Epoxyeicosatrienoic acids contribute with altered nitric oxide and endothelin-1 pathways to conduit artery endothelial dysfunction in essential hypertension.

Jeremy Bellien, Michele Iacob, Isabelle Remy-Jouet, Daniele Lucas, Christelle Monteil, Laurence Gutierrez, Cathy Vendeville, Yvonne Dreano, Alain Mercier, Christian Thuillez, et al.

Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Epoxyeicosatrienoic Acids Contribute With Altered Nitric Oxide and Endothelin-1 Pathways to Conduit Artery Endothelial Dysfunction in Essential Hypertension

Jeremy Bellien, Michele Iacob, Isabelle Remy-Jouet, Daniele Lucas, Christelle Monteil, Laurence Gutierrez, Cathy Vendeville, Yvonne Dreano, Alain Mercier, Christian Thuillez and Robinson Joannides

Circulation 2012, 125:1266-1275
doi: 10.1161/CIRCULATIONAHA.111.070680
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7322. Online ISSN: 1524-4539

Epoxyeicosatrienoic Acids Contribute With Altered Nitric Oxide and Endothelin-1 Pathways to Conduit Artery Endothelial Dysfunction in Essential Hypertension

Jeremy Bellien, PharmD, PhD; Michele Iacob, MD; Isabelle Remy-Jouet, PhD; Daniele Lucas, PharmD, PhD; Christelle Monteil, PharmD, PhD; Laurence Gutierrez, MSN; Cathy Vendeville, BSc; Yvonne Dreano, BSc; Alain Mercier, MD; Christian Thuillez, MD, PhD; Robinson Joannides, MD, PhD

Background—We sought to clarify, using functional and biological approaches, the role of epoxyeicosatrienoic acids, nitric oxide (NO)/reactive oxygen species balance, and endothelin-1 in conduit artery endothelial dysfunction during essential hypertension.

Methods and Results—Radial artery diameter and mean wall shear stress were determined in 28 untreated patients with essential hypertension and 30 normotensive control subjects during endothelium-dependent flow-mediated dilatation induced by hand skin heating. The role of epoxyeicosatrienoic acids and NO was assessed with the brachial infusion of inhibitors of cytochrome P450 epoxygenases (fluconazole) and NO synthase (N^G-monomethyl-L-arginine [L-NMMA]). Compared with controls, hypertensive patients exhibited a decreased flow-mediated dilatation in response to postischemic hyperemia as well as to heating, as shown by the lesser slope of their diameter–shear stress relationship. In controls, heating-induced flow-mediated dilatation was reduced by fluconazole, L-NMMA, and, to a larger extent, by L-NMMA+fluconazole. In patients, flow-mediated dilatation was not affected by fluconazole and was reduced by L-NMMA and L-NMMA+fluconazole to a lesser extent than in controls. Furthermore, local plasma epoxyeicosatrienoic acids increased during heating in controls (an effect diminished by fluconazole) but not in patients. Plasma nitrite, an indicator of NO availability, increased during heating in controls (an effect abolished by L-NMMA) and, to a lesser extent, in patients, whereas, inversely, reactive oxygen species increased more in patients (an effect diminished by L-NMMA). Plasma endothelin-1 decreased during heating in controls but not in patients.

Conclusions—These results show that an impaired role of epoxyeicosatrienoic acids contributes, together with an alteration in NO/reactive oxygen species balance and endothelin-1 pathway, to conduit artery endothelial dysfunction in essential hypertension.

Clinical Trial Registration—https://www.eudract.ema.europa.eu. Unique identifier: RCB2007-A001–10-53.

(*Circulation*. 2012;125:1266-1275.)

Key Words: arteries ■ endothelium-derived factors ■ epoxyeicosatrienoic acids ■ hypertension

The endothelium plays a crucial role in controlling vascular tone and homeostasis through the release of vasoactive factors.[1,2] In essential hypertension, endothelial dysfunction is present in both resistance and conduit arteries and constitutes an early independent predictor of cardiovascular events.[2,3] Moreover, it was prospectively shown that the normalization of blood pressure with the use of conventional antihypertensive drugs is associated with a greater reduction in the occurrence of cardiovascular events when conduit artery endothelial function is restored.[3] Therefore, the restoration of conduit artery endothelial function has emerged as a primary target to limit cardiovascular morbidity and mortality in patients with essential hypertension.

Received September 29, 2011; accepted February 2, 2012.

From the Department of Pharmacology, Rouen University Hospital, Rouen (J.B., M.I., C.T., R.J.); Institut National de la Sante et de la Recherche Medicale (INSERM) U1096, Rouen Medical School, University of Rouen, Rouen (J.B., M.I., I.R.-J., C.M., C.V., C.T., R.J.); Centre d'Investigation Clinique–INSERM 0204, Rouen University Hospital, Rouen (L.G.); Department of General Medicine, Rouen Medical School, University of Rouen, Institute for Research and Innovation in Biomedicine, Rouen (A.M.); and Department of Biochemistry, INSERM U613, ECLA, Brest University Hospital, Brest (D.L., Y.D.), France.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.111.070680/-/DC1.

Correspondence to Robinson Joannides, MD, PhD, Service de Pharmacologie, INSERM U1096, Institute for Research and Innovation in Biomedicine, CHU de Rouen, 76031 Rouen CEDEX, France. E-mail robinson.joannides@chu-rouen.fr

Circulation **is available at http://circ.ahajournals.org** **DOI: 10.1161/CIRCULATIONAHA.111.070680**

Clinical Perspective on p 1275

However, the mechanisms of endothelial dysfunction, although largely investigated in resistance arteries,[4–8] have been poorly documented at the level of the conduit arteries in human hypertension. In fact, to our knowledge, the role of a decrease in nitric oxide (NO) availability has only been reported in 1 study.[9] In addition, the evolution of the epoxyeicosatrienoic acid (EET) pathway has never been explored. This is particularly important because EETs, which are synthesized by endothelial cytochrome P450 epoxygenases, share with NO many cardiovascular protective properties, contributing notably to the regulation of vascular tone and remodeling. In addition, increasing experimental evidence suggests that a decrease in EET availability is involved in the pathophysiology of end-organ damage in arterial hypertension.[2,10] Furthermore, a role for the vasoconstrictor endothelin-1 (ET-1) and reactive oxygen species (ROS), which inactivate NO, in the endothelial dysfunction associated with arterial hypertension remains to be clarified at the level of the conduit arteries in humans.

In this context, the use of a sustained hyperemic stimulation induced by the hand skin heating method may be useful.[11] Indeed, we demonstrated in healthy volunteers by combining this method with functional approaches based on the local inhibition of cytochrome P450 epoxygenases and NO synthase that both pathways contribute to the regulation of conduit artery flow-mediated dilatation.[11] Moreover, because of the sustained and stable increase in blood flow, it is possible to draw local blood samples during heating and thus to putatively perform the biological identification of the endothelial factors released.[11]

In this context, the present study was designed to compare, with the use of functional and biological approaches, the role of EETs, NO/ROS balance, and ET-1 during endothelium-dependent flow-mediated dilatation in patients with essential hypertension and normotensive control subjects.

Methods

Subjects

The study was performed in a total of 58 subjects. All subjects were nonsmokers, must not have received hormone replacement therapies or statins for the last 6 months, and did not receive any medication at the time of the study.[12] Subjects with cardiac and/or cerebrovascular ischemic disease, heart failure, impaired renal function (estimated glomerular filtration rate <60 mL/min per 1.73 m^2 according to the Cockcroft-Gault equation), or other major pathologies were excluded from the study. Twenty-eight untreated patients with essential hypertension were recruited from general practitioners of the local clinical research network if supine systolic and diastolic blood pressures measured after 10 minutes of rest were consistently found to be ≥140/90 mm Hg 3 times at 1- to 2-week intervals. Secondary forms of hypertension were excluded by routine diagnostic procedures. Patients were enrolled if never treated (n=19) or reporting a medical history of discontinued pharmacological antihypertensive treatment interrupted for at least 3 months before the day of inclusion to exclude possible remnant effects of the drugs on endothelial function (n=9). Moreover, 30 volunteers frequency-matched for major vascular risk factors with the hypertensive patients (Table) were recruited by the Centre d'Investigation Clinique–Institut National de la Santé et de la Recherche Médicale 0204 and the Department of Pharmacology of Rouen University Hospital and were deemed healthy on the basis of the absence of familial history of essential hypertension and systolic and diastolic blood pressure values <140/90 mm Hg, as well as complete medical examination and routine laboratory tests. On the day of inclusion, systolic and diastolic blood pressures were measured after 10 minutes of rest on the dominant arm by means of a brachial cuff oscillometric device (Omron HEM-705CP) 3 times at 5-minute intervals. The study was approved by the local ethics committee (Committee for the Protection of Persons of Normandy), and all participants gave written informed consent. The study was conducted according to the Principles of Good Clinical Practice and the Declaration of Helsinki. The study was registered at https://www.eudract.ema.europa.eu under the unique identifier RCB2007-A001–10-53.

Table. Baseline Characteristics of Normotensive Control Subjects and Hypertensive Patients

Parameters	Normotensive Controls (n=30)	Hypertensive Patients (n=28)
Age, y	48±9	52±10
Male, n (%)	23 (77)	23 (82)
Body mass index, kg/m^2	25.9±3.6	27.5±4.0
Systolic blood pressure, mm Hg	124±8	163±15*
Diastolic blood pressure, mm Hg	76±7	100±9*
Heart rate, bpm	63±10	74±10*
Total cholesterol, mg/dL	209±32	217±30
LDL cholesterol, mg/dL	139±28	142±33
HDL cholesterol, mg/dL	51±13	52±18
Triglycerides, mg/dL	96±48	112±75
Fasting glucose, mg/dL	94±8	99±12
Creatinemia, mg/dL	0.91±0.16	0.92±0.19
Estimated GFR, mL/min	103±23	107±29
Blood viscosity, cP	4.1±0.6	4.1±0.4
Radial artery diameter, mm	2.435±0.352	2.465±0.447
Radial artery blood flow, mL/min	12.2±5.2	13.9±5.6
Radial artery mean wall shear stress, kPa	5.0±2.2	5.7±2.3

Values are mean±SD unless indicated otherwise. Statistical analyses of high-density lipoprotein (HDL) cholesterol and triglycerides were performed on log-transformed values, but the untransformed values are given in the table. LDL indicates low-density lipoprotein; GFR, glomerular filtration rate.

*$P<0.05$ vs normotensive controls.

General Procedure

Measurements were performed in the morning while subjects were in a supine position in a quiet air-conditioned room maintained at a constant temperature (22°C to 24°C). Radial internal diameter, blood flow, and digital arterial pressure were measured continuously on the nondominant arm with a high-precision echo tracking device coupled to a Doppler system (NIUS 02, Asulab) and a finger photoplethysmograph (Finapres System, Ohmeda).[11]

Assessment of Conduit Artery Endothelial Dysfunction in Essential Hypertension Using Postischemic Hyperemia and Hand Skin Heating

Peripheral conduit artery endothelial dysfunction has been demonstrated in essential hypertension with the classic method of postischemic hyperemia[9] but not with the hand skin heating method. Therefore, radial artery endothelial function was assessed in 12 hypertensive patients and 12 control subjects with the use of both methods and by measuring endothelium-independent dilatation.

Postischemic Hyperemia

An arterial occlusion cuff was placed on the wrist, was inflated 50 mm Hg above systolic blood pressure for 10 minutes, and was

deflated to allow postischemic hyperemia with the continuous measurements of all parameters. Postischemic hyperemia was characterized by the peak flow (maximal increase in blood flow) and the duration of the increase in flow (t1/2=time elapsing between peak hyperemia and return to 50% of this peak).

Hand Skin Heating

The hand skin temperature was modified by use of a water-filled thermo-controlled device (Polystat 1, Bioblock Scientific). The hand was introduced in the thermo-controlled tank by use of a thin watertight glove fixed to the device. The device was then filled with water, and the temperature was fixed to 34°C for 20 minutes. Then hand skin heating was performed by gradually increasing the water temperature from 34°C to 37°C, 40°C, and 44°C, with each level of temperature maintained for 7 minutes. Total blood viscosity was measured with a cone-plate viscometer (Ex100 CTB, Brookfield) at a shear rate of 241 s^{-1} at 37°C.[11] From the individual values of radial artery diameter (d), flow (Q), and total blood viscosity (μ), the mean arterial wall shear stress was calculated on the basis of a poiseuillean model {ie, $\tau=[(4\ \mu Q)/(\pi r^3)$, $(r=d/2)]$}, and the diameter–shear stress relationships were constructed in hypertensive patients and control subjects to compare these 2 groups at the same level of stimulus.[11]

Endothelium-Independent Dilatation

Radial artery endothelium-independent dilatation was assessed with the use of 0.3 mg sublingual glyceryl trinitrate.

Evolution of NO, EETs, ET-1, and ROS Pathways During Heating-Induced Flow-Mediated Dilatation in Essential Hypertension

The role of NO, EETs, ET-1, and ROS during heating was assessed in 16 hypertensive patients and 18 control subjects by functional and biological approaches.

Functional Approach

The forearm volume of each volunteer was measured by the water displacement method to adjust the doses of the pharmacological agents to be infused. A 27-gauge needle was inserted under local anesthesia (1% lidocaine) into the brachial artery of the nondominant arm to permit infusion of saline (0.9%) and pharmacological agents at a constant rate (1 mL/min) with the use of dual programmable syringe pumps (Vial Program 2, Becton Dickinson). Oral aspirin (500 mg; BMS Laboratory) was administered to block the production of vasomotor prostanoids.[11] To assess the functional role of the EET pathway in radial artery flow-mediated dilatation, heating was performed during the infusion of saline and the inhibitor of cytochrome P450 epoxygenases fluconazole (0.4–1.6 μmol/min per liter; Pfizer Holding).[11] To assess the role of the NO pathway and whether a contribution of the EET pathway may be unmasked under conditions of reduced NO availability, heating was performed during the infusion of saline, the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) (8–20 μmol/min per liter; Bachem), and fluconazole associated with L-NMMA.[11] Moreover, endothelium-independent dilatation in response to sodium nitroprusside (from 5–20 nmol/min per liter; 3 minutes each dose) was assessed.[11]

Biological Approach

In addition to the functional approach, a 4F catheter was inserted into the distal portion of the antecubital vein of the infused arm, when accessible, allowing blood sampling in the venous return at 34°C and 44°C.[11] Blood sampling was performed in the absence and in the presence of fluconazole for the quantification of EET plasma level by gas chromatography/mass spectrometry with negative-ion chemical ionization,[13] as well as in the absence and in the presence of L-NMMA for the quantification of nitrite plasma level, used as indicator of NO bioavailability, by a triiodide/ozone-based chemiluminescence assay.[14]

Moreover, ET-1 plasma levels were quantified with the use of a human ET-1 EIA kit,[15] and whole blood level of ROS was quantified by electron paramagnetic resonance spectroscopy (Miniscope MS-200, Magnettech).[11] An expanded description of the biological approach is available in the online-only Data Supplement.

Statistical Analysis

Statistics were performed with the SYSTAT package (SYSTAT 8.0; SPSS). Continuous variables are presented as mean±SD in the text and as mean±SEM in the figures, unless indicated differently. Qualitative variables are presented as absolute and relative frequencies.

Normality of data was assessed with the Shapiro-Wilk test, and a logarithmic transformation was used to normalize distributions, when appropriate. Differences between groups were assessed by Fisher exact test for categorical variables and by 2-sample *t* test for continuous variables. Analysis of flow-mediated dilatation induced by postischemic hyperemia was repeated with ANOVA with group as factor and with peak flow and duration of the increase in flow as covariates. Differences between groups for the diameter–shear stress relationship were assessed by ANOVA with the use of a generalized linear model with group, shear stress, and subject as factors. Differences between the effects of the inhibitors on the diameter–shear stress relationship were assessed within group by ANOVA with the use of a generalized linear model with inhibitor, shear stress, and subject as factors followed, in case of significance, by a Tukey test for post hoc pairwise comparisons. Differences within and between groups for the reduction in the magnitude of flow-mediated dilatation obtained with the inhibitors were assessed by ANOVA with the use of a generalized linear model with group, inhibitor, and subject as factors followed, in case of significance, by a Tukey test for post hoc pairwise comparisons. In addition, the impact of blood pressure levels on the magnitude of flow-mediated dilatation during saline infusion and on the absolute change in flow-mediated dilatation induced by fluconazole and L-NMMA was assessed by comparing these variables in the quartiles of diastolic blood pressure in the whole population with a Kruskal-Wallis test followed, in case of significance, by a Dunn test for post hoc pairwise comparisons. Differences between biological markers levels at 34°C and 44°C were assessed within groups under each inhibitor condition with a paired *t* test. Differences within and between groups for the variations of biological markers levels between 34°C and 44°C were assessed by ANOVA with a generalized linear model with group, inhibitor, and subject as factors followed, in case of significance, by a Tukey test for post hoc pairwise comparisons. Spearman rank correlation coefficient (r_s) was calculated to assess the correlation between diastolic blood pressure obtained the day of inclusion and functional and biological variables. Finally, Pearson correlation analysis was performed to determine the linear relationship between the variations in biological markers levels and the magnitude of flow-mediated dilation during heating. A value of $P\leq 0.05$ was considered statistically significant.

Results

There was no significant difference between hypertensive patients and normotensive controls for demographic and biological characteristics except for higher systolic and diastolic blood pressures and higher heart rate in hypertensive patients compared with normotensive controls (Table). In addition, the basal radial artery diameter, blood flow, and mean wall shear stress were similar in patients and controls.

Assessment of Conduit Artery Endothelial Dysfunction in Essential Hypertension With the Use of Postischemic Hyperemia and Hand Skin Heating

Peak flow (Figure 1A) was not significantly different, but the duration of the increase in flow (Figure 1B) and the magnitude of radial artery flow-mediated dilatation (Figure 1C) obtained with postischemic hyperemia were reduced in hy-

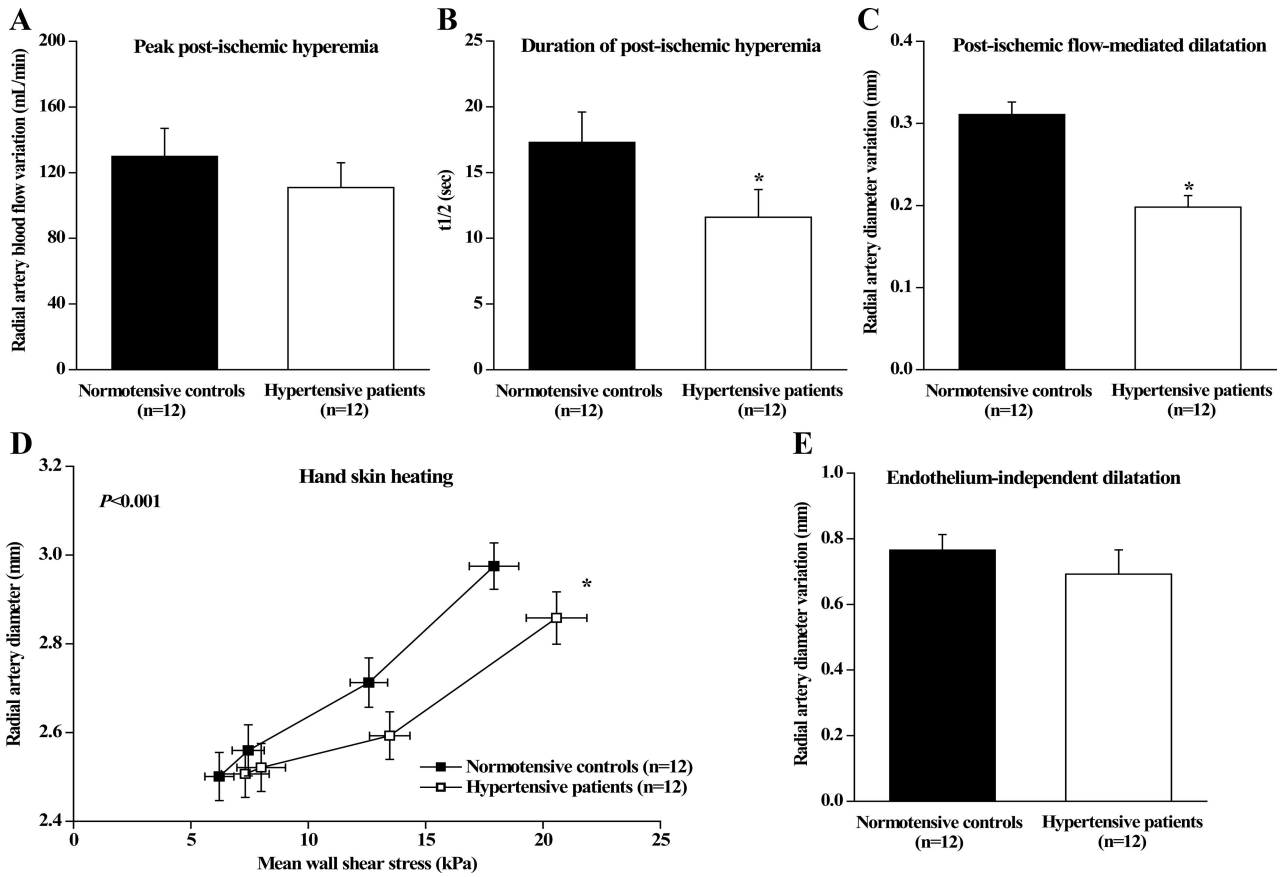

Figure 1. Bar graphs show (**A**) peak radial artery blood flow, (**B**) time to return to 50% from peak flow (t1/2), (**C**) magnitude of radial artery flow-mediated dilatation during postischemic hyperemia, and (**E**) radial artery endothelium-independent dilatation to glyceryl trinitrate in 12 normotensive controls and 12 essential hypertensive patients. **D**, Line graph shows radial artery diameter–mean wall shear stress relationships obtained during hand skin heating in the same subjects of the 2 groups. The altered radial artery flow-mediated dilatation in response to postischemic hyperemia in the presence of reduced hyperemia (ie, t1/2 of flow) in hypertensive patients compared with controls is confirmed with consideration of the flow stimulus (ie, wall shear stress) by the downward shift of the diameter–shear stress relationship. $P<0.001$, shear stress effect; *$P<0.05$ vs normotensive controls.

pertensive patients (n=12) compared with normotensive controls (n=12). The difference between groups in the magnitude of flow-mediated dilatation remained significant after adjustment for peak flow and duration of the increase in flow ($P<0.001$).

There was a downward shift of the radial artery diameter–mean wall shear stress relationship obtained during hand skin heating in hypertensive patients (n=12) compared with normotensive controls (n=12; Figure 1D). Thus, the magnitude of radial artery flow-mediated dilatation (from 34° to 44°C) was reduced in patients (0.373±0.132 mm) compared with controls (0.484±0.104 mm; $P=0.03$). The radial artery endothelium-independent dilatation in response to glyceryl trinitrate was not significantly different between groups (Figure 1E).

Evolution of NO and EET Pathways During Heating-Induced Flow-Mediated Dilatation in Essential Hypertension

Functional Approach

Compared with saline, fluconazole induced a downward shift of the radial artery diameter–mean wall shear stress relationship in normotensive controls (n=14) but not in hypertensive patients (n=11; Figure 2A). Thus, the magnitude of radial artery flow-mediated dilatation (from 34° to 44°C) was reduced by fluconazole in controls but not in patients (Figure 2B).

Compared with saline, L-NMMA and, to a larger extent, L-NMMA+fluconazole induced a downward shift of the diameter–shear stress relationship in normotensive controls (n=14; Figure 3A). Thus, the magnitude of radial artery flow-mediated dilatation was reduced by L-NMMA and, to a larger extent, by L-NMMA+fluconazole in controls (Figure 3B). In contrast, L-NMMA and L-NMMA+fluconazole induced a similar downward shift of the diameter–shear stress relationship in hypertensive patients (n=13). Thus, the magnitude of radial artery flow-mediated dilatation was reduced similarly by L-NMMA and L-NMMA+fluconazole in patients. Moreover, the decrease in flow-mediated dilatation induced by L-NMMA was lesser in patients than in controls.

In the entire population, there was a significant decrease in the magnitude of flow-mediated dilatation under saline infusion as well as in the absolute change in flow-mediated dilatation induced by fluconazole and L-NMMA with the increase in diastolic blood pressure (Figure 4A through 4C).

The radial artery endothelium-independent dilatation in response to sodium nitroprusside was not significantly differ-

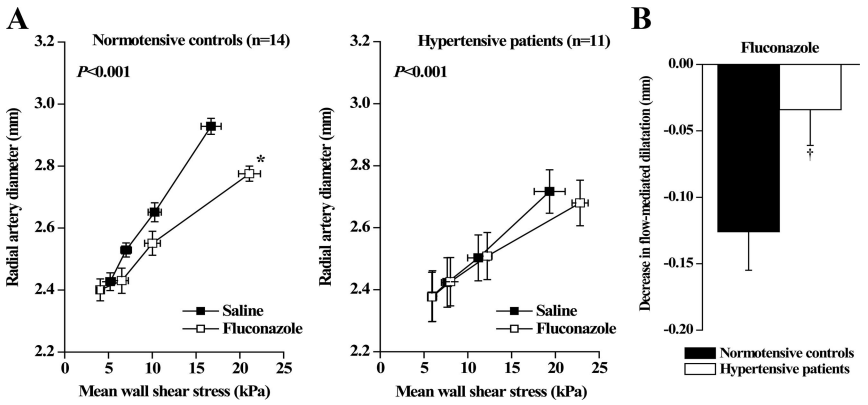

Figure 2. A, Line graphs show radial artery diameter–mean wall shear stress relationships obtained during hand skin heating in the presence of saline and fluconazole in normotensive controls and essential hypertensive patients. **B**, Bar graph represents the reduction obtained with fluconazole in the magnitude of radial artery flow-mediated dilatation compared with saline in the same subjects of the 2 groups. Fluconazole induced a significant downward shift of the diameter–shear stress relationship in normotensive controls but not in hypertensive patients. Thus, fluconazole reduced the magnitude of radial artery flow-mediated dilatation to a larger extent in normotensive controls than in hypertensive patients (**B**). $P<0.001$, shear stress effect; *$P<0.001$ vs saline; †$P<0.05$ vs normotensive controls.

ent in hypertensive patients and normotensive controls (Figure I in the online-only Data Supplement).

Biological Approach

The basal EET plasma level was not significantly different in normotensive controls (12.8±4.5 ng/mL; n=9) and in hypertensive patients (13.9±4.0 ng/mL; n=9; $P=0.27$). However, hand skin heating induced an increase in local plasma EET level only in controls (Figure 5A). This increase was reduced but not abolished by fluconazole. In the entire population, the variation in plasma EET level during heating was inversely correlated with diastolic blood pressure ($r_s=-0.61$, $P=0.009$).

The basal nitrite plasma level was lower in hypertensive patients (140±25 nmol/L; n=7) than in normotensive controls (176±30 nmol/L; n=7; $P=0.049$). Heating induced an increase in local plasma nitrite level in both groups, but this increase was lesser in hypertensive patients (Figure 5B). The increase in nitrite was abolished in both groups by L-NMMA. In the entire population, the variation in plasma nitrite level during heating was inversely correlated with diastolic blood pressure ($r_s=-0.71$, $P=0.013$).

The basal ET-1 plasma level was not significantly different under our experimental conditions in normotensive controls (2.5±0.8 pmol/L; n=9) and in hypertensive patients (3.2±1.0 pmol/L; n=9; $P=0.13$), but hand skin heating induced a decrease in local plasma ET-1 level in controls but not in patients (Figure 6A). In the entire population, there was a significant decrease in the variation of plasma ET-1 during heating with the increase in diastolic blood pressure ($r_s=0.65$, $P=0.009$; Figure 6B). Moreover, the decrease in ET-1 during heating in normotensive controls was not significantly affected by fluconazole (n=5) or by L-NMMA (n=5; Figure IIA and IIB in the online-only Data Supplement).

The basal ROS blood level was not significantly different between normotensive controls (34.6±6.9 μmol/L; n=7) and

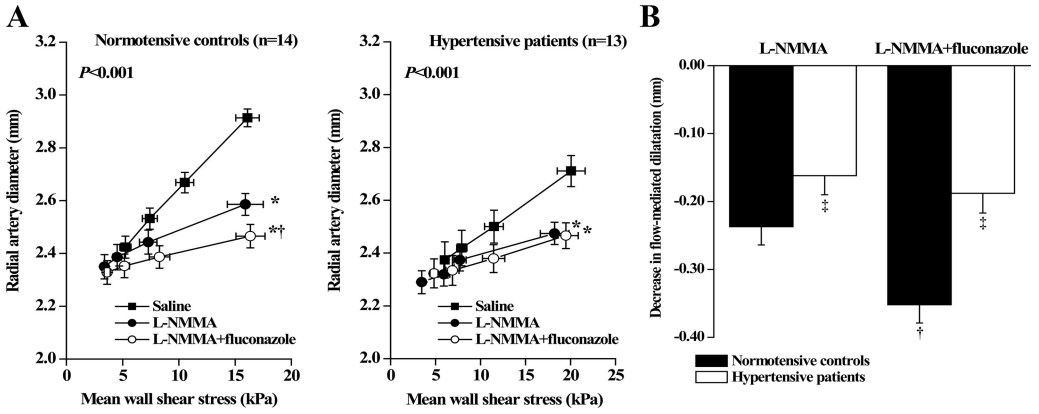

Figure 3. A, Line graphs show radial artery diameter–mean wall shear stress relationships obtained during hand skin heating in the presence of saline, N^G-monomethyl-L-arginine (L-NMMA), and L-NMMA+fluconazole in normotensive controls and essential hypertensive patients. **B**, Bar graph represents the reduction obtained with L-NMMA and L-NMMA+fluconazole in the magnitude of radial artery flow-mediated dilatation in the same subjects of the 2 groups. L-NMMA induced a downward shift of the diameter–shear stress relationship in normotensive controls and in hypertensive patients, but L-NMMA+fluconazole induced a more pronounced downward shift than L-NMMA alone only in controls. Thus, L-NMMA+fluconazole reduced the magnitude of radial artery flow-mediated dilatation to a larger extent than L-NMMA alone in controls but not in hypertensive patients. Moreover, the reduction in the magnitude of radial artery flow-mediated dilatation obtained with L-NMMA was more marked in controls than in patients. $P<0.001$, shear stress effect; *$P<0.001$ vs saline; †$P<0.01$ vs L-NMMA; ‡$P<0.05$ vs normotensive controls.

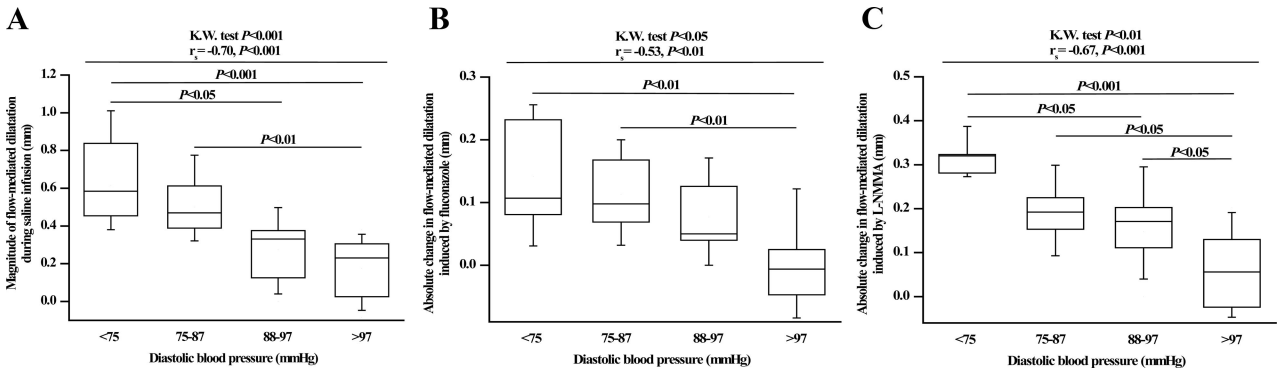

Figure 4. Box plots of (**A**) the magnitude of flow-mediated dilatation induced by hand skin heating during saline infusion and of the reduction in the magnitude of flow-mediated dilatation obtained with (**B**) fluconazole and with (**C**) N^G-monomethyl-L-arginine (L-NMMA) as a function of the quartiles of diastolic blood pressure in the population of patients with essential hypertension and normotensive controls. The magnitude of flow-mediated dilatation during saline infusion as well as the effects of the 2 inhibitors on this parameter decreased with the increase in diastolic blood pressure. Box plots with medians, first and third quartiles, and fifth and 95th percentiles are shown. K.W. indicates Kruskal-Wallis test; r_s, Spearman rank correlation coefficient.

hypertensive patients (34.8±13.6 μmol/L; n=7; P=0.98). Heating induced an increase in blood ROS level in both groups, but this increase was higher in hypertensive patients (Figure 7). The increase in ROS was reduced by L-NMMA in patients but not in controls and was abolished in both groups by L-NMMA+fluconazole. Moreover, the increase in ROS during heating was similarly reduced by fluconazole alone in normotensive controls and in hypertensive patients (Figure III in the online-only Data Supplement).

Finally, the magnitude of radial artery flow-mediated dilatation induced by heating during saline infusion in the population of hypertensive patients and normotensive controls was positively correlated with nitrite variation (n=14; r=0.65, P=0.008) and negatively correlated with the variations of ET-1 (n=18; r=−0.47, P=0.046) and ROS but did not reach statistical significance (n=14; r=−0.45, P=0.08; Figure IVA through IVC in the online-only Data Supplement). In addition, the magnitude of flow-mediated dilatation was negatively correlated with EET variation during heating but only in normotensive controls (n=9; r=−0.63, P=0.055; Figure IVD in the online-only Data Supplement).

Discussion

The major finding of this study is that the decrease in flow-mediated dilatation of peripheral conduit arteries in patients with essential hypertension is due to an imbalance between endothelium-derived relaxing and constricting factors, characterized by the abrogation of the EET pathway, an altered NO/ROS balance, and an associated absence of adaptation of the ET-1 pathway to flow increase.

First, we validated hand skin heating as a useful tool to detect conduit artery endothelial dysfunction in patients with essential hypertension. Indeed, we observed that the radial artery flow-mediated dilatation was decreased in patients in response to heating as well as in response to the more commonly used method of postischemic hyperemia, without modification in the smooth muscle cell reactivity. Interestingly, although peak flow was preserved, we noticed that there was a decrease in the duration of the increase in flow during postischemic hyperemia in our patients.[16] As stressed previously, this may be due to an imbalance between vasoconstrictor and vasodilator influences and thus may reflect the presence of an arteriolar endothelial dysfunction shortening the time to return to baseline tone and flow.[17] Although the difference between groups for flow-mediated dilatation persists after statistical adjustment, this reduced hyperemia probably contributed to the decrease in the endothelial response.[18] In this context, the downward shift of the diameter–shear stress relationship obtained in hypertensive patients

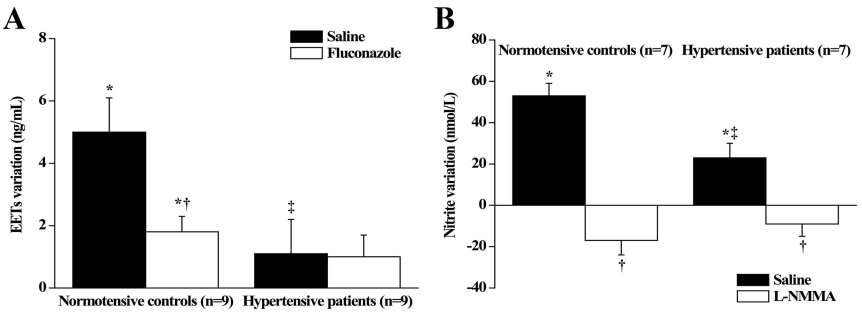

Figure 5. Bar graphs show variations in the plasma levels (**A**) of epoxyeicosatrienoic acids (EETs) during hand skin heating in the presence of saline and fluconazole and (**B**) of nitrite in the presence of saline and N^G-monomethyl-L-arginine (L-NMMA) in normotensive controls and patients with essential hypertension. EETs increased during heating in controls but not in hypertensive patients, and this increase was reduced by fluconazole. In addition, nitrite increased during heating in both groups but to a lesser extent in patients, and these increases were abolished by L-NMMA. *P<0.05 vs baseline; †P<0.05 vs saline; ‡P<0.05 vs normotensive controls.

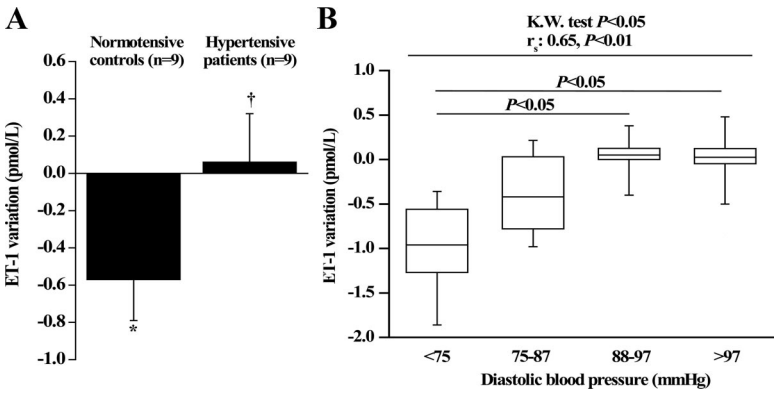

Figure 6. A, Bar graph shows variations in the plasma level of endothelin-1 (ET-1) during hand skin heating in the presence of saline in normotensive controls and patients with essential hypertension. During heating, plasma ET-1 decreased in controls but not in hypertensive patients. *$P<0.05$ vs baseline, †$P<0.05$ vs normotensive controls. **B**, Box plot of variations in ET-1 level during hand skin heating in the presence of saline as a function of the quartiles of diastolic blood pressure in the population of patients with essential hypertension and normotensive controls. The magnitude of ET-1 reduction during hand skin heating decreased with the increase in diastolic blood pressure. Box plot with medians, first and third quartiles, and fifth and 95th percentiles is shown. K.W. indicates Kruskal-Wallis test; r_s, Spearman rank correlation coefficient.

during heating compared with controls confirms, by directly taking into account the flow stimulus, the presence of this endothelial dysfunction.

In regard to the NO pathway, we confirmed in our normotensive controls the physiological role of NO in the regulation of conduit artery flow-mediated dilatation.[11,17] Indeed, the pharmacological inhibition of NO synthase with L-NMMA was associated with a downward shift of the diameter–shear stress relationship during heating. Accordingly, the local plasma nitrite level increased during heating under saline infusion, as shown previously during postischemic hyperemia,[19] but we also demonstrated that these nitrites originated from NO produced by NO synthase because the nitrite level was no longer increased in the presence of L-NMMA. In hypertensive patients, there was a lesser basal nitrite plasma concentration compared with control subjects, confirming biologically the decrease in basal NO availability during this disease.[4,5,9] In this respect, previous data have demonstrated an inverse correlation between nitrite level and the number of cardiovascular risk factors, including hypertension.[20] Moreover, L-NMMA reduced flow-mediated dilatation and local plasma nitrite level also increased during heating in patients, but these effects were significantly less marked than in control subjects, showing that an alteration in the NO pathway contributes to the endothelial dysfunction of conduit arteries during essential hypertension. These results are in agreement with a previous functional study demonstrating the absence of an inhibitory effect of L-NMMA on flow-mediated dilatation in patients with essential hypertension but in response to less sustained stimulation induced by postischemic hyperemia.[9]

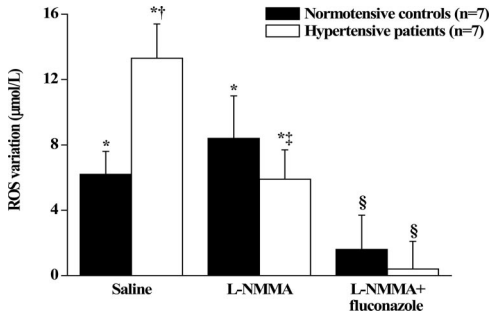

Figure 7. Bar graph shows variations in the whole blood level of reactive oxygen species (ROS) during hand skin heating in the presence of saline, N^G-monomethyl-L-arginine (L-NMMA), and L-NMMA+fluconazole in normotensive controls and patients with essential hypertension. ROS increased during heating in controls and, to a larger extent, in hypertensive patients. This increase was reduced by L-NMMA in patients but not in controls, whereas L-NMMA+fluconazole similarly reduced ROS in both groups. *$P<0.05$ vs baseline; †$P<0.05$ vs normotensive controls; ‡$P<0.05$ vs saline; §$P<0.05$ vs L-NMMA.

Furthermore, using the biological approach, we observed, in accordance with our previous results obtained in healthy subjects, that ROS increased during heating in both studied groups.[11] However, this increase was higher in patients than in controls, although basal levels were similar, and this between-group difference was abolished by L-NMMA. These results strongly support the hypothesis that increased ROS production originating from uncoupled NO synthase contributed to the endothelial dysfunction of conduit arteries in our patients, in accordance with several animal observations.[21] Conversely, a role for cytochrome P450 epoxygenases in this increased ROS production, a mechanism contributing to the endothelial dysfunction of resistance arteries in patients with coronary artery disease,[22] appears not to be involved. In fact, fluconazole either alone or associated with L-NMMA similarly reduced ROS production in both groups. Moreover, this last result is in accordance with our previously reported data suggesting that a physiological production of ROS by a cytochrome P450–dependent mechanism occurs in cutaneous resistance arteries to limit the skin arteriolar dilatation during heating.[11]

In regard to the EET pathway, we confirmed in controls the physiological role of cytochrome P450 epoxygenases in the regulation of conduit artery flow-mediated dilatation,[11,23] as illustrated by the downward shift of the diameter–shear stress relationship induced by fluconazole during heating, infused either alone or in the presence of L-NMMA. Moreover, we provided for the first time in humans in vivo strong evidence

that EETs are the factors produced by cytochrome P450 epoxygenases mediating this response. Indeed, we observed that the local EET plasma concentration increased during the flow stimulation and that this increase was impaired by fluconazole. Interestingly, the increase in EET level was reduced but not abolished by fluconazole, suggesting, as shown in animals, the presence of an intracellular storage form of EETs into phospholipids, which can be liberated on cell activation independently of cytochrome P450 epoxygenase activity.[24] In hypertensive patients, the basal EET plasma level was similar to that of control subjects according to 1 reported study, which demonstrated with the use of biological quantification that the alteration in basal EET availability can only be significantly detected in renovascular hypertensive patients.[25] However, we no longer observed in patients a reduction in flow-mediated dilatation with fluconazole alone or even associated with L-NMMA or an increase in the local EET plasma level during heating, demonstrating for the first time that, in addition to NO, an impairment in EET pathway contributes to the conduit artery endothelial dysfunction during essential hypertension. This impairment in EET pathway during arterial hypertension has been suggested previously from animal experiments and notably may be due to an increased degradation of EETs by soluble epoxide hydrolase.[2,10,26] Conversely, an increased role of cytochrome P450 epoxygenases in endothelium-dependent dilatation was reported at the level of forearm resistance arteries in patients with essential hypertension compared with normotensive controls, which compensates for the decrease in NO availability.[8] Whether these discrepancies are related to differences in the vascular beds studied or to the grade and/or duration of hypertension of patients remains to be investigated.

Furthermore, in accordance with most of the previous studies,[27,28] we observed a slightly higher basal ET-1 plasma level in our hypertensive patients compared with controls, although this was not significant under our experimental conditions. However, as stressed previously,[28] because endothelium-derived ET-1 has a predominantly paracrine action, its basal plasma level does not necessarily represent the degree of activation of the endothelin system. Most interesting, we observed that plasma ET-1 availability decreases during flow stimulation in controls but not in patients. It must be stressed that the studies evaluating the role of shear stress in the modulation of ET-1 release by the endothelium are controversial and are probably influenced by experimental conditions.[29] In fact, the level, duration, and steady or pulsatile application of shear stress as well as the cell type and species studied may influence the results.[29] Nonetheless, 1 study has demonstrated in vivo in healthy volunteers, with the use of local infusion of a blocker of the ET-1 type A receptor, that ET-1 acutely mediates the radial artery constriction associated with conditions of reduced flow induced by distal ischemia.[30] This result, suggesting an increased availability of ET-1 during conditions of reduced shear stress, supports our hypothesis (ie, a decreased availability of ET-1 with increased shear stress). Importantly, this mechanism appears to be lost in hypertensive patients and thus could contribute to aggravate the conduit artery endothelial dysfunction by exacerbating the vasoconstrictor influence of ET-1. Thus, the administration of newly developed ET-1 receptor antagonists, which have recently been shown to improve blood pressure control in resistant hypertension,[31] may also be useful to prevent residual conduit artery endothelial dysfunction during essential hypertension, as shown in forearm arterioles.[7] Furthermore, this absence of decrease in ET-1 during flow stimulation in patients may be related to the decrease in the availability of NO, which can acutely impair ET-1 release.[32] However, NO-independent mechanisms directly mediated by shear stress are probably involved, as observed previously in primary culture of human umbilical veins,[33] because the inhibition of NO synthase in our healthy controls did not abolish the decrease in ET-1 variation during heating. In the same way, the decreased availability of EETs is probably not involved because the inhibition of cytochrome P450 epoxygenases by fluconazole did not affect ET-1 variation in controls. Additional experiments are necessary to explore the interplay between EETs and ET-1 pathways, as stressed previously with the use of ET-1 receptor antagonists and/or infusion of synthetic EET analogues, which are currently under development but not available for human use.

Finally, in addition to demonstrating the presence of these alterations in EETs, NO, and ET-1 pathways in patients with essential hypertension at the level of the conduit arteries, our results show, in the entire population studied, a progressive impairment of these pathways, with the increase in blood pressure levels thus contributing to reduce endothelium-dependent flow-mediated dilatation.

In conclusion, our results demonstrate that an impaired role of EETs contributes, with an alteration in NO and ET-1 pathways, to conduit artery endothelial dysfunction in essential hypertension. In this context, restoring the EET pathway may be useful to prevent conduit artery endothelial dysfunction during essential hypertension; this thus represents a new therapeutic target that may be useful to improve the cardiovascular prognosis in patients.

Study Limitations

Average levels of total and low-density lipoprotein cholesterol were borderline high according to American Heart Association guidelines in our study participants. However, these levels were similar between groups and were associated with normal values of high-density lipoprotein cholesterol, thus probably not affecting sustained conduit artery flow-mediated dilatation.[34] Furthermore, blood sampling was performed in the venous return, and thus measured factors may originate from distal vascular compartments. However, the magnitude of flow-mediated dilatation was positively correlated with nitrite variation and negatively correlated with ET-1 and ROS variations, showing that a significant part of these factors originates from the studied conduit artery.[19] Finally, in an unexpected way, there was a negative correlation between the radial artery dilatation and EET variation in healthy subjects. Although dedicated experiments are warranted, this result may reflect a compensatory increased release of EETs, which may help to maintain the vasodilator function in subjects with incipient alteration in endothelial function, in particular during aging, whereas this mechanism is lost in hypertensive patients. Accordingly, we noticed that

the decrease in flow-mediated dilatation obtained with fluconazole was 25% higher in the subjects explored in the present study (≈125 μm) than in the younger healthy subjects (mean age of 25 years) previously explored (≈100 μm), whereas, at the same time, the magnitude of the dilatory response decreased by 20% in older subjects (620 versus 500 μm).[11] Furthermore, we cannot exclude that fluconazole affected pathways other than EETs. However, this appears unlikely under our experimental conditions because fluconazole reduced flow-mediated dilatation even when infused with L-NMMA and did not affect the decrease in plasma ET-1 during heating in control subjects, suggesting the absence of effects on NO and ET-1 pathways.

Acknowledgments

The authors thank the general practitioners of the research network of the General Medicine Department of Rouen University Hospital for recruitment of the patients with essential hypertension. The authors also thank Dr Jean-François Ménard (Department of Biometry, Rouen University Hospital) for his help regarding the statistical analysis and Richard Medeiros (Rouen University Hospital Medical Editor) for editing the final manuscript.

Sources of Funding

This study was supported by grants from the Institut National de la Santé et de la Recherche Médicale–Direction de l'Hospitalisation et de l'Organisation des Soins for translational research and from the Société Française d'Hypertension Artérielle.

Disclosures

None.

References

1. Vanhoutte PM, Shimokawa H, Tang EH, Feletou M. Endothelial dysfunction and vascular disease. *Acta Physiol (Oxf)*. 2009;196:193–222.
2. Bellien J, Joannides R, Richard V, Thuillez C. Modulation of cytochrome-derived epoxyeicosatrienoic acids pathway: a promising pharmacological approach to prevent endothelial dysfunction in cardiovascular diseases? *Pharmacol Ther*. 2011;131:1–17.
3. Modena MG, Bonetti L, Coppi F, Bursi F, Rossi R. Prognostic role of reversible endothelial dysfunction in hypertensive postmenopausal women. *J Am Coll Cardiol*. 2002;40:505–510.
4. Calver A, Collier J, Moncada S, Vallance P. Effect of local intra-arterial N^G-monomethyl-L-arginine in patients with hypertension: the nitric oxide dilator mechanism appears abnormal. *J Hypertens*. 1992;10:1025–1031.
5. Panza JA, Casino PR, Kilcoyne CM, Quyyumi AA. Role of endothelium-derived nitric oxide in the abnormal endothelium-dependent vascular relaxation of patients with essential hypertension. *Circulation*. 1993;87:1468–1474.
6. Taddei S, Virdis A, Ghiadoni L, Magagna A, Salvetti A. Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. *Circulation*. 1998;97:2222–2229.
7. Cardillo C, Campia U, Kilcoyne CM, Bryant MB, Panza JA. Improved endothelium-dependent vasodilation after blockade of endothelin receptors in patients with essential hypertension. *Circulation*. 2002;105:452–456.
8. Taddei S, Versari D, Cipriano A, Ghiadoni L, Galetta F, Franzoni F, Magagna A, Virdis A, Salvetti A. Identification of a cytochrome P450 2C9-derived endothelium-derived hyperpolarizing factor in essential hypertensive patients. *J Am Coll Cardiol*. 2006;48:508–515.
9. Ghiadoni L, Versari D, Magagna A, Kardasz I, Plantinga Y, Giannarelli C, Taddei S, Salvetti A. Ramipril dose-dependently increases nitric oxide availability in the radial artery of essential hypertension patients. *J Hypertens*. 2007;25:361–366.
10. Gao J, Bellien J, Gomez E, Henry JP, Dautreaux B, Bounoure F, Skiba M, Thuillez C, Richard V. Soluble epoxide hydrolase inhibition prevents coronary endothelial dysfunction in renovascular hypertension. *J Hypertens*. 2011;29:1128–1135.
11. Bellien J, Iacob M, Gutierrez L, Isabelle M, Lahary A, Thuillez C, Joannides R. Crucial role of NO and endothelium-derived hyperpolarizing factor in human sustained conduit artery flow-mediated dilatation. *Hypertension*. 2006;48:1088–1094.
12. Bertrand-Thiebault C, Masson C, Siest G, Batt AM, Visvikis-Siest S. Effect of HMGCoA reductase inhibitors on cytochrome P450 expression in endothelial cell line. *J Cardiovasc Pharmacol*. 2007;49:306–315.
13. Goulitquer S, Dréano Y, Berthou F, Corcos L, Lucas D. Determination of epoxyeicosatrienoic acids in human red blood cells and plasma by GC/MS in the NICI mode. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2008;876:83–88.
14. MacArthur PH, Shiva S, Gladwin MT. Measurement of circulating nitrite and S-nitrosothiols by reductive chemiluminescence. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2007;851:93–105.
15. Bruno CM, Neri S, Sciacca C, Caruso L. Plasma endothelin-1 levels in liver cirrhosis. *Int J Clin Lab Res*. 2000;30:169–172.
16. Lauer T, Heiss C, Preik M, Balzer J, Hafner D, Strauer BE, Kelm M. Reduction of peripheral flow reserve impairs endothelial function in conduit arteries of patients with essential hypertension. *J Hypertens*. 2005;23:563–569.
17. Joannides R, Haefeli W, Linder L, Richard V, Bakkali ElH, Thuillez C, Lüscher T. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. *Circulation*. 1995;91:1314–1319.
18. Joannidès R, Bakkali ElH, Richard V, Moore N, Thuillez C. Evaluation of the determinants of flow-mediated radial artery vasodilatation in humans. *Clin Exper Hypertens*. 1997;19:813–826.
19. Rassaf T, Heiss C, Hendgen-Cotta U, Balzer J, Matern S, Kleinbongard P, Lee A, Lauer T, Kelm M. Plasma nitrite reserve and endothelial function in the human forearm circulation. *Free Radic Biol Med*. 2006;41:295–301.
20. Kleinbongard P, Dejam A, Lauer T, Jax T, Kerber S, Gharini P, Balzer J, Zotz RB, Scharf RE, Willers R, Schechter AN, Feelisch M, Kelm M. Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans. *Free Radic Biol Med*. 2006;40:295–302.
21. Förstermann U, Li H. Therapeutic effect of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling. *Br J Pharmacol*. 2011;164:213–223.
22. Fichtlscherer S, Dimmeler S, Breuer S, Busse R, Zeiher AM, Fleming I. Inhibition of cytochrome P450 2C9 improves endothelium-dependent, nitric oxide-mediated vasodilatation in patients with coronary artery disease. *Circulation*. 2004;109:178–183.
23. Fischer D, Landmesser U, Spiekermann S, Hilfiker-Kleiner D, Hospely M, Müller M, Busse R, Fleming I, Drexler H. Cytochrome P450 2C9 is involved in flow-dependent vasodilation of peripheral conduit arteries in healthy subjects and in patients with chronic heart failure. *Eur J Heart Fail*. 2007;9:770–775.
24. Weintraub NL, Fang X, Kaduce TL, VanRollins M, Chatterjee P, Spector AA. Potentiation of endothelium-dependent relaxation by epoxyeicosatrienoic acids. *Circ Res*. 1997;81:258–267.
25. Minuz P, Jiang H, Fava C, Turolo L, Tacconelli S, Ricci M, Patrignani P, Morganti A, Lechi A, McGiff JC. Altered release of cytochrome p450 metabolites of arachidonic acid in renovascular disease. *Hypertension*. 2008;51:1379–1385.
26. Loch D, Hoey A, Morisseau C, Hammock BO, Brown L. Prevention of hypertension in DOCA-salt rats by an inhibitor of soluble epoxide hydrolase. *Cell Biochem Biophys*. 2007;47:87–98.
27. Saito Y, Nakao K, Mukoyama M, Imura H. Increased plasma endothelin level in patients with essential hypertension. *N Engl J Med*. 1990;322:205.
28. Elijovich F, Laffer CL, Amador E, Gavras H, Bresnahan MR, Schiffrin EL. Regulation of plasma endothelin by salt in salt-sensitive hypertension. *Circulation*. 2001;103:263–268.
29. Wang GX, Cai SX, Wang PQ, Ouyang KQ, Wang YL, Xu SR. Shear-induced changes in endothelin-1 secretion of microvascular endothelial cells. *Microvasc Res*. 2002;63:209–217.
30. Spieker LE, Lüscher TF, Noll G. ETA receptors mediate vasoconstriction of large conduit arteries during reduced flow in humans. *J Cardiovasc Pharmacol*. 2003;42:315–318.
31. Weber MA, Black H, Bakris G, Krum H, Linas S, Weiss R, Linseman JV, Wiens BL, Warren MS, Lindholm LH. A selective endothelin-receptor antagonist to reduce blood pressure in patients with treatment-resistant hypertension: a randomised, double-blind, placebo-controlled trial. *Lancet*. 2009;374:1423–1431.
32. Richard V, Hogie M, Clozel M, Löffler BM, Thuillez C. In vivo evidence of an endothelin-induced vasopressor tone after inhibition of nitric oxide synthesis in rats. *Circulation*. 1995;91:771–775.

33. Morawietz H, Talanow R, Szibor M, Rueckschloss U, Schubert A, Bartling B, Darmer D, Holtz J. Regulation of the endothelin system by shear stress in human endothelial cells. *J Physiol*. 2000;525:761–770.

34. Mullen MJ, Kharbanda RK, Cross J, Donald AE, Taylor M, Vallance P, Deanfield JE, MacAllister RJ. Heterogenous nature of flow-mediated dilatation in human conduit arteries in vivo: relevance to endothelial dysfunction in hypercholesterolemia. *Circ Res*. 2001;88:145–151.

CLINICAL PERSPECTIVE

Patients with essential hypertension are at increased cardiovascular risk even when their blood pressure levels are well controlled, and restoration of the endothelial function of conduit arteries has emerged as a therapeutic target that may help to prevent the development of cardiovascular complications. In the present study, we demonstrated that several mechanisms contribute to conduit artery endothelial dysfunction in hypertensive patients. In addition to nitric oxide/reactive oxygen species imbalance and alteration in the endothelin-1 pathway, a decrease in epoxyeicosatrienoic acid availability is involved. This is particularly important because epoxyeicosatrienoic acids are not only endothelium-derived relaxing factors, but they also play a major role in maintaining cardiovascular homeostasis by contributing to the regulation of vascular inflammation, cell proliferation, angiogenesis, and hemostasis. Consequently, a new class of pharmacological agents referred to as soluble epoxide hydrolase inhibitors is under development. Therefore, to increase epoxyeicosatrienoic acid availability by reducing their degradation may be particularly useful during essential hypertension. Thus, in addition to decreasing blood pressure, epoxyeicosatrienoic acids could restore the protective action of the endothelium in conduit arteries and prevent the development of atherosclerosis and cardiovascular complications.