Essential hypertension is characterized by an impairment of endothelial function. Although the hallmark of endothelial dysfunction is a reduced bioavailability of nitric oxide (NO), other mechanisms (such as an alteration in the production of prostanoids, an impairment of endothelium-dependent hyperpolarizations, as well as an increased release of endothelin-1) can individually, or in association, contribute to endothelial dysfunction.

Nebivolol is a third-generation β1-adrenergic receptor antagonist whose hemodynamic profile is different from that of classical β-blockers. Experimental models have demonstrated that nebivolol induces vasodilation through stimulation of endothelial NO bioactivity by a mechanism that is, so far, not completely known. Studies in humans evaluating forearm blood flow and endothelium-dependent vasodilation have provided considerable, even if indirect, evidence of the effect of nebivolol on NO bioactivity. These findings are supported by animal and human cell data showing that nebivolol directly stimulates NO release in endothelial cells. As far as the precise mechanisms involved in the vasodilatory effect of nebivolol, there is evidence indicating that endothelial NO synthase (eNOS) is involved because the production of NO from the endothelium can be abolished by eNOS inhibitors. At present, the mechanism by which nebivolol reduces circulating ADMA in hypertensive patients remains unclear, our ex vivo results suggest that the upregulation of DDAH2 expression may have a role.

Asymmetric dimethylarginine (ADMA) is a naturally occurring amino acid that circulates in plasma; it is excreted in urine, and found in tissues and cells. ADMA has aroused interest because it inhibits eNOS and, therefore, has the potential to

**BACKGROUND**

This study was conducted to evaluate (i) the effect of nebivolol, a selective β1-adrenergic receptor antagonist, on plasma concentration of asymmetric dimethylarginine (ADMA) and on flow-mediated dilation (FMD) in essential hypertensive patients; (ii) the effect of serum derived from the treated hypertensive patients on ADMA and on dimethylarginine dimethylaminohydrolase 2 (DDAH2), the enzyme that selectively degrades ADMA, in human umbilical vein endothelial cells (HUVECs).

**METHODS**

Forty healthy subjects and 40 matched essential hypertensive patients treated with atenolol and nebivolol according to a double-blind, randomized design participated in the study. Evaluation of brachial artery (BA) reactivity was performed by a longitudinal B-mode scan of the right BA. ADMA and L-arginine were measured by high-performance liquid chromatography. DDAH2 expression and endothelial nitric oxide synthase activity (eNOS) were also evaluated in HUVECs.

**RESULTS**

ADMA levels were significantly decreased and FMD increased only in patients receiving nebivolol (P < 0.01). Furthermore, in nebivolol group, we found a significant correlation between changes in ADMA levels and changes in FMD (P < 0.01). Sera derived from patients treated with nebivolol but not with atenolol decreased ADMA and increased DDAH2 expression and eNOS activity (P < 0.001) in HUVECs.

**CONCLUSIONS**

The results of this study demonstrate that the improvement of endothelial dysfunction induced by nebivolol in hypertensive patients may be related to its effect on circulating ADMA levels. Although the mechanism by which nebivolol reduces circulating ADMA in hypertensive patients remains unclear, our ex vivo results suggest that the upregulation of DDAH2 expression may have a role.
produce considerable biological effects, particularly endothelial dysfunction. An emerging role for ADMA as a novel cardiovascular risk factor has also been recently reported. ADMA is synthesized in cells when arginine residues in proteins are methylated by the action of protein arginine methyltransferases. Dimethylarginine dimethylaminohydrolase (DDAH) selectively degrades ADMA. Two isoforms exist: DDAH1 predominates in tissues containing neuronal NOS, whereas DDAH2 is more prevalent in tissues expressing eNOS. Recently, in an in vitro study we demonstrated that nebivolol increased the expression and activity of DDAH2 in endothelial cells.

Although nebivolol has been shown to reverse endothelial dysfunction and several studies have investigated nebivolol-induced vasodilating effect, the precise molecular mechanism(s) is still under investigation. In this study, we first compared the effect of nebivolol with atenolol, a traditional selective β1-adrenergic receptor blocker without vasodilating properties, on plasma ADMA concentrations and on brachial artery (BA) flow–mediated dilation (FMD) in patients with essential hypertension. Then, in ex vivo studies, we evaluated the effect of serum derived from nebivolol- and atenolol-treated hypertensive patients on DDAH2 expression, eNOS activity, and ADMA concentration in endothelial cells.

**METHODS**

**Patients.** Forty healthy subjects and 40 matched essential hypertensive patients participated in this study. Subjects with smoking history (more than five cigarettes/day), ethanol consumption (>80 g/day), total cholesterol >250 mg/dl, diabetes, body mass index (BMI) >30, secondary hypertension, cardiac, hepatic, and renal pathologies were excluded. Only postmenopausal women not taking hormone replacement therapy were included. Grade I essential hypertension was diagnosed according to the European Societies of Hypertension and Cardiology criteria. For this purpose, before enrollment into the study, blood pressure (BP) values were determined as the mean of three measurements made at 2-min intervals after the patients had been seated for 10 min (clinical BP). Grade I essential hypertensive patients without clinically evident organ damage were recruited if supine clinic arterial BP (after 10 min of rest) was consistently found to be >140/90 and <160/100 mm Hg and they were never treated (n = 31) or reported a history of discontinued pharmacological antihypertensive treatment (n = 9). Subjects were defined as normal if BP values were <130/80 mm Hg. The protocol was approved by the Ethical Committee of the University of Verona, and all patients gave their written consent to participate in this study.

**Experimental design.** Hypertensive patients were treated with atenolol (100 mg) or nebivolol (5 mg) once daily (n = 20 for each group) according to a double-blind randomized design. After 2 weeks of treatment, patients with clinical BP >140/90 mm Hg were excluded from the study. Additional clinic visits were scheduled at the end of the active treatment (4 weeks) and after 1 week of wash out.

Before and after each study period, 24-h ambulatory BP monitoring was recorded by a noninvasive oscillometric device (BR-102, Schiller, Switzerland). BP was recorded at 15-min intervals (daytime 7 AM to 11 PM) or 20-min intervals (night-time 11 PM to 7 AM). Before starting with ambulatory BP monitoring, sitting BP was also measured by standard sphygmomanometer.

Metabolic parameters and FMD were assessed at baseline and after 4 week of the active treatment period. ADMA and l-arginine were also assessed after the 1-week wash-out. Estimated glomerular filtration rate, used as an indicator for renal function, was derived from creatinine levels as previously described. Whole blood obtained from all patients after 12 h of fasting and 30 min after the antihypertensive therapy was collected in ethylenediamine tetraacetic acid or without additives and drawn into pyrogen-free blood-collection tubes. The samples were frozen and thawed only once.

**Evaluation of FMD.** Endothelial function was evaluated by measurement of FMD as previously described. A B-mode scan of the right BA was obtained in longitudinal section 5–10 cm above the elbow by the same operator using a 7.5-MHz linear array transducer that was held at the same point throughout the scan by a stereotactic clamp. Simultaneous electrocardiographic recordings were obtained and displayed on the ultrasound system (Philips, Eindhoven, The Netherlands) video monitor. Imaging studies were performed using lower-arm occlusion technique. After 1 min of acquisition (basal diameter), a BP cuff was inflated 50 mm Hg above the systolic pressure for 5 min; following deflation, the BA was imaged continuously for 3 min (endothelium-dependent dilation). A repeat baseline scan was obtained after a 15-min rest. Endothelium-independent dilation was obtained by the administration of sublingual glyceryl trinitrate (0.3 mg). FMD and glyceryl trinitrate–induced dilation were defined as the maximal percent change in BA diameter compared to baseline. Computer-assisted edge detection brachial analysis software (Medical Imaging Applications, Coralville, IA) was used to calculate BA diameters. The variability of FMD was calculated in healthy subjects 5 weeks after baseline determinations. The variability was 11% with a mean difference of 0.7% between the two measurements. Power analysis indicates that, assuming a two-tailed 5% test, the sample size is sufficient to detect a 1.5% improvement in FMD after therapy with 80% power.

**ADMA and l-arginine measurement.** Concentrations of ADMA and l-arginine were measured by high-performance liquid chromatography as previously described.

**Incubation of serum derived from hypertensive patients with HUVECs.** Human umbilical vein endothelial cells (HUVECs) obtained as described were incubated for 24 h with 0, 30, and 60% serum derived from untreated hypertensive patients or treated with nebivolol and atenolol. ADMA concentration in the supernatant and DDAH2 and eNOS expression and activity in cells were measured. To evaluate the relationship between DDAH2 expression and ADMA concentration in serum-treated HUVECs, the effect of small interference RNA (siRNA)-mediated knockdown of DDAH2 on ADMA concentration was also...
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Evaluating. Endothelin contamination of cell cultures involving the use of serum was routinely excluded with the chromogenic Limulus amebocyte lysate assay (Sigma).

eNOS activity. eNOS activity was determined as previously described.18,23

Real-time reverse transcriptase–PCR quantification of DDAH2 RNA and eNOS. Total RNA was extracted and real-time reverse transcriptase–PCR analysis was conducted as previously described.22 Primers were designed by Beacon Design 4.0 software (PREMIER Biosoft International, Palo Alto, CA) and synthesized by MWG Biotech AG (Ebersberg, Germany): DDAH2, sense 5′-GCTGCTAGAATCCACCTGAG-3′ and antisense 5′-GAGGCTTTTGGGACTCCATCG-3′, PCR fragment of 143 bp; β-actin, sense 5′-TTGGCAATGAGCGGTTCC-3′ and antisense 5′-GGGCTTTGCGGACTCCATCG-3′, PCR fragment of 143 bp; and eNOS, sense 5′-AGCACTGTGTTGGCGTAC-3′ and antisense 5′-GTGGCAATGAGCGGTTCC-3′, PCR fragment of 148 bp.

Transcript abundance, normalized to β-actin expression, was expressed as fold increase over a control sample (no addition of serum).

Western blotting analysis. Western blotting analysis was conducted by using the DDAH2 goat polyclonal antibody (ab1383) from Abcam (Cambridge, UK) as previously described.18 Immunoreactive band was visualized with a horseradish peroxidase–conjugated secondary antibody using an ECL detection kit (GE Biosciences Amersham, Piscataway, NJ) at ~33 kDa and quantified by densitometric analysis after normalization to β-actin.

siRNA-mediated knockdown of DDAH2 in HUVECs. siRNAs were designed by targeting the following DDAH2 sequences: (i) CTGGATCTGGCCAAAGCTCAA; (ii) TAGGATAGTATAGGCTGGAGACAGGTGAAGAA according to the siRNA design guidelines (www.qiagen.com). The sequence GACTCGAGCAGCTGGAGACAGGTGAAGAA was used as the scrambled siRNA control.

Transfection of HUVECs. Transfection of HUVECs by Transmessenger Transfection reagent (Qiagen) was optimized and performed as previously described.18

Statistical analysis. Statistical analysis was performed by one- or two-way analysis of variance with repeated measures followed by post-hoc Tukey’s test for multiple comparisons using the “SPSS 11” program for Macintosh. Statistical significance was inferred when \( P < 0.05 \).

RESULTS

Effect of nebivolol on ADMA plasma concentrations and on FMD in hypertensive patients

Age, sex, total cholesterol and HDL cholesterol, triglycerides, plasma glucose, and heart rate were similar and within a normal range between normotensive subjects and essential hypertensive patients, who differed in BP \( (P < 0.01) \). Basal levels of plasma ADMA resulted significantly higher whereas FMD was lower in hypertensive patients compared to normotensive subjects \( (P < 0.01) \) (Table 1). Age, gender, body mass index, and metabolic parameters were similar at baseline and remained unchanged throughout the treatment period between hypertensive patients randomized to receive nebivolol or atenolol (data not shown). At baseline, there were no significant differences in BP values, FMD, estimated glomerular filtration rate, and circulating levels of ADMA, l-arginine, and l-arginine/ADMA ratio between the two groups (Table 2).

Both nebivolol and atenolol significantly and similarly reduced clinic and ambulatory BP monitoring values \( (P < 0.01) \) (Table 2). At the end of the treatment only in the patients receiving nebivolol there was a significant decrease of plasma ADMA \( (P < 0.01) \) that was associated to a significant increase of l-arginine/ADMA ratio \( (P < 0.001) \); however, atenolol treatment was ineffective.

At baseline, FMD was similar in the two groups (Table 2); furthermore, we found a significant inverse correlation between circulating ADMA levels and FMD \( (r = 0.64, P < 0.001) \). Treatment with atenolol failed to improve FMD significantly (from 5.85 ± 2.1 to 6.11 ± 2.3), whereas nebivolol significantly improved FMD as compared to baseline (from 5.93 ± 1.9 to 7.52 ± 2.2, \( P < 0.01 \)). In both groups, the BA dilator response to glyceryl trinitrate was similar at

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Basal systemic, demographic, hemodynamic, humoral characteristics, asymmetric dimethylarginine (ADMA) and endothelial function for normotensive subjects and essential hypertensive patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Normotensive subjects</td>
</tr>
<tr>
<td>56.5 ± 7.7</td>
<td>55.9 ± 10</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>9/11</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.3 ± 2.7</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>120.7 ± 6.8</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>76.3 ± 5.1</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>75.1 ± 6.8</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>232 ± 12</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>63 ± 14</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>122 ± 17</td>
</tr>
<tr>
<td>Plasma glucose (mg/dl)</td>
<td>142 ± 35</td>
</tr>
<tr>
<td>ADMA (µmol/l)</td>
<td>0.37 ± 0.09</td>
</tr>
<tr>
<td>l-Arginine (µmol/l)</td>
<td>79.4 ± 16.6</td>
</tr>
<tr>
<td>l-Arginine/ADMA ratio</td>
<td>217.3 ± 44.0</td>
</tr>
<tr>
<td>Flow-mediated dilation (%)</td>
<td>8.21 ± 2.53</td>
</tr>
<tr>
<td>Brachial artery (mm)</td>
<td>4.08 ± 0.76</td>
</tr>
</tbody>
</table>

BMI, body mass index; BP, blood pressure.
* \( P < 0.01 \) vs. normotensive subjects.
Table 2 | Values of blood pressure, heart rate, asymmetric dimethylarginine (ADMA), l-arginine and endothelial function for hypertensive patients in treatment either with nebivolol or atenolol

<table>
<thead>
<tr>
<th></th>
<th>Nebivolol (baseline)</th>
<th>Nebivolol (4 weeks)</th>
<th>Nebivolol (wash-out)</th>
<th>Atenolol (baseline)</th>
<th>Atenolol (4 weeks)</th>
<th>Atenolol (wash-out)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinic SBP (mm Hg)</td>
<td>152.4 ± 8.1</td>
<td>133.0 ± 7.2*</td>
<td>145.5 ± 6.4</td>
<td>151.8 ± 7.7</td>
<td>134.2 ± 5.1*</td>
<td>144.2 ± 6.7</td>
</tr>
<tr>
<td>Clinic DBP (mm Hg)</td>
<td>96.1 ± 4.3</td>
<td>85.0 ± 3.1*</td>
<td>91.1 ± 4.5</td>
<td>96.5 ± 5.1</td>
<td>85.8 ± 3.6</td>
<td>92.1 ± 3.8</td>
</tr>
<tr>
<td>ABPM SBP (mm Hg)</td>
<td>140.2 ± 5.3</td>
<td>123.5 ± 4.9*</td>
<td>—</td>
<td>141.1 ± 6.1</td>
<td>123.7 ± 5.4*</td>
<td>—</td>
</tr>
<tr>
<td>ABPM DBP (mm Hg)</td>
<td>86.4 ± 3.6</td>
<td>72.2 ± 2.7*</td>
<td>—</td>
<td>87.2 ± 4.1</td>
<td>77.9 ± 3.1*</td>
<td>—</td>
</tr>
<tr>
<td>Clinic heart rate (beats/min)</td>
<td>76.5 ± 5.8</td>
<td>65.9 ± 5.2*</td>
<td>70.4 ± 5.4</td>
<td>77.3 ± 4.9</td>
<td>63.2 ± 5.1*</td>
<td>71.2 ± 5.4</td>
</tr>
<tr>
<td>ADMA (µmol/l)</td>
<td>0.62 ± 0.07</td>
<td>0.37 ± 0.06*</td>
<td>0.60 ± 0.07</td>
<td>0.61 ± 0.08</td>
<td>0.57 ± 0.01</td>
<td>0.60 ± 0.07</td>
</tr>
<tr>
<td>l-Arginine (µmol/l)</td>
<td>92.54 ± 15.82</td>
<td>97.11 ± 18.34</td>
<td>91.23 ± 16.45</td>
<td>86.88 ± 16.7</td>
<td>88.04 ± 22.2</td>
<td>91.1 ± 18.6</td>
</tr>
<tr>
<td>l-Arginine/ADMA ratio</td>
<td>150.2 ± 29.4</td>
<td>262.8 ± 51.1*</td>
<td>162.3 ± 24.9</td>
<td>144.84 ± 37.9</td>
<td>160.3 ± 48.7</td>
<td>151.7 ± 42.3</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73 m²)</td>
<td>98.8 ± 8.3</td>
<td>104.2 ± 8.6</td>
<td>—</td>
<td>101.7 ± 9.9</td>
<td>105.8 ± 8.5</td>
<td>—</td>
</tr>
<tr>
<td>FMD (%)</td>
<td>5.93 ± 1.9</td>
<td>7.52 ± 2.2*</td>
<td>—</td>
<td>5.85 ± 2.1</td>
<td>6.11 ± 2.3</td>
<td>—</td>
</tr>
<tr>
<td>GTN (%)</td>
<td>11.15 ± 2.7</td>
<td>11.36 ± 2.9</td>
<td>—</td>
<td>12.07 ± 2.6</td>
<td>12.30 ± 2.8</td>
<td>—</td>
</tr>
<tr>
<td>Brachial artery (mm)</td>
<td>4.06 ± 0.7</td>
<td>4.05 ± 0.6</td>
<td>—</td>
<td>4.02 ± 0.5</td>
<td>4.03 ± 0.8</td>
<td>—</td>
</tr>
</tbody>
</table>

ABPM, 24-h ambulatory blood pressure monitoring; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FMD, flow-mediated vasodilation; GTN, brachial artery dilator response to nitroglycerin; SBP, systolic blood pressure.

*P < 0.01 vs. baseline; †P < 0.01 vs. atenolol group.

baseline and was not significantly increased after treatment (Table 2). Similarly, resting BA (mm) remained unmodified after treatment in both groups (Table 2). In nebivolol group, we found a significant correlation between changes in circulating ADMA levels and changes in FMD ($r = 0.62$, $P < 0.01$) (Figure 1).

Effect of serum derived from hypertensive patients untreated or treated with nebivolol and atenolol on ADMA concentration, DDAH2 expression, and eNOS activity in HUVECs

Sera derived from hypertensive patients treated with nebivolol (but not with atenolol, data not shown) caused a dose-dependent decrease of ADMA concentration in conditioned medium of HUVECs compared to sera derived from untreated hypertensive patients ($P < 0.001$) (Figure 2).

Sera from patients treated with nebivolol but not with atenolol also induced a dose-dependent significant increase in DDAH2 mRNA levels in HUVECs (Figure 3a). This effect was due to transcriptional regulation as it was blocked by pretreatment with actinomycin D (1.0 µg/ml). Sera from nebivolol but not from atenolol-treated patients also induced a dose-dependent increase in DDAH2 protein expression in HUVECs (Figure 3b).

Transfection of HUVECs with anti-DDAH2 siRNA (target sequence: TAGGATGTATAGGAAGGAGA) but not scrambled siRNA abolished the ability of sera derived from patients treated with nebivolol to decrease ADMA concentration in HUVECs (Figure 4).

Finally, only sera from nebivolol-treated patients induced a significant dose-dependent increase in eNOS activity ($P$ from $0.01$ to $<0.001$) (Figure 5) without any variation of eNOS expression (data not shown).
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**Figure 3** | Effect of serum derived from patients treated for 4 weeks with nebivolol and atenolol on dimethylarginine dimethylaminohydrolase (DDAH2) expression after incubation with human umbilical vein endothelial cells (HUVECs). (a) Dose-dependent effect of serum at concentrations ranging from 0 to 60% of cell medium, and activity of actinomycin on DDAH2 mRNA evaluated by real-time reverse transcriptase-PCR after incubating with HUVECs for 12 h. Results were normalized to β-actin expression and were expressed as fold increase over a control sample (no addition of serum). Data are means ± s.d. of experiments performed in triplicate. *P < 0.01 vs. control. (b) Dose-dependent effect of serum on DDAH2 protein (35 kDa) evaluated by western blotting after incubating with HUVECs for 24 h. The figure shows a representative blot of different experiments and the average data were obtained by densitometric analysis. Results are means ± s.d. and are expressed as density in arbitrary units after normalization to β-actin (AU). *P < 0.01 vs. 0 (no addition of serum); **P < 0.001 vs. 0 (no addition of serum); †P < 0.001 vs. 30%.

**Figure 4** | Effect of transfection of human umbilical vein endothelial cells (HUVECs) by anti-dimethylarginine dimethylaminohydrolase 2 (DDAH2) small interference RNA (siRNA) on ability of serum from patients treated with nebivolol to reduce asymmetric dimethylarginine (ADMA) concentration in the medium. HUVECs transfected with DDAH2 siRNA or scrambled siRNA (sc siRNA) were incubated for 24 h with control (no treatment) and nebivolol-treated sera. *P < 0.01 vs. control.

**Figure 5** | Effect of serum derived from patients treated for 4 weeks with nebivolol and atenolol on eNOS activity after incubation with human umbilical vein endothelial cells (HUVECs). HUVECs were stimulated for 24 h at concentrations of serum ranging from 0 to 60% of cell medium. Data are means ± s.d. of experiments performed in triplicate. *P < 0.01 vs. serum derived from atenolol-treated patients; **P < 0.001 vs. serum derived from nebivolol-treated patients.

**DISCUSSION**

In agreement with previous reports, our results show that plasma ADMA was higher and l-arginine/ADMA ratio and FMD was lower in hypertensive patients than in healthy subjects. Increased plasma ADMA concentrations and reduced l-arginine/ADMA ratio have already been correlated with decreased FMD. Moreover, in spite of the same BP-lowering effect, we demonstrated that nebivolol, but not atenolol, induced a decrease in plasma ADMA that was paralleled by an increase in l-arginine/ADMA ratio. Another peculiar finding of this study is that long-term treatment with nebivolol was able to reverse endothelial dysfunction in essential hypertensive patients. Although the effect of nebivolol on endothelial function and NO bioactivity has been already evaluated in hypertensive patients, the results are still conflicting. Our data, even though in different experimental conditions, agree with previous studies demonstrating that nebivolol significantly increased the BA response to acetylcholine. At variance with our results, a recent study showed that nebivolol did not modify FMD in the BA. The reasons explaining this conflicting results are unclear; however, contrary to others, in this study nebulol was not shown to ameliorate the parameters exploring oxidative stress.

Consistent with previous findings, our results show that atenolol, despite similar BP-lowering effect, is ineffective in reversing endothelial dysfunction in hypertensive patients. On the other hand, in previous studies, atenolol (compared to nebivolol) failed to show antioxidant activity and to influence NO availability as well as to improve coronary flow reserve.

ADMA is an inhibitor of eNOS activity and, therefore, has the potential to produce considerable biological effects, particularly endothelial dysfunction. Some recent studies investigated the potential contribution of ADMA to endothelial dysfunction and demonstrated that in healthy subjects and in hypertensive patients, plasma ADMA levels and endothelium-dependent vasodilation were inversely related.
This relationship was independent of potential confounders because, in multivariate models, ADMA, but not other risk factors, retained an independent association with endothelial function. In this study, we confirmed the inverse correlation between plasma ADMA levels and FMD in all our hypertensive patients and more interestingly, in nebivolol-treated group, we found a significant correlation between changes in plasma ADMA levels and changes in FMD. Our results agree with previous findings showing that treatment with rosuvastatin in hypercholesterolemic patients led to a reduction of circulating ADMA levels and to an improvement of endothelial function; moreover, treatment with enalapril in patients with syndrome X was demonstrated to reduce plasma ADMA and increase endothelial NO availability.

Although the mechanisms of endothelial dysfunction are multifactorials, taken together, these results suggest that the reduction of plasma ADMA levels may be casually involved in the ability of nebivolol to reverse endothelial dysfunction. However, on the basis of the present results, we cannot exclude that the demonstrated antioxidative action of nebivolol may have contributed to FMD improvement.

The reason why nebivolol reduced circulating ADMA remains unclear, but it has been proposed that the elevation in plasma ADMA that occurs with vascular disease and risk factors is largely due to impaired concentration and/or activity of DDAH2. In this context, we recently reported that nebivolol decreased ADMA in the medium of HUVECs by augmenting DDAH2 expression and activity, and that the induction of eNOS activity by the drug was at least partially secondary to this effect. The results we obtained in ex vivo studies support this hypothesis. Incubation of HUVECs with serum derived from nebivolol-treated patients, in fact, caused a dose-dependent increase of DDAH2 expression which was associated to a contemporary fall in ADMA concentration and to an increase of eNOS activity. The fact that DDAH2-specific siRNA inhibited ADMA reduction demonstrates that the effect of serum of nebivolol-treated patients on ADMA concentration is DDAH2-mediated.

The effect of serum derived from nebivolol-treated group is likely to be due to the nebivolol and/or metabolites present in plasma. In this study, in fact, the blood was withdrawn from the patients ~30 min after oral administration, and pharmacokinetic studies have shown that the highest plasma concentration after taking nebivolol per os is reached after 30–60 min. It could be argued that a partial limitation of this study may be the lack of identification of metabolites of nebivolol. Actually, some metabolites of nebivolol and sera from mice treated with nebivolol were already shown to have similar pattern of activity on NO availability in endothelial cells as compared with nebivolol itself.

At present, there is no certainty about the precise mechanisms involved in the vasodilatory effects of nebivolol. The results of experimental studies suggest that nebivolol may stimulate NO bioactivity through phospholipase C activation, release of adenosine triphosphate, and a NO-cGMP-signaling transduction pathway. Moreover, the activation of Ca²⁺-activated K⁺ channels has also been proposed. A multiplicity of receptors has also been involved: 5-HT₁A, β₂- and β₃-adrenergic receptor, and estrogen receptor. Of particular interest, although performed with suprapharmacological doses, are the studies recently published by Ladage et al. who demonstrated an estradiol-agonistic action of nebivolol in HUVECs and in spontaneously hypertensive rats. Because experimental evidences suggest that the ability of estradiol to increase the expression and activity of eNOS is mediated by its effect on DDAH2 activity and metabolism of ADMA, a tentative explanation of our results could be that the effect of nebivolol on DDAH2/ADMA pathway may be at least partially mediated by estradiol receptors on HUVECs. Of course, the possibility that nebivolol influences DDAH2 expression through these receptors and/or mechanism awaits for appropriate verification in hypertensive patients.

In conclusion, the results of this study demonstrate that the improvement of endothelial dysfunction induced by nebivolol in hypertensive patients may be related also to its effect on circulating ADMA levels. Although the mechanism by which nebivolol reduces circulating ADMA in hypertensive patients is unclear, our ex vivo results suggest that the upregulation of DDAH2 expression may have a role. These results are consistent with the notion that vasodilating β-blockers may offer some advantage more than traditional β-blockers such as atenolol.

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