RESEARCH

Cardiovascular Therapeutics

Oxidative Stress Impairs Endothelial Function in Nondipper Hypertensive Patients

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SUMMARY

Aims: Essential hypertension, as well as other established cardiovascular risk factors, is associated with endothelial dysfunction. Hypertensive patients with a nondipper circadian pattern have a greater risk of cerebrovascular and cardiovascular complications in comparison with those with a dipper circadian pattern. In this study, we evaluated the association between nondipper pattern and endothelial function in patients with essential hypertension. **Methods:** We evaluated the forearm blood flow (FBF) response to intraarterial acetylcholine (ACh), an endothelium-dependent vasodilator, and sodium nitroprusside (SNP), an endothelium-independent vasodilator, infusions in 190 hypertensive patients stratified according to dipper and nondipper status. The FBF was measured by strain-gauge plethysmography. Effects of oxidative stress on FBF were evaluated by intraarterial infusion of vitamin C. Ambulatory BP monitorings were obtained by a validated oscillometric device (SpaceLabs 90207 Monitor Inc., Issaquah, WA, USA). **Results:** Systolic and diastolic blood pressures were higher during daytime and lower during nighttime in dipper subjects than in nondippers. The peak percent increase in ACh-stimulated FBF was higher in dippers than in nondippers (473% vs. 228%, $P < 0.001$). The FBF responses to SNP were similar in dipper and nondipper patients. The FBF response to ACh during coinfusion of vitamin C was higher in nondippers rather than in dipper hypertensives. **Conclusions:** Present data demonstrate that endothelium-dependent vasodilation is impaired in patients who have nondipper hypertension. The effects of vitamin C on impaired ACh-stimulated vasodilation support the hypothesis that oxidative stress contributes to endothelial dysfunction of nondipper hypertensive patients.

Introduction

Blood pressure (BP) is a quantitative trait with a normal, continuous distribution in the general population. Diseases related to cardiovascular system are the most common cause of morbidity and mortality in the industrialized countries. Essential hypertension is one of the most important risk factors for cardiovascular diseases and clinical outcomes [1]. In addition, hypertension is associated to target-organ damage such as left ventricular hypertrophy [2], microalbuminuria [3,4], or subclinical vascular impairment as endothelial dysfunction [5,6], an early marker of atherosclerosis.

In the last years, some studies showed that 24-h ambulatory monitored BP is a better predictor of cardiovascular events compared with office BP [7,8]. In addition, ambulatory BP monitoring (ABPM) can supply information on day/night BP variations and dipper status, a condition associated to an increased cardiovascular risk in hypertensive patients [9] as well as in general population [10–12]. However, only few papers have demonstrated a significant relationship between BP components or its

circadian rhythm and endothelial dysfunction; in particular, Higashi et al. compared, in a case-control study including a very small group of hypertensive patients, the effect of dipper and nondipper status on endothelial function [13]. On the other hand, there are some experimental evidences demonstrating that the reduced nitric oxide (NO) bioavailability, in hypertensive status, may be secondary to increased degradation by oxygen derived free radicals rather than decreased production by endothelial cells [14].

Thus, the aim of this study was to verify, in a large group of newly diagnosed hypertensive patients without clinical vascular damage, (1) the possible relationship between circadian BP variations and endothelial function evaluated by strain-gauge plethysmography and (2) the role of oxidative stress, evaluated by intraarterial infusion of vitamin C, on vascular reactivity.

Methods

Study Population

A total of 190 outpatients at Catanzaro University Hospital, 105 men and 85 women, aged 20–69 years (mean $\pm$ SD = 45 $\pm$ 10), with well-documented history of essential hypertension were included in the study. All patients were Caucasian and underwent physical examination and review of their medical history. Secondary forms of hypertension were excluded by systematic testing by a standard clinical protocol including renal US studies, computed tomography, renal scan, catecholamine, PRA, and aldosterone measurements. At the time of vascular evaluation, none of the patients had history or clinical evidence of angina, myocardial infarction, valvular heart disease, diabetes, hyperlipidemia, peripheral vascular disease, coagulopathy, or any disease predisposing them to vasculitis or Raynaud's phenomenon. Body mass index (BMI) ranged from 20 to 34 kg/m^2. All patients were newly diagnosed hypertensives and none of them had ever been previously treated with antihypertensive drugs.

The local ethics Committee approved the study, and all participants gave written informed consent for all procedures.

BP Measurements

Readings of clinic BP were obtained in the left arm of the supine patients, after 5 min of quiet rest, with a mercury sphygmomanometer. A minimum of three BP readings were taken on three separate occasions at least 2 weeks apart. Systolic and diastolic BP were recorded at the first appearance (phase I) and the disappearance (phase V) of Korotkoff sounds. Baseline BP values were the average of the last two of the three consecutive measurements obtained at intervals of 3 min. Patients with a clinic BP $\geq$140 mm Hg systolic and/or $\geq$90 mm Hg diastolic were defined as hypertensive.

ABPM was obtained by using a validated oscillometric device (SpaceLabs 90207 Monitor Inc., Issaquah, WA, USA). Recordings of BP and heart rate (HR) were taken every 15 min during the day (from 7:00 AM to 11:00 PM) and every 30 min during the night (from 11:00 PM to 7:00 AM). The dipper pattern was defined as a greater than 10% fall both in the systolic and in the diastolic BP to provide comparability with the definition used by Verdecchia [15]. BP monitorings were performed on a working day with the subjects performing usual daily activities and refraining from heavy physical exercise. Only recordings in which regular sleep was present were included in the analysis.

Forearm Blood Flow Measurements

All studies were performed at 9:00 AM after overnight fasting, with the subjects lying supine in a quiet, air-conditioned room (22–24°C). The subjects were instructed to continue their regular diet; caffeine, alcohol, and smoking were not allowed within at least 24 h before the study. Forearm volume was determined by water displacement. Under local anesthesia and sterile conditions, a 20-gauge polyethylene catheter (Vasculon 2, Baxter Healthcare Corp., Deerfield, IL, USA) was inserted into the brachial artery of the nondominant arm of each subject for evaluation of BP (Baxter Healthcare Corp.) and for drug infusion. This arm was slightly elevated above the level of the right atrium, and a mercury-filled silastic strain-gauge was placed on the widest part of the forearm. The strain-gauge was connected to a plethysmograph (model EC-4, D.E. Hokanson, Issaquah, Washington, DC) calibrated to measure the percent change in volume; this was connected to a chart recorder to obtain the forearm blood flow (FBF) measurements. A cuff placed on the upper arm was inflated to 40 mm Hg with a rapid cuff inflator (model E-10, D.E. Hokanson) to exclude venous outflow from the extremity. A wrist cuff was inflated to supra-systolic BP values 1 min before each measurement to exclude the hand blood flow.

The antecubital vein of the opposite arm was cannulated. The FBF was measured as the slope of the change in the forearm volume; the mean of at least three measurements was obtained at each time point.

Vascular Function

The protocol, previously described by Panza et al. [5], and subsequently used by our group [6], was employed for this study. All patients underwent measurement

of FBF and BP during intraarterial infusion of saline, acetylcholine (ACh), and sodium nitroprusside (SNP) at increasing doses. All participants rested 30 min after artery cannulation to reach a stable baseline before data collection; measurements of FBF and vascular resistance (VR), expressed in units, were repeated every 5 min until stable. Endothelium-dependent and endothelium-independent vasodilation were assessed by a dose–response curve to intraarterial ACh infusions (7.5, 15, and 30 $\mu g/mL^{-1}/min^{-1}$, each for 5 min) and SNP infusions (0.8, 1.6, and 3.2 $\mu g/mL^{-1}/min^{-1}$, each for 5 min), respectively. The sequence of administration of ACh and SNP was randomized to avoid any bias related to the order of drug infusion. The drug infusion rate, adjusted for forearm volume of each subject, was 1 mL/min. ACh (Sigma, Milan, Italy) was diluted with saline immediately before infusion. SNP (Malesci, Florence, Italy) was diluted in 5% glucose solution immediately before each infusion and protected from light with aluminum foil. The reproducibility of the method was tested by Bland–Altman plot.

Evaluation of Oxidative Stress by Infusion of Vitamin C

To evaluate the impact of oxidative stress on endothelium-dependent and endothelium-independent vasodilation in both dipper and nondipper hypertensive patients, ACh and SNP were infused under controlled conditions (saline infusion) and in the presence of intrabrachial vitamin C (24 mg/min), which was administered 5 min before the agonists and continued throughout. This vitamin C concentration has been shown to both protect human plasma from free radical-mediated lipid peroxidation [15] and improve impaired ACh-induced vasodilation in different setting of patients [14,16–20].

The infusion of each vascular active drug was started only after that both FBF and VR reached baseline values.

Statistical Analysis

Data are expressed as mean ± SD (normally distributed data), and comparisons among groups were made by one-way ANOVA, Student's t-test or the χ^2 test, as appropriate. Relationships between paired parameters were analyzed by Pearson product moment correlation coefficient. To test the independent relationship between endothelial function (peak percent increase on FBF) and dipper status, we constructed multiple linear regression models based on a series of traditional (age, gender, smoking, BP, BMI, cholesterol, and triglycerides) and emerging risk factors (fasting insulin and serum CRP). In the multivariate linear regression analysis, data are expressed as standardized regression coefficient (β) and *P*-value. All calculations were made with a standard statistical package (SPSS for Windows version 9.0.1, Chicago, IL, USA).

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as write.

Results

The baseline clinical characteristics of patients, stratified according to dipper status, are summarized in Table 1. As expected, systolic and diastolic BP were higher during daytime and lower during night-time in dipper subjects than in nondippers. There were no significant differences between groups about diurnal and nocturnal pulse pressure values. Nondipper group showed significant higher values of nocturnal HR, triglyceride, glucose, fasting insulin, and CRP.

Vascular Function

Intraarterial infusions of ACh induced a significant ($P < 0.001$) dose-dependent increase in FBF and decrease in forearm VR in both groups. However, nondipper subjects showed a significant lower ACh-stimulated vasodilation in comparison with dippers (Figure 1). In particular, the FBF increments from basal measurements at the three incremental doses of ACh were: 2.6 ± 1.3 (79%), 8.1 ± 4.9 (245%), 15.6 ± 8.2 mL × 100 mL^{-1} of tissue × min^{-1} (473%) in the dippers, and. 1.5 ± 1.1 (47%), 3.8 ± 2.4 (119%), 7.3 ± 4.1 mL × 100 mL^{-1} of tissue × min^{-1} (228%) in the nondippers. At the highest dose of ACh, VR were 7.3 ± 3.2 U in the dipper group and 12.6 ± 5.4 U in the nondippers ($P = 0.0001$). Similarly, during SNP infusions, a significant increase in FBF and a decrease in forearm VR were observed in both groups without any significant difference (Figure 1). No significant change in BP ($P = 0.358$ for nondippers, and $P = 0.700$ for dippers) or HR ($P = 0.317$ for nondippers and $P = 0.207$ for dippers) were observed during the intraarterial infusions of either ACh or SNP.

ACh and Vitamin C coinfusion

Vasodilatory response to ACh significantly increased during coinfusion of vitamin C in both dipper and nondipper patients. However, the improvement in ACh-stimulated vasodilation was significantly highest in nondipper subjects than in dippers (Figure 2), demonstrating that oxidative stress level are different in hypertensive patients showing the dipper or nondipper status. These data also suggest that reduced nitric oxide (NO) bioavailability, observed in nondipper patients, is attributable to reactive

Table 1 Demographic, biochemical and hemodynamic data in hypertensive patients

Parameters	All	Nondippers	Dippers	*P*
No.	190	109	81	
Gender, M/F	105/85	67/42	38/43	0.065
Age (years)	45 ± 10	46 ± 11	43 ± 09	0.115
BMI (kg/m^2)	27.5 ± 2.9	27.9 ± 2.8	27.0 ± 2.9	0.024
Smoking (%)	20	23	22	0.440
Systolic BP (mm Hg)	152 ± 16	152 ± 15	150 ± 16	0.345
Diastolic BP (mm Hg)	95 ± 11	95 ± 11	95 ± 10	0.987
Pulse pressure (mm Hg)	56 ± 12	57 ± 12	55 ± 11	0.223
Heart rate (bpm)	72 ± 9	72 ± 10	72 ± 9	0.888
24-h systolic BP (mm Hg)	141 ± 13	140 ± 11	142 ± 14	0.229
24-h diastolic BP (mm Hg)	85 ± 9	85 ± 9	85 ± 4	0.245
24-h pulse pressure (mm Hg)	56 ± 9	55 ± 9	57 ± 10	0.279
24-h heart rate (bpm)	72 ± 9	72 ± 8	73 ± 8	0.074
Diurnal systolic BP (mm Hg)	145 ± 13	143 ± 12	149 ± 15	0.007
Diurnal diastolic BP (mm Hg)	89 ± 9	87 ± 10	90 ± 9	0.041
Diurnal pulse pressure (mm Hg)	57 ± 10	56 ± 9	58 ± 11	0.094
Diurnal heart rate (bpm)	74 ± 9	75 ± 7	73 ± 8	0.069
Nocturnal systolic BP (mm Hg)	132 ± 12	134 ± 11	129 ± 13	0.006
Nocturnal diastolic BP (mm Hg)	79 ± 9	80 ± 10	77 ± 8	0.021
Nocturnal pulse pressure (mm Hg)	53 ± 9	54 ± 9	52 ± 10	0.189
Nocturnal heart rate (bpm)	65 ± 7	66 ± 6	64 ± 6	0.024
Total cholesterol (mg/dL)	204 ± 32	206 ± 33	199 ± 32	0.094
LDL cholesterol (mg/dL)	129 ± 33	132 ± 34	126 ± 30	0.196
HDL cholesterol (mg/dL)	51 ± 12	51 ± 12	50 ± 12	0.419
Triglyceride (mg/dL)	114 ± 39	120 ± 37	107 ± 40	0.032
Glycemia (mg/dL)	94 ± 11	95 ± 11	94 ± 11	0.570
hs-CRP (mg/L)	4.0 ± 2.4	4.3 ± 2.4	3.5 ± 2.5	0.027
Fasting insulin (μUI/L)	14 ± 7	16 ± 7	13 ± 7	0.0009
Forearm blood flow (mL × 100 mL^{-1} of tissue × min^{-1})	3.3 ± 0.7	3.2 ± 0.7	3.3 ± 0.7	0.504

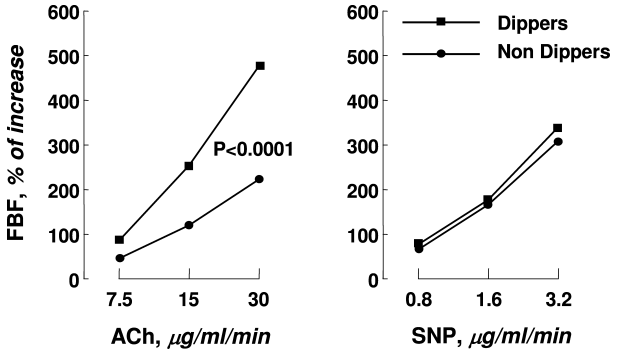

Figure 1 Responses of FBF to intraarterial infusion of ACh and SNP in dippers and non dippers hypertensives subjects.

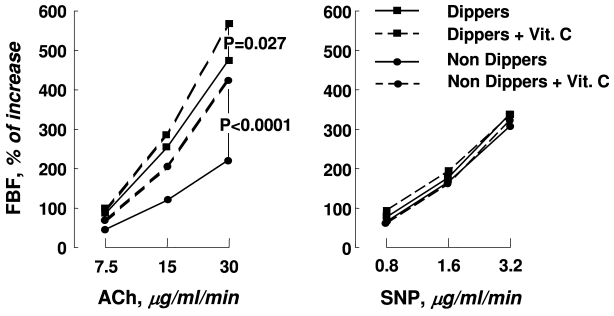

Figure 2 Responses of FBF during ACh and SNP infusions, before and during coinfusion of vitamin C in dipper and nondipper hypertensives subjects.

oxygen species (ROS) inactivation rather than reduced NO synthase activity.

Univariate Analysis

Results of the linear regression analyses are summarized in Table 2. In particular, we observed a significant linear relationship between endothelium-dependent vasodilation and gender and HDL-cholesterol; on the contrary, fasting insulin, CRP, age, BMI, triglyceride and glycemia showed an inverse relationship. No significant correlation was found with LDL-cholesterol, total cholesterol, and smoking.

Table 2 Correlation between peak increase in ACh-stimulated forearm blood flow and independent variables

Variables	Pearson's coefficient	*P*
Fasting insulin (μUI/L)	−0.373	0.0001
hs-CRP (mg/L)	−0.319	0.0001
Gender (F/M)	0.303	0.0001
Age (years)	−0.202	0.003
BMI (kg/m^2)	−0.172	0.009
HDL-cholesterol (mg/dL)	0.162	0.013
Triglyceride (mg/dL)	−0.144	0.023
Glycemia (mg/dL)	−0.133	0.033
LDL-cholesterol (mg/dL)	−0.078	0.142
Cholesterol (mg/dL)	−0.064	0.189
Smoke (n/y)	−0.045	0.269

Table 3 Independent predictors of peak increase in ACh-stimulated forearm blood flow

	Partial r (%)	Total r (%)	Significance (*P*)
Dipper status (%)	37.6	37.6	0.0001
Fasting insulin (μUI/L)	6.9	44.5	0.0001
hs-CRP (mg/L)	4.7	49.2	0.0001
Gender (male)	3.4	52.6	0.001
HDL-cholesterol (mg/dL)	1.0	53.6	0.045

Multivariate Analysis

To test the independence of the association between dipper status and the FBF response to ACh from other risk factors, we performed a multiple regression analysis that included traditional cardiovascular risk factors, CRP, and fasting insulin. Only dipper status ($\beta = 0.526$, $P = 0.0001$), fasting insulin ($\beta = -0.206$, $P = 0.0001$), CRP ($\beta = -0.207$, $P = 0.0001$), gender ($\beta = 0.177$, $P = 0.001$), and HDL-cholesterol ($\beta = -0.106$, $P = 0.045$) were independently related with peak percent increase of ACh-stimulated vasodilation. In particular, the dipper status was the strongest predictor of FBF, accounting for the 37.6% of its variation; the addition of fasting insulin, CRP, gender, and HDL-cholesterol explains another 16% of the variation. The final model accounts for the 53.6% of FBF variation (Table 3).

Discussion

The reduced forearm ACh-stimulated vasodilation in nondipper hypertensive patients in comparison with dipper subjects represents the major finding of this study. Of interest, the excess of oxidative stress, documented in nondipper subjects, may be considered the major novelty of our data; in fact, the intraarterial administration of vitamin C significantly improves the impaired endothelium-dependent vasodilation. Present data are in agreement with that previously reported by Higashi et al. [13], demonstrating that the absence of the BP circadian rhythm induces a significant reduction in NO bioavailability. In addition, our findings also demonstrate that dipper status affects only endothelium-dependent vasodilation, while endothelium-independent vasodilation is unaffected, at least in the first steps of hypertension; in keeping with this, our patients are newly diagnosed and without clinically evident vascular complications. Differently to Alioglu et al. [21], we observed a significant difference in CRP values between dipper and nondipper hypertensive patients. This discordance may be, probably, due to the difference in the study populations. In fact, our patients showed higher BP values and were untreated, while about 25% and 55% of Alioglu's population received, respectively, a statin therapy and ACE-inhibitors, both drugs known to interfere with vascular inflammation.

Previously published experimental [22] and human [23] data demonstrated that endothelial function progressively worsens with the increase of BP; differently, our experiences about a very large population of hypertensive patients [6,24–26] do not confirm these findings. Probably, this discordance is only apparent because all our patients are newly diagnosed, never treated and without clinical evidence of vascular damage, contrary to those investigated by others that showed a history of chronically elevated BP and in treatment, for many years, with one or more antihypertensive agents [23]. So that, it should be of considerable importance to recognize the real association existent between hemodynamic load and endothelial dysfunction. With the exclusion of our previous data [26], demonstrating a significant and negative linear relationship between pulse pressure and endothelium-dependent vasodilation, at this moment there are not other evidences about the correlation between BP levels and the entity of endothelial dysfunction. In our opinion, these arguments are clinically relevant since they are in agreement with the results of this study, in which we have found that the absence of BP circadian rhythm is significantly associated with endothelial dysfunction, irrespective of 24-h BP values.

At this moment, it is not possible to hypothesize if the absence of circadian rhythm induces endothelial dysfunction or vice versa. Probably, even if speculative, it is likely that this is a bidirectional phenomenon involving NO bioavailability and BP circadian rhythmicity. With reference to the first assumption, different BP components, such as flow pulsatility, interfering with both shear stress and oxidative stress, may be considered as responsible of reduced NO bioavailability [26]. Nevertheless,

it is plausible that oxidative stress itself influences the nondipper status reducing physiological night-time BP decrease. In this study, the nocturnal systolic BP was higher in nondippers than in dippers. In keeping with this, it is possible to hypothesize that the inappropriate nocturnal BP reduction represents the main mechanism by which endothelium-dependent vasodilation is damaged in nondipper patients. Consistent with this hypothesis, even if we did not directly measure the oxidative stress, it is possible to speculate that systolic BP elevation reduces ACh-stimulated vasodilation reducing NO bioavailability as consequence of low shear stress. In fact, it is demonstrated that systolic BP elevation is associated with continuous oscillatory shear stress that causes a sustained activation of pro-oxidant processes resulting in redox-sensitive gene expression [27]. In addition, this condition also promotes the activation of the nuclear factor-kappa B, resulting in an increase of oxidative stress [28]. Taken together, laminar flow-mediated shear stress rather than oscillatory shear stress appears to exert protective antiatherosclerotic vascular effects. The improvement observed in ACh-stimulated FBF during coinfusion of vitamin C in nondippers represent a compelling evidence to confirm the role of ROS in the appearance of endothelial dysfunction. With the reference to the second assumption, recent data, obtained by Kunieda et al. in an animal model, support the possible role of NO in the regulation of BP circadian rhythmicity; in fact, they demonstrated that a reduced NO production is responsible of an impairment of BP circadian rhythm that is restored with a passive NO donor [29]. Consistent with this hypothesis, even if we did not directly measure the oxidative stress, it is possible to speculate that, in our nondipper patients, an NO reduced bioavailability, due to increased production of ROS, induces a loss of BP circadian rhythm. These mechanisms, even if they remain still too speculative, are consistent with previous experimental findings.

Conclusions

In conclusion, we demonstrated that (1) the nondipper status when compared with patients showing a normal circadian variation is associated with a reduced endothelium-dependent vasodilation and (2) the endothelial dysfunction is due, at least in part, to increased oxidative stress, which can be improved by the administration of intrabrachial vitamin C. Differently from that of Higashi et al. [13], our study is not a case-control one and was performed in a larger group of never-treated hypertensive patients. Thus, these characteristics confer to our results a reliable interesting clinical importance, contributing to explain the high risk for cardiovascular disease or stroke observed in hypertensive patients showing an abnormal decline in nocturnal BP [14,30].

Considering the key role of normal endothelium in antiproliferative and antiatherosclerotic processes, we suggest that endothelial dysfunction in hypertensive nondipper patients may confer an increased risk of macrovascular diseases. As previously reported by us [6] and others [31,32] endothelial dysfunction, an early step in the appearance and progression of atherosclerosis, is a powerful independent predictor of cardiovascular events. In keeping with this, it should be clinically relevant in hypertensive patients (1) to reach informations about the BP circadian rhythm to stratify the global cardiovascular risk and (2) to administrate a pharmacological treatment aimed to reduce especially the systolic component of BP and to improve endothelial dysfunction.

Study Limitation

The major limitation of this study is that we did not measured F-2 isoprostan, oxidized LDL or peroxynitrite, which could provide additional information about oxidative stress.

Author Contribution

Raffaele Maio: design, data analysis, data interpretation, drafting article

Maria Perticone: design, data analysis, data interpretation, drafting article, critical revision

Angela Sciacqua: data analysis, data interpretation, statistics, critical revision

Eliezer J Tassone: data collection, data analysis, data interpretation

Paola Naccarato: data collection, data analysis, data interpretation, statistics

Chiara Bagnato: data collection

Gianmarco Iannopollo: data collection

Giorgio Sesti: design, data analysis, data interpretation, drafting article, critical revision

Francesco Perticone: design, data analysis, data interpretation, drafting article, approval

Conflict of Interest

The authors declare no conflict of interests.

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