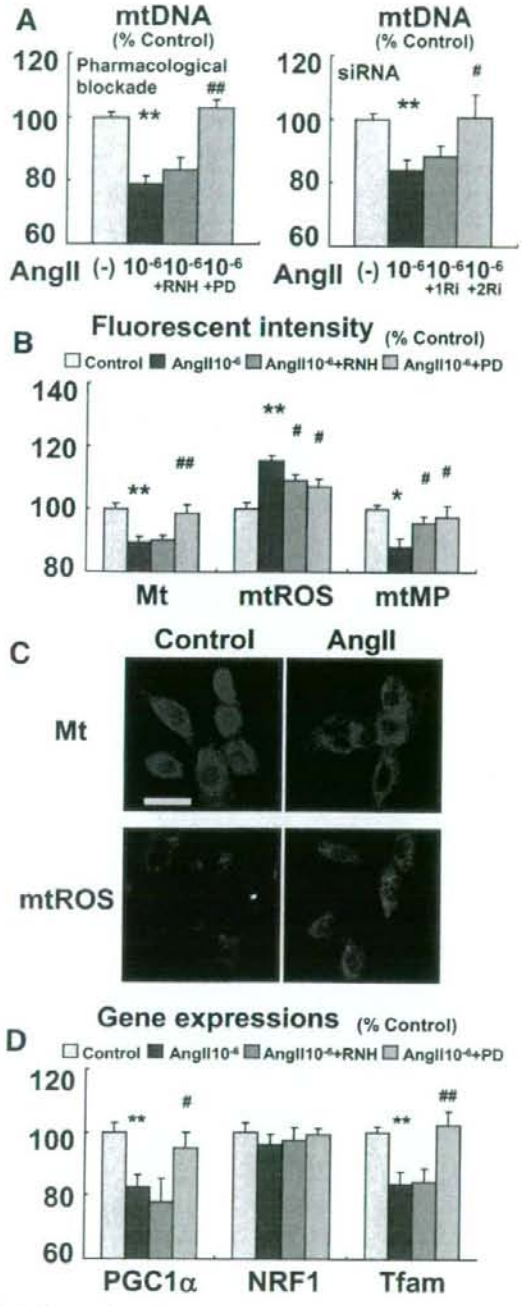

FIG. 2. Pharmacological blockade or targeted silencing of AT2R but not AT1R in C2C12 cells recovers mitochondrial biogenesis reduced by AngII. The AT1R blocker, RNH-6270 (RNH, 10^{-5} mol/l), or the AT2R blocker, PD-123319 (PD, 10^{-5} mol/l), was added to C2C12 cells previous to the treatment with AngII (10^{-6} mol/l) for 48 h (*A–D*). Targeted disruption of AT1R (1Ri) or AT2R (2Ri) by small interfering RNA (siRNA) was also performed (*A*). *A*: Mitochondrial DNA copy number (mtDNA) estimated by quantitative PCR analysis ($n = 12$ per group). *B*: Mitochondrial density (Mt), mitochondrial ROS production (mtROS), and mitochondrial membrane potential (mtMP) estimated with the aid of fluorescent probes ($n = 12$). *C*: Microscopic analysis of the cells. C2C12 cells were stained with MitoTracker Green (green, a probe for mitochondria) or MitoSOX Red (red, a probe for mitochondria-derived ROS) and observed with a confocal microscope. Scale bar: 100 μm. *D*: Expression of genes involved in mitochondrial biogenesis ($n = 12$). The values were standardized to those for the control. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the AngII-treated group. (Please see http://diabetes.diabetesjournals.org/cgi/content/full/db08-0949 for a high-quality digital representation of this figure.)

Microarray analysis. Microarray analysis of the skeletal muscle of AngII-infused mice was performed. Details of the methods used are provided in the supplemental materials.

Statistical analysis. Statistical analysis was performed by standard methods. Details of the methods used are provided in the supplemental materials.

RESULTS

AngII reduces mitochondrial content in association with increased mitochondrial ROS production and lowers mitochondrial membrane potential in C2C12 cells. To investigate the effect of AngII on mitochondrial content, ROS production, membrane potential, and ATP production, we treated C2C12 myotubular cells with or without 10^{-8}–10^{-6} mol/l AngII for 48–96 h. Mitochondrial DNA copy number, which is considered to be a surrogate marker of mitochondrial content, showed a dose-dependent reduction (22% reduction at 10^{-6} mol/l, $n = 12$, $P < 0.01$, Fig. 1*A*) in the AngII-treated groups. The magnitude of reduction was similar for 48 and 96 h of incubation with AngII. Mitochondrial mass, estimated by means of fluorescent staining, also decreased as a result of AngII treatment (9% reduction at 10^{-6} mol/l, Fig. 1*B*), in association with a significant increase in mitochondrial ROS (mtROS) (27% increase, Fig. 1*B*). Mitochondrial membrane potential (mtMP) was lowered (6% decrease, Fig. 1*B*) by AngII, whereas cellular ATP content was not significantly changed (Fig. 1*C*).

Blockade of AT2R but not AT1R reverses mitochondrial reduction in C2C12 cells caused by AngII, whereas blockade of either AT1R or AT2R suppresses AngII-induced changes in mtROS and mtMP. To determine the receptor responsible for the effects of AngII on mitochondria, C2C12 cells were subjected to pharmacological and genetic blockade of their receptors. Pharmacological blockade of AngII by the AT1R blocker RNH-6270 (10^{-5} mol/l) or AT2R blocker PD-123319 (10^{-5} mol/l) revealed that the decline in mitochondrial content was completely reversed by blockade of AT2R (Fig. 2*A* and *B*). Consistent results were obtained by genetic blockade of these receptors by siRNA in that the silencing of AT2R completely reversed the decrease in mitochondrial content, whereas that of AT1R did not affect it (Fig. 2*A*). We confirmed that the siRNAs achieved >80% reduction in the expression and protein levels of the receptors by using quantitative PCR and Western blotting (data not shown). On the other hand, the increase in mtROS and the decrease in mtMP induced by AngII were partially, but significantly, suppressed by blockade of either AT1R or AT2R (Fig. 2*B*). Using a confocal microscope, we were able to confirm that the fluorescent probes were distributed specifically in mitochondria (Fig. 2*C*).

We also examined the expressions of genes involved in mitochondrial biogenesis (PGC1α, NRF1, and mitochondrial transcription factor A [Tfam]) and found that both PGC1α and Tfam were decreased in the AngII-treated group (18% and 16% decrease, respectively, $n = 12$, $P < 0.01$, Fig. 2*D*); however, expression of NRF1 was not affected by AngII. Consistent with its effects on mitochon-

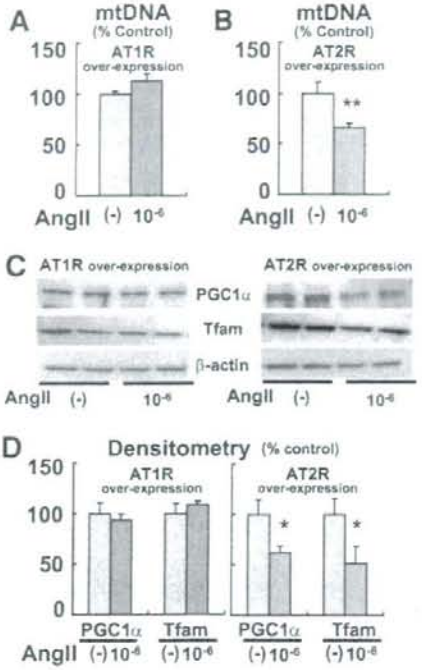

FIG. 3. Overexpression of AT2R in C2C12 cells augments AngII-induced reduction in mitochondrial biogenesis. Angiotensin II receptors (AT1R or AT2R) were overexpressed in C2C12 cells by CAG promoter–driven expression vectors previous to the treatment with AngII (10^{-6} mol/l) for 48 h (*A–D*). Mitochondrial DNA copy number (mtDNA) estimated by quantitative PCR analysis in C2C12 cells overexpressing AT1R (*A*) or AT2R (*B*) (n = 12 per group). *C*: Western blots of proteins that are related to mitochondrial biogenesis (PGC1α and Tfam). *D*: Densitometry of the Western blots. The density of the blots for PGC-1α and Tfam was normalized by that for the internal control (β-actin) (n = 8). The values were standardized to those for the control. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

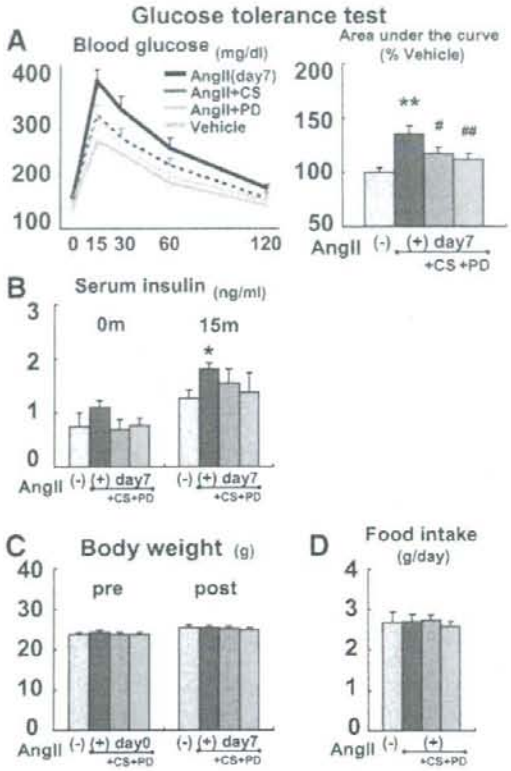

FIG. 4. Exogenous administration of AngII in mice for 1 week provokes glucose intolerance without changes in body weight or food intake. The 8-week-old male C57bl/6 mice were treated with a subpressor dose (0.1 μg/g body wt/day) of AngII or PBS (vehicle) by means of a subcutaneously implanted osmotic pump. The AT1R blocker, CS-866 (CS), or the AT2R blocker, PD-123319 (PD), was orally administered (*A–D*). *A*: Blood glucose levels (mg/dl) at 0, 15, 30, 60, and 120 min after intraperitoneal injection of 2.0 g/kg body wt glucose and area under the curve of the glucose level (% vehicle) (n = 18 per group). *B*: Serum insulin levels during glucose tolerance test at 0 and 15 min (0m, 15m). *C*: Body weight of mice before and 1 week after the implantation of osmotic pumps (pre, post). *D*: Food intake of mice during the experiment. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the AngII-treated group.

drial content (Fig. 2*A* and *B*), AT2R blockade by PD-123319 completely reversed the AII-induced decrease in PGC1α and Tfam, but AT1R blockade by RNH-6270 did not affect their expressions (Fig. 2*D*).

These results indicate that, under the present experimental condition using C2C12 cells, AT1R-dependent signal pathways have effects on mtROS and mtMP without any change in mitochondrial content, whereas AT2R-dependent pathways influence mitochondrial biogenesis, mtROS, and mtMP. The expression levels of the receptors can thus be expected to determine the effect of AngII on mitochondria.

Overexpression of AT2R in C2C12 cells augments AngII-induced reduction in mitochondrial biogenesis. Next, we performed overexpression of the receptors by using the CAG promoter–driven expression vectors in C2C12 cells (Fig. 3*A–D*). In the AT1R overexpression group, mtDNA was not significantly changed as a result of treatment with AngII (Fig. 3*A*); however, in the AT2R overexpression group, mtDNA showed a major decrease as the result of treatment with AngII (44% decrease, n = 12, $P < 0.01$, Fig. 3*B*). Western blot analysis confirmed that PGC1α and Tfam protein levels in the AT2R overexpression group were significantly diminished by AngII (Fig. 3*C* and *D*). These results were also compatible with those for their gene expressions under pharmacological blockades (Fig. 2*D*).

Exogenous administration of AngII in mice for 1 week provokes glucose intolerance without changes in body weight or food intake. To explore the effects of AngII on muscle mitochondria and glycemic control in vivo, C57bl/6 mice were subjected to chronic infusion of AngII by means of an osmotic pump, combined with the pharmacological blockade of AT1R by CS-866 or of AT2R by PD-123319. We compared the effect on the four groups: control (vehicle implanted), AngII infusion, AngII infusion with AT1R blockade, and AngII infusion with AT2R blockade. In the AngII-treated groups, glucose levels after the glucose challenge were significantly higher than in the control (33% elevation in area under the curve of the glucose level, n = 18, $P < 0.01$; Fig. 4*A*), and the AngII-induced change in glycemic control was significantly suppressed by blockade of either AT1R or AT2R (13% or 17% suppression, n = 18, $P < 0.05$ and 0.01, respectively; Fig.

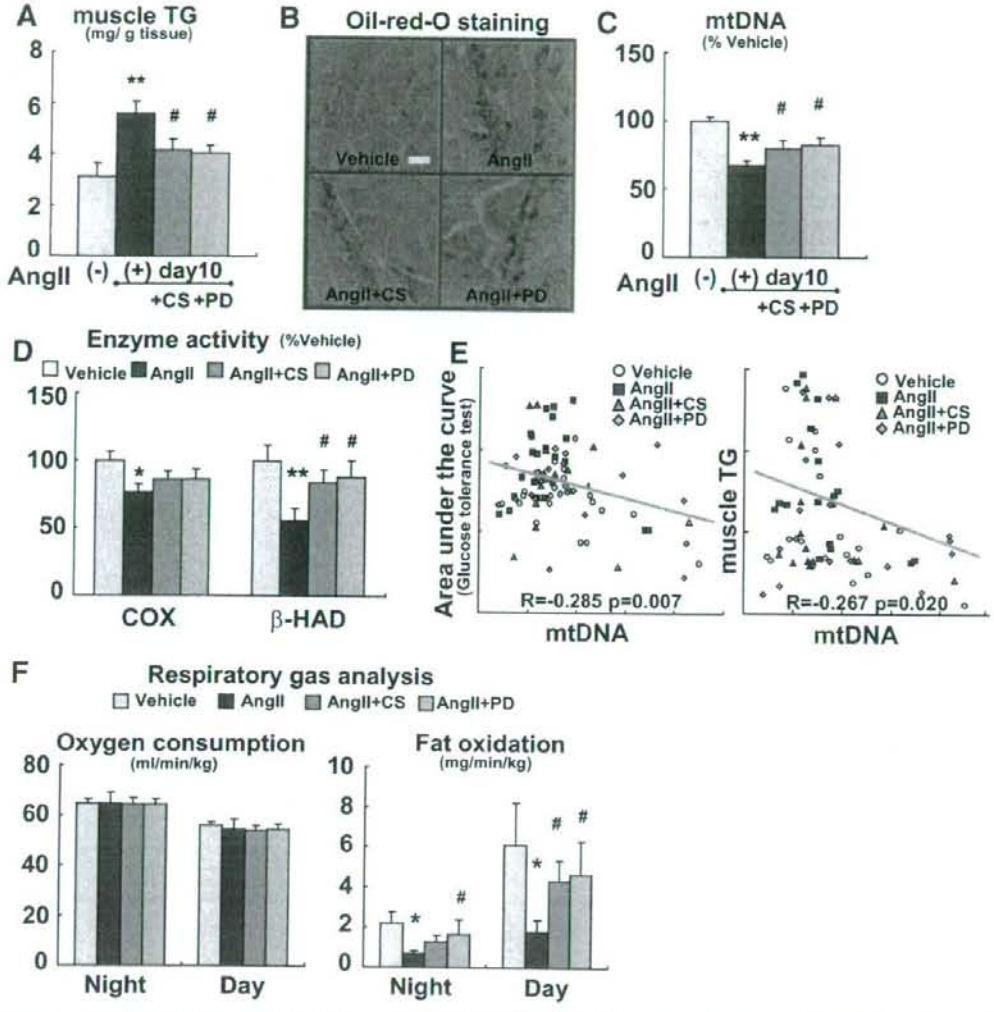

FIG. 5. AngII reduces mitochondrial content and increases triglyceride (TG) content in the skeletal muscle in both AT1R- and AT2R-dependent manners. *A*: Muscle triglyceride content (muscle triglyceride) measured in the quadriceps (n = 8 per group). *B*: Histological analysis of AngII-induced lipid accumulation in muscle by Oil-red-O staining. Neutral lipids were stained in red. Scale bar: 100 μm. *C*: mtDNA of the quadriceps estimated by quantitative PCR analysis (n = 12). *D*: Enzyme activity in the muscle of the mitochondrial proteins, cytochrome C oxidase (COX), and β-hydroxyacyl-CoA dehydrogenase (β-HAD) (n = 12). *E*: The relationship between area under the curve of the glucose levels in the glucose tolerance test and mtDNA, or between muscle triglyceride and mtDNA. Symbols were distinguished according to the treatment. *F*: Oxygen consumption and fat oxidation analyzed by indirect calorimetry. $^*P < 0.05$, $^{**}P < 0.01$ vs. control; $\#P < 0.05$ vs. the AngII-treated group. (Please see http://diabetes.diabetesjournals.org/cgi/content/full/db08-0949 for a high-quality digital representation of this figure.)

4*A*). In the AngII-infused groups, the serum insulin concentrations at 0 and 15 m of the glucose challenge showed a parallel increase with glucose levels (Fig. 4*B*). There were no significant changes in body weight or food intake in any of the four groups (Fig. 4*C* and *D*).

AngII reduces mitochondrial content and increases triglycerides in the skeletal muscle in both AT1R- and AT2R-dependent manners. We then examined the muscle triglyceride level in the quadriceps of the mice and found that they were higher in the AngII group (78% increase, n = 8, $P < 0.01$, Fig. 5*A*) than in the control group. Pharmacological blockade of either AT1R or AT2R caused a significant reduction in the muscle triglyceride level compared with that in the AngII group (26% or 28% reduction, respectively, n = 8, $P < 0.05$, Fig. 5*A*). To confirm that lipids were accumulated in the intramyocellular region, we performed Oil-red-O staining and found that AngII infusion increased intramyocellular lipids, and the increase was suppressed by blockade of either AT1R or AT2R (Fig. 5*B*). We also found that the ceramide content in the muscle was parallel to the triglyceride level (data not shown). The copy number of muscle mitochondrial DNA was reduced in the AngII group, and this change was also significantly diminished by blockade of either AT1R or AT2R (Fig. 5*C*). Furthermore, COX and β-HAD activities in the muscle, which represent electron transport and β-oxidative function of mitochondria, respectively, were significantly reduced in the AngII group, and

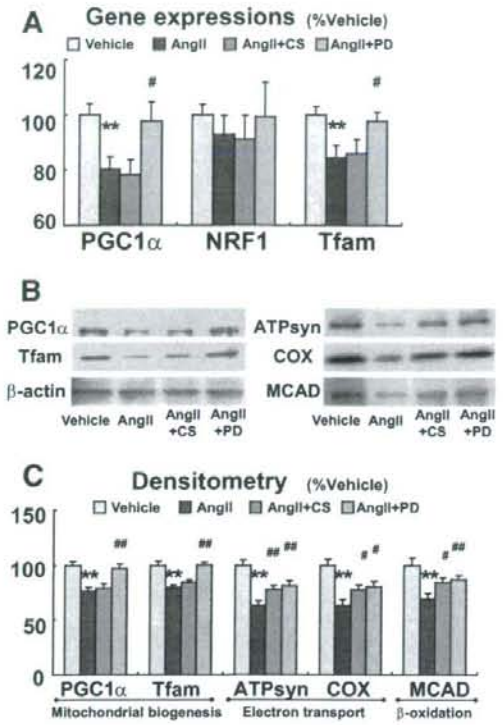

FIG. 6. Blockade of AT2R but not AT1R in AngII-infused mice reverses reduction in expression and protein levels of molecules involved in mitochondrial biogenesis. *A*: Expression of genes involved in mitochondrial biogenesis in the skeletal muscle of the mice (n = 12 per group). *B*: Western blots of proteins that are related to mitochondrial biogenesis (PGC1α and Tfam), electron transport (ATPsyn and COX), and β-oxidation (MCAD). *C*: Densitometry of the Western blots. The density of the blot was normalized by that for the internal control (β-actin) (n = 8). The values were standardized to those for the control. $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the AngII-treated group.

this change was diminished by blockade of either AT1R or AT2R, in a manner that was parallel to the mitochondrial content (Fig. 5*D*). These experiments also showed that mtDNA correlated significantly with the area under the curve of the glucose tolerance test ($R = -0.285$, $n = 72$, $P < 0.01$) and muscle triglyceride ($R = -0.267$, $n = 72$, $P < 0.05$) in the quadriceps (Fig. 5*E*). Respiratory gas analysis was used to estimate oxygen consumption and fat oxidation. Although oxygen consumption measured over 24 h showed no significant changes in the four groups (Fig. 5*F*), a dramatic decrease in fat oxidation was observed in the AngII-infused group (69% at night and 70% during the day, $n = 8$, $P < 0.05$, Fig. 5*F*), and this change was abrogated by blockade of either AT1R or AT2R and to a greater extent by the AT2R blockade (41% or 46% recovery at night, respectively, $n = 8$, $P < 0.05$, Fig. 5*F*).

Blockade of AT2R but not AT1R in AngII-infused mice reverses reduction in expression and protein levels of molecules involved in mitochondrial biogenesis. Consistent with the result for C2C12 cells (Fig. 2*D*), AT2R blockade by PD-123319 in AngII-infused mice reversed the reduction in the expression in PGC1α and Tfam, but blockade of AT1R by CS-866 did not affect their expressions (Fig. 6*A*). Western blot analysis and densitometry of the blots confirmed this result (Fig. 6*B* and *C*). On the other hand, the AngII-induced reduction in mitochondrial proteins involved in electron transport or fatty acid oxidation (ATP synthase [ATPsyn], COX, and medium-chain fatty acyl-CoA dehydrogenase [MCAD]) were all suppressed by blockade of either AT1R or AT2R (Fig. 6*B* and *C*), and the manner of changes in the protein levels was parallel to that seen in mtDNA (Fig. 5*B*).

These results indicate that the AngII-induced reduction in muscle mitochondrial content in mice is caused by AT2R-dependent suppression of mitochondrial biogenesis and also by AT1R-dependent mechanisms that are not directly related to their biogenesis.

Microarray analysis of the skeletal muscle of AngII-infused mice. Detailed results of microarray analysis are presented in the supplemental materials.

DISCUSSION

In the study reported here, we found that AngII reduced mitochondrial content in cultured myotubular cells and skeletal muscle of mice. In addition, the AngII-infused mice showed a decrease in fat oxidation that was associated with an increase in intramuscular triglyceride content and impaired glucose tolerance. These findings imply that the cardiovascular hormone AngII, which has been thought previously to act mainly on the cardiovascular system, may have novel roles in the regulation of mitochondria and lipid metabolism in the skeletal muscle.

To determine the receptor responsible for the effects of AngII on mitochondrial content, we used pharmacological blockade and RNA interference of the receptors in C2C12 myotubes, which exhibited substantial expressions of both AT1R and AT2R. We found that the decrease in mitochondrial content induced by treatment with AngII for 48 h could be reversed by the pharmacological and genetic blockades of AT2R but not by those of AT1R. Consistent with this finding, AngII decreased the expression levels of PGC1α and Tfam, which positively regulate mitochondrial biogenesis, in an AT2R-dependent manner. We further confirmed that this reduction was attributable to AT2R but not to AT1R, by means of overexpression of the receptors in C2C12 cells. In the skeletal muscle of AngII-infused mice, on the other hand, the pharmacological blockade of either AT1R or AT2R partially but significantly reversed the AngII-induced reduction in mitochondrial content. The change in muscle mitochondrial content was parallel to the mitochondrial protein levels of ATPsyn, COX, and MCAD and the enzyme activity of COX and β-HAD. While still in this in vivo situation, the AngII-induced decrease in expression and protein levels of PGC1α and Tfam was prevented by the pharmacological blockade of AT2R but not by that of AT1R. Therefore, the difference in the protein levels under the AT1R blockade was observed between PGC1α/Tfam and ATPsyn/COX/MCAD.

These results imply that the in vitro and in vivo regulation of mitochondria by AngII was somewhat different under the present experimental conditions. AngII reduced mitochondrial content predominantly via an AT2R-dependent direct suppression of mitochondrial biogenesis in the cultured myocytes. On the other hand, mitochondrial content in the skeletal muscle in mice appeared to be determined by a complex combination of factors. Cytokines and hormones released from other tissues, ROS production, and nutritional availability are all known to influence mitochondrial DNA copy number in vivo (25).

For example, the plasma level of the insulin-sensitizing hormone adiponectin from adipose tissue has been reported to be decreased by AngII infusion via AT1R (26). Because adiponectin has been shown to increase mitochondrial number (27), decreased adiponectin levels via AT1R might affect mitochondria in the in vivo condition. These kinds of mechanisms can explain the difference, which we found in the present study, between the results for AT1R blockade under the in vitro and in vivo experimental conditions.

The finding that the expression and protein levels of molecules involved in mitochondrial biogenesis in skeletal muscle of AngII-infused mice were not modulated by AT1R suggests that AT1R-dependent pathways reduce mitochondrial content by a way other than via the reduction of mitochondrial biogenesis. Therefore, we propose that mitochondrial degradation was involved in the regulation of mitochondrial content in the present study. Mitochondria are degraded in lysosomes by a process known as "mitophagy" (28). Previous studies have shown that AT1R-dependent pathways augment ROS production, which is known to promote mitophagy and reduce mitochondrial content in rat kidney (29,30). Moreover, in our experiments, an AT1R-dependent increase in lysosomes has been observed in AngII-treated C2C12 cells (data not shown). These findings together suggest that the AngII-induced ROS production via AT1R would augment muscle mitophagy and that an AT1R blockade can be expected to protect muscle mitochondria from various insults that lead to mitophagy.

Chronic AngII infusion in rodents has been shown to reduce glucose uptake in muscle and provoke insulin resistance (31). Consistent with this finding, our study demonstrated that 1-week exogenous administration of a subpressor dose of AngII with a subcutaneously implanted osmotic pump exacerbated glycemic control in C57bl/6 mice without causing changes in food intake or suppression in insulin secretion. The AngII-induced glucose intolerance in mice was accompanied by a reduction in mitochondrial content and an increase in triglyceride levels in the skeletal muscle. Previous studies have shown a strong relationship between accumulation of intramuscular triglycerides and insulin resistance (32,33). Although triglycerides themselves are thought to be biologically inactive, accumulating muscle triglyceride levels lead to an increase in intramuscular fatty acids, which has been shown to inhibit insulin signaling via phosphorylation of serine residues in insulin receptor substrate 1 (34). Other lipid metabolites, such as long-chain fatty acyl coenzyme A, diacylglycerols, and ceramides, have also been shown to impair muscle insulin signal directly (35).

Muscle triglycerides are believed to increase in association with a reduction in fat oxidation; indeed, a significant relationship between reduced fat oxidation and increased triglyceride levels has been demonstrated by means of percutaneous biopsy of the vastus lateralis muscle in insulin-resistant subjects (36). Moreover, reduced muscle mitochondrial content has been shown to correlate with decreased fat oxidation and insulin resistance in nondiabetic subjects with a family history of type 2 diabetes (37). The fact that PGC1α-dependent pathways shift fuel substrates for oxidation from carbohydrates to lipids, in addition to promoting mitochondrial biogenesis, can explain the relationship between mitochondrial content and fat oxidation in muscle (38). These studies point to the importance of mitochondrial content in skeletal muscle as an upstream element in the pathogenesis of intramuscular lipids and insulin resistance.

The findings in the present study lead us to hypothesize that administration of AngII in mice causes glucose intolerance at least partly by reducing the mitochondrial content in skeletal muscle, which results in reduced fat oxidation and subsequent accumulation of intramuscular lipids. In support of this notion, we identified significant relationships among mitochondrial content in quadriceps on the one hand, and intramuscular triglyceride and the index of glucose intolerance, expressed as area under the curve of glucose levels after glucose challenge, on the other. The results of the in vivo energy expenditure, which showed that the AngII infusion in mice did not change the oxygen consumption, indicate that the reduction in muscle mitochondrial content by AngII was not mediated through changes in chronic physical activity. However, it is possible that AngII first impairs glycemic control and subsequently reduces mitochondrial content, because hyperglycemia itself has been known to decrease mitochondrial content (39,40). Future studies should specifically focus on the time course of the AngII-induced decrease in mitochondrial content and deterioration of glycemic control, as well as their causal relationship.

To summarize, we have demonstrated that AngII causes a reduction in mitochondrial content in cultured myotubular cells and the skeletal muscle in mice. Exogenous administration of AngII with an osmotic pump in mice provoked glucose intolerance, which is associated with reduced mitochondrial content, decreased fat oxidation, and increased intramuscular triglyceride levels. Putting these findings together suggests that the cardiovascular hormone AngII, which has been thought to act mainly on the cardiovascular system, can also regulate mitochondrial content and lipid metabolism in the skeletal muscle, and thus affect glycemic control. AngII-infused mice are likely to reduce muscle mitochondrial content through both AT1R and AT2R by different mechanisms: through AT1R-dependent augmentation of mitochondrial degradation and AT2R-dependent direct suppression of their biogenesis.

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REFERENCES

1. Kelley DE, He J, Menshikova EV, Ritov VB: Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* 51:2944–2950, 2002
2. Ritov VB, Menshikova EV, He J, Ferrell RE, Goodpaster BH, Kelley DE: Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. *Diabetes* 54:8–14, 2005
3. Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI: Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med* 350:664–671, 2004
4. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ: Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC1 and NRF1. *Proc Natl Acad Sci U S A* 100:8466–8471, 2003
5. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstråle M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC: PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34:267–273, 2003
6. Morino K, Petersen KF, Dufour S, Befroy D, Frattini J, Shatzkes N, Neschen S, White MF, Bilz S, Sono S, Pypaert M, Shulman GI: Reduced

mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. *J Clin Invest* 115:3587–3593, 2005
7. Petersen KF, Dufour S, Shulman GI: Decreased insulin-stimulated ATP synthesis and phosphate transport in muscle of insulin-resistant offspring of type 2 diabetic parents. *PLoS Med* 2:e233, 2005
8. Lowell BB, Shulman GI: Mitochondrial dysfunction and type 2 diabetes. *Science* 307:384–387, 2005
9. Kim JA, Wei Y, Sowers JR: Role of mitochondrial dysfunction in insulin resistance. *Circ Res* 102:401–414, 2008
10. Horiuchi M, Akishita M, Dzau VJ: Recent progress in angiotensin II type 2 receptor research in the cardiovascular system. *Hypertension* 33:613–621, 1999
11. Stump CS, Hamilton MT, Sowers JR: Effect of antihypertensive agents on the development of type 2 diabetes mellitus. *Mayo Clin Proc* 81:796–806, 2006
12. Perkins JM, Davis SN: The renin-angiotensin-aldosterone system: a pivotal role in insulin sensitivity and glycemic control. *Curr Opin Endocrinol Diabetes Obes* 15:147–152, 2008
13. Jandeleit-Dahm KA, Tikellis C, Reid CM, Johnston CI, Cooper ME: Why blockade of the renin-angiotensin system reduces the incidence of new-onset diabetes. *J Hypertens* 23:463–473, 2005
14. Leiter LA, Lewanczuk RZ: Of the renin-angiotensin system and reactive oxygen species type 2 diabetes and angiotensin II inhibition. *Am J Hypertens* 18:121–128, 2005
15. Wei Y, Sowers JR, Clark SE, Li W, Ferrario CM, Stump CS: Angiotensin II-induced skeletal muscle insulin resistance mediated by NF-kappaB activation via NADPH oxidase. *Am J Physiol Endocrinol Metab* 294:E345–E351, 2008
16. Wei Y, Sowers JR, Nistala R, Gong H, Uptergrove GM, Clark SE, Morris EM, Szary N, Manrique C, Stump CS: Angiotensin II-induced NADPH oxidase activation impairs insulin signaling in skeletal muscle cells. *J Biol Chem* 28:35137–35146, 2006
17. Folli F, Kahn CR, Hansen H, Bouchie JL, Feener EP: Angiotensin II inhibits insulin signaling in aortic smooth muscle cells at multiple levels: a potential role for serine phosphorylation in insulin/angiotensin II crosstalk. *J Clin Invest* 100:2158–2169, 1997
18. Janke J, Engeli S, Gorzelniak K, Luft FC, Sharma AM: Mature adipocytes inhibit in vitro differentiation of human preadipocytes via angiotensin type 1 receptors. *Diabetes* 51:1699–1707, 2002
19. Kouyama R, Suganami T, Nishida J, Tanaka M, Toyoda T, Kiso M, Chiwata T, Miyamoto Y, Yoshimasa Y, Fukamizu A, Horiuchi M, Hirata Y, Ogawa Y: Attenuation of diet-induced weight gain and adiposity through increased energy expenditure in mice lacking angiotensin II type 1a receptor. *Endocrinology* 146:3481–3489, 2005
20. Mitsuishi M, Miyashita K, Itoh H: cGMP rescues mitochondrial dysfunction induced by glucose and insulin in myocytes. *Biochem Biophys Res Commun* 367:840–845, 2008
21. Niwa H, Yamamura K, Miyazaki J: Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108:193–199, 1991
22. Li Y, Kishimoto I, Saito Y, Harada M, Kuwahara K, Izumi T, Takahashi N, Kawakami R, Tanimoto K, Nakagawa Y, Nakanishi M, Adachi Y, Garbers DL, Fukamizu A, Nakao K: Guanylyl cyclase-A inhibits angiotensin II type 1A receptor-mediated cardiac remodeling, an endogenous protective mechanism in the heart. *Circulation* 106:1722–1728, 2002
23. Folch J, Lees M, Sloane Stanley GH: A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226:497–509, 1957
21. Bass A, Brdiczka D, Eyer P, Hofer S, Pette D: Metabolic differentiation of distinct muscle types at the level of enzymatic organization. *Eur J Biochem* 10:198–206, 1969
25. Shadel GS: Expression and maintenance of mitochondrial DNA: new insights into human disease pathology. *Am J Pathol* 172:1445–1456, 2008
26. Ran J, Hirano T, Fukui T, Saito K, Kageyama H, Okada K, Adachi M: Angiotensin II infusion decreases plasma adiponectin level via its type 1 receptor in rats: an implication for hypertension-related insulin resistance. *Metabolism* 55:478–488, 2006
27. Civitarese AE, Ukropcova B, Carling S, Hulver M, DeFronzo RA, Mandarino L, Ravussin E, Smith SR: Role of adiponectin in human skeletal muscle bioenergetics. *Cell Metab* 4:75–87, 2006
28. Kim I, Rodriguez-Enriquez S, Lemasters JJ: Selective degradation of mitochondria by mitophagy. *Arch Biochem Biophys* 462:245–253, 2007
29. de Cavanagh EM, Toblli JE, Ferder L, Piotrkowski B, Stella I, Inserra F: Renal mitochondrial dysfunction in spontaneously hypertensive rats is attenuated by losartan but not by amlodipine. *Am J Physiol Regul Integr Comp Physiol* 290:R1616–R1625, 2006
30. de Cavanagh EM, Piotrkowski B, Basso N, Stella I, Inserra F, Ferder L, Fraga CG: Enalapril and losartan attenuate mitochondrial dysfunction in aged rats. *FASEB J* 17:1096–1098, 2003
31. Shiuchi T, Iwai M, Li HS, Wu L, Min LJ, Li JM, Okumura M, Cui TX, Horiuchi M: Angiotensin II type-1 receptor blocker valsartan enhances insulin sensitivity in skeletal muscles of diabetic mice. *Hypertension* 43:1003–1010, 2004
32. Pan DA, Lillioja S, Kriketos AD, Milner MR, Baur LA, Bogardus C, Jenkins AB, Storlien LH: Skeletal muscle triglyceride levels are inversely related to insulin action. *Diabetes* 46:983–988, 1997
33. Perseghin G, Scifo P, De Cobelli F, Pagliato E, Battezzati A, Arcelloni C, Vanzulli A, Testolin G, Pozza G, Del Maschio A, Luzi L: Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: a 1H–13C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. *Diabetes* 48:1600–1606, 1999
34. Griffin ME, Marcucci MJ, Cline GW, Bell K, Barucci N, Lee D, Goodyear LJ, Kraegen EW, White MF, Shulman GI: Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes* 48:1270–1274, 1999
35. Kraegen EW, Cooney GJ: Free fatty acids and skeletal muscle insulin resistance. *Curr Opin Lipidol* 19:235–241, 2008
36. Kelley DE, Goodpaster B, Wing RR, Simoneau JA: Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am J Physiol* 277:E1130–E1141, 1999
37. Befroy DE, Petersen KF, Dufour S, Mason GF, de Graaf RA, Rothman DL, Shulman GI: Impaired mitochondrial substrate oxidation in muscle of insulin-resistant offspring of type 2 diabetic patients. *Diabetes* 56:1376–1381, 2007
38. Gerhart-Hines Z, Rodgers JT, Bare O, Lerin C, Kim SH, Mostoslavsky R, Alt FW, Wu Z, Puigserver P: Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha. *EMBO J* 26:1913–1923, 2007
39. Civitarese AE, Smith SR, Ravussin E: Diet, energy metabolism and mitochondrial biogenesis. *Curr Opin Clin Nutr Metab Care* 10:679–687, 2007
40. Schrauwen-Hinderling VB, Roden M, Kooi ME, Hesselink MK, Schrauwen P: Muscular mitochondrial dysfunction and type 2 diabetes mellitus. *Curr Opin Clin Nutr Metab Care* 10:698–703, 2007

"Pulse" Treatment With High-Dose Angiotensin Blocker Reverses Renal Arteriolar Hypertrophy and Regresses Hypertension

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Abstract—One ultimate goal of hypertension therapy is to cause permanent reversal ("regression") of already established hypertension. Our aim was to examine whether high-dose "pulse" treatment with a renin-angiotensin system inhibitor could cause regression of established hypertension and to link this action to reversal of arteriolar hypertrophy and changes in vascular matrix metalloproteinase activities. First, 16-week-old male spontaneously hypertensive rats (n=60) were pulse treated for 2 weeks with high-dose angiotensin-converting enzyme inhibitor (enalapril), angiotensin receptor blocker (candesartan), calcium channel blocker (nifedipine), or vasodilator (hydralazine) with or without salt restriction, and the long-term effects on blood pressure were examined. Second, spontaneously hypertensive rats were treated with angiotensin receptor blocker or calcium channel blocker, and the effects on renal gene expressions, arteriolar structure, and vascular matrix metalloproteinase were compared. Treatment of spontaneously hypertensive rats with different antihypertensive agents caused apparently similar reductions in blood pressure during the course of the pulse treatment, within the limitations of the tail-cuff method. After cessation of medications, blood pressure in the rats treated with renin-angiotensin system inhibitor remained reduced by >30 to 40 mm Hg for 4 months. No such effect was seen with calcium channel blocker or vasodilator. The 2-week angiotensin receptor blocker treatment induced a marked reversal of the arteriolar hypertrophy specifically in the small (30 to 100 μm) renal arterioles, together with increased expression and activity of matrix metalloproteinase-13. In conclusion, transient high-dose pulse treatment with angiotensin receptor blocker caused changes in vascular matrix metalloproteinase activity, specific reversal of renal arteriolar hypertrophy, and regression of hypertension in spontaneously hypertensive rats. **(*Hypertension*. 2009;53:83-89.)**

Key Words: angiotensin receptor blocker ■ calcium channel blocker ■ regression ■ spontaneously hypertensive rat ■ MMP ■ renal arteriolar hypertrophy

It has been estimated that ≈26.4% of the adult world population in the year 2000 had hypertension, and the number was projected to increase to 29.2% by the year 2025.[1] Because hypertension is a major risk factor for diseases such as stroke, coronary artery disease, heart failure, kidney disease, and vascular disease, the medical, economic, and social consequences of the current epidemic of hypertension are considerable.[2]

One strategy for managing this disease is "prevention" of the development of hypertension. Previous studies by Harrap et al,[3] Richer et al,[4] and other groups, including our own[5–7], have shown that treatment of young (4- to 6-week-old) prehypertensive spontaneously hypertensive rats (SHRs) with a renin-angiotensin system (RAS) inhibitor is effective in permanently attenuating the later development of hypertension. In other words, transient administration of a RAS inhibitor, if given before hypertension was fully established, was found to be effective for hypertension prevention in SHR. The feasibility of using transient RAS inhibition to prevent the development of hypertension in human patients has been confirmed recently by Julius et al[8] in the landmark Trial of Preventing Hypertension.

A different strategy would be to aim for "regression" of already established hypertension. Importantly, Smallegange et al[9] reported that transient treatment of adult SHRs with a high-dose angiotensin-converting enzyme inhibitor (ACEI), together with a low-salt diet, was effective in causing a sustained reduction of blood pressure even if administration of the drug was started at 16 weeks, well after hypertension was established in the SHR model. These results suggested that high-dose RAS inhibition could indeed be effective in the reversal or regression of already established hypertension. Potentially, this could have a great clinical benefit, because it could mean that patients with established hypertension could well be "cured" by appropriate transient therapy.

At present, it is unclear whether regression of hypertension is an effect that is specific to RAS inhibitors or is generally

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seen with high doses of other antihypertensive agents, such as calcium channel blockers (CCBs) or vasodilators. The molecular mechanism is also undefined, in particular, the relationship with renal arteriolar hypertrophy, which is thought to play an important role in the maintenance of hypertension in SHRs.[10,11]

The objective of this study was, therefore, to test the hypothesis that high-dose pulse treatment with a RAS inhibitor but not a CCB would cause regression of established hypertension and to link this action to reversal of arteriolar hypertrophy and changes in vascular matrix metalloproteinase (MMP) activities. The specific aim of the first experiment was to compare the efficacy of a total of 8 antihypertensive pulse regimens (with and without a low-salt diet) in inducing regression. In the second experiment, we examined whether high-dose pulse treatment could affect the renal arteriolar hypertrophy found in SHRs, as well as cause changes in activities of the vascular matrix metalloproteinases (MMPs). Our results suggest that high-dose pulse treatment with a RAS inhibitor causes changes in arteriolar MMP activity, leading to reversal of arteriolar hypertrophy and, ultimately, to regression of hypertension in the SHR model.

Methods

Animal Treatment Protocols

The studies were conducted using 16-week-old male Wistar-Kyoto (WKY) rats (WKY/Izm) and SHRs (SHR/Izm) obtained from Sankyo Laboratory Services (Tokyo, Japan). All of the experiments were approved by the institutional review committee and performed in accordance with the Keio University School of Medicine Animal Experimentation Guidelines.

Experiment 1

SHRs were randomly divided into 10 groups as follows (n=6 per group). Rats in group 1 were control SHRs. Rats in groups 2 to 5 were treated from 16 to 18 weeks with the ACEI enalapril maleate in drinking water (20 mg/kg per day), the ARB candesartan cilexetil dissolved in the drinking water[7] (50 mg/kg per day), the vasodilator hydralazine (25 mg/kg per day), or the CCB nifedipine in chow (50 mg/kg per day). Rats in groups 6 to 10 were treated identically to groups 1 to 5 but were also treated from 16 to 18 weeks with a low-salt diet (0.05% Na). All of the interventions were discontinued at age 18 weeks, and the rats were observed without any medication for a further 18 weeks, then euthanized at age 36 weeks.

Experiment 2

WKY rats and SHRs were randomly divided into 4 groups as follows (n=6 per group). Rats in group 1 were control WKY rats. Rats in group 2 were control SHRs. Rats in groups 3 and 4 (ARB and CCB groups) were treated with either the ARB candesartan (50 mg/kg per day) or the CCB nifedipine (50 mg/kg per day), as described above, then euthanized at the end of the 2-week pulse treatment at age 18 weeks.

Assays

The systolic blood pressure and heart rate of awake animals were measured by tail-cuff plethysmography using a Natsume KN-210 manometer (Natsume, Inc). Twenty-four-hour urine collection was performed in metabolic cages, and urine albumin excretion was determined by a direct competitive ELISA (Nephrat). Other biochemical assays are described in the online data supplement (available at http://hyper.ahajournals.org).

Histological Studies

The kidneys and thoracic aortas were removed and fixed in 4% paraformaldehyde, then embedded in paraffin blocks. In experiment 2, tissue samples were also obtained from the mesentery, heart, and brain, for the examination of mesenteric, cardiac, and cerebral arterioles. Details of the histological assessment are described in the online data supplement.

Preparation of RNA and Real-Time RT-PCR and Microarray Analysis

Kidney RNA was prepared for real-time RT-PCR and microarray analysis, as described in detail in the online data supplement.

In Situ Zymography and Immunofluorescence Staining

High-resolution, high-sensitive zymography was performed using the protocol of Ahmed et al,[12] with minor modifications. Immunofluorescence staining of vascular MMP expression was performed using standard protocols (for details, see the online data supplement).

Statistics

Results were expressed as the means±SEMs. Statistical comparisons were made by ANOVA, followed by Scheffe's posthoc test. P values <0.05 were considered statistically significant.

Results

Experiment 1

Effects of Pulse Treatment With Antihypertensive Agents on Systolic Blood Pressure in SHRs

The changes in systolic blood pressure in the different groups are shown in Figure 1. At age 16 weeks, before the initiation of the pulse treatment, hypertension had been fully established, ie, the blood pressure in the different groups had reached the plateau value of ≈220 mm Hg. Treatment with the different antihypertensive agents caused a decrease in blood pressure to ≈150 mm Hg during the duration of the 2-week pulse therapy. After discontinuation of the antihypertensive medication, the blood pressure rapidly reverted to control values in the CCB- and vasodilator-treated groups. In clear contrast, the blood pressure in the ACEI- and ARB-treated groups were maintained at values of ≤180 mm Hg (a difference of >30 to 40 mm Hg). Essentially similar results were found in groups 6 to 10, which had been exposed to a low-salt diet from age 16 to 18 weeks (Figure 1B).

Effects of Pulse Treatment With Antihypertensive Agents on Cardiovascular Hypertrophy, Parameters of Renal Function, and Plasma Renin Activity/Plasma Aldosterone Concentration at Age 36 Weeks in SHRs

As expected from the sustained decrease in blood pressure, the heart weight:body weight ratios, aortic media:lumen ratios, and renal arteriolar media:lumen ratios were decreased at the end of the study (age 36 weeks) in the rats previously treated with pulse ACEI or ARB (data not shown). No significant differences in blood urea nitrogen, plasma creatinine, plasma renin activity, plasma aldosterone concentration, or the oxidative marker plasma lipid peroxides were found in the different groups at age 36 weeks.

Experiment 2

Short-Term Effects of Pulse Treatment With ARB or CCB on Cardiovascular Hypertrophy and Parameters of Renal Function at Age 18 Weeks in WKY Rats and SHRs

In experiment 2, we examined the short-term effects of the 2-week pulse treatment on cardiovascular hypertrophy and

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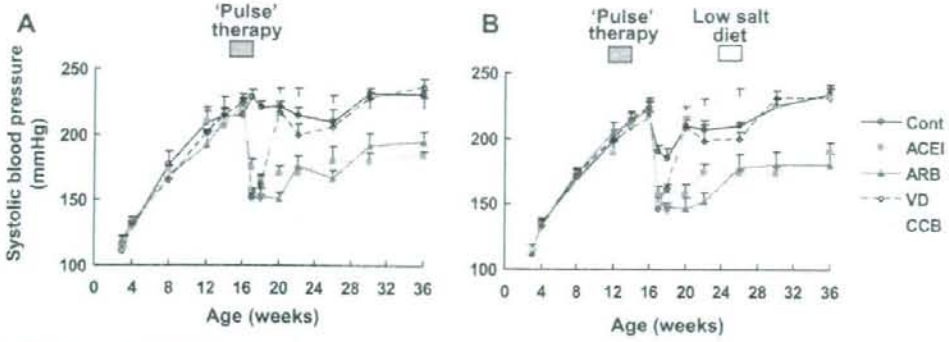

Figure 1. Effects of pulse treatment with antihypertensive agents, with and without a low-salt diet, on systolic blood pressure in SHRs. A, Results without a low-salt diet. B, Results with a low-salt diet. Cont indicates control; ACEI, treated with pulse enalapril; ARB, treated with pulse candesartan; Vo, treated with pulse hydralazine; and CCB, treated with pulse nifedipine from age 16 to 18 weeks. Systolic blood pressures in the ACEI and ARB groups were significantly ($P<0.01$) decreased vs the Cont groups at all time points from age 20 weeks onward in both A and B (symbols have been omitted for the sake of clarity).

parameters of renal function in 4 groups of rats: normotensive WKY rats, control SHRs, and SHRs treated with either ARB or CCB for 2 weeks, and data were obtained immediately at the end of the pulse treatment (age 18 weeks). Pulse treatment with ARB was associated with a small decrease in heart weight:body weight ratios compared with CCB, but the results did not attain statistical significance. Similarly, aortic media:lumen ratios were not significantly changed in the rats treated with ARB or CCB. In contrast, the media:lumen ratios in the small (30 to 100 μm) arterioles were markedly reduced by the ARB pulse treatment but not by the CCB (Figure 2). Of interest, the decreases in media:lumen ratios were found to be specific for renal small arterioles and were not found in larger renal arterioles (100 to 300 μm) or arterioles from other tissues, namely, the mesentery, heart, and brain (Table S1). Urine albumin excretion was significantly decreased in the SHRs treated with ARB (WKY: 7.2±2.6 mg/d; SHR: 9.2±3.0 mg/d; SHR+ARB: 0.8±0.4 mg/d [$P<0.05$ vs SHR]; SHR+CCB: 3.6±1.2 mg/d), but no significant differences in blood urea nitrogen, plasma creatinine, or plasma lipid peroxides were found in the different groups.

Microarray Analysis of Differences in Renal Gene Expressions in SHRs Treated With Pulse ARB or CCB for 2 Weeks

The differences in expression of a total of 28 000 genes in the kidneys of SHRs treated with ARB or CCB were examined using the Affymetrix rat 230 2.0 gene expression array. A total of 1345 genes were elevated in the ARB-treated rats compared with the CCB-treated rats, whereas 5671 were reduced. Several extracellular matrix–related genes, including type IV procollagen and MMP-15, were elevated in the ARB-treated rats, whereas MMP-9, tissue inhibitor of matrix metalloproteinase (TIMP)-2, and TIMP-3 gene expressions

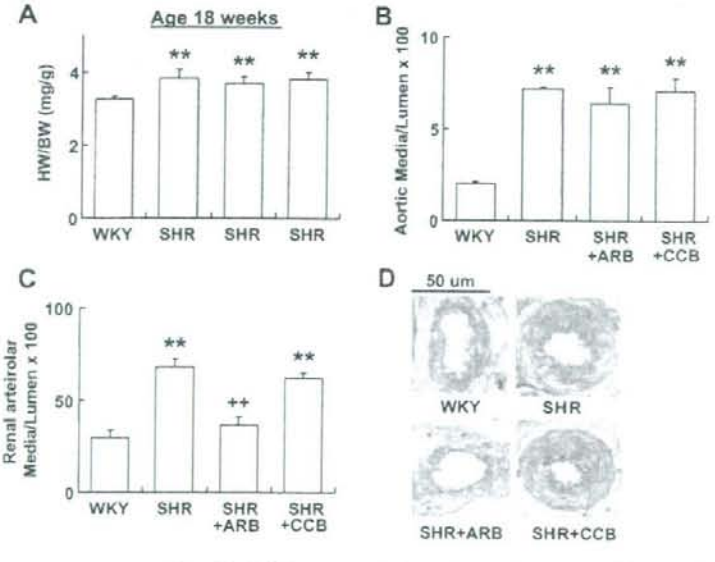

Figure 2. Short-term effects of pulse treatment with ARB or CCB on cardiovascular hypertrophy at age 18 weeks in SHRs. A, Heart weight:body weight ratios. B, Aortic media:lumen ratios. C, Media:lumen ratios of renal small arterioles (30 to 100 μm). D, Representative photomicrographs of renal arterioles in the different groups. WKY indicates untreated WKY rats; SHR, untreated SHRs; SHR+ARB, SHRs treated with pulse candesartan; SHR+CCB, SHRs treated with pulse nifedipine. **$P<0.01$ vs WKY rats; ++$P<0.01$ vs SHRs.

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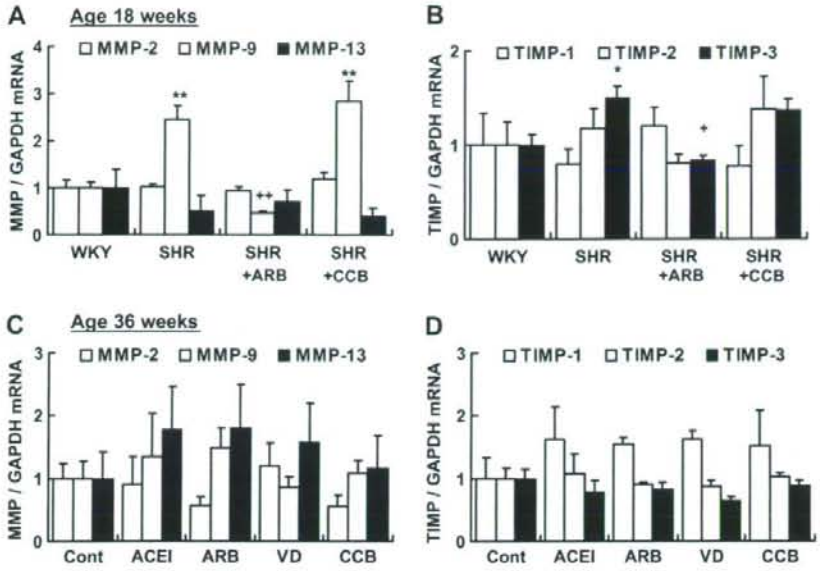

Figure 3. Effects of pulse treatment with ARB or CCB on renal gene expressions in SHRs. A, MMP-2, MMP-9, and MMP-13 mRNA at age 18 weeks. B, TIMP-1, TIMP-2, and TIMP-3 mRNA at age 18 weeks. C, MMP-2, MMP-9, and MMP-13 mRNA at age 36 weeks. D, TIMP-1, TIMP-2, and TIMP-3 mRNA at age 36 weeks. Abbreviations of groups as in Figures 1 and 2. $^{*}P<0.05$, $^{**}P<0.01$ vs WKY rats; $+P<0.05$, $++P<0.01$ vs SHRs.

were decreased in the ARB-treated group (Table S2). Among the genes of the RAS, only renin mRNA was increased in the ARB-treated group, which was expected as a feedback response to inhibition of the RAS.

Real-Time RT-PCR Analysis of the Short-Term Effects of Pulse Treatment With ARB or CCB on Renal Gene Expressions at Age 18 Weeks in WKY Rats and SHRs

To confirm the results of the microarray analysis, the differences in the gene expression of MMP-2, MMP-9, MMP-13, TIMP-1, TIMP-2, and TIMP-3 were assessed by real-time RT-PCR. As shown in Figure 3, pulse treatment of SHRs with ARB caused a significant decrease in MMP-9 and TIMP-3 mRNA expression, whereas no significant effect was seen with CCB, findings that were consistent with the results of the microarray analysis. Values of MMP-13 mRNA expression were somewhat higher in the ARB-treated rats compared with CCB at the end of the pulse treatment (18 weeks), but the results did not attain statistical significance. No significant differences were found in any of these genes between the different groups at age 36 weeks.

Short-Term Effects of Pulse Treatment With ARB or CCB on Vascular MMP Activity and Expression at Age 18 Weeks in WKY Rats and SHRs

High-resolution, high-sensitivity in situ zymography was performed to examine the activity of MMPs in the renal microvasculature. As shown in Figure 4, degradation of type I collagen (type I collagenolytic activity) in the renal arterioles was found to be clearly increased in the SHRs treated with ARB but not with CCB. Parallel experiments were performed in the presence of an MMP-13 inhibitor, because MMP-13 is known to be the predominant MMP involved in degradation of type I collagen in the rat, which lacks the MMP-1 gene. The type I collagenolytic activity in the vasculature was inhibited by the MMP-13 inhibitor, confirming that the changes seen reflected MMP-13 activity. In the case of type IV collagenolytic activity, MMP-9–dependent degradation of type IV collagen was decreased in ARB-treated rats but not in the CCB-treated rats. Both type I and type IV collagenolytic activities were completely inhibited by the broad-spectrum MMP inhibitor 1-10 phenanthrolene (data not shown). Examination of MMP-13, MMP-2, and MMP-9 expression by immunofluorescence staining showed a similar trend to the results of in situ zymography (Figure 5).

Discussion

The main findings of this study were as follows: (1) pulse treatments with ARB and ACEI (with or without concomitant low-salt diet treatment) were equally effective in causing a long-term reduction in blood pressure; (2) the reductions in blood pressure were accompanied by long-term reductions in cardiac and vascular hypertrophy; (3) the pulse treatment caused a remarkable regression of renal arteriolar hypertrophy in the course of just 2 weeks; and (4) these changes were associated with changes in expression and activity of vascular MMPs in the kidney.

The fact that long-term reductions in blood pressure were seen with an ARB and ACEI but not with the CCB or vasodilator is of interest in view of the widespread use of these agents for the treatment of hypertension. Concerning the dose of ARB or ACEI required to obtain regression, we are performing a companion study to examine the effects of

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