

## ORIGINAL ARTICLE

# Role of the nitric oxide metabolic pathway and prostanoids in the pathogenesis of endothelial dysfunction and essential hypertension in young men

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The aim of this study was to explore the role of selected prostanoids and the nitric oxide (NO) metabolic pathway in the pathogenesis of endothelial dysfunction (ED) in young men with and without essential hypertension (HTN). A total of 70 men aged 18–40 years old (23 hypertensive and 47 normotensive) were investigated. Initial metabolite concentrations of the NO pathway (asymmetric dimethylarginine, L-arginine and symmetric dimethylarginine), selected cardiovascular risk markers (serum lipids, creatinine, glucose and high-sensitivity C-reactive protein), oxidative stress markers (malonyldialdehyde, thiol index and nitrotyrosine) and prostanoids (thromboxane B<sub>2</sub> (TxB<sub>2</sub>) and 6-keto-prostaglandin F (PGF)-1- $\alpha$ ) were measured. Ultrasound assessment of endothelial function (flow-mediated vasodilation (FMD)) of the brachial artery was studied before and after intravenous infusion of L-arginine (16.0 g). All measurements were repeated after oral administration of indomethacin (75 mg per day) for 2 days. The prevalence of ED was similar in both hypertensive and normotensive groups. A lower baseline plasma level of 6-keto-PGF-1- $\alpha$  and a higher baseline of TxB<sub>2</sub> were observed in the hypertensive group. A different response to indomethacin assessed by prostanoid levels was observed and was dependent on the presence of HTN. No significant differences in metabolites of the NO pathway were observed at either baseline or following indomethacin treatment. In hypertensive patients, L-arginine and indomethacin had a synergistic positive effect on FMD. ED in young normotensive men primarily depends on NO deficiency. In young hypertensive men, disorders in prostanoid metabolism have important roles in decreasing NO bioavailability. The high prevalence of ED in potentially healthy subjects suggests that the ultrasound FMD measurement is an important tool in the stratification of cardiovascular risk. *Hypertension Research* (2011) 34, 79–86; doi:10.1038/hr.2010.169; published online 7 October 2010

**Keywords:** endothelial dysfunction; nitric oxide; oxidative stress; prostanoids

## INTRODUCTION

The endothelium has a pivotal role in the regulation of vascular tone. Endothelial dysfunction (ED) resulting from decreased activity of nitric oxide (NO)-dependent metabolic pathways, which may be a crucial pathophysiological link that leads to the development of cardiovascular complications of arterial hypertension (HTN).<sup>1</sup> Studies in hypertensive subjects have shown that ED exists in resistance vessels and is manifested by a decreased response to vasodilatory agents.<sup>2–4</sup> ED may be detected in hypertensive subjects, as well as in healthy individuals with cardiovascular risk factors or in those genetically predisposed to developing arterial HTN. Furthermore, it has been shown that ED is an independent risk factor for future cardiovascular events.<sup>5–9</sup>

A deficiency of NO has complex etiology; one of the most important causes is enhanced NO degradation by reactive oxygen species (ROS). Impaired activity of NO-dependent metabolic pathways in the endothelium may constitute an important pathophysiological step in the development of cardiovascular disease and is considered to have a causative role in the impairment of flow-mediated vasodilation (FMD).<sup>10,11</sup>

Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of NO synthase (NOS) and may thus decrease NO bioavailability. ADMA is an important marker of cardiovascular risk<sup>12,13</sup> and may mediate ED in patients with coronary artery disease, arterial HTN, hyperlipidemia, congestive heart failure,<sup>14</sup> chronic kidney disease<sup>15</sup> and/or cerebral stroke.<sup>16</sup> ADMA and symmetric dimethylarginine (SDMA; without inhibitory action on NOS) are produced from methylated nuclear proteins, especially histones.<sup>13</sup>

Disorders involving the biotransformation of arachidonic acid derivatives (prostanoids) are also postulated to be an important factor in ED pathogenesis.<sup>17</sup> An imbalance between the vasoconstrictive action of some prostanoids (such as thromboxane) and the vasodilatory action of others (for example, prostacyclin) could also have an important role in the pathogenesis of HTN. ED may develop in normotensive subjects and might be eliminated by inhibition of cyclooxygenase (COX), which could be due to the decreased lipid peroxidation, resulting in a reduction in oxidative stress.<sup>18</sup>

Viridis *et al.*<sup>19</sup> analyzed the role of the decreased availability of NO and ROS and activity of each isoform of COX in the development of

ED. In addition, rats exposed to an endotoxin had a significant impairment in endothelial function, which was rescued after treatment with a COX-2 inhibitor. The authors suggested that NO was scavenged by ROS produced via the COX-2 pathway. Regardless of enhanced NOS activity, a decrease of NO bioavailability is explained by the coincident generation of ROS, which transforms NO into peroxynitrites (ONOO<sup>-</sup>). Formation of ONOO<sup>-</sup> *in vivo* has been attributed to the reaction between the free radicals superoxide (O<sub>2</sub><sup>-</sup>) and NO (reviewed in Pacher *et al.*<sup>20</sup>). Nitrotyrosine formation is an ONOO<sup>-</sup>-dependent mechanism, and its level is commonly used as a marker of ONOO<sup>-</sup> production.

HTN might be associated with altered expression in the NO and prostanoids pathways,<sup>21</sup> as early as 30 years of age; however, the role of prostanoids appears to increase with age.<sup>22</sup> Campia *et al.*<sup>23</sup> performed a study in healthy and hypertensive subjects with or without lipid disorders and demonstrated a crucial role of prostanoids in the regulation of vascular tone. Vasoconstrictive prostanoids could also limit endothelium-mediated vasodilatation. The deficiency of NO might be more common in young subjects (under the age of 30 years) and may be reversible by administration of L-arginine<sup>24</sup>; however, in elderly subjects (over 60),<sup>25</sup> disturbances of the prostanoid pathway might be more important.<sup>25</sup> The results of a study performed by Bode-Boger,<sup>26</sup> however, oppose this hypothesis by showing a significant improvement of endothelial function in elderly subjects after administration of L-arginine.

The aim of this study was to assess the role of NO and prostanoids in the pathogenesis of ED in young men with and without HTN. We investigated (1) whether decreased bioavailability of NO in subjects with ED results from its enhanced degradation by ROS produced during lipid peroxidation or by its decreased synthesis due to competitive inhibition of NOS; (2) if administration of L-arginine (a substrate for NOS) increases NO bioavailability (that is, improving FMD) by enhancing its synthesis, which is inhibited by competitive NOS inhibitors; (3) whether administration of indomethacin (a non-selective COX inhibitor) reduces peroxidation of lipids, resulting in an increase of NO bioavailability and (4) if there are any differences between hypertensive and normotensive subjects in the pathogenesis of ED.

## METHODS

All experiments were conducted and approved in accordance with the guidelines of the Bioethics Committee at Wrocław Medical University and adhered to the principles of the Declaration of Helsinki and Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects (revised 13 November 2001, effective 13 December 2001).

## Patients

Male volunteers (mean age, 18–40 years) from the university community in Wrocław, Poland, were recruited to participate in this study from leaflets distributed in the campus area in Wrocław. A total of 23 subjects with HTN and 47 normotensive subjects were included in the study. The recruited subjects were either healthy, normotensive individuals or had a history of untreated essential HTN for at least last 6 months. Exclusion criteria for the study were secondary HTN, coexisting with diabetes mellitus, chronic inflammatory diseases, infections, smoking, malignancies and mental disorders. Subjects with unknown histories of HTN and elevated blood pressure observed at two consecutive screening visits underwent a 24-h ambulatory blood pressure monitoring using a Welch Allyn ABPM 6100S (Welch Allyn Poland & Baltic States, Poznan, Poland). If HTN was confirmed,<sup>27</sup> subjects were given subsequent clinical evaluation to exclude secondary HTN. All blood pressure measurements were taken using a sphygmomanometer (Welch Allyn, DS66 Trigger Aneroid with a set of adult cuffs, size 10–12 cm).

## Study protocol

A scheme of the study protocol is shown in Figure 1a. Each subject was given a medical examination. At 06:30 hours on the day after the medical exam, fasting blood was drawn from the antecubital vein. To avoid platelet activation, blood was drawn without venous stasis. The following parameters were measured:

1. Metabolites of the NO pathway (ADMA, SDMA and L-arginine)
2. Prostanoids levels (thromboxane B<sub>2</sub> (TxB<sub>2</sub>) and 6-keto-PGF-1- $\alpha$ )
3. Markers of oxidative stress (malondialdehyde (MDA), reduced glutathione (GSH), oxidized glutathione (GSSG), thiol index (GSH/GSSG) and nitrotyrosine)
4. Concentrations of total cholesterol, low-density lipoprotein and high-density lipoprotein cholesterol, triglycerides, serum creatinine, plasma glucose, high-sensitivity C-reactive protein, thyroid-stimulating hormone, thyrotropin, K<sup>+</sup> and uric acid.

After the blood was drawn, the vasodilatory reactivity of the brachial artery after reversible ischemia (FMD) was measured to diagnose ED. The cutoff point for ED was established as <10% change in the diameter of the brachial artery in response to reactive hyperemia compared with the baseline value. Subjects were assigned to one of four subgroups according to the presence of HTN and ED (Figure 1b):

- (1) Normal blood pressure and no ED: HTN(-)/ED(-)
- (2) Normal blood pressure but with ED: HTN(-)/ED(+)
- (3) HTN without ED: HTN(+)/ED(-)
- (4) HTN with ED: HTN(+)/ED(+)

Following the baseline FMD testing, the subjects were administered 16.0 g L-arginine (Fresenius SE, Homburg, Germany) intravenously, followed by a second FMD measurement.

After testing, all subjects were given 75 mg of slow-release indomethacin *per os*. (Metindol Retard, Polfa Warszawa, Poland) daily for 2 days, after which all procedures were repeated.

## Procedure

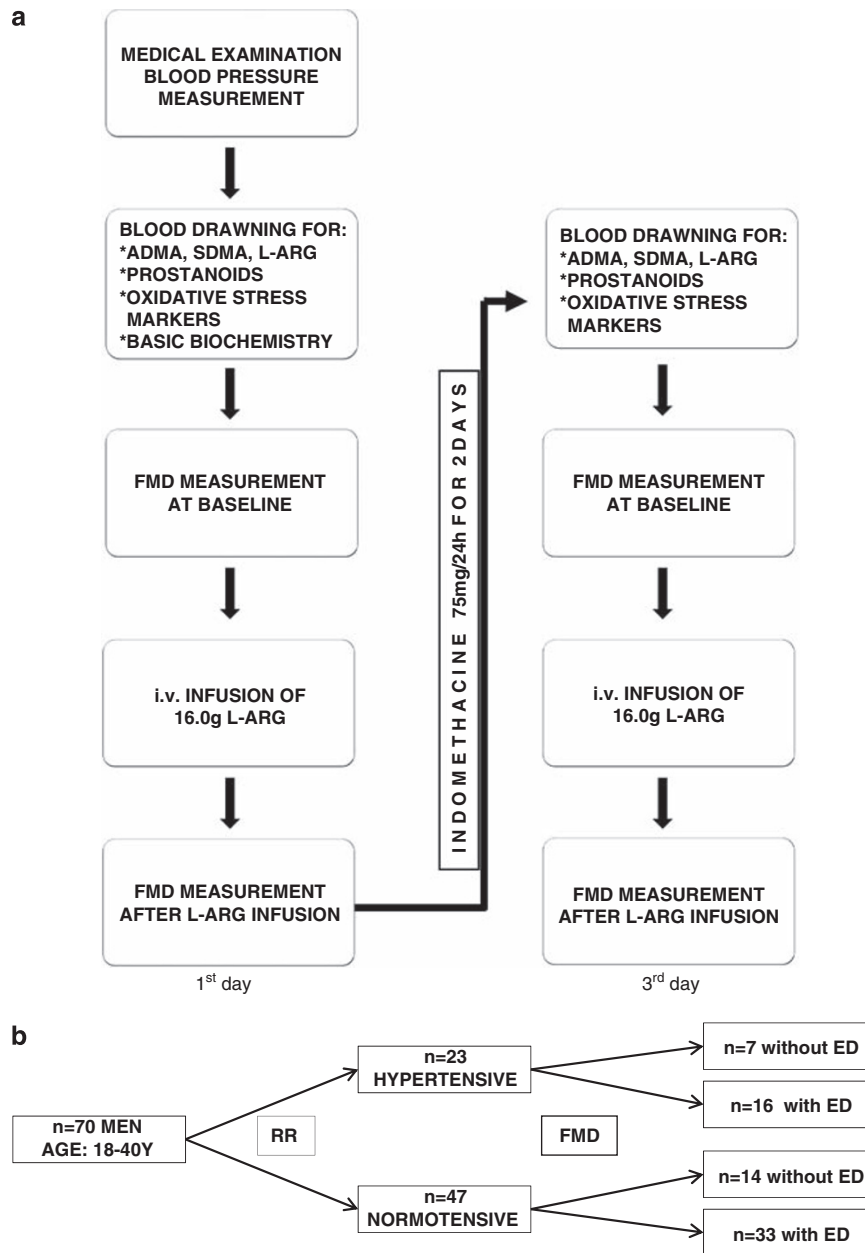
**Measurements of endothelial function.** Ultrasound assessment of FMD in response to reactive hyperemia was performed according to the method described by Celermajer *et al.*<sup>28</sup> using the ALOKA model SSD 5500 duplex ultrasound machine (ALOKA GmbH, Willich, Germany) with a 7–14-MHz linear array transducer and the ALOKA probe holder MP-AH 0001 for immobilization of the arm/forearm. This was done to improve the accuracy of the FMD measurement.

**Biochemical tests.** EDTA plasma (2.7 ml) was separated, immediately centrifuged (6000 r.p.m. for 15 min at 4 °C) and frozen at -80 °C for evaluation of ADMA, SDMA, L-arginine levels and markers of oxidative stress. Whole blood was used only for assessment of the thiol index, as recommended. Blood for assessment of prostanoids levels was drawn (v=2.7 ml) into sodium citrate tubes with 500  $\mu$ l of 5 mmol l<sup>-1</sup> crystalline acetylsalicylic acid (Sigma-Aldrich Sp. z o.o., Poznań, Poland) in 0.9% saline solution to inhibit *ex vivo* platelet activity. Blood was immediately centrifuged (6000 r.p.m. for 15 min at 4 °C), and plasma was separated and stored at -80 °C until analysis. The blood for the remaining biochemical analyses was allowed to clot. It was then centrifuged for subsequent analyses.

**Assessment of ADMA, SDMA and L-arginine levels.** Plasma concentrations of L-arginine, N<sup>G</sup>,N<sup>G</sup>-dimethylarginine (ADMA) and N<sup>G</sup>,N<sup>G</sup>-dimethylarginine (SDMA) were measured by high-performance liquid chromatography, and precolumn derivatization was measured with *o*-phthalaldehyde by a previously published method.<sup>13</sup>

**Markers of oxidative stress.** MDA levels were assessed using a lipoperoxidation marker using a colorimetric assay (LPO-586, BIOXYTECH, OXIS International, Inc., Beverly Hills, CA, USA). In this method, a reaction of *N*-methyl-*O*-2-phenylindole with MDA is used and results in the synthesis of a chromogenic product ( $\lambda_{max}$ =586 nm). Addition of HCl inhibits cross-reactivity for hydroxyalkenals; thus, the results reflect only the level of MDA.<sup>29,30</sup>

The thiol index (GSH/GSSG), the ratio of reduced to oxidized glutathione, was measured using a colorimetric assay (GSH/GSSG-412 BIOXYTECH) described by Gunthenberg.<sup>31</sup>



**Figure 1** (a) Scheme of study protocol. First, medical examination of each subject was performed, followed by blood pressure measurement. Next, blood samples for subsequent analyses were drawn, and a baseline flow-mediated vasodilation (FMD) measurement was performed. Subsequently, 16.0g of L-arginine was administered intravenously, followed by a second ultrasound FMD measurement. Each subject was then told to take an oral slow-release indomethacin tablet (75 mg per day for 2 days). On the day 3, the protocol was repeated. (b) Schematic of the subject categorization according to the values of blood pressure (normotensive vs. hypertensive) and FMD of the brachial artery (with endothelial dysfunction (ED) vs. without ED).

Peroxynitrite production was determined by measuring nitrotyrosine levels using an enzyme immunoassay for nitrotyrosine (BIOXYTECH).<sup>32,33</sup>

**Measurement of prostanoids level.** Plasma concentrations of prostanoids (6-keto-PGF-1- $\alpha$  as a marker of prostacyclin synthesis and TxB<sub>2</sub> reflecting thromboxane formation) were measured using commercial ELISA kits (Assay Designs-EIA kit 6-keto-PGF-1-a and TxB<sub>2</sub> enzyme immunoassay EIA kit; BIOMIBO, Warszawa, Poland).

**Other biochemical analyses.** Concentrations of total cholesterol, low-density lipoprotein and high-density lipoprotein fractions and triglycerides, serum creatinine, fasting plasma glucose, high-sensitivity C-reactive protein, thyroid-stimulating hormone, thyrotropin, K<sup>+</sup> and uric acid were measured by standard

laboratory assays. Serum insulin levels were measured with a commercial AxSYM Insulin Assay (Abbott Laboratories Poland Sp. z o.o., Warszawa, Poland) based on the Microparticle Enzyme Immunoassay (MEIA) technology. The formulas used to calculate the homeostasis model assessment for insulin resistance (HOMA-IR) and to calculate the homeostasis model assessments for insulin secretion (HOMA- $\beta$ ) were

1.  $HOMA\ IR = \frac{\text{fasting insulin } (\mu U\ ml^{-1}) \times \text{fasting glucose } (mmol\ l^{-1})}{22.5}$
2.  $HOMA\ \beta = \frac{20 \times \text{fasting insulin } (\mu U\ ml^{-1})}{\text{fasting glucose } (mmol\ l^{-1}) - 3.5}$ .

**Statistical analysis.** Data are expressed as the mean  $\pm$  s.e.m. The differences between two continuous parameters were assessed using a Mann-Whitney

U-test or a Student's *t*-test, followed by a Shapiro-Wilk test and Levene's test as appropriate. For comparison of more than two groups, an ANOVA followed by Tukey's test or a Kruskal-Wallis test (for non-parametric statistics) was performed. Correlations were assessed with a Pearson Moment or Spearman's test as appropriate. All calculations were performed using Statistica 6.0 StatSoft (StatSoft Poland Sp. z o.o., Kraków, Poland).

## RESULTS

### Blood pressure

Blood pressure was significantly higher in hypertensive subjects ( $n=23$ ) compared with those who were normotensive ( $n=47$ ; systolic blood pressure=148.1 vs. 124.5 mm Hg, diastolic blood pressure=91.4 vs. 78.3 mm Hg, mean arterial pressure=110.3 vs. 93.7 mm Hg, pulse pressure=56.7 vs. 46.2 mm Hg, respectively;  $P<0.05$ ).

### Baseline characteristics of investigated population

All four subgroups had very similar demographic characteristics. There were no significant differences in the basic biochemistry between analyzed subgroups or in other markers of cardiovascular risk (Table 1). Insulin levels and HOMA-IR values were non-significantly higher in hypertensive subjects, and HOMA- $\beta$  was non-significantly lower in subgroups with ED (Table 1). None of the recruited patients were treated with any drugs, and none suffered from any chronic disease.

### Endothelial function at baseline and after administration of L-arginine and indomethacin

There were no significant differences in FMD of the brachial artery at baseline between hypertensive and normotensive subjects. There was, however, a significant increase in FMD values in response to intravenous infusion of L-arginine in both subgroups with ED, independent of the presence of HTN (Figure 2a). Administration of indomethacin

resulted in an improvement of endothelial function in subgroups with ED. Infusion of L-arginine after pretreatment with indomethacin resulted in further improvement of endothelial function, but only in hypertensive subjects, even in those with normal FMD values at baseline. No significant changes in FMD values in subgroups without HTN (compared with the values before L-arginine infusion and after indomethacin treatment) were observed (Figure 2a).

### ADMA, SDMA and L-arginine levels

There were no significant differences in baseline levels of ADMA, SDMA, L-arginine or L-arginine/ADMA ratio in the hypertensive vs. normotensive groups (ADMA=0.43  $\pm$  0.01 vs. 0.40  $\pm$  0.010  $\mu\text{mol l}^{-1}$ , SDMA=0.22  $\pm$  0.01 vs. 0.23  $\pm$  0.01  $\mu\text{mol l}^{-1}$ , L-arginine=35.8  $\pm$  3.7 vs. 33.2  $\pm$  1.9  $\mu\text{mol l}^{-1}$ , respectively). No significant differences in the concentrations of these metabolites between all four investigated subgroups were observed.

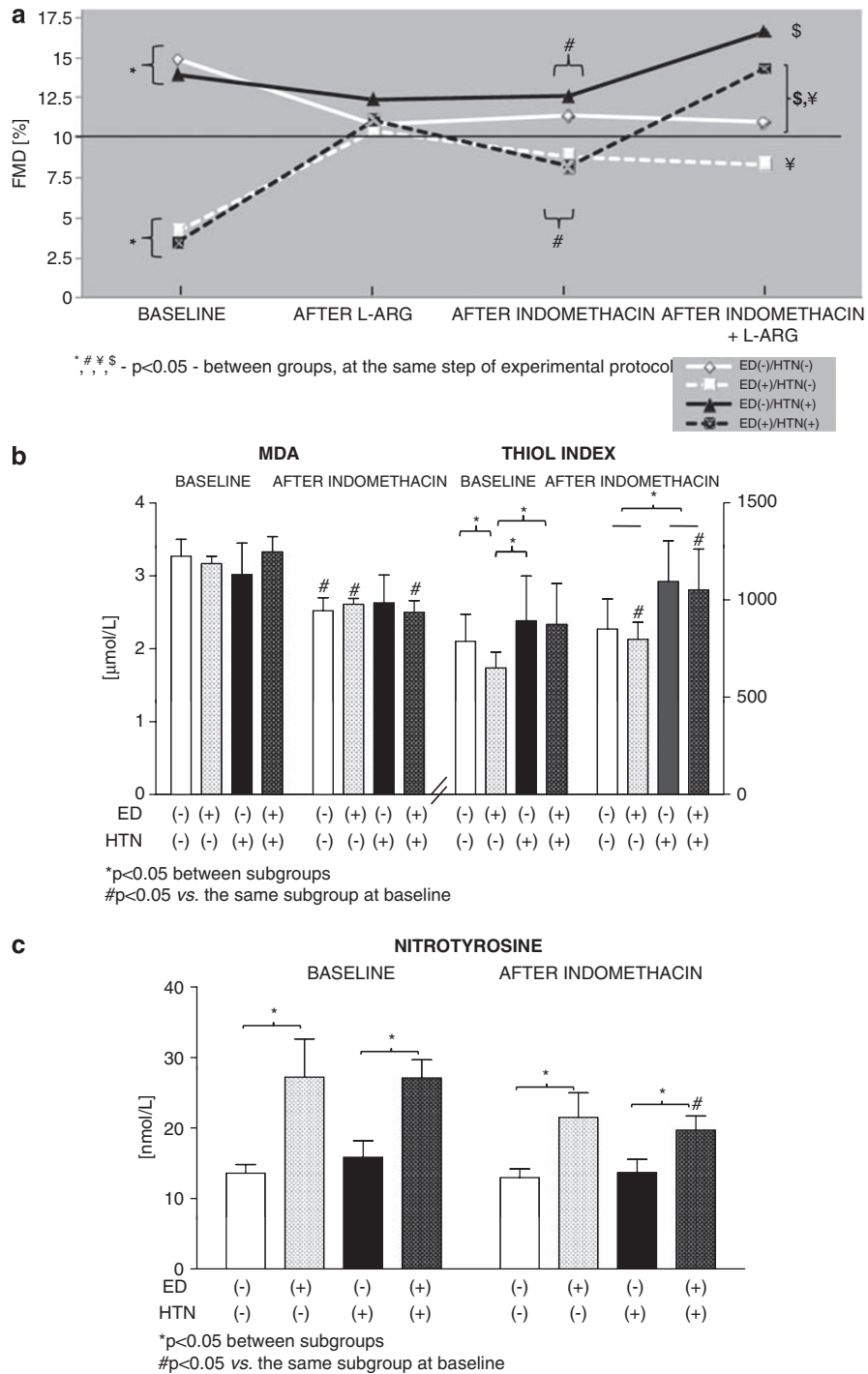
### Markers of oxidative stress

The baseline levels of MDA were similar in all subgroups and decreased after treatment with indomethacin (Figure 2b). A significantly lower baseline thiol index was observed in subgroups with ED compared with those with appropriate vasodilatory function of the endothelium. In subgroups with HTN, the thiol index was higher compared with those who were normotensive. Treatment with indomethacin led to an increase in the thiol index and a decrease in MDA levels in all subgroups, which was statistically significant only in subgroups with ED (Figure 2b). ONOO<sup>-</sup> production, as determined by nitrotyrosine levels, was significantly higher in subgroups with ED, independent of the presence of HTN. A significant decrease in nitrotyrosine levels after treatment with indomethacin was observed only in the subgroup with ED and HTN (Figure 2c).

**Table 1** Demographic characteristics of investigated population divided according to presence of hypertension (HTN) and endothelial dysfunction (ED)

Parameter	ED(-)/HTN(-), n=14 (mean $\pm$ s.e.m.)	ED(+)/HTN(-), n=33 (mean $\pm$ s.e.m.)	ED(-)/HTN(+), n=7 (mean $\pm$ s.e.m.)	ED(+)/HTN(+), n=16 (mean $\pm$ s.e.m.)	P-value
Age (years)	25.5 $\pm$ 1.21	26.5 $\pm$ 1.24	23.3 $\pm$ 2.1	26.9 $\pm$ 1.9	NS
BMI (kg m <sup>-2</sup> )	25.0 $\pm$ 0.52	26.4 $\pm$ 0.84	26.5 $\pm$ 1.7	26.8 $\pm$ 1.0	NS
WHR (1)	0.93 $\pm$ 0.01	0.92 $\pm$ 0.02	0.93 $\pm$ 0.03	0.96 $\pm$ 0.01	NS
SBP (mm Hg)	118.8 $\pm$ 3.13	126.8 $\pm$ 1.93	149.1 $\pm$ 2.4* <sup>†</sup>	147.6 $\pm$ 3.7* <sup>†</sup>	<0.05
DBP (mm Hg)	77.1 $\pm$ 1.99	78.8 $\pm$ 1.39	93.3 $\pm$ 2.4* <sup>†</sup>	90.7 $\pm$ 2.2* <sup>†</sup>	<0.05
MAP (mm Hg)	91.0 $\pm$ 2.2	94.8 $\pm$ 1.39	111.9 $\pm$ 2.3* <sup>†</sup>	109.7 $\pm$ 2.4* <sup>†</sup>	<0.05
PP (mm Hg)	41.8 $\pm$ 2.3	48.0 $\pm$ 1.63	55.8 $\pm$ 2.0* <sup>†</sup>	57.0 $\pm$ 3.0* <sup>†</sup>	<0.05
HR (b.p.m.)	71.4 $\pm$ 2.5	76.6 $\pm$ 2.35	78.2 $\pm$ 4.4	74.6 $\pm$ 2.1	NS
hsCRP (mg l <sup>-1</sup> )	1.83 $\pm$ 1.03	2.37 $\pm$ 0.49	2.04 $\pm$ 0.22	1.97 $\pm$ 0.18	NS
Fasting glucose (mg dl <sup>-1</sup> )	88.4 $\pm$ 3.98	87.1 $\pm$ 1.85	86.4 $\pm$ 3.5	91.4 $\pm$ 1.5	NS
GFR (ml min <sup>-1</sup> )	140.7 $\pm$ 9.52	140.7 $\pm$ 4.25	149.4 $\pm$ 11.5	141.1 $\pm$ 6.2	NS
Uric acid (mg dl <sup>-1</sup> )	6.54 $\pm$ 0.41	6.14 $\pm$ 0.24	5.9 $\pm$ 0.35	5.9 $\pm$ 0.4	NS
TCh (mg dl <sup>-1</sup> )	177.2 $\pm$ 10.3	183.3 $\pm$ 7.45	172.1 $\pm$ 10.5	190.1 $\pm$ 10.5	NS
TG (mg dl <sup>-1</sup> )	116.1 $\pm$ 21.3	110.5 $\pm$ 9.8	145.0 $\pm$ 35.1	137.6 $\pm$ 18.7	NS
HDL (mg dl <sup>-1</sup> )	50.1 $\pm$ 3.31	48.2 $\pm$ 1.97	49.5 $\pm$ 3.1	47.5 $\pm$ 2.1	NS
LDL (mg dl <sup>-1</sup> )	104.8 $\pm$ 8.2	116.9 $\pm$ 6.9	93.5 $\pm$ 5.3	110.1 $\pm$ 6.6	NS
TSH (mIU l <sup>-1</sup> )	2.55 $\pm$ 0.38	2.25 $\pm$ 0.18	2.82 $\pm$ 0.6	1.75 $\pm$ 0.23	NS
K <sup>+</sup> (mEq l <sup>-1</sup> )	4.05 $\pm$ 0.06	4.15 $\pm$ 0.08	4.35 $\pm$ 0.26	4.23 $\pm$ 0.10	NS
Insulin ( $\mu\text{U ml}^{-1}$ )	7.55 $\pm$ 1.47	8.16 $\pm$ 0.71	11.6 $\pm$ 3.4	13.2 $\pm$ 3.2	NS
HOMA-IR	1.52 $\pm$ 0.25	1.82 $\pm$ 0.17	2.53 $\pm$ 0.85	2.32 $\pm$ 0.45	NS
HOMA- $\beta$	205.2 $\pm$ 103.7	108.9 $\pm$ 15.1	197.4 $\pm$ 42.4	140.6 $\pm$ 31.5	NS

Abbreviations: BMI, body mass index; b.p.m, beats per minute; DBP, diastolic blood pressure; GFR, glomerular filtration rate; HDL, high-density lipoprotein; HOMA- $\beta$ , homeostasis model assessments for insulin secretion; HOMA IR, homeostasis model assessment for insulin resistance; HR, heart rate; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; MAP, mean arterial pressure; NS, not significant; PP, pulse pressure; SBP, systolic blood pressure; TCh, total cholesterol; TG, triglycerides; TSH, thyroid stimulating hormone; WHR, waist hips ratio.  
\* $P<0.05$  vs. ED(-)/HTN(-); <sup>†</sup> $P<0.05$  vs. ED(+)/HTN(-).



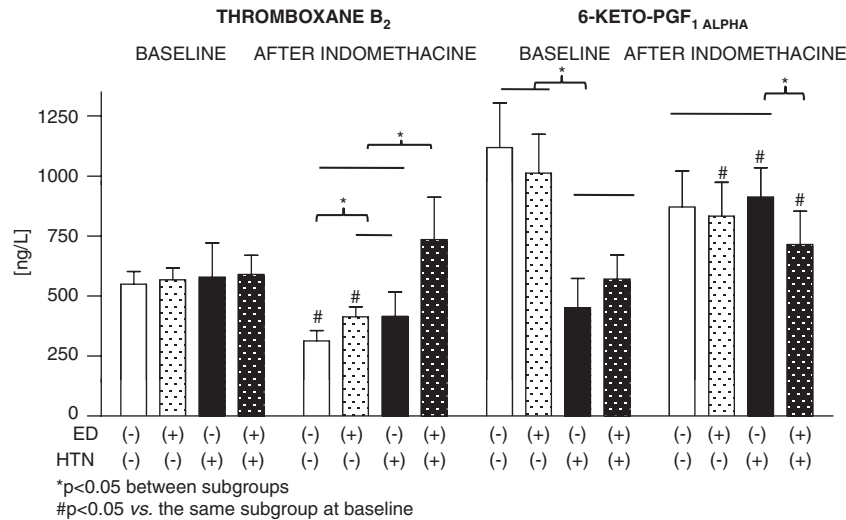
**Figure 2** (a) Characteristics of changes in vascular reactivity (flow-mediated vasodilation) in response to L-arginine and/or indomethacin in the following subgroups: (1). ED(-) and HTN(-),  $n=14$ . (2). ED(+) and HTN(-),  $n=33$ . (3). ED(-) and HTN(+),  $n=7$ . (4). ED(+) and HTN(+),  $n=14$ . \*, #, ¥, \$  $P < 0.05$  between groups, at the same step of experimental protocol. (b) Markers of oxidative stress at baseline and after administration of indomethacin. Means were compared between the four subgroups separated according to endothelial function and blood pressure at each step of the protocol. \* $P < 0.05$  between subgroups; # $P < 0.05$  vs. the same subgroup at baseline. (c) Comparison of nitrotyrosine levels and its changes between subgroups after treatment with indomethacin. \* $P < 0.05$  between subgroups; # $P < 0.05$  vs. the same subgroup at baseline. ED, endothelial dysfunction; HTN, hypertension; MDA, malonyldialdehyde; Thiol index, reduced glutathione (GSH)/oxidized glutathione (GSSG).

### Prostanoids

The hypertensive group was characterized by a significantly lower plasma concentration of prostacyclin (6-keto-PGF-1- $\alpha$  levels:  $534.6 \pm 78.6 \text{ ng l}^{-1}$  vs.  $1043.6 \pm 126.1 \text{ ng l}^{-1}$   $P < 0.05$ ) and a slightly

higher baseline plasma level of  $\text{TxB}_2$  ( $584.7 \pm 68.6 \text{ ng l}^{-1}$  vs.  $561.8 \pm 38.5 \text{ ng l}^{-1}$ , without statistical significance). Furthermore, a significant increase in plasma prostacyclin levels was observed in subjects with HTN in response to indomethacin. Conversely, a





**Figure 3** Prostanoids at baseline and after administration of indomethacin. Means were compared at the same step of the experimental protocol between the four subgroups separated according to endothelial function and blood pressure. \* $P < 0.05$  between subgroups; # $P < 0.05$  vs. the same subgroup at baseline. ED, endothelial dysfunction; HTN, hypertension.

decrease in plasma prostacyclin levels was observed in the normotensive group (Figure 3). Finally, thromboxane was decreased in normotensive subgroups after treatment with indomethacin (Figure 3).

## DISCUSSION

To our knowledge, this is the first demonstration that vascular reactivity assessed by FMD is characterized by a different pattern of regulatory mechanisms in young men with essential HTN compared with normotensive subjects. The improvement of endothelial function in hypertensive subjects after L-arginine infusion was significantly potentiated by indomethacin pretreatment. Given that a blockage of the prostanoids pathway and the concurrent activation of NO synthesis are necessary to restore appropriate endothelial function, a cascade relationship between these two pathways is possible. Activation of the prostanoid pathway in hypertensive subjects may increase oxidative stress by increasing the formation of lipid peroxides, which subsequently scavenge NO. Therefore, administration of L-arginine alone is not sufficient to restore appropriate FMD because of persistent NO scavenging. Inhibition of COX reduces peroxidation of lipids (as assessed by MDA measurements) and increases the serum anti-oxidative capacity (thiol index), resulting in increased NO bioavailability. Given that a synergistic improvement of FMD after administration of indomethacin is potentiated further by administration of L-arginine in hypertensive subjects, a causative role of the increased oxidative stress in reducing NO bioavailability could be postulated in these subjects. A decrease in nitrotyrosine levels in these subjects after treatment with indomethacin confirms this hypothesis. Thus, our observation is consistent with earlier findings that a balance between NO and ROS is crucial for maintenance of vascular function.<sup>34,35</sup> According to a study by Taddei *et al.*,<sup>36</sup> COX is responsible for ROS formation, which subsequently decreases NO bioavailability. Ge *et al.*<sup>37</sup> postulated that HTN might be associated with higher COX-1 expression and that non-selective blockage of COX may result in lower synthesis of vasoconstrictive prostanoids. Long-term oxidative stress enhances COX-2 expression. This enhancement could be a reason for the enhanced synthesis of vasoconstrictive prostanoids and ROS, which actively decrease NO bioavailability.

This relationship, however, was not observed in normotensive subjects. Modulation of one pathway was demonstrated to be sufficient to restore FMD in the presence of ED. Moreover, in normotensive subjects, no synergy between the drugs was observed.

Contrary to other investigators, Schlaich *et al.*<sup>38</sup> did not find significant differences in plasma levels of ADMA and L-arginine between healthy and hypertensive subjects.<sup>39</sup> In our current study, we did not observe HTN-dependent differences in concentrations of ADMA, L-arginine or the L-arginine/ADMA ratio. Infusion of L-arginine resulted in a significant improvement of FMD in both groups with ED during the first day of the experiment, independent of the presence of HTN. Nevertheless, an inhibition of COX resulted in a further, synergistic improvement of FMD only in hypertensive subjects, indicating that oxidative stress is a primary mechanism of decreased NO bioavailability.

Takiuchi *et al.*<sup>40</sup> showed that impaired vasodilatory function of coronary and peripheral vessels in subjects with HTN results from elevated plasma levels of ADMA. The results of our study are consistent with these findings, as we did not observe significant differences in ADMA plasma levels in young men, regardless of the presence of HTN. Thus, decreased NO bioavailability resulted from increased NO degradation, not from decreased NO synthesis (that is, increased ADMA levels).

Another novel finding in our study was the trend in the changes of thromboxane and prostacyclin levels after COX inhibition by indomethacin. An increase in 6-keto-PGF-1- $\alpha$  plasma levels was more common in hypertensive subjects; however, in normotensive subjects, a decrease in plasma PGI was more frequent. The observed increase in prostacyclin production despite treatment with nonsteroidal anti-inflammatory drug could be explained by activation of the peroxisome proliferator-activated receptor (PPAR)- $\alpha$  and - $\gamma$  by some anti-inflammatory drugs.<sup>41,42</sup> Given that the peroxisome proliferator-activated receptor response element is present within the promoter region of the COX-2 gene, increased expression of COX-2 might be responsible. Nevertheless, the presence of such regulation only in hypertensive subjects needs further investigation.

Independent of the presence of HTN, a similar prevalence of ED was observed. These findings suggest independent mechanisms that

lead to the development of ED and HTN. ED is observed in subjects with HTN as well as in healthy subjects with increased cardiovascular risk.<sup>43</sup> The phenotype of endothelial function, however, is different in hypertensive subjects compared with normotensive men. Administration of indomethacin potentiates response to L-arginine only in hypertensive subjects. This observation could indicate that HTN is associated with COX-dependent oxidative stress, which may lead to impairment of FMD due to increased NO scavenging. Elevated insulin levels and HOMA-IR values in hypertensive subjects could indicate an important role of metabolic disorders in the pathogenesis of HTN in young men. The increase in insulin levels and HOMA-IR values, however, did not reach statistical significance in our study. To verify this hypothesis, a larger population of subjects should be investigated.

To date, a 'normal range' of FMD values has not been established; therefore, the selection of the cut-off point is arbitrary and fluctuates from study to study (from 5 to 15%). In one study performed on young healthy men,<sup>44</sup> the mean FMD value was  $9.7 \pm 1.4\%$ . In other studies,<sup>45,46</sup> it has been shown that normal FMD values are higher in younger male subjects, and a regression model to assess the cut-off point for ED was calculated [FMD =  $25.5 - (0.17 \times \text{age}) - (2.6 \times \text{resting vessel diameter})$ ]. In this study, ED was recognized when the increase in FMD was <10% of the increase in the diameter of the artery, which was likely because the investigated population included young men. Nevertheless, it is necessary to clearly establish a normal range of FMD values that takes into account gender and age.

### Limitations

In this study, the vasodilation protocol following nitroglycerine was not investigated. As cardiovascular risk factors increase in number, smooth muscle dysfunction becomes apparent, and the nitroglycerin response is progressively impaired independently of ED.<sup>47</sup> This should be taken into account when studying patients with either coronary or systemic atherosclerosis.<sup>48</sup> Given that only young male subjects were investigated, we did not include administration of nitroglycerine in the study protocol.

### Perspectives

The results of our study indicate different mechanisms underlying the regulation of vascular tone that are dependent on the presence of HTN. Moreover, the ED present in the two groups evaluated was shown to be triggered by different mechanisms. A divergent response to nonsteroidal anti-inflammatory drugs dependent on the presence of HTN may have important clinical implications, which will allow for the selection of patients for whom the treatment with non-steroidal anti-inflammatory/antiplatelet drugs or other strategies to reduce oxidative stress in the primary prevention of cardiovascular events will be beneficial. Hypertensive subjects with concomitant ED may constitute this group; however, further research is required to confirm this hypothesis. Given that the prevalence of ED in potentially healthy subjects is quite high, it might be reasonable to consider the assessment of FMD as an important tool in the stratification of cardiovascular risk in this group.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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