FOXOs = Forkhead box, subgroup O family
H_2O_2 = Hydrogen peroxide
HMG-CoA = 3-hydroxy-3 methylglutaryl coenzyme A
HOCl = Hypochlorous acid
ICAM-1 = Intercellular adhesion molecule-1
IgG = Immunoglobulin G
IgM = Immunoglobulin M
JNK = Janus-family tyrosine kinase
LDL = Low-density lipoprotein
LOX-1 = Lectin-like receptor for ox-LDL
MAPK = Mitogen-activated protein kinase
MCP-1 = Monocyte chemoattractant/chemotactic protein
MDA = Malondialdehyde
MMP = Matrix metalloproteinase
NADPH = Nicotinamide adenine dinucleotide phosphate
NAFLD = Non-alcoholic fatty liver disease
Nef2 = Nuclear factor [erythroid-derived 2]-like 2
NF-kb = Nuclear factor-kappaB,
NO = Nitric oxide
NOS = Nitric oxide synthases
NOX2 = NADPH oxidase 2 (gp91phox)
NPC1L1 = Niemann-Pick C1-Like 1
$O_2^{\cdot -}$ = Superoxide anion
$OH^{\cdot}$ = Hydroxyl radical
ox-LDL = Oxidized LDL
PI3K = Phosphoinositide 3-kinase
PPARγ = Peroxisome proliferator-activated receptor gamma
RHI = Reactive hyperemia index
ROS = Reactive oxygen species
S1P = Sphingosine 1-phosphate
SR-B1 = Scavenger receptor type B1
TBARS = Thiobarbituric acid reactive substances
TG = Triglyceride
Th = T helper
TRL = Triglyceride-rich lipoproteins
VEGF = Vascular endothelial growth factor
VCAM = Vascular cell adhesion molecule
VLDL = Very low-density lipoproteins
ZOF = Zucker Obese fatty

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Received: September 01, 2009 Revised: October 01, 2009 Accepted: October 03, 2009

特集　透析患者における運動・リハビリ療法

臨牀透析 2011 Vol.27 No.10
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［各　論］

Ⅲ 透析患者に対する運動療法の適応と禁忌

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要旨 血液透析（HD）患者の健康状態を維持・向上させるための手段として，HD患者に対する運動療法の重要性が高まっている．運動機能が著しく低下したHD患者や合併症を有するHD患者が運動療法を行う場合には，まず運動に対する安全性の確保が優先される．そのため，HD患者の病態を十分に把握し，運動療法の適応があるか否かを判断しなければならない．運動療法を安全に長期間行うために，医療従事者は心血管疾患や透析アミロイドーシスといった合併症を評価し，患者に見合った運動様式や負荷量の運動療法を処方する．HD患者に対する運動療法は，有酸素運動と筋力増強運動を併用した運動プログラムを，非HD日あるいはHD中に行うことが推奨される．

〈key point〉

はじめに

血液透析（hemodialysis；HD）患者に対して有酸素運動あるいは筋力増強運動などの運動療法を実施すると，患者の運動機能やquality of lifeが有意に向上することが知られている[1]．生涯にわたってHD治療を必要とする末期腎不全患者は，年々高齢化が進んでいることから，健康状態を維持・向上させるための一つの手段としてHD患者に対する運動療法の重要性が高まっている．運動療法を効果的に行うためには，一定以上の負荷の運動を適切な頻度で行う必要があるが，運動機能が著しく低下したHD患者や合併症を有するHD患者に運動療法を行う場合は，まず運動に対する安全性の確保が優先される．運動療法を安全に長期間継続するためには，医療従事者はHD患者が有する合併症を十分に把握したうえで，患者に見合った運動療法を処方する必要がある．

Key words 血液透析，運動療法，合併症

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0910-5808/11/頁/JCOPY

I．運動療法の適応

ここでの重要なポイント

- 米国腎臓財団の Kidney Disease Outcomes Quality Initiative は，すべての HD 患者に対して運動療法を推奨している．
- 病態の急激な変化や新たな合併症を生じた場合には，医学的管理を優先し，その病態が改善してから運動療法を再開する．

HD患者の運動機能

HD 患者の運動機能は，同年代の健常成人の 60〜80 %に低下していると報告されている[2]．また，多くの HD 患者は運動機能の低下，すなわち筋力や持久力の低下によって，歩行や階段昇降あるいは上肢の動作に困難を感じており[3),4]，身体活動量は健常成人よりも著しく低下している[5]．運動機能の低下は日常生活活動（activities of daily living；ADL）を制限し，ADL の制限が運動機能のさらなる低下をもたらすという悪循環を形成することから，この悪循環を断ち切るためには，運動療法が効果的な手段となりうる．このことは，米国腎臓財団の Kidney Disease Outcomes Quality Initiative が 2005 年の指針で，すべての HD 患者に対して運動療法を推奨していることと一致する[6]．

全身状態から運動療法の可否を判断

HD 患者の多くは運動療法のよい適応となるが，実際に運動療法を処方するにあたっては，患者の全身状態から運動療法の可否を判断することになる．とくに，非代償性心不全の患者や栄養状態が不良な患者は，運動療法の実施が困難な場合が多い．このような患者は，運動療法の導入前に病態の変化を注意深く観察し，その変化を把握したうえで運動療法を開始する．また，病態の急激な変化や新たな合併症を生じた場合には，薬物療法などの医学的管理を優先し，その病態が改善してから運動療法を再開する．骨関節障害に対して外科的治療を必要とする場合，あるいは関節痛や痺れが増悪している時期は運動療法を実施すべきではない．

II．運動療法のリスク管理

ここでの重要なポイント

- 心血管疾患における運動療法の禁忌やリスクの層別化を参考にして，HD 患者に対して適切な運動療法を処方する．
- 運動中は，血圧，心拍数，心電図，兆候および自覚症状をモニタリングし，運動が過負荷にならないように注意する．

1）HD 患者の合併症

HD 患者は，末期腎不全に加えて心血管疾患や骨関節障害などの合併症を重複して有することが多いため，その障害像も多様である．とくに注意を要する合併症として，虚血性心疾患や心不全および透析アミロイドーシスが挙げられる．

心血管疾患

HD 患者は，心血管疾患を高率に合併し運動療法の実施を妨げるため，運動に対する心血管疾患のリスクを正確に評価する必要がある．また，透析アミロイドーシスは，骨関節障害や末梢神経障害を生じるため，関節痛や痺れを悪化させないように，その障害部位や重症度を十分に把握する．さらに，末期腎不全に起因する筋障害や安静による廃用性の筋力低下は，運動機能を著しく低下させ ADL を制限するため，運動療法の実施に際してその評価が重要となる．

透析アミロイドーシス

2）運動療法の禁忌とリスクの層別化

運動療法を処方する場合，初めに合併症や運動能力の程度に基づいて，HD 患者の運動に対するリスクを評価して，患者一人ひとりの運動プログラムを決定する．とくに，複数の合併症を有している患者に対しては，合併症を管理するための優先順位を決めてから運動療法を実施する．

患者一人ひとりの運動プログラムを決定

HD 患者は，程度の差こそあれ慢性心不全の状態といえるため，運動を実施する際には，心不全における運動療法の禁忌や（**表 1**）〔参考 URL[1]〕，American Association of Cardiovascular and Pulmonary Rehabilitation が提唱したリスクの層別化を参考にするとよい[7]．なお，HD 患者の体重増加に関しては，通常以上の体重増加があり，さらに血圧の上昇，心胸郭比の増大，下腿浮腫の出現などを認めた場合には，心負荷の増大を考えて運動療法の実施を慎重に判断する．

体重増加

表 1　心不全を合併する血液透析患者に対する運動療法

絶対的禁忌	・不安定狭心症，または低負荷で誘発される心筋虚血（ゆっくりとした平地歩行で誘発される） ・過去 1 週間以内における心不全症状（呼吸困難，易疲労性など）の増悪 ・未治療の運動誘発性重症不整脈（心室細動，持続性心室頻拍） ・手術適応のある重症弁膜症，とくに大動脈弁狭窄症 ・急性全身性疾患または発熱 ・運動療法が禁忌となるその他の疾患（中等症以上の大動脈瘤，重症高血圧，血栓性静脈炎，2 週間以内の塞栓症，重篤な多臓器障害など）
相対的禁忌	・NYHA Ⅳ度の心不全，または強心薬を静脈内投与している心不全 ・運動によって自覚症状が悪化する場合（疲労，めまい，発汗多量，呼吸困難など） ・運動によって収縮期血圧が低下する場合 ・運動誘発性の中等症不整脈（非持続性心室頻拍，頻脈性心房細動など） ・高度房室ブロック
禁忌とならないもの	・低心機能症例（左室駆出率の低下） ・高齢であること ・埋め込み型除細動器（ICD）装着例

NYHA：New York Heart Association classification，ICD：implantable cardioverter defibrillator

〔心血管疾患におけるリハビリテーションに関するガイドライン（2007 年改訂版）（参考 URL[1]）を基に筆者作成〕

表2　運動中のモニタリング項目と運動療法の中止基準

モニタリング項目	中止基準
収縮期血圧	安静時より40 mmHg以上の上昇，または10 mmHg以上の低下
心拍数	130拍/min以上への上昇
心電図	明らかな虚血性ST-T変化，発作性心房細動，心室性期外収縮頻発，R on T型心室性期外収縮，または心室頻拍の出現
兆候	チアノーゼ，顔面蒼白，冷汗，または運動失調の出現
自覚症状	狭心痛，動悸，呼吸困難，息切れ，めまい，ふらつき，倦怠感，または下肢疼痛の出現

3）運動療法の中止基準

運動中は，血圧，心拍数，心電図，兆候および自覚症状をモニタリングし，身体に対して運動の過負荷を示す所見が認められた場合には，いったん運動を中止する（**表2**）．そして，合併症の悪化や新たな合併症の出現がないことを確認したうえで，運動の負荷強度や頻度を下げるなどの調節を行う．

Ⅲ．運動療法の実際

ここでの重要なポイント

- HD患者に対する運動療法として，有酸素運動と筋力増強運動を併用した運動プログラムが推奨される．
- 非HD日あるいはHD中は，HD患者の心循環動態が安定し自覚症状が少ないことから，運動療法に適した時間帯である．

HD患者に運動療法を処方する際には，運動様式や負荷量に加えて，運動を行う時間帯を考慮する．

1）運動様式と負荷量

運動療法として，有酸素運動と筋力増強運動を併用した運動プログラムが推奨されており，負荷強度や頻度は患者の運動機能や合併症に応じて個別に設定する．

有酸素運動

有酸素運動として，ウォーキングやエルゴメータを使用した運動が行われる．有酸素運動の負荷量を設定する目安は，運動後に強い疲労が残らない程度とし，自覚的運動強度（Borg指数）が「楽である」から「や

用語解説

● Borg指数

運動負荷試験中に生じる自覚症状を定量化するために作成された指標であり，現在では運動処方や生活指導などにも用いられている．運動の自覚的な強度を6〜20までの15段階で示し，数値が大きいほど運動はきついことになる．数値を10倍するとおよその心拍数になり，11から13に当たる強度がほぼ嫌気性代謝閾値に相当するとされる．

やきつい」の11〜13になるような強度の運動を処方する[8]．運動中は血圧や心拍数が過度に上昇しないことを確認して，週3回以上の運動を目標とする．筋力増強運動は，重錘，ウエイトマシンあるいはゴムチューブなどの器具を用いて行うが，骨関節障害を有する場合には，運動負荷量の調節が容易なゴムチューブが適している．負荷量は，10〜15回反復できる強さを設定し，1セット当り10〜15回，1日1〜3セットを行う[9]．

筋力増強運動

2）運動を行う時間帯

非HD日

非HD日は，HD日と比べて血圧や心拍数が安定し自覚症状が少ないことから，比較的強い運動が実施できる．しかし，HD患者には週3回のHD治療を行うという時間的制約があることから，患者には自宅で家事の合間やテレビを見ながら，座位や立位で行えるような筋力増強運動を指導することが多い．また，HD日に運動療法を実施する場合は，心循環動態が安定するHD開始から2時間以内のHD中に行うと，比較的安全に運動療法を実施することができる．とくに，HD中の運動療法は，医療従事者の監視下で，運動中のバイタルサインや自覚症状の変化を詳細に観察できるため，合併症を有し運動機能が低下した高齢HD患者に対しても安全に実施できる方法と思われる[10]．具体的には，持ち運び可能なエルゴメータをベッド上あるいはベッドサイドに固定して有酸素運動を行う[10]．HD前後の時間を利用して運動療法を行う場合は，患者の心循環動態が安定し自覚症状に問題がないことを確認したうえ

HD日

ワンポイント**アドバイス**

腰痛を訴える患者にはどの程度まで運動を勧めたらよいのでしょうか？

腰痛のあるHD患者に対する運動の可否は，その腰痛の原因によるため，まずは専門医による判断が必要である．腰痛の原因として，緊急の薬物治療や外科的治療を要する骨関節障害がないことを確認したうえで，運動療法や生活指導といった保存療法を開始する．専門医から保存療法を指示された場合は，腰痛が生じてから2日以内は腰痛の変化を観察し，続いて患者の状態に合った運動療法を処方する．

腰痛に対する運動療法として，胸筋や背筋をストレッチして過剰な筋緊張を和らげ，加えて腹筋や背筋の筋力増強運動を行って体幹の安定性を高めると効果的である．ストレッチは，気持ちよくリラックスできる程度の強さで，息を吐きながら10秒程度筋肉を伸長する．筋力増強運動は，腰痛を悪化させないように細心の注意を払い，無理のない負荷で行う．生活指導は，腰椎の生理的前彎を保持するために，日常生活における姿勢や身体の使い方を指導する．具体的には，① 座位や立位の姿勢をとる際は前かがみにならない，② 椅子には深く腰掛ける，③ 眠るときには股関節と膝関節を屈曲させた姿勢をとる，といった指導を行う．運動療法と生活指導を同時に行うと，腰痛の緩和にはより効果的である．

で，医療従事者の厳重な監視下で実施することが望ましい．

おわりに

HD 患者に対する運動療法の有用性は高いにもかかわらず，日常診療に運動療法を取り入れている透析施設は少ない．HD 患者が運動療法を安全に実施できれば，その効果は顕著であることから，今後は多くの透析施設が日常診療の一環として運動療法を実施していくことが望まれる．

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Summary

Indications and contraindications for exercise training for maintenance hemodialysis patients

Toshiki Kutsuna * and Takashi Masuda **

Because exercise training is known to be one effective means for improving the overall condition of patients undergoing maintenance hemodialysis (HD), the study titled, Kidney Disease Outcomes Quality Initiative by the National Kidney Foundation states that all HD patients should engage in exercise training. Medical staff members must thoroughly evaluate the physical condition and circumstances associated with comorbidity in HD patients. This must be accomplished to judge whether or not they can safely perform exercises. In addition, it is important to monitor blood pressure, heart rate, electrocardiograms and other symptoms during exercise routines to prevent cardiac overload. It has been reported that exercise training can be safely performed by HD patients on non-HD days or during HD sessions, because most of these patients are hemodynamically stable and feel fewer symptoms at those times. Both aerobic and resistance exercises have also been demonstrated to be effective interventions to augment decreased exercise capacity and reduced muscle strength in HD patients.

Key words：hemodialysis, exercise training, comorbidity

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Endogenous hydrogen peroxide up-regulates the expression of nitric oxide synthase in the kidney of SHR

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Background and method **Both nitric oxide synthase (NOS) expression and oxidative stress are elevated in the tissues of spontaneously hypertensive rats (SHR) compared with Wistar–Kyoto rats (WKY). The purpose of the present study was to determine the relationship between the endothelial and neuronal NOS (eNOS and nNOS) expression and oxidative stress in the kidney of SHR and WKY.**

Results **Plasma and urinary hydrogen peroxide (H_2O_2) and nitrate/nitrite (NO_x), the renal NADPH oxidase activity and eNOS and nNOS expressions were all higher in SHR than in WKY. Although the treatment with either the NADPH oxidase inhibitor, apocynin or the superoxide dismutase mimetic, tempol for 8 weeks decreased the systolic blood pressure (SBP) and inhibited the renal NADPH oxidase activity in SHR, apocynin decreased but tempol increased the plasma and urinary H_2O_2 and NO_x and the eNOS and nNOS expressions in the renal cortex and medulla of SHR. In contrast to SHR, neither apocynin nor tempol affected these parameters in WKY. H_2O_2 administered intravenously for 1 week in WKY increased plasma and urinary H_2O_2 and NO_x and the eNOS and nNOS expressions in the renal cortex and medulla in a dose-dependent manner without changing the renal NADPH oxidase activity.**

Conclusion **These results indicate that oxidative stress up-regulates the NOS expression in the kidney of SHR compared with WKY; and that endogenous H_2O_2 is a mediator of the up-regulation of the NOS expression in the kidney of SHR. *J Hypertens* 29:1167–1174 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Journal of Hypertension 2011, 29:1167–1174

Keywords: hydrogen peroxide, hypertension, kidney, NADPH oxidase, nitric oxide synthase, oxidative stress, superoxide dismutase

Abbreviations: eNOS, endothelial NOS; H_2O_2, hydrogen peroxide; nNOS, neuronal NOS; NOS, nitric oxide synthase; NO_x, nitrate/nitrite; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite; ROS, reactive oxygen species; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; SOD, superoxide dismutase; WKY, Wistar–Kyoto rats

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Received 8 November 2010 Revised 25 February 2011
Accepted 7 March 2011

Introduction

Nitric oxide is a vasodilatory factor synthesized by three isoforms of nitric oxide synthase (NOS): endothelial, neuronal and inducible NOS (eNOS, nNOS and iNOS) [1]. The eNOS and nNOS expression is elevated in the kidney and vessels of spontaneously hypertensive rats (SHR) compared with normotensive Wistar–Kyoto rats (WKY) [2,3]. The NOS activity and nitric oxide production were also elevated in these tissues of SHR [4,5], and NOS blockade caused marked increases in blood pressure in SHR [6]. However, nitric oxide-mediated vasodilatory responses were impaired in SHR [7].

Oxidative stress is involved in several pathophysiological conditions, including hypertension, hypercholesterolemia and diabetes [8,9]. Superoxide anion (O_2^-) is generated by NADPH oxidase [8,10] and metabolized into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). Oxidative stress and reactive oxygen species (ROS) are elevated in SHR [11,12] due to increased NADPH oxidase activity [5,13] and decreased SOD activity [14]. O_2^- and ROS can interact rapidly and irreversibly with nitric oxide to yield peroxynitrite ($ONOO^-$), thereby restricting the half-life, diffusion distance and bioactivity of nitric oxide in tissues [15]. Both the inhibitor of NADPH oxidase, apocynin and the SOD mimetic, tempol reduced blood pressure and improved the impaired vasodilatory responses in SHR [5,16–18].

Several mechanisms are speculated to regulate the NOS expression in a hypertensive state. Shear stress increased the eNOS expression in cultured endothelial cells [19]. Nitric oxide exerted a negative-feedback influence on the eNOS expression in cultured endothelial cells [20,21], and an antioxidant therapy mitigated the up-regulated eNOS and nNOS expression in SHR [4] suggesting that oxidative stress may up-regulate the NOS expression to compensate ROS-mediated nitric oxide inactivation. Additionally, H_2O_2 directly increased the eNOS expression in cultured endothelial cells [22]. However, the precise mechanism that up-regulates the NOS expression in the kidney of SHR has not been clarified yet. To determine the role of oxidative stress in the regulation of the renal NOS expression, the present

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study compared the effects of apocynin and tempol on the NOS expression in the kidney of SHR and WKY, and also examined the effect of exogenous H_2O_2 on the renal NOS expression in WKY.

Methods

Animal and experimental protocol

Male SHR/Izm and WKY/Izm were obtained from SLC (Shizuoka, Japan) and used in the different experimental protocols. These rats were housed in a facility at the Tohoku University School of Medicine and had free access to standard laboratory chow and water while housed at a controlled temperature (24°C) with a 12-h light, 12-h dark cycle. All protocols involving rats were reviewed and received prior approval by the Animal Welfare Committee at the Tohoku University School of Medicine.

Protocol 1: Effect of apocynin and tempol in SHR and WKY. Five-week old SHR and WKY were randomly divided into three groups ($n = 6$ in each group): a control group, an apocynin group or a tempol group, and treated with vehicle, apocynin (2 mmol/l) or tempol (2 mmol/l) in drinking water for 8 weeks, respectively.

Protocol 2: Effect of H_2O_2 administration in WKY. Twelve-week old WKY were anesthetized with ether anesthesia and placed on a temperature-controlled surgical table to maintain body temperature at 37°C during the operation process. A catheter was implanted into the right jugular vein. The rats were returned to individual metabolism cages and allowed to recover for 2 days after the surgery. To prevent vascular damage at the end of the catheter, physiological saline was infused continuously at 12 ml/day to dilute H_2O_2. After 2 days, rats were randomly divided into three groups ($n = 6$ in each group): a control group, a low-dose H_2O_2 group (1.2 μmol/kg per day) and a high-dose H_2O_2 group (4.0 μmol/kg per day). The physiological saline and H_2O_2 was infused continuously for 1 week. These H_2O_2 doses were selected to obtain urinary excretion and plasma concentrations of H_2O_2 equal to those in SHR.

Blood pressure measurement and preparation of plasma and urinary samples

The systolic blood pressure (SBP) was monitored by the tail-cuff method (Model UR-5000; Ueda, Tokyo, Japan). The rats were placed in individual metabolism cages on the day before the final experimental day, and urine samples were collected on ice. On the final experimental day, the rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and blood samples were collected by decapitation. These samples were centrifuged for 5 min by 1500 r.p.m., and separated from the sediments and stored at −80°C.

Measurement of biochemical parameters in plasma and urinary samples

Creatinine and urea nitrogen were measured by a standard autoanalysis technique (BML, Tokyo, Japan). H_2O_2 was measured using an Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Molecular Probes, Eugene, Oregon, USA) [23]. Nitrate/nitrite (NO_x) was measured by Griess reagent method [24] using Nitrate/Nitrite Colorimetric Assay kit (Cayman Chemical Company, Ann Arbor, Michigan, USA).

Preparation of tissue samples

The kidney and thoracic aorta were quickly removed after decapitation. The kidney was hemisected and sectioned into the cortex, the inner stripe of the outer medulla, and the inner medulla. These tissues were homogenized in a 100 mmol/l potassium buffer (pH 7.25) containing 30% glycerol, 1 mmol/l dithiothreitol, and 0.1 mmol/l phenylmethylsulfonyl fluoride. The samples were snap-frozen in liquid nitrogen and stored at −80°C. The protein concentration of the samples was measured using the Bradford method [25].

Measurement of the renal NADPH oxidase activity

The NADPH oxidase activity was measured as an index of O_2^- generation by the lucigenin-enhanced chemiluminescence method [26]. Proteins of the renal cortical and medullary samples (200 μg) were resuspended respectively in 1 ml Krebs-Hepes buffer (mmol/l: NaCl 119, Hepes 20, KCl 4.6, $CaCl_2$ 1.2, Na_2HPO_4 0.15, KH_4PO_4 0.4, $MgSO_4$ 1.0, $NaHCO_3$ 25 and glucose 5.5). Chemiluminescence was recorded by a tube luminescencer (PSN AB-2200; ATTO, Tokyo, Japan) every 60 s for 5 min after adding lucigenin (10 μmol/l; Sigma–Aldrich, St Louis, Missouri, USA). The NADPH oxidase activity was determined by deducting the background value from the value obtained after adding NADPH (100 μmol/l) and expressed as counts per minute (CPU)/mg of protein.

Immunoblot analysis

Proteins of the samples (50 μg) were separated by electrophoresis on an 8.5% sodium dodecyl sulfate polyacrylamide gel. The proteins were transferred electrophoretically to a nitrocellulose membrane in a transfer buffer consisting of 25 mmol/l Tris–HCl, 192 mmol/l glycine, and 20% methanol. The membrane was blocked by immersion into a buffer (TBST-20) containing 10 mmol/l Tris–HCl, 150 mmol/l NaCl, 0.08% Tween-20, and 10% nonfat dry milk. The membrane was then incubated with primary antibodies raised against eNOS, nNOS, iNOS (BD Transduction Laboratories, San Jose, California, USA) or nitrotyrosine as an index of $ONOO^-$ formation (Santa Cruz Biotechnology, Santa Cruz, California, USA). The membrane was rinsed several times with TBST-20 buffer and then incubated with a horseradish peroxidase-conjugated goat antimouse IgG (Santa Cruz Biotechnology). After several washes in TBST-20, the immunoblots were developed using an enhanced chemiluminescence kit (Super Signal; Thermo Fisher Scientific, Waltham, Massachusetts, USA). The relative

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intensities of the bands at the 140 kDa for eNOS, 155 kDa for nNOS, 130 kDa for iNOS and 70 kDa for nitrotyrosine were quantified using Image J software (version 1.40, National Institutes of Health, Bethesda, Maryland, USA). The band intensities for each protein were normalized to those for β-actins as an internal standard, and the band intensity in the control group was assigned a value of 1.

Statistical analysis

Data are presented as the means ± SEM. Data were analyzed by repeated-measure ANOVA, followed by Tukey test for multiple comparison among the groups. Paired data were analyzed by the Student's *t*-test. A value of P less than 0.05 was considered to indicate statistical significance.

Results

Protocol 1: effect of apocynin and tempol in SHR and WKY

The SBP and plasma and urinary parameters in the three groups of SHR and WKY are shown in Table 1. The SBP, plasma and urinary H_2O_2 and NO_x were significantly higher in the control SHR group than in the control WKY group ($P < 0.01$), but the plasma creatinine and urea nitrogen and creatinine clearance were not significantly different between these control groups. In SHR, both apocynin and tempol significantly decreased the SBP. Tempol significantly decreased the plasma creatinine and increased creatinine clearance, but apocynin did not affect them. Apocynin significantly decreased the plasma and urinary H_2O_2 and NO_x, but tempol significantly increased them. In WKY, neither apocynin nor tempol affected the SBP or plasma and urinary parameters.

The NADPH oxidase activities in the renal cortex and outer medulla were significantly higher in the control SHR group than in the control WKY group (27 743 ± 507 vs. 6720 ± 339 counts/min per mg protein in the cortex, 21 038 ± 513 vs. 6326 ± 343 counts/min per mg protein in the outer medulla; $P < 0.01$). The activities in the inner medulla were barely detectable in SHR and WKY. In SHR, both apocynin and tempol significantly inhibited the NADPH oxidase activities by 72 and 56% in the renal cortex and by 71 and 55% in the outer medulla. In WKY, apocynin significantly inhibited the NADPH oxidase activities by 48% in the renal cortex and by 43% in the outer medulla, but tempol did not significantly affect the activities.

The eNOS expression in the renal cortex, the outer medulla, the inner medulla and aorta was significantly higher in the control SHR group than in the control WKY group (Fig. 1a). The nNOS expression in the three sections of the kidney and aorta was also significantly higher in the control SHR than in the control WKY (Fig. 1b). The iNOS expression in the three sections of the kidney and aorta was barely detectable and not significantly different among the control SHR and WKY groups (data not shown). The nitrotyrosine levels in the three sections of the kidney and aorta were significantly higher in the control SHR than in the control WKY (Fig. 1c).

The eNOS expression in the renal cortex, the outer medulla, the inner medulla and aorta of SHR was significantly decreased by apocynin, but was significantly increased by tempol (Fig. 2a). The eNOS expression in the three sections of the kidney and aorta of WKY was not significantly affected by apocynin or tempol (Fig. 2b). The nNOS expression in the renal cortex, the outer medulla, the inner medulla and aorta of SHR was significantly decreased by apocynin, but was significantly increased by tempol (Fig. 3a). The expression of nNOS protein in the three sections of the kidney and aorta of WKY was not significantly affected by apocynin or tempol (Fig. 3b). The iNOS expression in the three sections of the kidney and aorta of both SHR and WKY was not significantly affected by apocynin or tempol (data not shown). The nitrotyrosine levels in the renal cortex, the outer medulla, the inner medulla and aorta of SHR were significantly decreased by apocynin and tempol (Fig. 4a). The nitrotyrosine levels in the three sections of the kidney and aorta of WKY were significantly decreased by apocynin but not affected by tempol (Fig. 4b).

Protocol 2: effects of H_2O_2 administration in WKY

Effects of H_2O_2 administration on the SBP and plasma and urinary parameters in WKY are shown in Table 2. The SBP was significantly increased by H_2O_2 administered at the high dose but not at the low dose. The plasma

Table 1 Effect of apocynin and tempol on the SBP and biochemical parameters in SHR and WKY

	SHR			WKY		
	Control ($n = 6$)	Apocynin ($n = 6$)	Tempol ($n = 6$)	Control ($n = 6$)	Apocynin ($n = 6$)	Tempol ($n = 6$)
SBP (mmHg)	224 ± 3	202 ± 3**	196 ± 3**	153 ± 3	150 ± 3	151 ± 3
Plasma creatinine (mg/dl)	0.17 ± 0.01	0.17 ± 0.01	0.13 ± 0.01**	0.19 ± 0.01	0.18 ± 0.01	0.18 ± 0.01
Creatinine clearance (ml/min)	2.50 ± 0.51	2.33 ± 0.46	3.65 ± 0.46*	2.15 ± 0.31	2.11 ± 0.38	2.29 ± 0.35
Plasma urea nitrogen (mg/dl)	17.86 ± 0.60	20.50 ± 0.85	17.68 ± 0.58	18.88 ± 0.63	17.05 ± 0.83	20.75 ± 0.84
Plasma H_2O_2 (umol/l)	3.51 ± 0.18	2.62 ± 0.23 *	5.45 ± 0.26**	1.71 ± 0.22	1.51 ± 0.24	1.69 ± 0.22
Urine H_2O_2 (nmol/day)	26.45 ± 1.24	21.25 ± 1.06*	38.16 ± 1.69**	19.73 ± 1.46	17.69 ± 1.61	19.16 ± 1.51
Plasma NO_x (umol/l)	16.55 ± 0.39	12.43 ± 0.36**	19.80 ± 0.45**	9.78 ± 0.39	9.14 ± 0.41	10.12 ± 0.38
Urine NO_x (umol/day)	0.53 ± 0.03	0.33 ± 0.03**	0.72 ± 0.33**	0.30 ± 0.03	0.29 ± 0.03	0.32 ± 0.03

Values are means ± SEM. SHR, spontaneously hypertensive rats; WKY, Wistar–Kyoto rats. * $P < 0.05$ vs. the control group. ** $P < 0.01$ vs. the control group.

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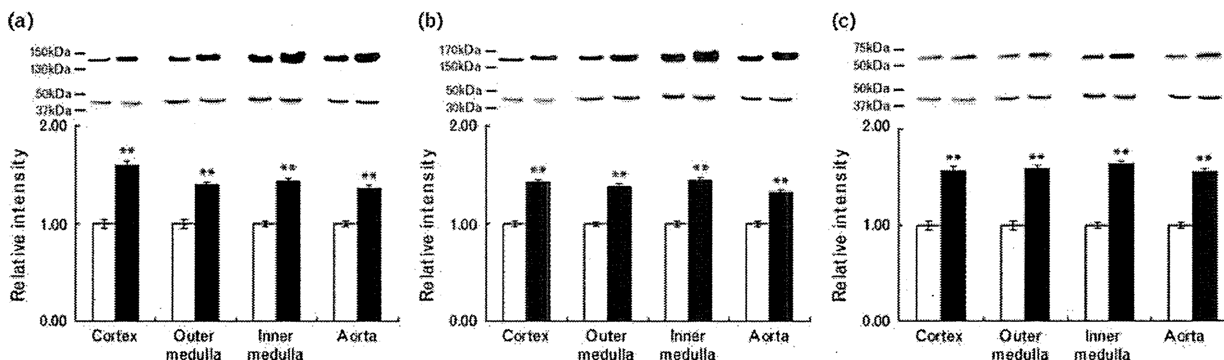
Fig. 1



Basal levels of eNOS, nNOS and nitrotyrosine in SHR and WKY. The levels of eNOS (a), nNOS (b) and nitrotyrosine(c) proteins in the renal cortex, the outer medulla, the inner medulla and aorta were compared between the control WKY group (open bars) and control SHR group (closed bars) ($n = 6$ in each group). Top panel shows representative immunoblots, middle panel shows the immunoblots β-actins and bottom panel shows data of the densitometric analysis. $^{**}P < 0.01$ vs. the control WKY group. NOS, nitric oxide synthase; SHR, spontaneously hypertensive rats; WKY, Wistar–Kyoto rats.

creatinine and urea nitrogen were not affected by H_2O_2 administration. The plasma and urinary H_2O_2 and NO_x were significantly increased by H_2O_2 administration in a dose-dependent manner. The NADPH oxidase activities in the renal cortex and outer medulla were not affected by H_2O_2 administration (data not shown).

The eNOS expression in the renal cortex, the outer medulla, the inner medulla and aorta of WKY was significantly increased by H_2O_2 administration in a dose-dependent manner (Fig. 5a). The nNOS expression in the three sections of the kidney and aorta of WKY was also significantly increased by H_2O_2 administration in a dose-dependent manner (Fig. 5b). The iNOS expression or nitrotyrosine level in the three sections of the kidney and aorta of WKY was not significantly affected by H_2O_2 administration (data not shown).

Discussion

To determine the role of oxidative stress in the regulation of the renal NOS expression, the present study first compared the effect of apocynin and tempol on the NOS expression in the kidney of SHR and WKY. Both the renal NOS expression and oxidative stress were elevated in SHR compared with WKY. Although both apocynin and tempol decreased the blood pressure, inhibited the renal NADPH oxidase activity and reduced the renal nitrotyrosine expression in SHR, the effect of these drugs on the H_2O_2 and nitric oxide production and the renal NOS expression was quite the opposite. The plasma and urinary H_2O_2 and NO_x, and the renal eNOS and nNOS expressions were decreased by apocynin but increased by tempol in SHR. In contrast to SHR, they were not affected by apocynin or tempol in WKY. The effect of H_2O_2 administration was further examined in WKY, and exogenous H_2O_2 increased the renal NOS expression without changing the renal NADPH oxidase activity.

NADPH oxidase is considered to be the major source of O_2^- generation in hypertensive processes [17]. O_2^- interacting with nitric oxide forms $ONOO^-$; this causes inactivity of nitric oxide and further impairs vasodilatation. $ONOO^-$ interacting with tyrosine residues in proteins forms nitrotyrosine which induces tissue injury [15]. Therefore, the nitrotyrosine levels can be used as an index of $ONOO^-$ formation. The renal cortical and medullary NADPH oxidase activities, plasma and urinary H_2O_2, and the renal and aortic nitrotyrosine levels were all higher in SHR than in WKY, in agreement with previous studies [5,13,27]. We also measured the total SOD activity in the renal cortex and medulla and found that these activities were not significantly different between SHR and WKY (data not shown). Additionally, a previous study reported that the catalase and glutathione peroxidase activities in the renal cortex and medulla were not significantly different between SHR and WKY [28]. Therefore, elevated ROS levels in the kidney of SHR may be dependent on the O_2^- generation by NADPH oxidase.

Chronic apocynin treatment reduced the blood pressure and inhibited the expression of NADPH oxidase subunits in SHR [5,17]. Acute or chronic tempol treatment reduced the blood pressure and inhibited the NADPH oxidase activity by metabolizing O_2^- into H_2O_2 in SHR [18,29,30]. In the present study, the nitrotyrosine levels were alleviated by both apocynin and tempol treatments in the kidney and aorta of SHR. On the basis of these results, the antihypertensive effects of apocynin and tempol in SHR may have been caused by lowering O_2^- generation and ameliorating O_2^--induced nitric oxide inactivation. In spite of the antihypertensive and antioxidative effects, the effects of apocynin and tempol on the NOS expression and nitric oxide production were quite the opposite, and were accompanied by changes in

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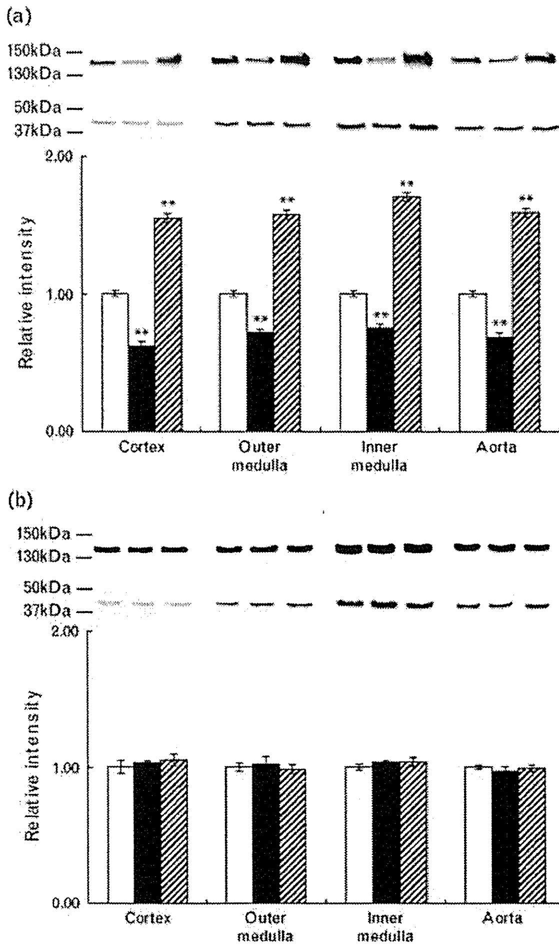
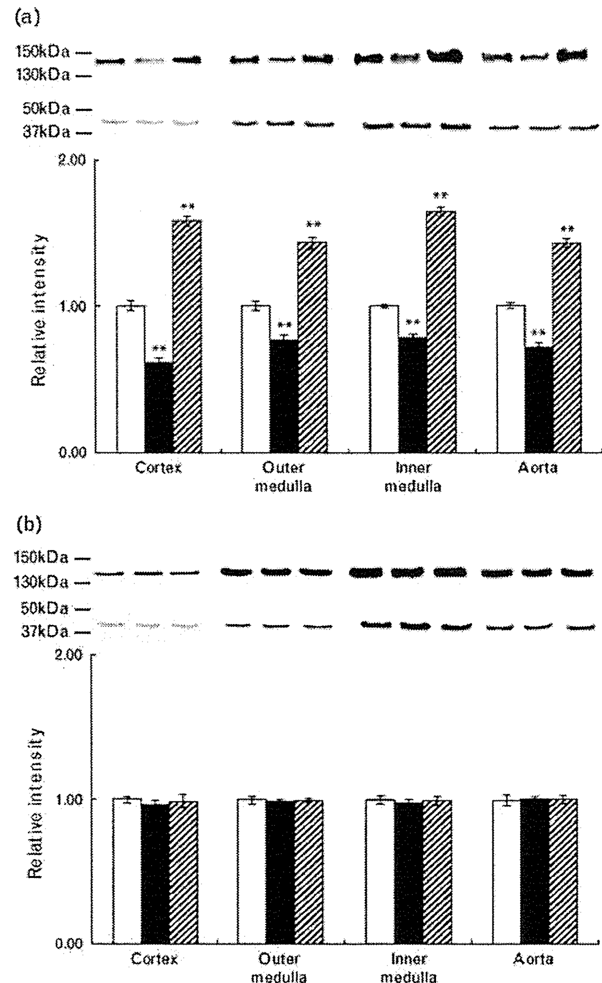
Fig. 2



Effects of apocynin and tempol on the eNOS expression in SHR and WKY. The levels of eNOS protein in the renal cortex, the outer medulla, the inner medulla and aorta of SHR (a) and WKY (b) were compared among the control group (open bars), the apocynin group (closed bars) and tempol group (hatched bars) ($n = 6$ in each group). Top panel shows representative immunoblots, middle panel shows the immunoblots β-actins and bottom panel shows data of the densitometric analysis. $^{**}P < 0.01$ vs. the control group. NOS, nitric oxide synthase; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.

Fig. 3



Effects of apocynin and tempol on the nNOS expression in SHR and WKY. The levels of nNOS protein in the renal cortex, the outer medulla, the inner medulla and aorta of SHR (a) and WKY (b) were compared among the control group (open bars), the apocynin group (closed bars) and tempol group (hatched bars) ($n = 6$ in each group). Top panel shows representative immunoblots, middle panel shows the immunoblots of β-actins and bottom panel shows data of the densitometric analysis. $^{**}P < 0.01$ vs. the control group. NOS, nitric oxide synthase; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.

the plasma and urinary H_2O_2 in SHR. Neither apocynin nor tempol affected H_2O_2, NO_x or NOS expression in WKY indicating that the effects of these drugs on the NOS expression and nitric oxide production are specific in SHR with elevated oxidative stress.

To our knowledge, there has been no study which compared the effects of apocynin and tempol on the renal NOS expression in SHR, although it has been reported that the antioxidant lazanoid mitigated the up-regulation of NOS expression in tissues of SHR [3]. The present results indicating that apocynin decreased the nitric oxide production and NOS expression in the renal cortex, the outer medulla and inner medulla of SHR are not consistent with those of previous studies. The NOS activity in the aorta of SHR was increased by apocynin treatment for 4 weeks [31], but was not affected by apocynin treatment for 6 weeks [17]. Apocynin treatment for 3 weeks normalized the elevated juxtaglomerular nNOS expression in SHR [32], and apocynin treatment for 1 week reduced the perivascular nitric oxide concentration in the mesenteric arteries of SHR [5]. In contrast to apocynin, the effect of chronic tempol treatment on the NOS expression in the renal cortex, the outer medulla and inner medulla of SHR has not been reported [33]. Thus, the present study reported for the first time

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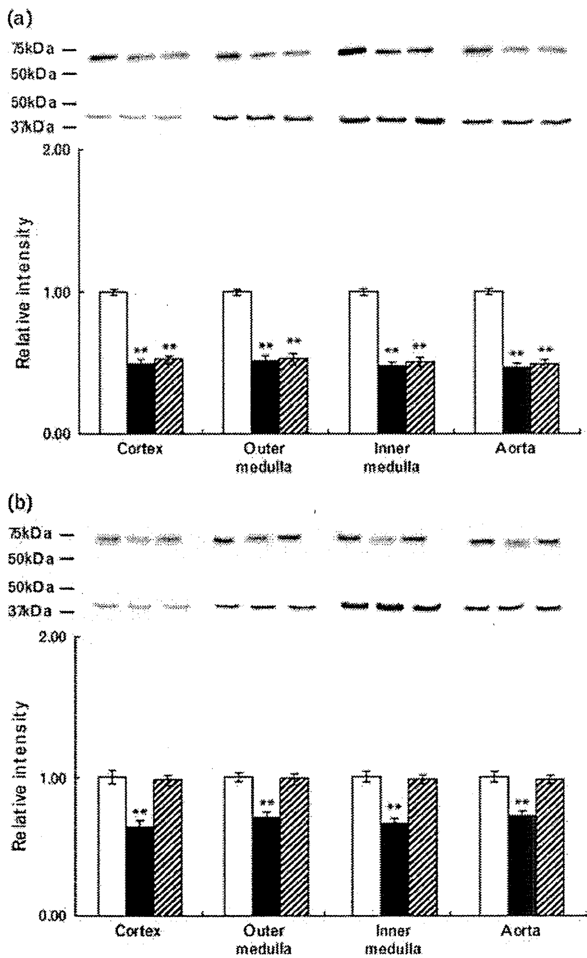
Fig. 4



Effects of apocynin and tempol on the levels of nitrotyrosine in SHR and WKY. The levels of nitrotyrosine in the renal cortex, the outer medulla, the inner medulla and aorta of SHR (a) and WKY (b) were compared among the control group (open bars), the apocynin group (closed bars) and tempol group (hatched bars) ($n = 6$ in each group). Top panel shows representative immunoblots, middle panel shows the immunoblots β-actins and bottom panel shows data of the densitometric analysis. $^{**}P < 0.01$ vs. the control group. SHR, spontaneously hypertensive rats; WKY, Wistar–Kyoto rats.

Table 2 Effect of exogenous H_2O_2 on the SBP and biochemical parameters in WKY

	Control ($n = 6$)	Low dose ($n = 6$)	High dose ($n = 6$)
SBP (mmHg)	145±3	156±3	159±3*
Plasma creatinine (mg/dl)	0.20±0.01	0.21±0.01	0.22±0.01
Plasma urea nitrogen (mg/dl)	13.40±0.61	13.90±0.68	13.60±0.65
Plasma H_2O_2 (umol/l)	1.88±0.24	2.24±0.27	3.16±0.29†,‡
Urine H_2O_2 (nmol/day)	18.63±1.46	54.12±1.39†	74.65±2.16†,‡
Plasma NO_x (umol/l)	9.49±0.40	17.74±0.41†	22.02±0.38†,‡
Urine NO_x (umol/day)	0.32±0.03	0.51±0.03†	0.83±0.04†,‡

Values are means ± SEM. WKY, Wistar–Kyoto rats. $^{*}P < 0.05$ vs. the control group. $^{†}P < 0.01$ vs. the control group. $^{‡}P < 0.01$ vs. the low dose H_2O_2-treated group.

that chronic tempol treatment could increase the renal NOS expression in SHR.

Several mechanisms such as shear stress [19], nitric oxide inactivation [20,21], oxidative stress [4] and H_2O_2 [22] are speculated to up-regulate the NOS expression in the hypertensive state. In the present study, apocynin decreased but tempol increased the renal NOS expression and nitric oxide production in SHR, although both drugs reduced the blood pressure, inhibited the renal NADPH oxidase activity and decreased the nitrotyrosine expression. Therefore, the opposite effects of these drugs on the renal NOS expression can not be explained only by shear stress (blood pressure), the renal NADPH oxidase-produced O_2^- or nitric oxide inactivation ($ONOO^-$). The opposite effects of these drugs on the renal NOS expression and nitric oxide production were accompanied by changes in the plasma and urinary H_2O_2, suggesting that H_2O_2 may up-regulate the renal NOS expression in SHR.

To determine the role of H_2O_2 in the regulation of the renal NOS expression, the present study further examined the effect of exogenous H_2O_2 in WKY. Although H_2O_2 administration at the high dose caused a slight increase in the blood pressure, H_2O_2 increased the eNOS and nNOS expression and nitric oxide production in a dose-dependent manner without changing the renal NADPH oxidase activity and nitrotyrosine levels. These results indicate that the H_2O_2-increased NOS expression and nitric oxide production were independent of O_2^- generation or blood pressure. Previous studies reported that direct infusion of H_2O_2 into the left renal artery increased the urine volume and induced transient massive proteinuria in the infused kidney of Munich-Wistar rats [34], and that chronic infusion of H_2O_2 for 5 days into the renal medulla significantly increased the blood pressure in Sprague–Dawley rats [35]. In agreement with the present results, H_2O_2 increased the eNOS expression and activity in cultured endothelial cells by changing the rate of gene transcription and altering mRNA processing and stability [22]. H_2O_2 increased the perivascular nitric oxide concentration in rat mesenteric arteries [6]. However, other studies have yielded conflicting results on the effect of H_2O_2 on vasomotor tone: vasoconstrictor [36], vasodilator [37] or biphasic vasomotor [38,39] effects depending on the vascular beds and the experimental conditions. In addition to the eNOS expression, we also observed that H_2O_2 administration increased the levels of phosphorylated eNOS at Ser1177 but decreased the ratio of phosphorylated eNOS to total eNOS in the renal cortex, the medulla and aorta of WKY (data not shown).

High pressure induced O_2^- production in isolated arteries via NADPH oxidase activation [10], and oscillatory shear stress increased O_2^- and H_2O_2 production, which stimulated the eNOS expression in cultured

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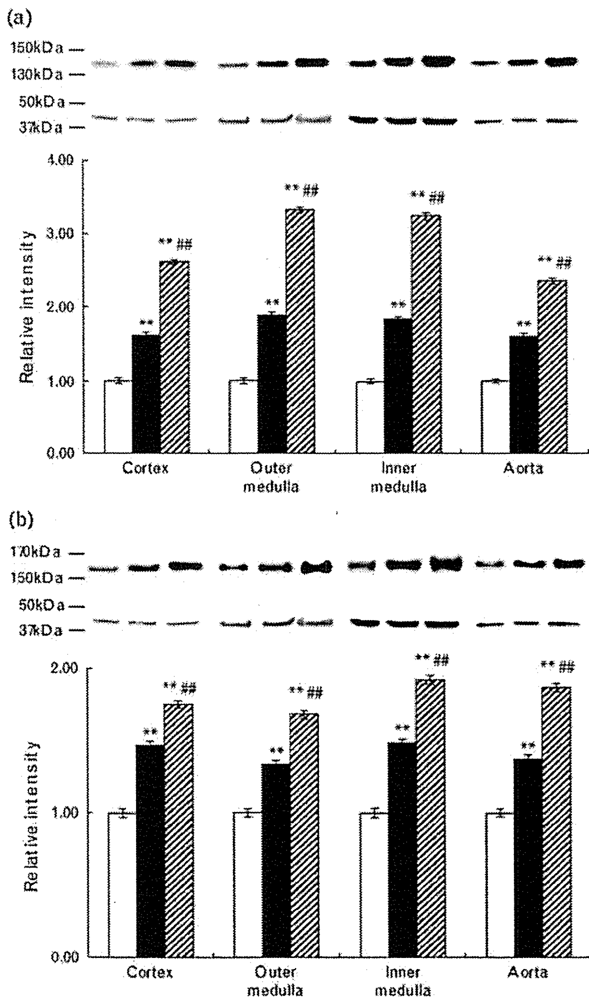
Fig. 5



Effect of exogenous H_2O_2 on the NOS expression in WKY. The levels of eNOS (a) and nNOS (b) proteins in the renal cortex, the outer medulla, the inner medulla and aorta of WKY were compared among the control group (open bars), the low-dose H_2O_2 group (closed bars) and high-dose H_2O_2 group (hatched bars) ($n = 6$ in each group). Top panel shows representative immunoblots, middle panel shows the immunoblots β-actins and bottom panel shows data of the densitometric analysis. $^{**}P < 0.01$ vs. the control group. $^{\#\#}P < 0.01$ vs. the low-dose H_2O_2 group. NOS, nitric oxide synthase; SHR, spontaneously hypertensive rats; WKY, Wistar–Kyoto rats.

endothelial cells [40]. As well as endothelial cells, increases of the renal perfusion pressure stimulated H_2O_2 and nitric oxide productions in the renal medulla of Sprague–Dawley rats [41], whereas nitric oxide-mediated vasodilatory responses were impaired in SHR [7]. Taken together with the present results, SOD may alleviate ROS-mediated nitric oxide inactivation through metabolizing O_2^- into H_2O_2; this causes the increase of H_2O_2-induced NOS expression and further improves the impaired vasodilatory responses in a hypertensive state. Nitric oxide also has various renal effects including the regulation of renal hemodynamics, the inhibition of tubular Na reabsorption, tubuloglomerular feedback and sympathetic nerve activity [42]. In agreement with the present results, tempol treatment for 2 weeks in SHR increased the glomerular filtration rate (GFR) by 17% [18]. In addition, tempol treatment for 7 weeks in SHR selectively increased the renal medullary blood flow by approximately 50% [43]. The tempol-increased GFR and renal medullary blood flow in SHR might be mediated in part through the ameliorated nitric oxide bioactivity because of the increase of H_2O_2-induced NOS expression and the decrease of O_2^--induced nitric oxide inactivation in the kidney.

In conclusion, the renal NOS expression and nitric oxide production were elevated in SHR. Although both apocynin and tempol decreased the blood pressure and inhibited the renal NADPH oxidase activity in SHR, apocynin decreased but tempol increased the renal NOS expression and nitric oxide production together with the plasma and urinary H_2O_2. Exogenous H_2O_2 increased the renal NOS expression and nitric oxide production in WKY. These results indicate that among ROS, endogenous H_2O_2 may be a mediator of the up-regulation of the renal NOS expression and nitric oxide production in SHR.

Acknowledgements

The work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology (Nos. 20590694 and 20300184).

There are no conflicts of interest.

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Lung (2012) 190:29–33
DOI 10.1007/s00408-011-9334-z

Effect of Aging on Cough and Swallowing Reflexes: Implications for Preventing Aspiration Pneumonia

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Received: 1 August 2011 / Accepted: 25 September 2011 / Published online: 28 October 2011
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Abstract The impairment of airway protective reflexes, i.e., swallowing and cough reflexes, is thought to be one of the major causes for aspiration pneumonia in older people. Restoration of cough and swallowing reflexes in the elderly is key to preventing aspiration pneumonia in the elderly. Although, the medical literature has asserted that cough and swallowing are controlled primarily by the brainstem, recent advances in human brain imaging has provided evidence that cortical and subcortical structures play critical roles in cough and swallowing control. Because of their nature, reflexive cough and swallowing activate both sensory and motor areas in the cortex. In both protective reflexes, the sensory component, including sensory cortex in reflexive circuits, seems to be more vulnerable to aging than the motor component, including the motor cortex. Therefore, the strategy to restore cough and swallowing reflexes should be focused on compensations of sensory components in these reflexive circuits. Remedies to enhance sensory nerve terminals and sensory cortical areas related to these reflexes might be useful to prevent aspiration pneumonia in the elderly.

Keywords Cough reflex · Swallowing reflex · Capsaicin · TRPV1

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Introduction

Aspiration is defined as the misdirection of either oropharyngeal or gastric contents into the lower respiratory tract, that is, the act of taking foreign material into the lungs. This can cause an assortment of pulmonary syndromes determined by the quantity and nature of the aspirated material, the frequency of aspiration, and the host factors that predispose the patient to aspiration and to modifying the response [1]. Pulmonary aspiration syndromes include aspiration pneumonitis, aspiration pneumonia, diffuse aspiration bronchiolitis, airway obstruction, lung abscess, exogenous lipoid pneumonia, chronic interstitial fibrosis, and *Mycobacterium fortuitum* pneumonia. Among these syndromes, one of most frequent and important is aspiration pneumonia [1].

We opened an aspiration clinic in Tohoku University Hospital in 2005. Most patients were referred by other doctors inside of the hospital. Figure 1 shows the comorbidities of patients suspected of aspiration from April 2005 to March 2011. Although, the background diseases are diversely distributed, the common mechanism for aspiration has to be identified to develop a strategy to prevent aspiration.

Increased incidence of aspiration in the elderly is a consequence of a number of age-related factors, including comorbid illnesses, daily medications, and the aging process itself [2]. These factors combine to adversely affect upper and lower respiratory tract host defenses against invading pathogens [3]. Among these factors, the major compromises of mechanical airway clearance, such as impaired cough and swallowing reflexes, probably present the most severe general hazards. The progressive loss of cough and swallowing reflexes with aging has been suggested as leading to aspiration pneumonia [4], a most common pneumonia in the elderly [5].

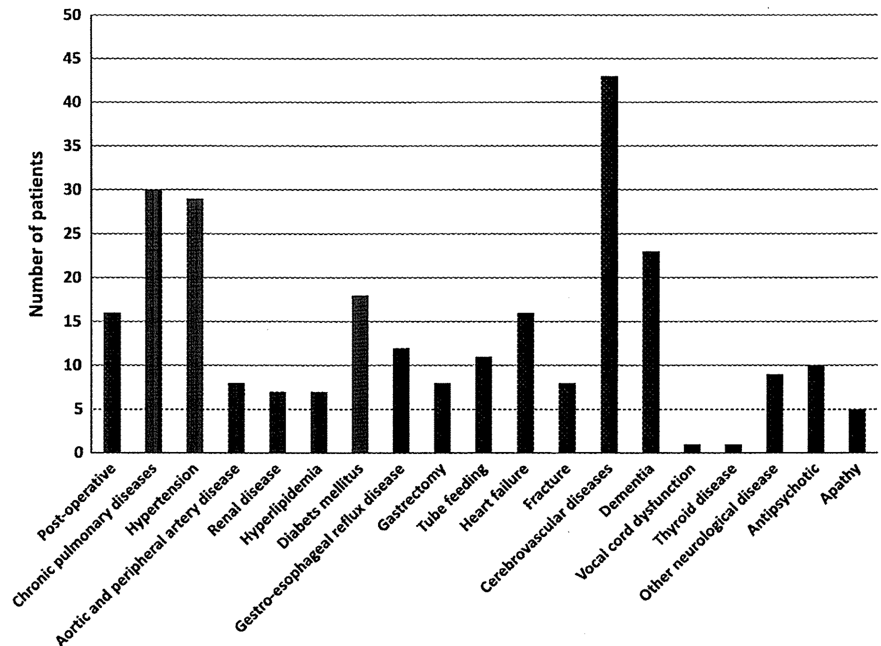

Fig. 1 Background conditions of aspiration in patients who had been seen at our aspiration clinic between April 2005 and March 2011. One patient had multiple diseases

Aging and Airway Protective Reflexes

Although impaired cough and swallowing reflexes have been shown in patients suffering from aspiration pneumonia [6–8], re-evaluation of age-related changes in protective reflexes in individuals who led active daily lives shows that both reflexes do not decrease with the advance of age [9, 10]. This suggests that involutional and degenerative changes of aging often result in marginally compensated protective reflexes [11].

Both cough and swallowing reflexes are well-integrated and have the afferent limb consisting of receptors and afferent nerves, the central cough or swallowing center in the brainstem, and the efferent limb consisting of motor nerves supplying the muscles used in coughing or swallowing. Moreover, recent advances in neuroscience indicated the importance of supra medullary structures in cough and swallowing reflexes [12–16]. Since the effect of aging on protective reflexes has been evaluated mostly by net performance, it is of important to study its effect on each component of the reflexive network in order to develop the remedies that will restore the protective reflexes.

Cortical Control of Airway Protective Reflexes

For many years the medical literature has asserted that cough and swallowing are controlled primarily by the brainstem. However, advances in human brain imaging have provided evidence that cortical and subcortical structures play a critical role in controlling cough and swallowing [12–16]. Because of its nature, either reflexive cough or swallowing activates both sensory and motor areas in the cortex (Fig. 2). The most consistent areas in neuroimaging studies include the primary sensorimotor cortex, sensory motor integration areas, the insula, the anterior cingulated cortex, and supplementary motor areas [17–19].

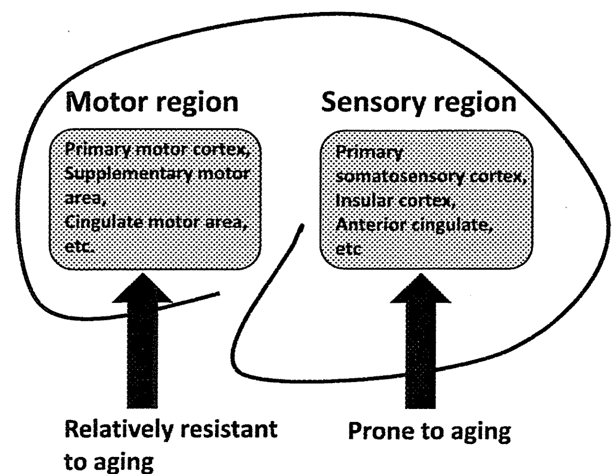

Fig. 2 Cortical areas related to cough and swallowing reflexes. Sensory regions of the cortex are prone to aging-related deficits

Malandraki and colleagues [20] found that by the functional MRI technique, the sensory processing areas in the cortical areas involved in swallowing were deteriorated by aging rather than the motor processing areas.