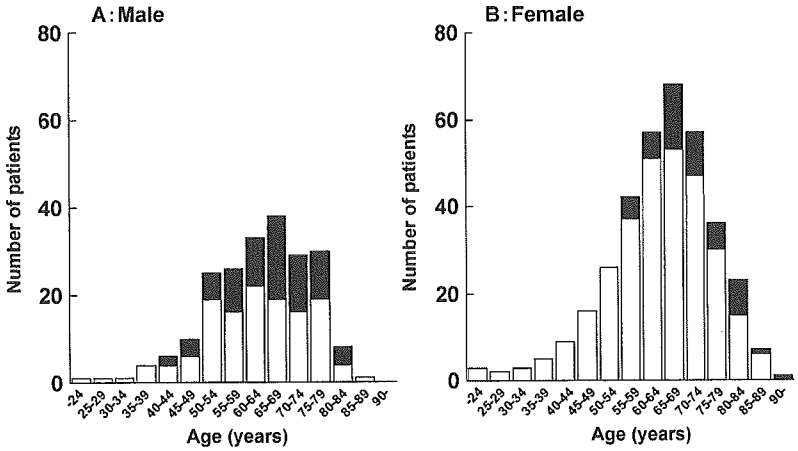

Fig. 2 Number of male (A) and female (B) patients with coronary heart disease (CHD, ■) or without CHD (□)

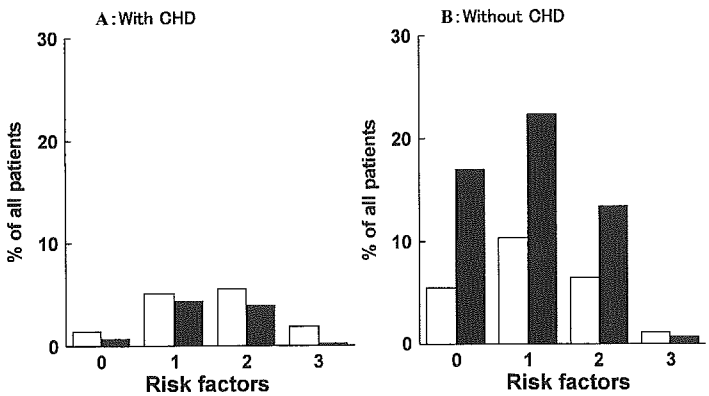

Fig. 3 Number of risk factors (smoking, diabetes mellitus and hypertension) in the patients with (A) and without (B) coronary heart disease (CHD)

Data are % of all patients (n=569).
□: Male, ■: Female

糖 (BS) およびヘモグロビン A_{1c} (HbA_{1c}) についても調査した．データは平均値±標準偏差で表示した．統計学的解析は Student's t-test を用い，危険率5%未満を有意差ありと判定した．本研究は浜松医科大学倫理委員会の承認の下に施行した．

成　績

調査した患者のうち男性は213例 (37.4%)，女性は356例 (62.6%) であり，女性患者が男性患者の1.7倍を占めた．対象患者の年齢は63.9±12.0歳であり男女間に有意な差異は認められなかった (Table 1)．対象患者における pravastatin 服用期間は1年以内の頻度が最も高く経時的に減少する傾向が認められた (Fig. 1)．また平均服用期間は55.5±44.6月であった．対象患者の既往歴では高血圧が最も多く全体の61.0%であった．次いで糖尿病が34.9%，虚血性心疾患が23.2%，喫煙が16.5%であった (Table 1)．対象患者の年齢分布では男女ともに65歳から69歳にピークが認められ，49歳以下の患者は全体の10.7%であった．虚血性心疾患の既往のある患者は男性では40歳から認められたのに対し，女性では55歳からであった (Fig. 2)．

虚血性心疾患の既往の有無について調べたところ，男性患者の37.6% (全体の14.1%) と女性患者の14.6% (全体の9.1%) では虚血性心疾患の既往を有していた．すなわち全対象患者の23.2%が二次予防目的のスタチン使用であった (Table 1)．一方，全対象患者のうち22%においては，虚血性心疾患の既往がなく，かつ喫煙歴，糖尿病，高血圧のいずれも有していなかった．その中で女性患者は97例 (17.1%) を占めた (Fig. 3)．

Pravastatin 服用開始前後における血清脂質値の調

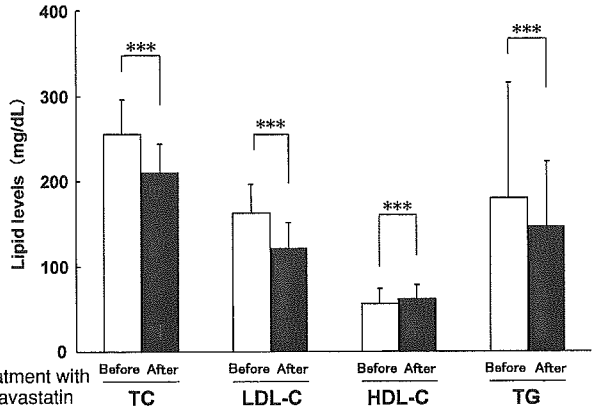

Fig. 4 Lipid profiles in the patients before (□) and after (■) the treatment with pravastatin
TC : Total cholesterol, LDL-C : LDL cholesterol, HDL-C : HDL cholesterol, TG : triglyceride, $***p<0.001$

査によると，対象患者の TC は 255±41 mg/dL から 210±34 mg/dL へと 17.6%有意に低下した．同様に LDL-C および TG はそれぞれ 25.5%および 18.7%有意に減少した．一方 HDL-C は 8.7%有意に増加した (Fig. 4)．さらに対象患者を性別および虚血性心疾患の既往によって層別化したところ，TC，LDL-C および TG は性別および虚血性心疾患の既往にかかわらずいずれの群においても有意に低下した (Fig. 5)．また HDL-C は女性で心血管疾患の既往がある群を除き有意に増加した．さらに男女ともに虚血性心疾患の既往がある群では既往なし群に比べ，また虚血性心疾患の既往にかかわらず，女性に比べ男性においてより低い TC レベルから pravastatin の投与が開始されていた (Fig. 5 A)．

Table 2 に pravastatin 服用患者における服用開始前および服用後の臨床検査値を，糖尿病既往あり群となし群に分けて示した．糖尿病既往なし群では，いずれの検査値においても服用前後で有意な差は認められなかった．一方，糖尿病既往あり群では pravastatin 服用後では，BUN および s-Cre は有意に高値を，HbA_{1C} は有意に低値を示した．

考　察

本研究では，わが国においてスタチンがどのような背景を持つ患者に使用されているかを推測する目的で，浜松医科大学附属病院において pravastatin を投与されている患者の背景を調査し，さらに本薬剤が血清脂質値に及ぼす影響について検討した．

今回は pravastatin 服用患者の 569 症例の背景について調査した．この症例数は浜松医科大学附属病院における pravastatin 処方数の 98%にあたる．今回の対象患者において虚血性心疾患既往歴のある患者は全体の 23%のみであった．現在までに行われている大規模臨床試験から，虚血性心疾患の二次予防におけるスタチン投与の有用性は明確に示されているが，一次予防の場合には二次予防の場合に比べその有用性が低くなることが知られている[4]．今回の調査から，わが国におけるスタチン投与患者の多くが，比較的有用性の低いと考えられる一次予防であると推察された．また女性で虚血性心疾患，糖尿病，高血圧の既往および喫煙歴のない患者が全体の 17%占めていた．虚血性心疾患に対するスタチン投与の有用性は，患者のベースラインリスクに依存することが明らかにされており[9]，虚血性心疾患の絶対リスクが欧米諸国に比べ低いわが国において一次予防，とくに高コレステロール血症のみを有する女性患者など，低リスク群に対するスタチンの有用性は十分に証明されているとは言えない．今後 EBM の観点からも医療経済的な視点からも，日本人におけるスタチン投与の有用性の検証が必要であると思われる．

今回の対象患者のうち 478 症例 (全症例の 84%) において，pravastatin 開始および調査時の血清脂質値が調査可能であった．Pravastatin 開始時の TC および LDL-C はそれぞれ 255 mg/dL および 162 mg/dL であった．この値は欧米および日本で行われた大規模臨床試験でのスタチン開始時での値とほぼ同値かやや低い値である[4~6,10~12]．今回，pravastatin の投与によって TC は 18%，LDL-C は 26%有意に低下した．Pravastatin を用いた大規模臨床試験における TC および LDL-C の低下率はそれぞれ 20%および 25%程度であることから[5,6,11,12]，それらの試験同様，本研究結果は pravastatin の良好なコレステロール低

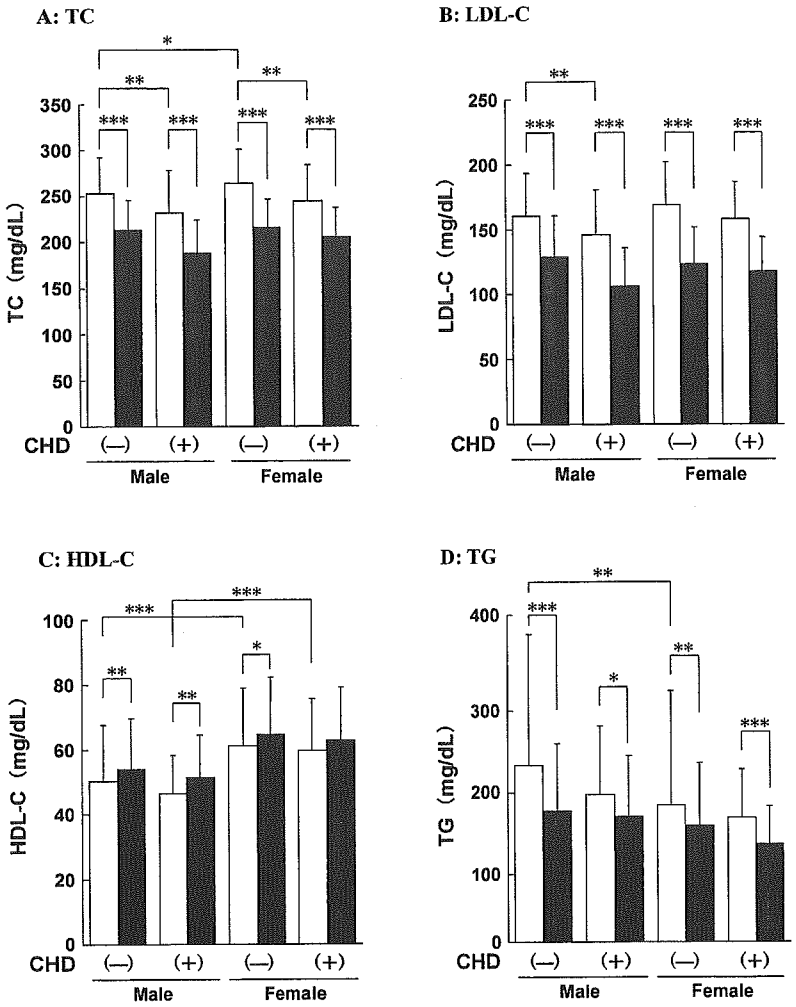

Fig. 5 Lipid profiles before (□) and after (■) the treatment with pravastatin in male and female patients with or without coronary heart disease (CHD)

TC : Total cholesterol, LDL-C : LDL cholesterol, HDL-C : HDL cholesterol, TG : triglyceride, $*p<0.05$, $**p<0.01$, $***p<0.001$

Table 2 Laboratory data before and after the treatment with pravastatin in the patients with or without diabetes mellitus

Laboratory data	Diabetes mellitus	Number of patients	Before pravastatin	After pravastatin
AST	−	220	24.3±12.7	23.0±10.0
	+	132	22.8±9.2	22.8±13.9
ALT	−	221	23.0±10.0	21.7±16.8
	+	129	22.8±12.4	22.2±20.6
CPK	−	192	106±83	108±58
	+	116	99.8±93.3	115±106
BUN	−	208	16.4±5.2	16.9±6.2
	+	130	16.8±7.0	18.4±9.1**
s-Cre	−	199	0.819±0.300	0.838±0.336
	+	131	0.770±0.367	0.909±0.615***
BS	−	107	104±19	105±23
	+	118	163±74	153±84
HbA_{1c}	−	53	5.57±0.49	5.59±0.54
	+	104	7.64±1.74	7.37±1.61*

Values are mean ± SD, $*p<0.05$, $**p<0.01$, $***p<0.001$

AST : L-asparate aminotransferase, ALT : L-alanine aminotransferase, CK : creatine kinase, s-Cre : serum creatinine, BUN : blood urea nitrogen, BS : blood glucose, HbA_{1c} : hemoglobin A_{1c}

Table 3 Demographic characteristics of the patients in the quartile treatment periods with pravastatin

	Periods with pravastatin [month]			
	0.9—22.1	22.1—50.8	50.9—87.2	87.5—174.5
Number of patinets	118	119	119	118
Male	56 (48%)	40 (34%)	49 (41%)	33 (28%)
Age* [years]	59.4±13.2	64.2±11.7	64.6±11.9	65.4±11.0
Smoking*	23 (19%)	21 (18%)	18 (15%)	16 (14%)
Risk factors				
Coronary heart disease	36 (31%)	30 (25%)	23 (19%)	21 (18%)
Diabetes mellitus	39 (33%)	34 (29%)	41 (34%)	48 (41%)
Hypertension	63 (53%)	79 (66%)	72 (61%)	76 (64%)

Values are number of patients or mean±SD.
( ) : % of numbers in the quartile treatment periods with pravastatin.
*Data at the point of the survey are presented.

下作用を示すものである．

今回興味深いことに，男女ともに虚血性心疾患の既往がある群では既往なし群に比べ，pravastatin はより低値の TC レベルから処方が開始されていることが明らかとなった．また虚血性心疾患の既往にかかわらず，女性に比べ男性でより低い TC から pravastatin の処方が開始されていた．このことは，処方者が虚血性心疾患発症リスクを考慮し，男性や虚血性心疾患の既往のある患者に対して，より低い TC から投与を開始したものと考えられる．

スタチン投与による臨床検査値の変動は，糖尿病の既往なし群では認められなかった．糖尿病を有する患者で pravastatin 服用後において HbA_{1c} が有意に低下していた．本研究では糖尿病の治療開始時期などの調査は行っていないため，HbA_{1c} が低下した理由は明らかではないが，pravastatin 服用期間中に糖尿病の治療が開始されたのではないかと思われる．さらに糖尿病を有する患者において腎機能検査値（s-Cre, BUN）の有意な上昇を認めた．このメカニズムは明らかではないが，糖尿病の合併症として腎機能障害の頻度は高く，非糖尿病患者群では pravastatin 投与によっても s-Cre と BUN の有意な変化は認められないことから，糖尿病の自然経過を反映するものかもしれない．

今回の調査は浜松医科大学附属病院の pravastatin 服用患者を対象とした．本研究結果は大学病院のような特定機能病院のものであり，直接わが国全体の処方動向と一致するものではないかもしれない．一般病院や診療所などにおける同様な調査の結果と併せて考慮する必要があるだろう．

さらに本研究では 2002 年 6 月から 1 年間の期間に pravastatin を投与されているほぼ全患者について調査し，2002 年 6 月からさかのぼって平均 4.5 年間の投与期間について調査した．したがって調査対象には，長期間投与されている患者と比較的最近投与が開始されている患者が混在している（Fig. 1）．このうちとくに長期間にわたって投与されている患者についてのデータの解釈には慎重でなければならない．すなわち数年前に投与が開始され，2002 年の 6 月から 1 年間の期間のいずれかの時点でも引き続き，pravastatin が投与されている患者は，数年前に投与開始となった患者の一部分と考えられ，死亡例，当該医療機関への来院を中止したもの，来院は続けているとしても副作用や十分な効果がみられないために投与を中止または変更したもの，または逆に血清脂質の正常化などの理由で治療を中止したものなどは，本研究の調査対象には含まれていない．これらの理由で調査対象に含まれていない患者の背景と，調査対象に含まれている長期にわたって投与が続けられている患者の背景が相違する可能性は否定できない．Pravastatin 服用期間に対して対象患者の背景因子を検討したところ，年齢および虚血性心疾患の既往率以外の因子に関しては明らかな傾向は認められなかった（Table 3）．平均年齢は服用期間が長くなるほど高い傾向が認められた．さらに虚血性心疾患の既往患者の割合は服用期間が短いほど増加する傾向が認められた．この理由として長期投与患者では虚血性心疾患発症にともなう他剤への変更または患者の死亡や転院が潜在する可能性が考えられる．したがって，今回の調査結果では pravastatin 服用患者の虚血性心疾患既往率を低く見積もっている可能性は否定できない．一方でこの結果は，最近になって pravastatin は一次予防に比べ二次

予防に対し積極的に用いられるようになったことを示しているのかもしれない．

結　論

本研究の対象患者においてpravastatinは血清コレステロール値を有意に低下しており，本剤の高脂血症治療における臨床的有用性が確認された．さらに処方者は心血管疾患発症リスクを考慮し，男性や虚血性心疾患の既往のある患者に対して，より低いTC値から投与を開始していることが明らかとなった．

一方，本研究では比較的虚血性心疾患発症リスクが低いと考えられる患者に対してpravastatin処方頻度が高いことが明らかとなった．虚血性心疾患の既往がない女性など低リスク患者に対するスタチン使用の有用性についてはいまだ十分に証明されているとは言えず，今後このような患者群に対するスタチン投与のエビデンス構築が必要と考えられる．

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文　献

1) Castelli WP, Garrison RJ, Wilson PW, Abbott RD, Kalousdian S, Kannel WB. Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study. *JAMA* 1986；**256**：2835-8.
2) Stamler J, Wentworth D, Neaton JD. Is relationship between serum cholesterol and risk of premature death from coronary heart disease continuous and graded? Findings in 356,222 primary screenees of the Multiple Risk Factor Intervention Trial (MRFIT). *JAMA* 1986；**256**：2823-8.
3) 清原裕地．日本動脈硬化学会・日本糖尿病学会合同委員会．地域住民中の糖尿病者における循環器疾患発症とその危険因子の関連―久山町研究―．*糖尿病合併症* 2000；**14**：80-4.
4) Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, McKillop JH, Packard CJ. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. *N Engl J Med* 1995；**333**：1301-7.
5) Sacks FM, Pfeffer MA, Moye LA, et al. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators. *N Engl J Med* 1996；**335**：1001-9.
6) Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. *N Engl J Med* 1998；**339**：1349-57.
7) Saito I, Folsom AR, Aono H, Ozawa H, Ikebe T, Yamashita T. Comparison of fatal coronary heart disease occurrence based on population surveys in Japan and the USA. *Int J Epidemiol* 2000；**29**：837-44.
8) 渡邉裕司．Evidence Based Medicine（EBM）と臨床薬理．エビデンスを使う．循環器領域―臨床決断とエビデンス．*臨床薬理* 2003；**34**：223-7.
9) 日本クリニカル・エビデンス編集委員会（監修）．*クリニカル・エビデンス ISSUE9 日本語版*．日経BP社，2004：166-8.
10) Pravastatin use and risk of coronary events and cerebral infarction in Japanese men with moderate hypercholesterolemia：the Kyushu Lipid Intervention Study. *J Atheroscler Thromb* 2000；**7**：110-21.
11) Ito H, Ouchi Y, Ohashi Y, Saito Y, Ishikawa T, Nakamura H, Orimo H. A comparison of low versus standard dose pravastatin therapy for the prevention of cardiovascular events in the elderly：the pravastatin anti-atherosclerosis trial in the elderly (PATE). *J Atheroscler Thromb* 2001；**8**：33-44.
12) Matsuzaki M, Kita T, Mabuchi H, Matsuzawa Y, Nakaya N, Oikawa S, Saito Y, Sasaki J, Shimamoto K, Itakura H, J-LIT Study Group. Japan Lipid Intervention Trial. Large scale cohort study of the relationship between serum cholesterol concentration and coronary events with low-dose simvastatin therapy in Japanese patients with hypercholesterolemia. *Circ J* 2002；**66**：1087-95.

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ARTICLE

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Angiotensin-II-induced oxidative stress elicits hypoadiponectinaemia in rats

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Abstract *Aims/hypothesis:* Hypertension, endothelial dysfunction and insulin resistance are associated conditions that share oxidative stress and vascular inflammation as common features. Adiponectin is an abundant plasma adipokine that plays a physiological role in modulating lipid metabolism and exerts a potent anti-inflammatory activity. We hypothesised that adiponectin levels decrease in response to oxidative stress and that this may promote the development of hypertension, endothelial dysfunction and insulin resistance. *Methods:* Rats were infused with angiotensin II (AngII) or its vehicle, either alone or in combination with tempol (4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl), a membrane-permeable metal-independent superoxide dismutase mimetic, or tetrahydrobiopterin (BH4), one of the most potent naturally occurring reducing agents and an essential cofactor for nitric oxide synthase activity. Heart rate, systolic blood pressure, body weight and serum levels of adiponectin were measured on day 7 of treatment, and then the animals were killed. Vessel tone and superoxide production were measured ex vivo in thoracic vascular rings. The expression of adiponectin mRNA in adipose tissue was assessed by Northern blotting, and in 3T3-L1 adipocytes exposed to H_2O_2 by real-time PCR. The expression of NAD(P)H oxidase subunit mRNAs in the rats was assessed by RT-PCR and real-time PCR. *Results:* Hypertension and endothelial dysfunction were induced in rats by infusion of AngII and reversed by administration of tempol. Plasma concentrations of adiponectin and adipose tissue levels of adiponectin mRNA were decreased in AngII-infused rats, and this effect was prevented by cotreatment with tempol or BH4. The production of superoxide anions (O_2^-) was significantly increased in the aortae of AngII-treated rats, and this increase was prevented by the administration of tempol or BH4. Levels of mRNAs that encode NAD(P)H oxidase components, including p22phox, gp91phox, p47phox and Rac1, were similarly increased in adipose tissue, aortae and hearts of AngII-infused rats. Cotreatment of rats with tempol or BH4 reversed AngII-induced increases in NAD(P)H oxidase subunit mRNAs. Fully differentiated 3T3-L1 adipocytes, also exhibited diminished adiponectin mRNA levels when exposed to low concentrations of H_2O_2. *Conclusions/interpretation:* Our results demonstrate that AngII-induced oxidative stress and endothelial dysfunction are accompanied by a decrease in adiponectin gene expression. Since antioxidants were observed to prevent the actions of AngII, and H_2O_2 on its own suppressed adiponectin expression, we conclude that adiponectin gene expression is negatively modulated by oxidative stress. Plasma adiponectin levels may provide a useful indicator of oxidative stress in vivo, and suppressed levels may contribute to the proinflammatory and metabolic derangements associated with type 2 diabetes, coronary artery disease and the metabolic syndrome.

Keywords Adiponectin · Angiotensin II · Metabolic syndrome · Oxidative stress · Tempol · Tetrahydrobiopterin

Abbreviations AngII: angiotensin II · BH4: tetrahydrobiopterin · AT1: angiotensin type 1 · GAPDH: glyceraldehyde-3-phosphate dehydrogenase · NO: nitric oxide · PSS: physiological salt solution · ROS: reactive oxygen species

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Introduction

Adiponectin is an important adipocytokine that is secreted by adipocytes and circulates at relatively high levels in the

bloodstream. Adiponectin has potent anti-inflammatory and atheroprotective effects on vascular tissue, and has an insulin-sensitising effect on tissues involved in glucose and lipid metabolism. Adiponectin levels are decreased in patients with, and animal models of, obesity, diabetes and coronary artery disease [1–5]. This observation, combined with the fact that adiponectin has a number of vascular protective effects [6–10], suggests that the decreased plasma adiponectin levels associated with obesity and diabetes may contribute to the development of vascular disease in these patients. However, the mechanism by which adiponectin levels are decreased remains unknown. Vascular endothelial dysfunction plays a pivotal role in the pathogenesis of atherosclerosis and increases the risk of future cardiovascular events [11, 12]. Adiponectin stimulates nitric oxide (NO) production in vascular endothelial cells [13]. In addition, hypoadiponectinaemia has been linked to endothelial dysfunction in humans [14, 15]. Thus, the observed relationship between insulin resistance and vascular endothelial cell dysfunction may be related to reduced levels of adiponectin.

Angiotensin II (AngII) exerts multiple effects on the cardiovascular system, including elevation of blood pressure, vascular endothelial dysfunction, and cardiovascular hypertrophy. AngII-induced cardiovascular alterations may be the result of free radical production [16]. Through its type 1 (AT1) receptor, AngII stimulates the overexpression of cytosolic proteins involved in the activation of NAD(P)H oxidase within vascular endothelial cells, smooth muscle cells and leucocytes [17, 18], which favours the production of reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide and hydroxyl radicals.

AngII also induces insulin resistance via oxidative stress [19]. Recent clinical trials suggest that blockade of the renin–angiotensin system, either by inhibition of angiotensin-converting enzyme (ACE) [20, 21] or blockade of the AT1 receptor [22], may substantially reduce the risk of developing type 2 diabetes, although the mechanism responsible for this effect has yet to be elucidated. Given that AngII inhibits the adipogenic differentiation of human adipocytes via the AT1 receptor [23], and that the expression of AngII-forming enzymes in adipose tissue is inversely correlated with insulin sensitivity [24], it has been suggested that blockade of the renin–angiotensin system might prevent the development of diabetes by promoting adipocyte differentiation. The increased production of AngII by large, insulin-resistant adipocytes inhibits the recruitment of preadipocytes, resulting in the increased storage of lipid in muscle and other tissue, thereby decreasing insulin sensitivity.

In the present study we examined the influence of AngII infusion on adiponectin expression in rats. Based on the finding that AngII infusion elicits a significant and profound decrease in circulating adiponectin, we investigated the possibility that oxidative stress might underlie AngII-induced hypoadiponectinaemia by examining the effect of tempol (4-hydroxy-2,2,6,6,-tetramethylpiperidin-1-oxyl) [25, 26], a membrane-permeable superoxidase dismutase mimetic, and tetrahydrobiopterin (BH4) [27, 28], one of the most potent naturally occurring reducing agents and an essential cofactor for enzymatic NO synthase activity.

Materials and methods

Animals and experimental protocol The present experiment was reviewed and approved by the Committee on Ethics of Animal Experiments and conducted according to the Guidelines for Animal Experiments, Dokkyo University Faculty of Medicine.

Seven-week-old male Sprague–Dawley rats (Tokyo Experimental Animals, Tokyo, Japan) were randomly divided into six experimental groups of eight rats. The rats were infused with AngII or its vehicle (distilled water), either alone (AngII and control groups, respectively) or in combination with tempol (AngII-tempol and tempol groups, respectively) or BH4 (AngII-BH4 and BH4 groups, respectively). AngII (Sigma, St Louis, MO, USA) was infused subcutaneously using an osmotic pump (model 2002; Alza Corporation, Palo Alto, CA, USA) at a dose of 300 $ng \cdot kg^{-1} \cdot min^{-1}$ for 7 days. Tempol (Wako Pure Chemical Industries, Tokyo, Japan) and BH4 (sapropterin; a generous gift from Daiichi Suntory Pharma, Tokyo, Japan) were administered in the drinking water (2 and 0.2 mg/ml, respectively), 24 h before and during the 7-day period of AngII infusion.

Vessel collection and adipose tissue preparation On day 7 of treatment, the heart rate and systolic blood pressure of the rats were measured using the tail cuff method. The rats were anaesthetised with intraperitoneally administered pentobarbital, and the chest was opened. With the heart still beating, heparin (150 IU) was given via intracardiac injection. The thoracic aorta was removed en bloc and placed in cold Krebs–Henseleit solution. Extravascular tissue was rapidly removed, and the vessel lumen was flushed with solution. Some of the aortas were cut into three 5-mm ring segments for use in studies of vasoreactivity and superoxide anion production.

Adipose tissue was also obtained from the peritoneal fat pad in order to measure the levels of mRNAs encoding adiponectin and NADPH oxidase-related proteins.

Measurement of adiponectin levels in serum Serum concentrations of adiponectin were determined by ELISA using a kit for the measurement of rat/mouse adiponectin (Otsuka Pharmaceuticals, Tokyo, Japan).

Organ chamber experiments Organ chamber experiments were performed as previously described [29]. Animals were anaesthetised with pentobarbital and then exsanguinated. The thoracic aortas were carefully dissected, and all perivascular tissue removed under a microscope in a physiological salt solution (PSS) of the following composition (in mmol/l): NaCl 121, KCl 4.7, $NaHCO_3$ 24.7, $MgSO_4$ 12.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, glucose 5.8; aerated with 95% O_2, 5% CO_2. In some experiments, the endothelium was denuded by gentle rubbing of the luminal surface with an appropriate silk. The rings of each thoracic aorta (5 mm in length) were mounted vertically between two hooks in organ chamber myographs (Medical Supply Company, Tokyo, Japan), which were filled with PSS and kept at

37°C. Isometric tension was measured with force transducers (Nihon Kohden, Tokyo, Japan). Each preparation was stretched to an optimal length in a stepwise manner, at which point the force induced by 118 mmol/l KCl was maximal and constant. After equilibration for at least 30 min, the rings were precontracted with prostaglandin F_2 (3–10 μmol/l). Once a stable contraction was achieved, the rings were exposed to acetylcholine (10^{-10} to 10^{-5} mol/l) to evaluate endothelial vasodilator function. Endothelium-independent relaxation in response to sodium nitroprusside (10^{-11} to 10^{-6} mol/l) was examined in endothelium-denuded rings.

Measurement of vascular superoxide anion production Superoxide anion production was measured using lucigenin (bis-*N*-methylacridinium nitrate) chemiluminescence, as previously described [29]. Briefly, the thoracic aortas were carefully dissected, and all perivascular tissue and contaminating blood products were removed in PSS under a microscope, after which the aortas were placed in HEPES-buffered PSS. In a preliminary study we confirmed that no adhesion of inflammatory cells to the endothelium occurred (data not shown). Scintillation vials containing 1 ml HEPES-buffered PSS with 5 μmol/l lucigenin were placed into a scintillation counter (Luminescence Reader BLR 301; Aloka, Tokyo, Japan). To validate our method we used tiron (4,5-dihydroxy-1,3-benzene disulphonic acid; 10 mmol/l), a superoxide scavenger, in all experiments. After dark adaptation, background counts were recorded for 3 min, after which three vascular segments (5 mm in length) from each thoracic aorta were added to each vial. Scintillation counts were then recorded every minute for 10 min and the respective background counts subtracted. The vessels were then dried for determination of dry weight. Lucigenin counts were expressed as counts per minute per milligram of dry weight. The measurements were also performed in the presence of the NAD(P)H oxidase inhibitor apocynin (100 μmol/l), which inhibits the assembly of the components of the enzyme [30, 31].

Measurement of levels of adiponectin and NADPH oxidase mRNAs in adipose tissue Standard Northern blotting was used to investigate the expression of adiponectin mRNA in adipose tissue, as previously described [32]. After probing for adiponectin, filters were stripped and re-probed for the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Radioactivity on the blots was quantified using an image analyser (BAS2000; Fuji Film, Tokyo, Japan). The expression of p22phox, gp91phox, p47phox, Rac1 and GAPDH mRNAs was analysed by RT-PCR, as previously described [33]. The NAD(P)H oxidase components were quantified by amplification of cDNA using an ABI Prism 7000 real-time thermocycler (Applied Biosystems, Foster City, CA, USA). Copy numbers of the transcripts were obtained from standard curves generated from rat p22phox, gp91phox, p47phox and Rac1 templates [34].

Cell culture and RT-PCR The 3T3-L1 pre-adipocytes (American Type Culture Collection, Manassas, VA, USA) were grown to confluence in DMEM containing 25 mmol/l glucose, as described previously [35]. Forty-eight hours following confluence, the cells were induced to differentiate into adipocytes 48 h after confluence by changing the medium to DMEM supplemented with 10% FCS, 5 μg/ml recombinant human insulin, 0.5 mmol/l isobutylmethylxanthine and 0.25 μmol/l dexamethasone for 48–72 h. The cells were used 9 or 10 days after the induction of differentiation, when more than 90% of the cells exhibited an adipocyte phenotype. The addition of glucose oxidase at concentrations of up to 100 mU/ml (type II from *Aspergillus niger*, 20,000 U/g solid in non-oxygen-saturated conditions; Sigma) to serum-free DMEM supplemented with 0.5% RIA-grade bovine serum albumin was used to generate H_2O_2 [35]. Total RNA was isolated from the cells and reverse transcribed. Adiponectin mRNA was quantified by amplification of cDNA using an ABI Prism 7000 real-time thermocycler. Cell respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan. To examine the cytotoxic effect of glucose oxidase, the cells were incubated (37°C) with MTT (0.4 mg/ml) for a further 60 min after exposure to glucose oxidase. Culture medium was removed by aspiration, and the cells were solubilised in DMSO. The extent of reduction of MTT to formazan within cells was quantified by the measurement of OD_{550}. Values were compared with those obtained for the control cells (no glucose oxidase).

Statistical analysis Data are expressed as means±SEM. Differences between two experiments were compared by Student's *t*-tests. Differences between three experiments were determined by two-way ANOVA and Bonferroni's multiple comparison test. A p value of 0.05 was considered statistically significant.

Results

Body weight and haemodynamic parameters The infusion of AngII alone elicited a profound pressor effect during the 7-day treatment period (48.8% increase in systolic blood pressure vs vehicle-infused rats; $p<0.05$) (Table 1). The

Table 1 Body weight and systolic blood pressure of the rats following infusion

	Control group	AngII group	Tempol group	AngII–tempol group	BH4 group	AngII–BH4 group
Body weight (g)	313±12	258±8**	295±10	275±9*	321±12	282±10
Systolic blood pressure (mmHg)	125±8	186±11**	123±11	142±7	125±7	136±12

Data are means±SEM
* $p<0.05$, ** $p<0.01$ vs control group

AngII-induced increase in blood pressure was accompanied by a 17.5% decrease in body weight ($p<0.01$). The unrestricted administration of either tempol (2 mmol/l) or BH4 (0.2 mg/ml), both of which are anti-oxidants, had no significant effect on rat body weight or systolic blood pressure. However, each agent effectively prevented the weight loss and pressor actions of AngII (Table 1).

Angiotensin-II-induced endothelial dysfunction Acetylcholine induced relaxation of aortic rings in an endothelium-dependent manner (Fig. 1). The vasorelaxation of aortic rings from AngII-infused rats was significantly impaired compared with that of rings from vehicle-infused control rats (Fig. 1a). This impairment was characterised by a ≈30% reduction in maximal acetylcholine-induced vasorelaxation and a marked rightward shift in the acetylcholine concentration–response curve. In contrast, AngII did not diminish the maximal vasorelaxant action of sodium nitroprusside, an endothelium-independent (NO-mediated) vasodilator, and caused only a small rightward shift in the concentration–response relationship (Fig. 1b). Since acetylcholine-induced vasorelaxation is mediated by NO in this system, our findings are consistent with the view that AngII promotes vasoconstriction by reducing levels of endothelial-derived NO, rather than diminishing the smooth muscle response to NO. Tempol and BH4 significantly ameliorated AngII-induced endothelial dysfunction (Fig. 1a), and had no effect on the endothelium-independent vasorelaxation induced by sodium nitroprusside (Fig. 1b). Of note, the tempol- and BH4-mediated improvements in endothelial vasodilator function were abolished in the presence of L-NAME (N^G-nitro-L-arginine methyl ester; 100 μmol/l), indicating that tempol and BH4 exert their beneficial effects through the restoration of NO bioactivity (data not shown).

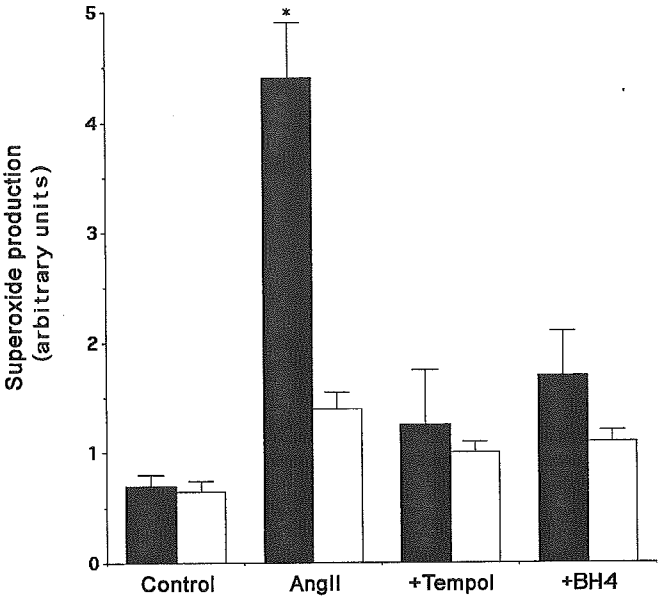

Fig. 2 Superoxide production in thoracic aortic rings in the absence (*closed bars*) and presence (*open bars*) of apocynin. Long-term treatment with tempol or BH4 suppressed the AngII-induced endothelial production of superoxide anions. The AngII-induced increase in superoxide production was acutely and significantly attenuated in the presence of apocynin (100 μmol/l). Results are expressed as means± SEM. Six to eight rings were used to determine the mean values. *$p<0.01$ vs the control value

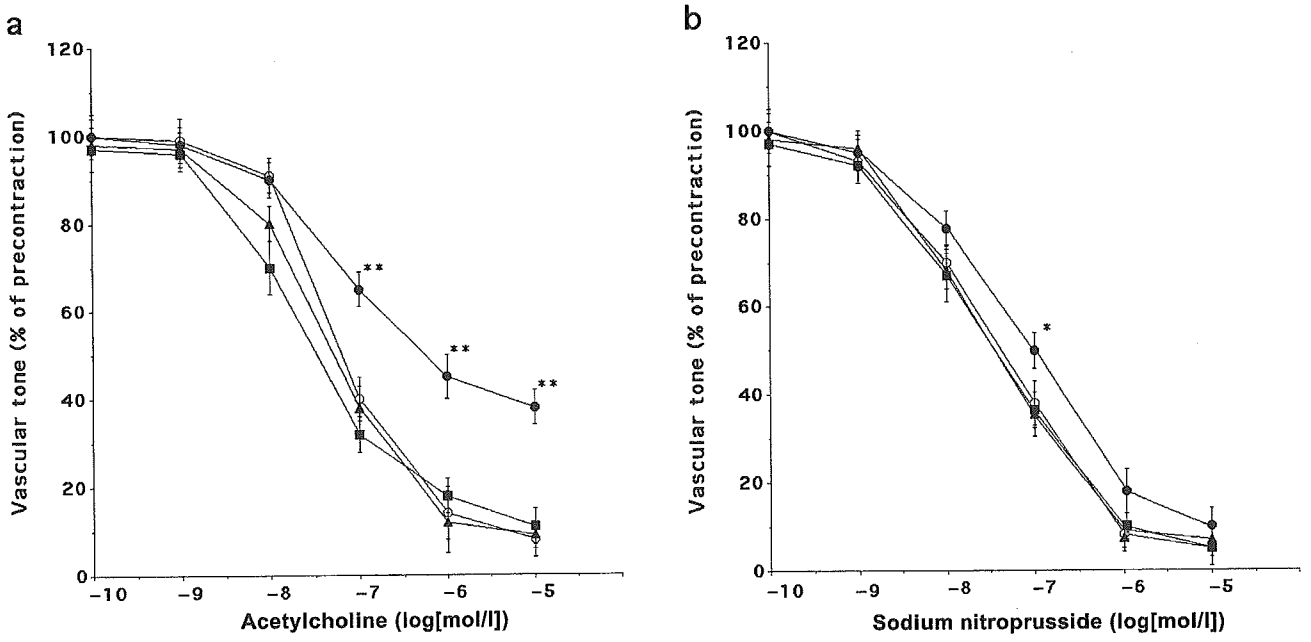

Fig. 1 Endothelium-dependent relaxation in response to acetylcholine (**a**) and endothelium-independent relaxation in response to the NO donor sodium nitroprusside (**b**) in thoracic aortic rings from control animals (*open circles*) and rats treated with AngII, either alone (*closed circles*) or in combination with tempol (*closed triangles*) or BH4 (*closed squares*). The data represent the means±SEM of six to eight vascular rings. *$p<0.05$, **$p<0.01$ vs the control value

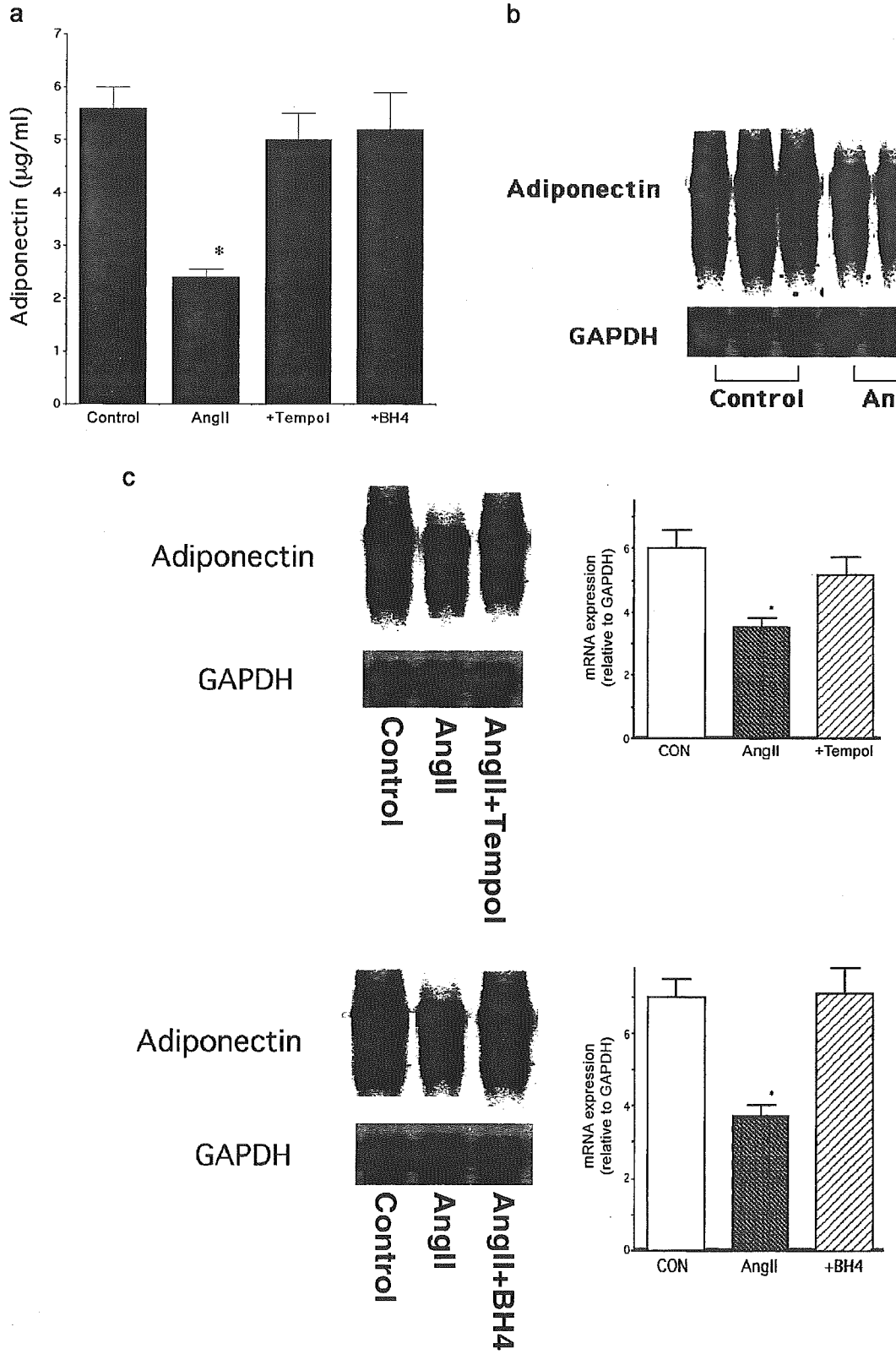

Fig. 3 **a** Plasma adiponectin levels in adipose tissue as determined by ELISA using a kit for the measurement of rat/mouse adiponectin. The results are expressed as means±SEM (n=7). **b**, **c** Adiponectin mRNA levels in adipose tissue as assessed by northern blot analysis. The results are expressed as means±SEM (n=3). *p<0.01 vs the control value

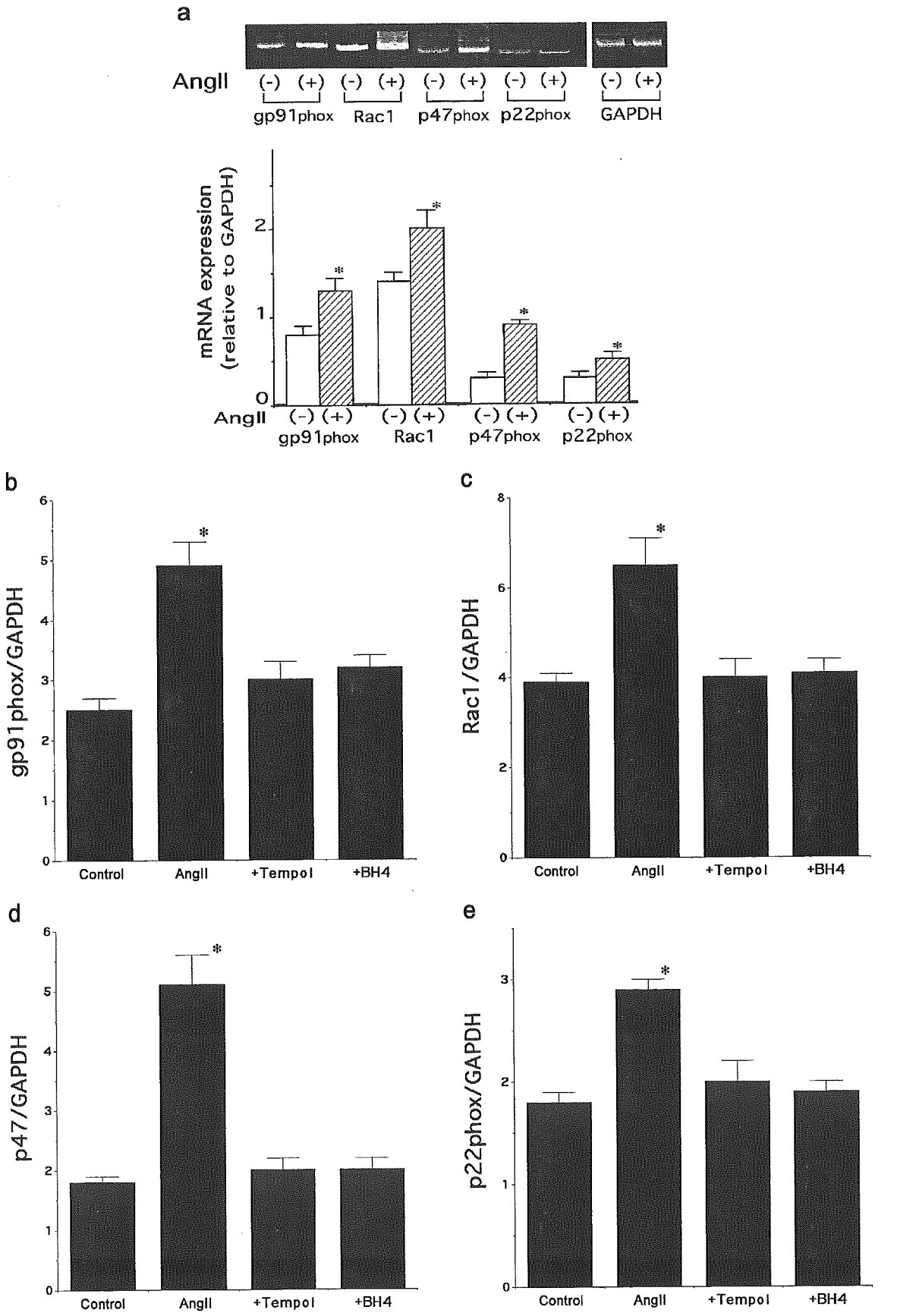

Fig. 4 **a** Expression of p22phox, gp91phox, p47phox and Rac1 in adipose tissue as assessed by RT-PCR in control rats and AngII-infused rats. **b–e** Expression of the NAD(P)H oxidase subunits gp91phox (**b**), Rac1 (**c**), p47phox (**d**), and p22phox (**e**) as evaluated by real-time PCR. Expression of the subunits was increased in AngII-infused rats, and treatment with tempol or BH4 significantly suppressed their upregulation. The results are expressed as means±SEM (n=4). *p<0.01 vs the control value

Angiotensin-II-induced superoxide production Low levels of superoxide were produced in vitro by aortic rings from control rats (Fig. 2). Infusion of AngII for 7 days produced a sixfold increase in vascular superoxide production, which was normalised by endothelial denudation (data not shown). Apocynin, an NAD(P)H oxidase inhibitor, markedly inhibited endothelial superoxide production in AngII-infused animals (Fig. 2). Similarly, cotreatment with tempol or BH4 significantly suppressed the AngII-induced production of superoxide anions. These results suggest that AngII induces endothelium-dependent superoxide production, predominately through NAD(P)H oxidase.

Plasma adiponectin levels and adiponectin mRNA levels in adipose tissue The AngII group had a significantly lower plasma adiponectin level than the control group (2.35±0.24 vs 5.60±0.44 μg/ml, $p<0.005$); however, concomitant treatment with tempol or BH4 restored plasma adiponectin concentrations (Fig. 3a).

Abundant adiponectin mRNA was detected by Northern blot analysis in abdominal adipose tissue from control (vehicle-infused) rats. Infusion of AngII reduced adiponectin mRNA levels by ≈50% (Fig. 3b), and concomitant treatment with tempol or BH4 prevented this reduction (Fig. 3c).

Angiotensin-II-induced upregulation of NAD(P)H oxidase The expression of mRNAs encoding p22phox, gp91phox, p47phox and Rac1 was significantly higher in the adipose tissue of rats in the AngII group than in the adipose tissue of the control rats (Fig. 4a). This increase was suppressed by concomitant treatment with tempol or BH4 (Fig. 4b–e). Treatment with tempol or BH4 alone had no effect on the expression of the transcripts for the NAD(P)H oxidase subunits.

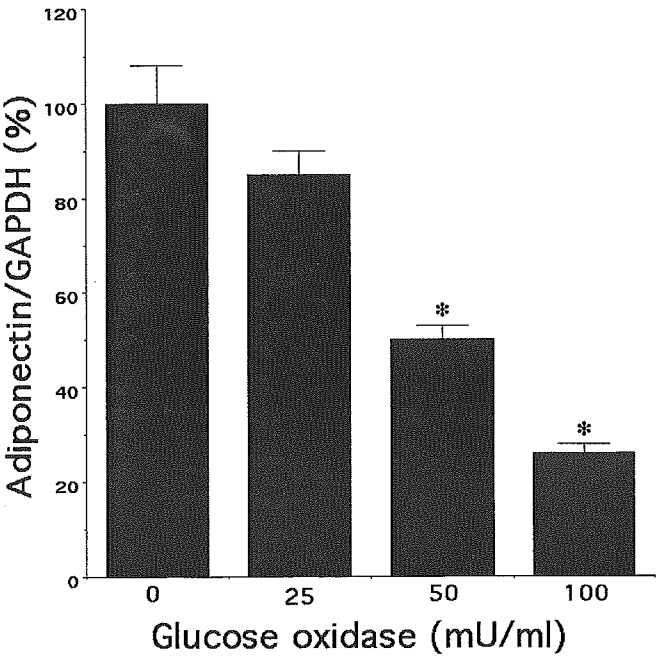

Fig. 5 Adiponectin mRNA levels in adipocytes exposed to H_2O_2. Fully differentiated 3T3-L1 adipocytes were serum-starved for 6 h and then exposed to H_2O_2 generated by adding different concentrations of glucose oxidase to the medium for 16 h. Total RNA was isolated from the cells and reverse transcribed. Quantification of adiponectin was performed by real-time PCR. The results are expressed as means±SEM (n=4). * $p<0.01$ vs the control value

Adiponectin mRNA expression in adipocytes following exposure to H_2O_2 To determine whether oxidants mediate the AngII-induced downregulation of adiponectin expression, we investigated this effect of AngII on adipocytes in culture following exposure to oxidative stress. Fully differentiated 3T3-L1 adipocytes were continuously exposed to H_2O_2 by the addition of glucose oxidase to the culture medium. Adiponectin mRNA levels were quantified by real-time PCR 16 h after exposure to H_2O_2. As shown in Fig. 5, glucose oxidase reduced adiponectin mRNA levels in a concentration-dependent manner. Given that cell respiration, measured by the MTT assay, was not significantly diminished even at the highest concentration of glucose oxidase used (data not shown), the reduction of mRNA by H_2O_2 cannot be explained by cytotoxicity.

Discussion

This study is the first to report hypoadiponectinaemia in a mammal as a consequence of chronic in vivo exposure to AngII. The results suggest a causal relationship between the AngII-mediated upregulation of NAD(P)H oxidase (with a resulting increase in ROS) and the impairment of adiponectin production. To the best of our knowledge, this is the first study to implicate a role for oxidative stress in the pathogenesis of hypoadiponectinaemia.

Clinical and laboratory studies have demonstrated that endothelial dysfunction is an important early step in atherosclerosis [36]. The endothelial dysfunction associated with long-term AngII treatment is primarily caused by an increase in NAD(P)H-oxidase-mediated vascular superoxide production [37–39]. The finding that tempol and BH4 restore endothelial function confirms that this is the case [25–28].

The key finding in the present investigation was that AngII infusion decreases circulating levels of adiponectin and reduces the expression of adiponectin mRNA in adipose tissue, the primary source of this adipokine. Suppression of adiponectin gene expression was prevented in AngII-infused rats by cotreatment with tempol or BH4, suggesting the involvement of ROS. Since adiponectin has multiple vasoprotective actions [6–10], decreased plasma adiponectin levels during AngII infusion may contribute to endothelial dysfunction, insulin resistance and cardiovascular pathophysiology. The suppression of adiponectin by AngII may be attributed to the upregulation of NADPH oxidase in adipose and vascular tissues, leading to the production of superoxide and derived species. We consider the increased production of superoxide anions to be primarily caused by the AngII-induced upregulation of NAD(P)H oxidase subunits, because it has been demonstrated that AngII-induced NAD(P)H oxidase activation is closely coupled to the increased expression of the enzyme in rats [40].

Support for the view that ROS can directly suppress adiponectin gene expression was provided by the results of our cell culture studies. Fully differentiated 3T3-L1 adipocytes were continuously exposed to H_2O_2, generated by glucose oxidase supplementation of the culture medium. At the highest concentration of glucose oxidase tested, it is estimated that cells may be exposed to concentrations of H_2O_2 of up to ~25 μmol/l [35]. This H_2O_2 exposure resulted in a concentration-dependent, substantial reduction in adiponectin mRNA expression. This finding reveals that oxidative stress within adipose tissue is sufficient to trigger hypoadiponectinaemia. The molecular mechanisms by which H_2O_2 and perhaps other ROS mediate the suppression of adiponectin mRNA levels await elucidation; diminished adiponectin gene transcription and accelerated adiponcectin mRNA degradation are viable possibilities.

Blockade of the AT1 receptor and inhibition of ACE both increase plasma levels of adiponectin. As demonstrated in the present study, AngII decreases circulating levels of adiponectin in vivo [41, 42], but does not regulate adiponectin levels in 3T3-L1 adipocytes in vitro [43]. Increased expression of NAD(P)H oxidase and increased ROS production might be localised to macrophages that invade adipose tissue, at least in obese animals, and this may be because of communication between macrophages and adipocytes in vivo.

Increasing evidence suggests that AngII is involved in the pathogenesis of a wide spectrum of cardiovascular diseases and insulin resistance [19, 37]. The present study demonstrates that oxidative stress induces hypoadiponectinaemia. Adiponectin levels are decreased in patients with obesity, diabetes and coronary artery disease. Obesity may result in increased oxidative stress in accumulated fat tissue, and patients with diabetes and coronary artery disease have high levels of oxidative stress. Thus, in addition to treating the underlying disease, it may be important to reduce oxidative stress to restore adiponectin levels and vascular integrity.

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References

1. Arita Y, Kihara S, Ouchi N et al (1999) Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun 257:79–83
2. Ouchi N, Kihara S, Arita Y et al (1999) Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. Circulation 100:2473–2476
3. Hotta K, Funahashi T, Arita Y et al (2000) Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. Arterioscler Thromb Vasc Biol 20:1595–1599
4. Kumada M, Kihara S, Sumitsuji S et al (2003) Association of hypoadiponectinemia with coronary artery disease in men. Arterioscler Thromb Vasc Biol 23:85–89
5. Ouchi N, Kihara S, Arita Y et al (2000) Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. Circulation 102: 1296–1301
6. Ouchi N, Kihara S, Arita Y et al (2001) Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. Circulation 103:1057–1063
7. Arita Y, Kihara S, Ouchi N et al (2002) Adipocyte-derived plasma protein adiponectin acts as a platelet-derived growth factor-BB-binding protein and regulates growth factor-induced common postreceptor signal in vascular smooth muscle cell. Circulation 105:2893–2898
8. Matsuda M, Shimomura I, Sata M et al (2002) Role of adiponectin in preventing vascular stenosis: the missing link of adipovascular axis. J Biol Chem 277:37487–37491
9. Okamoto Y, Kihara S, Ouchi N et al (2002) Adiponectin reduces atherosclerosis in apolipoprotein E-deficient mice. Circulation 106:2767–2770
10. Kubota N, Terauchi Y, Yamauchi T et al (2002) Disruption of adiponectin causes insulin resistance and neointimal formation. J Biol Chem 277:25863–26866
11. Suwaidi JA, Hamasaki S, Higano ST et al (2000) Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. Circulation 101:948–954
12. Schachinger V, Britten MB, Zeiher AM (2000) Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation 101:1899–1906
13. Hattori Y, Suzuki M, Hattori S, Kasai K (2003) Globular adiponectin upregulates nitric oxide production in vascular endothelial cells. Diabetologia. 46:1543–1549
14. Shimabukuro M, Higa N, Asahi T et al (2003) Hypoadiponectinemia is closely linked to endothelial dysfunction in man. J Clin Endocrinol Metab 88:3236–3240
15. Tan KC, Xu A, Chow WS et al (2004) Hypoadiponectinemia is associated with impaired endothelium-dependent vasodilation. J Clin Endocrinol Metab 89:765–769
16. Laursen JB, Rajagopalan S, Galis Z (1997) Role of superoxide in angiotensin II-induced but not catecholamine-induced hypertension. Circulation 95:588–593
17. Wolf G (2000) Free radical production and angiotensin. Curr Hypertens Rep 2:167–173
18. Griendling KK, Sorescu D, Ushio-Fukai M (2000) NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res 86:494–501
19. Ogihara T, Asano T, Ando K et al (2002) Angiotensin II-induced insulin resistance is associated with enhanced insulin signaling. Hypertension 40:872–879
20. Hansson L, Lindholm LH, Niskanen L et al (1999) Effect of angiotensin-converting-enzyme inhibition compared with conventional therapy on cardiovascular morbidity and mortality in hypertension: the Captopril Prevention Project (CAPP) randomised trial. Lancet 353:611–616
21. Yusuf S, Gerstein H, Hoogwerf B et al (2001) Ramipril and the development of diabetes. JAMA 286:1882–1885
22. Dahlof B, Devereux RB, Kjeldsen SE (2002) Cardiovascular morbidity and mortality in the losartan intervention for endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. Lancet 359:995–1003
23. Janke J, Engeli S, Gorzelniak K, Luft FC, Sharma AM (2002) Mature adipocytes inhibit in vitro differentiation of human preadipocytes via angiotensin-type 1 receptors. Diabetes 51:1699–1707
24. Gorzelniak K, Engeli S, Janke J, Luft FC, Sharma AM (2002) Hormonal regulation of the human adipose-tissue renin–angiotensin system: relationship to obesity and hypertension. J Hypertens 20:965–973
25. Schnackenberg CG, Wilcox CS (1999) Two-week administration of tempol attenuates both hypertension and renal excretion of 8-Iso prostaglandin F2alpha. Hypertension 33:424–428

26. Schnackenberg CG, Wilcox CS (2001) The SOD mimetic tempol restores vasodilation in afferent arterioles of experimental diabetes. Kidney Int 59:1859–1864
27. Hong HJ, Hsiao G, Cheng TH, Yen MH (2001) Supplementation with tetrahydrobiopterin suppresses the development of hypertension in spontaneously hypertensive rats. Hypertension 38: 1044–1048
28. Vasquez-Vivar J, Kalyanaraman B, Martasek P et al (1998) Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. Proc Natl Acad Sci U S A 95:9220–9225
29. Higashi M, Shimokawa H, Hattori T et al (2003) Long-term inhibition of Rho-kinase suppresses angiotensin II-induced cardiovascular hypertrophy in rats in vivo: effect on endothelial NAD(P)H oxidase system. Circ Res 93:767–775
30. Mukai Y, Shimokawa H, Higashi M et al (2002) Inhibition of renin–angiotensin system ameliorates endothelial dysfunction associated with aging in rats. Arterioscler Thromb Vasc Biol 22:1445–1450
31. Kashiwagi A, Shinozaki K, Nishio Y et al (1999) Endothelium-specific activation of NAD(P)H oxidase in aortas of exogenously hyperinsulinemic rats. Am J Physiol 277:E976–E983
32. Hattori Y, Kasai K, Gross SS (2004) NO suppresses while peroxynitrite sustains NF-kappaB: a paradigm to rationalize cytoprotective and cytotoxic actions attributed to NO. Cardiovasc Res 63:31–40
33. Hattori Y, Gross SS (1993) GTP cyclohydrolase I mRNA is induced by LPS in vascular smooth muscle: characterization, sequence and relationship to nitric oxide synthase. Biochem Biophys Res Commun 195:435–441
34. Lassegue B, Sorescu D, Szocs K et al (2001) Novel gp91phox homologues in vascular smooth muscle cells: nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. Circ Res 88:888–894
35. Tirosh A, Potashnik R, Bashan N, Rudich A (1999) Oxidative stress disrupts insulin-induced cellular redistribution of insulin receptor substrate-1 and phosphatidylinositol 3-kinase in 3T3-L1 adipocytes. A putative cellular mechanism for impaired protein kinase B activation and GLUT4 translocation. J Biol Chem 274: 10595–10602
36. Shimokawa H (1999) Primary endothelial dysfunction: atherosclerosis. J Mol Cell Cardiol 31:23–37
37. Rajagopalan S, Kurz S, Munzel T et al (1996) Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation: contribution to alterations of vasomotor tone. J Clin Invest 97:1916–1923
38. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW (1994) Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ Res 74:1141–1148
39. Pagano PJ, Clark JK, Cifuentes-Pagano ME (1997) Localization of constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. Proc Natl Acad Sci U S A 94:14483–14488
40. Fukui R, Ishizaka N, Rajagopalan S et al (1997) p22phox mRNA expression and NADPH oxidase activity are increased in aortas from hypertensive rats. Circ Res 80:45–51
41. Furuhashi M, Ura N, Higashiura K et al (2003) Blockade of the renin–angiotensin system increases adiponectin concentrations in patients with essential hypertension. Hypertension 42:76–81
42. Agata J, Nagahara D, Kinoshita S et al (2004) Angiotensin II receptor blocker prevents increased arterial stiffness in patients with essential hypertension. Circ J 68:1194–1198
43. Fasshauer M, Klein J, Neumann S, Eszlinger M, Paschke R (2002) Hormonal regulation of adiponectin gene expression in 3T3-L1 adipocytes. Biochem Biophys Res Commun 290:1084–1089

Supplementation with tetrahydrobiopterin prevents the cardiovascular effects of angiotensin II-induced oxidative and nitrosative stress

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Objective The pteridine cofactor tetrahydrobiopterin (BH4) has emerged as a critical determinant of endothelial nitric oxide synthase (eNOS) activity. When BH4 availability is limited, eNOS does not produce nitric oxide (NO) but instead generates superoxide. BH4 may reverse endothelial dysfunction due to cardiovascular disease, including atherosclerosis, coronary artery disease and hypertension. In this study, the influence of BH4 on cardiovascular parameters and the production of free radicals following angiotensin II (Ang II) infusion was assessed.

Methods BH4 (20 mg/kg per day in drinking water) was administered with Ang II (300 ng/kg per min subcutaneously, osmotic pump) for 7 days in Sprague–Dawley rats. In addition, BH4 was also given in vehicle-infused rats.

Results Treatment with BH4 significantly prevented some of the effects of Ang II, such as impaired vascular responses to acetylcholine, hypertension and increases in heart weight index values. Treatment with BH4 also significantly reduced Ang II-induced increases in inducible NO synthase expression, nitrotyrosine immunostaining, NO production and superoxide anion formation in rats.

Conclusion These results indicate that BH4 might prevent the development of hypertension and myocardial hypertrophy, as well as the Ang II-induced production of superoxide and NO, thereby reducing the production of peroxynitrite. Therefore, BH4 may protect against the cardiovascular manifestations of oxidative and nitrosative stress in this experimental model of Ang II-mediated hypertension. *J Hypertens* 23:1375–1382 © 2005 Lippincott Williams & Wilkins.

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Introduction

Angiotensin II (Ang II) exerts multiple effects on the cardiovascular system; specifically, it causes hypertension and myocardial hypertrophy. It has been suggested that the production of free radicals might be responsible for Ang II-induced cardiovascular manifestations [1]. An association between Ang II-mediated stimulation of the type I receptor and overexpression of several cytosolic proteins involved in activation of NAD(P)H oxidase in vascular endothelial cells, as well as smooth muscle cells and leukocytes, has been demonstrated [2,3]. Ang II might also induce nitric oxide synthase (iNOS), which causes nitric oxide (NO) overproduction within the vasculature. The simultaneous production of NO and O_2^- results in a diffusion-limited production of the reactive species, peroxynitrite ($ONOO^-$) [4]. Peroxynitrite is a powerful oxidant, which produces a wide array of tissue-damaging effects, including lipid peroxidation, enzyme and ion-channel inactivation via protein oxidation and nitration and inhibition of mitochondrial respiration [4].

Depletion of endothelial NO has been shown to play a fundamental role in the pathogenesis of atherosclerosis. There is a large body of evidence to suggest that high levels of superoxide due to NO inactivation play a large role in the development of atherosclerosis *in vivo* [5]. Recently, it has been shown that endothelial NO synthase (eNOS) generates O_2^- when limited amounts of tetrahydrobiopterin (BH4) are available [6–8]. In support of a critical role for BH4 in mediating O_2^- formation from eNOS, reduced BH4 levels due to inhibition of GTP cyclohydrolase I, the rate-limiting enzyme of BH4 synthesis in cells and intact vessel segments, has been shown to result in reduced NO generation and increased levels of O_2^- and hydrogen peroxide, as well as impaired vascular relaxation [9–11]. In addition, supplementation of cellular BH4 increases

the ability of NO synthase to generate NO [12–14]. These findings suggest a role for correction of BH4 levels through BH4 supplementation in preventing the development of cardiovascular disease.

The aim of the present study was to evaluate whether supplementation with BH4 might protect against the cardiovascular manifestations of Ang II exposure in rats.

Methods

Animal treatment

The present experiments were reviewed and approved by the Committee on Ethics of Animal Experiments and were conducted according to the Guidelines for Animal Experiments, Dokkyo University Faculty of Medicine.

Seven-week-old male Sprague–Dawley rats (Tokyo Experimental Animals, Tokyo, Japan) were assigned randomly to one of four experimental groups of eight rats. Ang II or its vehicle (distilled water) were infused without BH4 (Ang II and control groups, respectively), or with BH4 (Ang II + BH4 and BH4 groups, respectively). Ang II (Sigma Chemical Co., St. Louis, Missouri, USA) was infused subcutaneously using an osmotic pump (model 2002; Alza Corporation, Cupertino, California, USA) at a dose of 300 ng/kg per min for 7 days. BH4 (Sapropterin, a generous gift from Daiichi Santory Pharma Co., Ltd., Tokyo, Japan) was administered in the drinking water (20 mg/kg per day) starting 24 h before and throughout the 7-day period of Ang II infusion.

Vessel harvesting and organ preparation

On day seven of treatment, we measured heart rates and systolic blood pressure using the tail-cuff method. The rats were anesthetized via intraperitoneally administered pentobarbital and the chest was opened. With the heart still beating, heparin (150 IU) was given via intracardiac injection. The thoracic aorta was removed *en bloc* and placed in cold Krebs–Henseleit solution. Extravascular tissue was rapidly removed and the vessel lumen was flushed with solution. In some rats, the aorta was cut into three 5-mm ring segments for use in studies of vasoreactivity as well as superoxide anion production. Hearts were also removed from the rats in order to weigh and measure mRNA levels of NOSs and the various components of the NADPH oxidase system.

Organ chamber experiments

Organ chamber experiments were performed as described previously [15]. The animals were anesthetized with pentobarbital and killed by exsanguination. The thoracic aortas were carefully dissected and any perivascular tissue removed under microscopy in physiological salt solution (PSS) as follows (in mmol/l): NaCl, 121: KCl, 4.7; $NaHCO_3$, 24.7; $MgSO_4$, 12.2; $CaCl_2$, 2.5; KH_2PO_4, 1.2; and glucose, 5.8. The solution was aerated with 95% O_2 and 5% CO_2. In some experiments, the endothelium was denuded using gentle rubbing of the luminal surface with an appropriate silk. The 5 mm thoracic aorta segments were mounted vertically between two hooks in organ chamber myographs (Medical Supply Co., Toyko, Japan), which were filled with PSS and maintained at 37°C. Isometric tension was measured with force transducers (Nihon Kohden Co., Tokyo, Japan). Each preparation was stretched in a stepwise manner to an optimal length, at which point the force induced by 118 mmol/l KCl became maximal and constant. After achieving equilibration for at least 30 min, the rings were then pre-contracted using prostaglandin F_2. After a stable contraction was achieved, the rings were then exposed to acetylcholine (ACh) in order to evaluate the magnitude of endothelial vasodilation. Endothelium-independent relaxation as a result of exposure to sodium nitroprusside (SNP) was examined in rings without endothelium.

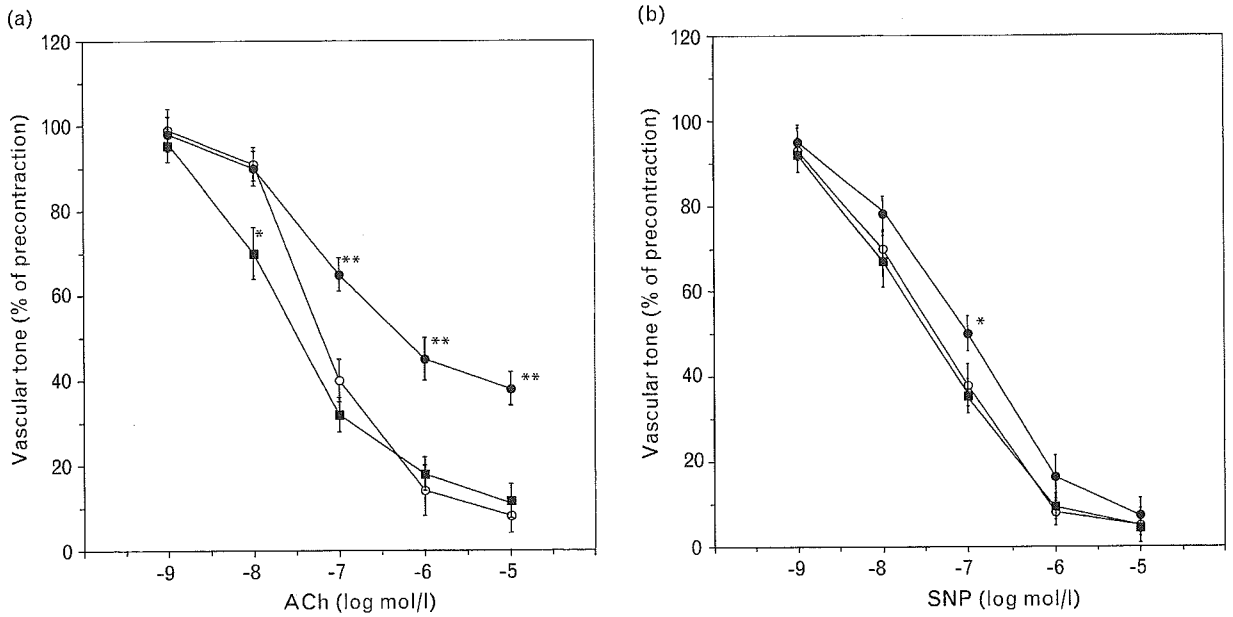
Measurement of vascular superoxide anion production

Superoxide anion production was measured using lucigenin chemiluminescence, as described previously [15]. Briefly, each thoracic aorta was carefully dissected and perivascular tissue and blood contaminants removed under microscopy in PSS, prior to placement in HEPES-buffered PSS. In a preliminary study, we confirmed no adhesion of inflammatory cells to the endothelium (data not shown). Scintillation vials containing 1 ml HEPES-buffered PSS with 5 μmol/l lucigenin (bis-*N*-methylacridinium nitrate) were placed into a scintillation counter (Luminescence Reader BLR 301; ALOKA, Tokyo, Japan). We used Tiron (10 mmol/l), a superoxide scavenger, in all experiments to confirm the validity of our technique with lucigenin. After dark adaptation, background counts were recorded for 3 min, after which three vascular segments (5 mm in length) from each thoracic aorta were added to each vial. Scintillation counts were then recorded every minute for 10 min and the respective background counts subtracted. The vessels were then dried for determination of dry weight. Lucigenin counts were expressed as counts/min per milligram of dry weight. Measurements were also performed in the presence of apocynin (100 μmol/l), a NAD(P)H oxidase inhibitor, which inhibits the assembly of NAD(P)H oxidase [16,17].

Measurement of plasma NO

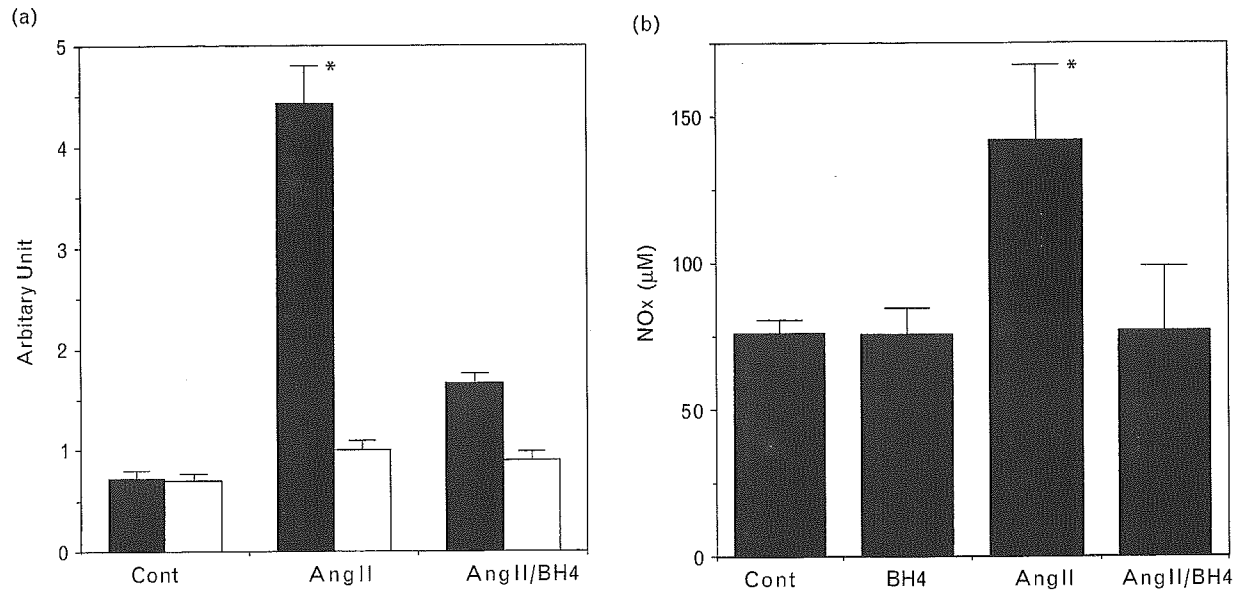
Plasma nitrite and nitrate levels (NO_2^- and NO_3^-, respectively) were measured with an automated NO detector/high-performance liquid chromatography system (ENO10; Eicom Co., Kyoto, Japan). Nitrite and nitrate in the dialysate were separated using a reverse-phase separation column and nitrate was reduced to nitrite in a reduction column. Nitrite was mixed with a Griess reagent, after which absorbance at 540 nm was measured by flow-through spectrophotometry.

Fig. 1



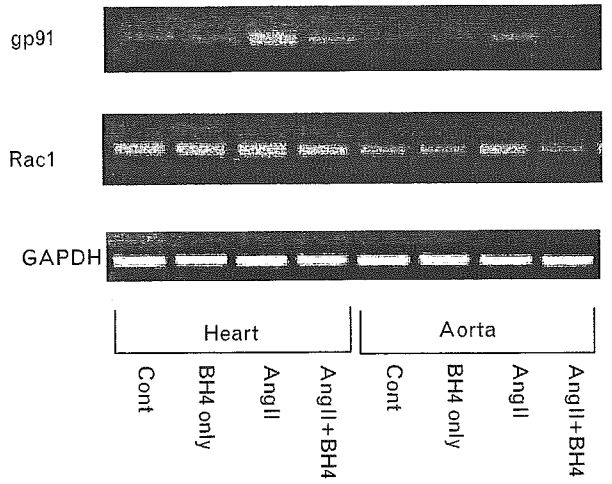
Endothelium-dependent relaxation in response to acetylcholine (ACh) (a), and endothelium-independent relaxation in response to sodium nitroprusside (SNP) (b), in thoracic aortic rings isolated from control animals (open circles), rats treated with angiotensin II (Ang II; closed circles), and rats treated with Ang II and tetrahydrobiopterin (Ang II + BH4; closed squares). Data represent means ± SEM of $n = 6–8$ vascular rings. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control values.

Fig. 2



(a) Superoxide production in thoracic aorta rings in the absence (closed column) and presence (open column) of apocynin. Long-term treatment with tetrahydrobiopterin (BH4) suppressed angiotensin II (Ang II)-induced endothelial production of superoxide anions. Ang II induced an increase in superoxide production, which was acutely and significantly attenuated in the presence of apocynin (100 μmol/l). Results are expressed as means ± SEM of of $n = 6$ vascular rings. (b) Plasma NO_x (nitrite + nitrate) levels in rats. Plasma NO_x levels were markedly increased in Ang II-infused rats, and treatment with BH4 significantly suppressed this upregulation. Results are expressed as means ± SEM of $n = 8$ rats. $^{*}P < 0.01$ compared with control values.

Fig. 3



Cardiac and vascular expression of gp91phox (gp91) and Rac1 was assessed by reverse transcription-polymerase chain reaction (RT-PCR). Treatment with tetrahydrobiopterin (BH4) suppressed the mRNA expression of gp91phox and Rac1. Cont, control; Ang II, angiotensin II.

Expression of NOSs and NADPH oxidase in the aorta

Expression of eNOS, iNOS, gp91phox, Rac1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was analyzed by reverse transcription-polymerase chain reaction (RT-PCR), as described previously [18]. Quantification of NAD(P)H oxidase components, including nox1, nox4, gp91phox and p22phox, was also performed by amplification of cDNA using an ABI Prism 7000 real-time thermocycler. Message copy numbers were obtained from standard curves generated from genuine rat nox1, nox4, gp91phox and p22phox templates [19]. The method used for Western blot analysis was the same as that described in a previous report [20], using ECL reagents (Amersham Biosciences, Toyko, Japan) for chemiluminescence. Anti-eNOS monoclonal antibody was obtained from BD Transduction Laboratories (San Diego, California, USA) and anti-iNOS polyclonal antibody was from Cell Signaling Technology (Beverly, Massachusetts, USA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, California, USA).

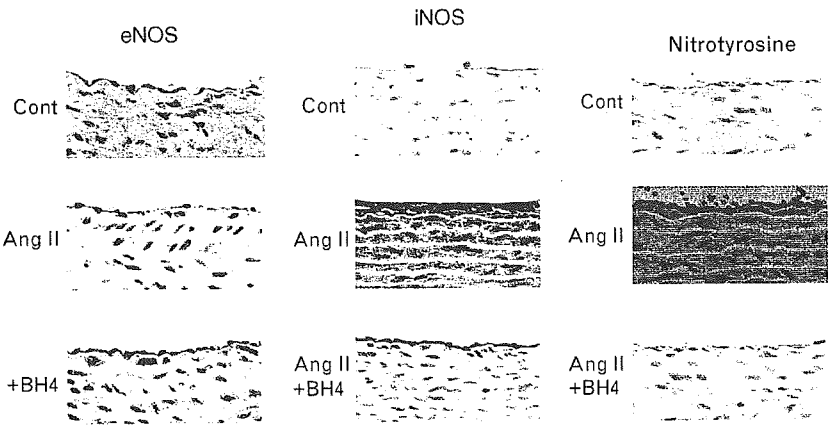
Immunohistochemical analysis of NOSs and nitrotyrosine expression

Paraffin sections of 2 μm thickness were prepared for analysis using the avidin–biotin–horseradish peroxidase complex method. The sections were de-paraffinized and incubated for 15 min in 3% hydrogen peroxide (H_2O_2) to block endogenous peroxidase activity. After treatment with the blocking serum, the sections were incubated with 1:100 dilutions of monoclonal antibodies against eNOS, iNOS (Transduction Laboratories, Lexington, Kentucky, USA) and nitrotyrosine (Upstate Biotechnology, Lake Placid, New York, USA) for 60 min. The sections were then rinsed with Tris-buffered saline with 0.1% Tween 20 and incubated with biotinylated secondary antibody against mouse immunoglobulin (Dako, Glostrup, Denmark) for 20 min. After being rinsed with Tris-buffered saline with 0.1% Tween 20, the sections were incubated with horseradish peroxidase-conjugated streptavidin for 20 min, followed by incubation with the peroxidase substrate solution, diaminobenzidine. The sections were then counterstained with hematoxylin and examined under light microscopy.

BH4 concentration in plasma, aorta and heart

Biopterin concentrations were measured in plasma, the aorta and the heart, using high-pressure liquid

Fig. 4



Immunohistochemical staining for endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and nitrotyrosine in aortic tissue from control (Cont), angiotensin II (Ang II)- and Ang II and tetrahydrobiopterin (Ang II + BH4)-treated rats. Similar immunohistochemical profiles were observed the aortic samples of 4–5 rats from each group.

chromatography (HPLC) analysis, as described previously by Fukushima and Nixon [21]. BH4 concentrations were determined by calculating the difference between total BH4, BH2 and oxidized biopterin and alkaline-stable biopterin (BH2 and oxidized biopterin).

Statistical analysis

Data are expressed as mean values ± SEM. Differences between two experiments were compared by Student's *t*-tests. Differences between three experiments were determined by two-way ANOVA and Bonferroni's multiple comparison testing. A P value of 0.05 was considered statistically significant.

Results

Body weight and hemodynamic parameters

Throughout the 7-day treatment period, the Ang II group demonstrated lower mean body weights than the control group. During this period, the Ang II group also exhibited a significant increase in systolic arterial pressure, compared with the control group (Table 1). Systolic blood pressure in the Ang II + BH4 group was significantly lower than that of the Ang II group, and comparable to that of the control group and the BH4 (only) group.

The mean heart weight index (mg/kg body weight) was significantly greater in the Ang II group, compared with control and BH4 (only) groups. The development of cardiac hypertrophy was inhibited by concomitant treatment with BH4 (Ang II + BH4 group), as determined by comparison with the control and BH4 (only) groups.

Angiotensin II-induced endothelial dysfunction

Endothelium-dependent relaxation upon exposure of aortas isolated from Ang II-infused rats to ACh was significantly impaired, compared to that observed in controls (Fig. 1a). Concomitant treatment with BH4 significantly ameliorated Ang II-induced endothelial dysfunction (Fig. 1a). Administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME) (100 μmol/l) abolished vasodilatation, indicating that this endothelial function was mediated by a NO-dependent mechanism (data not shown).

In contrast to the substantial impairment of maximal relaxation in response to acetylcholine in Ang II-infused rats, maximal relaxation in response to the NO donor compound SNP did not change as a result of Ang II infusion. This indicates that the ability of smooth muscle to relax in response to NO was not impaired. There was, however, a small but significant shift to the right in the SNP dose–response curve in Ang II-treated animals, which was improved by BH4 treatment (Fig. 1b).

Angiotensin II-induced superoxide production

A small amount of superoxide production was noted in control animals. Angiotensin II infusion for 1 week markedly increased vascular superoxide production (Fig. 2a). Endothelial denudation normalized superoxide production in Ang II-infused rats, with a remaining production of superoxide anions in vascular smooth muscle cells (data not shown). An NAD(P)H oxidase inhibitor, apocynin, markedly inhibited endothelial superoxide production in Ang II-infused animals. Importantly, concomitant oral treatment with BH4 significantly suppressed the Ang II-induced endothelial production of superoxide anions (Fig. 2a).

Plasma levels of nitrate/nitrite

Plasma levels of nitrate/nitrite were increased in the Ang II group, indicating upregulation of NO production after Ang II infusion (Fig. 2b). There was a marked decrease in nitrate/nitrite levels in the Ang II + BH4 group, which were similar to those of the control and BH4 (only) groups (Fig. 2b).

Angiotensin II-induced upregulation of NAD(P)H oxidase

mRNA expression of gp91phox and Rac1 was significantly increased in the thoracic aortas and hearts of Ang II-infused rats, compared with control animals (Fig. 3). Concomitant treatment with BH4 significantly suppressed this upregulation. Similarly, mRNA expression of gp91phox, nox1, nox4 and p22phox, in the thoracic aorta, as assessed by RealTime PCR, were all significantly increased, and concomitant treatment with BH4 significantly suppressed this upregulation (data not shown). Treatment with BH4 alone had no effect on NAD(P)H oxidase subunit mRNA expression.

Immunohistochemistry

Figure 4 shows representative sections of aortic tissue immunostained for eNOS, iNOS and nitrotyrosine. eNOS expression was similar among control and Ang II-infused rats, but slightly increased in Ang II + BH4-infused rats. iNOS immunoreactivity was significantly greater among the Ang II group, compared with the control group, and BH4 treatment decreased this increased iNOS expression (Ang II + BH4 group)

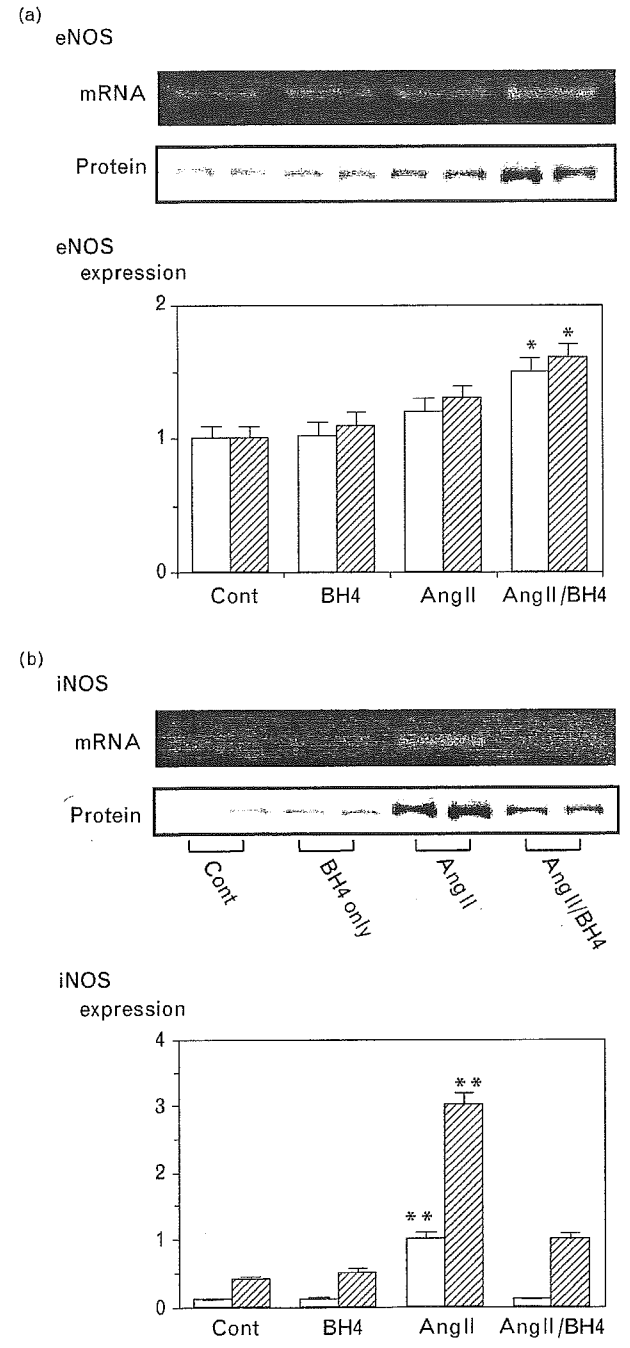
Table 1 Body weight and hemodynamic parameters of control rats and those treated with tetrahydrobiopterin (BH4), angiotensin II (Ang II), or Ang II + BH4

	Control	BH4	Ang II	Ang II + BH4
Body weight (g)	313 ± 12	321 ± 12	258 ± 8**	282 ± 10
Systolic blood pressure (mmHg)	125 ± 8	125 ± 7	186 ± 11**	136 ± 12
Heart weight index (mg/kg body weight)	3.2 ± 0.13	3.1 ± 0.11	3.9 ± 0.065**	3.3 ± 0.075

Data are mean ± SE. **$P < 0.01$ versus control group.

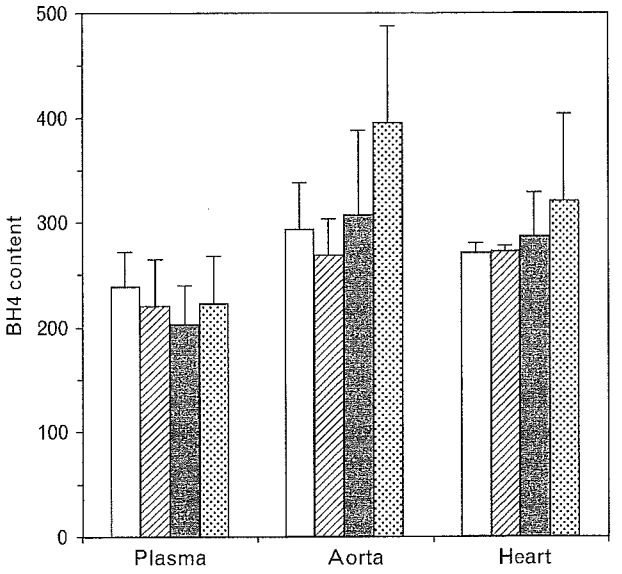
(Fig. 4). Similarly, nitrotyrosine staining, an indicator of peroxynitrite formation, was clearly observed in the Ang II group, but barely visible in the control and Ang II + BH4 groups (Fig. 4).

Fig. 5



mRNA and protein levels of (a) endothelial nitric oxide synthase (eNOS) and (b) inducible nitric oxide synthase (iNOS) in aortic tissue from control, tetrahydrobiopterin (BH4), angiotensin II (Ang II)- and Ang II + BH4-treated rats were evaluated by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. Levels of mRNA (open bars) and protein (hatched bars) are shown in the lower panels. Results are expressed as means ± SEM of $n = 5$ aortas. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control values.

Fig. 6



Tetrahydrobiopterin (BH4) content in plasma (pmol/ml), aortic and heart tissue (pmol/g tissue) from control, BH4 (only)-treated, angiotensin II (Ang II)-treated, and Ang II and tetrahydrobiopterin (Ang II + BH4)-treated rats. Control, white bars; BH4, hatched bars; Ang II, gray bars; Ang II + BH4, dotted bars. Results are expressed as means ± SEM of $n = 6$ rats.

mRNA and protein levels of eNOS and iNOS in aortic tissue

iNOS mRNA levels were barely detectable in the control group and the BH4 (only) group, while they were clearly increased in the Ang II group, a finding that was largely reversed by treatment with BH4 (Ang II + BH4) (Fig. 5). Similarly, iNOS protein levels were increased in the Ang II group, while low levels were observed in the other groups (Fig. 5). eNOS mRNA and protein levels were significantly greater in the Ang II + BH4 group, compared with the other groups (Fig. 5), which is compatible with our immunohistochemistry results regarding eNOS expression.

BH4 content of plasma, as well as aortic and heart tissue

BH4 levels in plasma, aortic and heart tissue did not differ between the control group and the Ang II group (Fig. 6). Even among rats treated with BH4, significant differences in BH4 levels were not observed, although marginally increased BH4 levels were observed in the aortic and heart tissue of Ang II + BH4-treated rats (Fig. 6). Ang II-infused rats had a reduced plasma BH4 to total biopterin ratio (81%), but this returned to normal (90%) following BH4 supplementation.

Discussion

In this study, BH4 prevented the development of hypertension and myocardial hypertrophy associated with long-term infusion of Ang II, and prevented