Original Article

Effect of direct renin inhibition on vascular function after long-term treatment with aliskiren in hypertensive and diabetic patients

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Objective: We tested the hypothesis that chronic treatment with the direct renin inhibitor aliskiren improves vascular function in resistance and conduit arteries of type two diabetic and hypertensive patients.

Method: Sixteen patients with mild essential hypertension and with a previous diagnosis of noninsulin-dependent diabetes mellitus were included in the study. Patients were then randomized to aliskiren (150 mg once daily, $n = 9$), or ramipril (5 mg once daily, $n = 7$). Each patient underwent a biopsy of the subcutaneous tissue and small arteries were dissected and mounted on a pressurized micromyograph to evaluate endothelium dependent vasorelaxation in response to acetylcholine ± *N* omega-nitro-L-arginine methyl ester hydrochloride in vessels precontracted with norepinephrine. Endothelial function has been quantified also in large conduit arteries by flow-mediated dilation.

Results: A similar office blood pressure-lowering effect was observed with the two drugs, although changes in DBP were not statistically significant in the ramipril group. Aliskiren significantly improved endothelium-dependent relaxation in subcutaneous resistance arteries, as well as increased flow-mediated dilation in conduit arteries, whereas the effects induced by ramipril did not reach statistical significance. Only aliskiren significantly increased the expression of p1177-endothelial nitric oxide synthase in the endothelium. Both aliskiren and ramipril had a negligible effect on markers of oxidative stress.

Conclusion: Aliskiren restored endothelial function and induced a more prompt peripheral vasodilation in hypertensive and diabetic patients possibly through the increased production of nitric oxide via the enhanced expression and function of the active phosphorylated form of endothelial nitric oxide synthase.

Keywords: aliskiren, diabetes, endothelium, hypertension, nitric oxide, renin, resistance arteries, vasodilation

Abbreviations: ACE, angiotensin-converting enzyme; Ang II, angiotensin II; ARB, angiotensin II receptor antagonist; BP, blood pressure; DRI, direct renin inhibitor; eNOS, endothelial nitric oxide synthase; FMD, flow-mediated dilation; L-NAME, *N* omega-nitro-L-arginine methyl ester hydrochloride; NIDDM, noninsulin dependent diabetes mellitus; PWV, pulse wave velocity; RAAS, renin–angiotensin–aldosterone system

INTRODUCTION

The renin–angiotensin–aldosterone system (RAAS) plays a key role in blood pressure (BP) regulation. Experimental and clinical evidence has indicated that RAAS activation is involved in the pathogenesis of hypertension and related target organ damage. In particular RAAS is involved in a different range of physiological and pathological processes including vasoconstriction, oxidative stress, inflammation, fibrosis, vascular growth, and hypertrophy leading to endothelial dysfunction and vascular remodeling [1,2]. Endothelial dysfunction is considered as fundamental to the development of hypertension, vascular remodeling, and atherosclerosis [3]. Furthermore, endothelial dysfunction as well as arterial stiffness and remodeling are important determinants of cardiovascular events in patients with arterial hypertension, since arterial stiffness and endothelial dysfunction progress in proportion to cardiovascular risk factors burden, including aging, hypertension, and diabetes [3,4]. In turn, type 2 diabetes contributes to the impaired endothelial function and promotes arterial stiffness, particularly in hypertensive patients [3,5]. Available evidence has demonstrated an association

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between diabetes and RAAS activation, in particular has been described a prominent involvement of angiotensin II (Ang II) in diabetic complications, including nephropathy and cardiovascular dysfunction [6]. Moreover, RAAS participates in the mechanisms through which hyperglycemia and insulin resistance may affect vascular function [1–3,5].

It has been demonstrated that RAAS blockade obtained with angiotensin-converting enzyme (ACE) inhibitors or Ang II receptor antagonists (ARBs) is useful in terms of vascular protection for the management of hypertension [7,8] and the related vascular structural alterations, particularly when associated with diabetes. Indeed, beyond the BP reduction the effects of these drugs include the reversing of arterial stiffness and remodeling, mainly in high risk hypertensive and diabetic patients [7,8]. Nevertheless, conflicting evidence exists on the role of RAAS blockers on vascular function especially in the peripheral resistance arteries, since these drugs do not always improve endothelium-dependent vasodilation in both experimental and clinical studies [9–11].

Possibly these controversial results might be explained by different causes including the not complete RAAS blockade in arterioles during ACE inhibition and ARBs treatment. Significantly, plasma Ang II concentration increases after ARBs treatment despite a decrease in aldosterone levels, and may have a detrimental effect on vascular function [12]. Furthermore, plasma renin activity (PRA), which is associated to cardiovascular disease development, is increased by ACE inhibition and ARB treatment [12]. Therefore, an alternative strategy to block RAAS avoiding the secondary increase of plasma renin or Ang II may be potentially clinically relevant.

Aliskiren is a direct renin inhibitor (DRI) that blocks the RAAS at the first limiting step in the pathway, reducing PRA and the circulating levels of angiotensin, and aldosterone. Experimental and clinical studies [13–17] have demonstrated the beneficial effects of aliskiren on BP reduction as well as on endothelial function in patients with essential hypertension. However, conflicting data were reported on the protective role of aliskiren on vascular function in high cardiovascular risk patients such as the hypertensive patients with ischemic heart disease [16]. Moreover, it has been shown that the combination of aliskiren with ACE inhibitors may improve endothelial function in type 1 diabetic patients [15], although several clinical data have discouraged this association particularly in type 2 diabetic patients [13]. Furthermore, the results of the major clinical trials in heart failure [18–20] suggested that such combination therapy is associated with more adverse events without an increase in benefit particularly in diabetic patients. Although in most of these studies a greater incidence of side effects, such as renal failure, hyperkalemia, and hypotension were reported, the ultimate mechanisms of such adverse effects have not been fully elucidated and remain elusive. Thus the meaning and mechanism of PRA blockade remains less clear in terms of vascular protection, particularly in higher cardiovascular risk patients such as the hypertensive and diabetic patients. We previously reported that aliskiren may improve vascular remodeling in hypertensive and diabetic patients [21]. However, the role of aliskiren on vascular function remains not fully described particularly in resistance arteries of diabetic patients. Thus, the aim of this study is to evaluate the effect of long-term treatment with aliskiren on vascular function in hypertensive and type 2 diabetic patients who are at higher risk to develop cardiovascular disease.

METHODS

Patients and trial design

This was a randomized, comparative, and single-blind study. The study protocol was approved by the Ethics Committee of Sant'Andrea Hospital, Faculty of Medicine and Psychology, University of Rome, and by the Ethics Committee of the University of Brescia Medical School. Each participant provided informed consent. The procedures followed were in accordance with institutional guidelines. Sixteen patients, aged between 30 and 70 years, with mild essential hypertension (sitting DBP between 90 and 99 mmHg or sitting SBP between 140 and 159 mmHg at the end of a 3-week placebo run-in period) [22] and with a previous diagnosis of non-insulin dependent diabetes mellitus (NIDDM), with or without ongoing oral hypoglycemic therapy, were enrolled in the study from a population of individuals who were part of a clinical trial that we reported previously [21]. The details of the inclusion criteria and protocol of the study were reported previously [21]. The demographic characteristics of the patients are summarized in Table 1. Briefly, nine patients had never received antihypertensive medication. In seven patients, previous antihypertensive therapy was withdrawn 2 weeks or less before enrollment. The characteristics of previous antihypertensive therapy were similar in the two groups (Table 1). Patients previously treated with RAAS blockers as well as patients with secondary forms of hypertension or with any disease that could have interfered with the study protocol, were excluded. NIDDM was assessed according to the Guidelines of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus [23].

Patients were randomly assigned to one of the two active treatments (aliskiren 150 mg once daily, $n = 9$; or ramipril 5 mg once daily, $n = 7$). After 2 weeks of active treatment, if BP was more than 130/85 mmHg, the dose of aliskiren or ramipril was doubled (300 mg once daily and 10 mg once daily, respectively). Open-label hydrochlorothiazide 12.5 mg once daily was added if BP was uncontrolled after 6 weeks, and doubled after 10 weeks of treatment if BP still remained uncontrolled.

Visits for patients re-evaluation were scheduled at 6 and 12 months after enrollment.

Venous blood samples were obtained with the participants in the supine position, after a wash-out period of 2 weeks or less, for standard hematology and serum biochemistry tests (including triglycerides and total cholesterol), at baseline and 12 months after enrollment. BP was measured using a standard sphygmomanometer.

Evaluation of endothelial function

Endothelial function of resistance arteries

The functional study of resistance arteries was performed by individuals unaware of the groups to which samples belonged. Gluteal subcutaneous biopsy (3-cm long, 0.5-cm wide, 1.5-cm deep) was obtained under local anesthesia (2% lidocaine) at baseline and at the end of the study (12 months) as described previously [21,24]. Small arteries (≈100–280 μm of average diameter in relaxed conditions; 2-mm long) were

TABLE 1. Demographic data

	Group randomized to ramipril, $n=7$		Group randomized to aliskiren, $n=9$	
	Basal	12 Months	Basal	12 Months
Age (year)	60 ± 11	+1	54 ± 6	+1
Sex (male/female)	5/2		8/1	
Body weight (kg)	95.9 ± 16.2	94.1 ± 14.0	102.6 ± 15.4	99.8 ± 14.5*
BMI (kg/m^2)	32.8 ± 4.61		34.8 ± 3.84	
Peripheral SBP (mmHg)	151 ± 10.6	121 ± 12.1**	153 ± 8.9	128 ± 7.3***
Peripheral DBP (mmHg)	84.7 ± 12.22	78.6 ± 7.48	94.2 ± 7.17	81.4 ± 6.31**
Peripheral mean blood pressure (mmHg)	107 ± 10.41	92.6 ± 8.86*	114 ± 6.39	97.0 ± 5.68***
Known duration of hypertension (year)	3.25 ± 2.5	+1	2.2 ± 0.5	+1
No. of patients who never received antihypertensive Treatment (before randomization)	3 (43%)		6 (67%)	
No. of antihypertensive medication per patient	0.6		1.0	
No. of patients on CCBs	1 (14%)		2 (22%)	
No. of patients on β-blockers	2 (29%)		0	
No. of patients on α-blockers	1 (14%)		2 (22%)	
No. of patients on diuretics	3 (43%)		1 (11%)	
Known duration of diabetes mellitus (year)	4.3 ± 5.1	+1	5.3 ± 8.4	+1
Number of patients on oral hypoglycemic therapy	6/7 (86%)		8/9 (89%)	
Number of patients on lipid-lowering agents	3/7 (43%)		5/9 (56%)	
Smokers (yes/no/ex)	2/4/1		3/4/2	
Fasting glucose (mg/dl)	137.6 ± 16.9	142.3 ± 33.7	138.9 ± 25.4	135.9 ± 49.8
Hemoglobin A1c (%)	7.9 ± 1.2	6.9 ± 0.6	7.4 ± 1.2	7.2 ± 1.6
Serum creatinine (mg/dl)	0.78 ± 0.08	0.82 ± 0.13	0.77 ± 0.13	0.80 ± 0.13
Cholesterol (mg/dl)	207 ± 34.1	212 ± 21.7	202 ± 52.7	181 ± 44.4
Triglycerides (mg/dl)	191 ± 31.8	184 ± 84.0	166 ± 75.8	162 ± 50.6

Data are expressed as mean ± SD. CCBs, calcium channel blockers.
*$P < 0.05$.
**$P < 0.01$ vs. basal.
***$P < 0.001$.

dissected from the subcutaneous fat of the biopsy samples and mounted on a pressurized myograph immediately after biopsy. Vessels were equilibrated and relaxed for 30 min or less in Krebs saline solution. Endothelium-dependent and endothelium-independent relaxations were assessed by the dilatory responses to acetylcholine hydrochloride (1 nmol/l to 100 μmol/l) ± *N* omega-nitro-L-arginine methyl ester hydrochloride (L-NAME) (100 μmol/l) and sodium nitroprusside (SNP; 10 nmol/l to 1 mmol/l), respectively, in vessels precontracted with norepinephrine (1 μmol/l).

Flow-mediated dilation

In six patients per group, high-resolution ultrasound was used to measure changes in brachial artery diameter, according to the previously described technique [25]. Ultrasound studies were performed in the morning, after the patients had rested in the supine position for 30 min in a quiet room with optimal temperature conditions. We recommended that patients eat a light meal before the exam and to avoid drinking coffee or tea in the 2 h before the study.

In all patients, it was possible to record good quality scans using a 7.5-MHz linear array ultrasound probe (7.5 MHz, Philips IE33: Philips: North America Corporation, Andover, Massachusetts, USA) carried out by two dedicated physician. Scans of the brachial artery approximately 5 cm above the elbow were obtained in longitudinal section and the transducer was maintained in a fixed position relative to the patient's arm by using a mechanical support, which allowed micrometric movements of the probe [25]. This device allows a perpendicular visualization of the artery walls, which is necessary to assess the real measure of the diameter and its changes induced by reactive hyperemia and by glyceryl trinitrate (GNT). Ultrasound images of the brachial artery were analyzed by dedicated software [26] (FMD Studio, CNR-Pisa, Pisa, Italy), allowing the measurement of the vessel diameter as the instantaneous distance between the two lines corresponding to the proximal and distal artery walls (with a frequency of 25 frames per second). Flow-mediated dilation (FMD) and GNT-induced dilation were determined as the percentage diameter change relative to baseline measurements. Arterial flow velocity was measured by means of a pulsed Doppler signal, with the sample volume placed in the center of the artery. Flow increase was induced by inflation of a BP cuff placed around the arm up to 50 mmHg above the SBP, and after 5 min of arterial occlusion, the cuff was deflated. An interval of 20 min was allowed for vessel recovery and sublingual GNT (spray, 40 μg) was administered. Scans were recorded twice at baseline, before and up to 120 s after the release of occlusion and up to 4 min after the administration of sublingual GNT. Brachial blood flow was calculated from Doppler flow velocity measurements. A continuous ECG trace was recorded throughout the test to have a reference point of the cardiac cycle.

Evaluation of pulse wave velocity

In six patients per group, pulse wave velocity (PWV) was measured at the carotid and femoral locations using the foot-to-foot velocity method [27]. Waveforms were obtained transcutaneously over the common carotid artery and the right femoral artery, and the time delay [transit time (*t*)] was measured between the feet of the two waveforms (Complior SP; Alam Medical, Saint Quentin Fallavier, France). The distance (*D*) covered by the waves is assimilated to the distance measured between the two recording sites (carotid–femoral distance). PWV is calculated as PWV = *D* (m)/*t* (s); all calculations, including measurement of parameters over 5–10 cardiac cycles, are automated. We used 80% of this distance as pulse wave travelled distance (*D*) and calculated PWV by the formula [*D* (m)/*t* (s)] × 0.80;

accordingly, an increase of PWV, at least 10 m/s, was considered as macrovascular target organ damage.

In all patients, applanation tonometry was also performed using a SphygmoCor device (AtCor Medical, West Ryde, New South Wales, Australia), as described previously [28]. Briefly, the applanation probe is positioned on the radial artery (right arm), and optimal applanation is obtained using visual inspection and following built-in quality control indices. BP is measured again using an Omron 705C oscillometric device (Omron Healthcare Europe BV, Hoofddorp, The Netherlands) and radial waveforms calibrated using brachial SBP and DBP measured before and after applanation. The central aortic waveform was calculated by the device software using the generalized transfer function for calculation of central SBP, DBP, and pulse pressure (PP) [28].

Echocardiographic parameters

In a subgroup of patients, left ventricular (LV) mass index and LV relative wall thickness were measured by transthoracic echocardiography. Echocardiography followed a standardized procedure. End diastolic LV diameter, septal, and posterior wall thickness were measured according to American Society of Echocardiography recommendations. LV mass was calculated using the formula recommended by international recommendations [29], and LV mass index was calculated by adjustment for height at the 2.7 power

Immunohistochemistry for endothelial nitric oxide synthase, nitrotyrosine, lectin-like oxidized LDL receptor 1 on resistance arteries

Part of vessels dissected from gluteal biopsy were immediately fixed with 10% formalin (pH 7.2–7.4), glutaraldehyde fixative solution for 24 h. Due to very limited size (1 mm length 4 mm) of the arterioles, all samples were processed manually. Therefore, the vessels were dehydrated through graded ethanol, and then embedded in paraffin. Sections (3 μm) were deparaffinized, rehydrated and stained with routine staining, hematoxylin–eosin (H&E), following standard techniques and visualized using an optical microscope. For immunohistochemistry, consecutive sections (3 μm) of arterious ring were deparaffinized in xylene and rehydrated through graded alcohol series. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide. The sections were then incubated at 4 °C overnight with primary antibody. Primary antibodies were the following: anti-eNOS (endothelial nitric oxide synthase) (1 : 300, anti mouse monoclonal eNOS; Novus Biologicals, Cambridge, UK, catalog number: NBP1-51582); antiphospho eNOS (1 : 75, anti rabbit policlonal eNOS phospho S1177; Abcam, Cambridge, UK, catalog number: ab75639); anti-lectin-like oxidized LDL receptor 1 (LOX-1) (1 : 100, anti rabbit policlonal anti LOX-1; Abcam, catalog number: ab126538); anti-nitrotyrosine (1 : 150, anti rabbit policlonal anti-nitrotyrosine; Abcam, catalog number: ab42789). After incubation, tissue sections were rinsed with PBS. The reaction was amplified with the LSAB2+ System-HRP (DAKO, Carpinteria, California, USA). A positive immunoreaction was identified after incubation with 3,3′-diaminobenzidine and counterstaining with Mayer hematoxylin. Negative controls were obtained omitting the primary antibody. Two independent investigators blinded to the treatment, analyzed the immunostaining for each antigen with a Leica microscope (Leitz Camera, Wetzlar, Germany) and, successively, 5-Mp (24-bit color depth) images of arterious ring (five images with same area, 40× objective), were captured by a computerized digital camera (Olympus Camedia 5050; Olympus, Tokyo, Japan) using SPOT (Diagnostic Instruments, Sterling Heights, Michigan, USA); immunostaining was quantified by a pathologist, using a computerized imaging software (Image J; NIH, Bethesda, Maryland, USA) and expressed as percentage (ratio of immunostaining area to total area).

Statistical analysis

All data are expressed as mean ± SEM, unless otherwise stated. To evaluate differences between and among groups data were analyzed by two-way ANOVA or one-way ANOVA followed by Newman–Keuls test, and Student paired and unpaired *t* tests as appropriate. *P* less than 0.05 was considered statistically significant.

RESULTS

Demographic data

Demographic data of patients enrolled in this study are synthetically reported in Table 1. Briefly, the two groups were similar at baseline with respect to BP values, metabolic control, and renal function. Known duration of previous antihypertensive treatment was also similar in both groups. Peripheral SBP was significantly and equally reduced by both treatments, whereas peripheral DBP was significantly reduced only in patients receiving aliskiren (Table 1). Parameters of central BP were similar in both groups and were not modified after 1 year of treatment (Table 2).

TABLE 2. Central hemodynamic parameters, pulse wave velocity, flow-mediated dilation, echocardiographic data

	Ramipril-treated group, $n = 6$		Aliskiren-treated group, $n = 6$	
	Basal	12 Months	Basal	12 Months
Central SBP (mmHg)	125.5 ± 2.377	117.4 ± 7.541	129.5 ± 7.215	127.2 ± 6.779
Central DBP (mmHg)	82.17 ± 4.651	72.80 ± 3.707	84.67 ± 3.921	85.33 ± 6.270
Central mean blood pressure	100.0 ± 3.540	91.80 ± 4.398	103.7 ± 4.470	102.5 ± 6.190
cfPWV (m/s)	9.552 ± 0.5463	10.00 ± 0.9157	9.292 ± 0.6238	9.562 ± 0.7285
Pulse pressure (mmHg)	63.00 ± 5.939	57.60 ± 6.038	55.00 ± 3.882	54.00 ± 3.225
ΔFMD (%)		−16.20 ± 14.17		112.1 ± 49.9*
Ejection fraction (%)	62.50 ± 1.478	60.80 ± 3.693	65.17 ± 1.990	64.50 ± 3.557
Left ventricular mass (g/m$^{2.7}$)	37.24 ± 4.355	33.40 ± 4.130	47.72 ± 5.750	45.00 ± 5.196

Data are expressed as mean ± SEM. ΔFMD, magnitude of flow-mediated dilation variation after 12 months; cfPWV, carotid–femoral pulse wave velocity.
*$P < 0.05$ vs. ramipril group.

Endothelial function

Nitric oxide bioavailability is traditionally quantified in humans by evaluating the endothelium induced relaxation in response to acetylcholine ± L-NAME in resistance arteries as well as by evaluating FMD in large arteries *in vivo*. Acetylcholine-dependent relaxations were similarly impaired in peripheral resistance arteries of patients randomized to both treatments (Fig. 1a). After 1 year, acetylcholine-induced relaxation was significantly improved only in patients randomized to aliskiren as compared with pretreatment (+50%, $P < 0.05$; maximal vasodilatation +84%, $P < 0.01$) and not in patients randomized to ramipril. This difference in endothelial function was also evident by comparing the acetylcholine-induced relaxation's curves in both treatment's groups after 1 year (maximal vasodilation +26% in aliskiren vs. ramipril-treated group, $P < 0.05$, Fig. 1b). Endothelial independent relaxation in response to SNP was similarly preserved in all the groups (Fig. 1c).

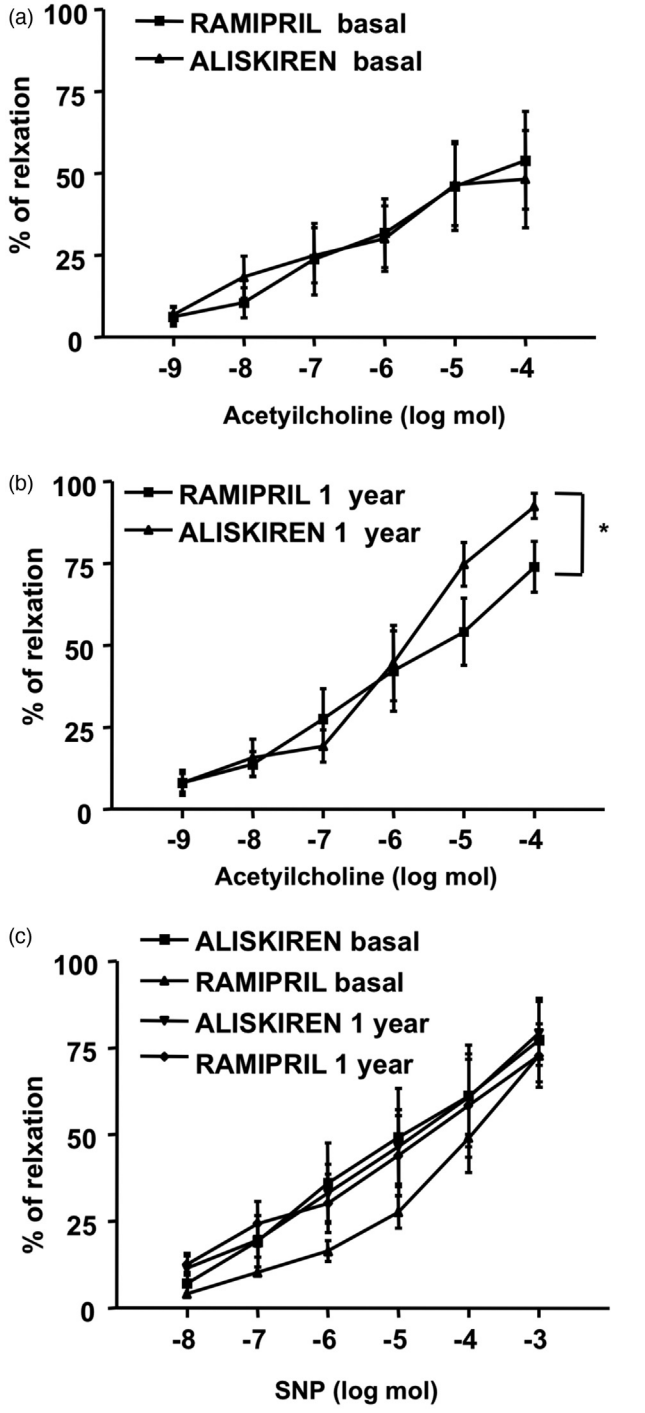

FIGURE 1 Dose–response curves to acetylcholine and sodium nitroprusside in resistance arteries precontracted with norepinephrine before and after aliskiren and ramipril treatment. *$P < 0.05$.

Acetylcholine-induced vasodilation was significantly blunted by L-NAME only in hypertensive and diabetic patients after aliskiren treatment (Fig. 2c and d), and not in ramipril-treated group (Fig. 2a and b). Taken together these data suggest that aliskiren improved endothelial function through the increased nitric oxide bioavailability.

FMD was performed in six patients from each group. FMD was similar in patients randomized to both ramipril and aliskiren before treatment and improved after 1 year of treatment only in aliskiren treated group. The analysis of repeated measurement showed that aliskiren-treated patients had a significant improved magnitude of FMD variation (ΔFMD) after 12 months as compared with ramipril-treated patients (ΔFMD in aliskiren-treated group +112.1 ± 49.9% vs. ΔFMD in ramipril-treated group −16.2 ± 14.17%, respectively, $P < 0.05$) (Fig. 3). Taken together those data suggest that only the DRI may improve endothelial function in diabetic and hypertensive patients after 1-year treatment independently of BP control.

Pulse wave velocity and echocardiographic parameters

PWV and PP, both indexes of large arteries stiffness, were similar in both groups before treatment and were not modified by both drugs after 1 year in diabetic and hypertensive patients (Table 2).

Ejection fraction and LV mass were similarly preserved in both groups and did not change significantly after 1 year of treatment (Table 2).

Endothelial nitric oxide synthase expression and reactive oxygen species production

To further analyze the effect of aliskiren on endothelial function we investigated the expression of the active phosphorylated form of eNOS (S1177-p-eNOS), presented as a ratio with respect to the total eNOS. S1177-p-eNOS was similar in resistance arteries from both groups before treatment and it was increased more than 25% only in aliskiren-treated patients as compared with pretreatment ($P < 0.01$; Fig. 4) and in ramipril-treated patients as well ($P < 0.05$).

To study the effect of aliskiren on reactive oxygen species production we evaluated markers of oxidative stress in the vasculature. Both nitrotyrosine and LOX-1 were similar in patients before treatment and did not change significantly after 1 year of treatment (Figs. 5 and 6), although nitrotyrosine showed a NS trend in reduction only in patients treated with aliskiren.

Taken together those data suggest that the improvement of endothelial function induced by aliskiren is associated to

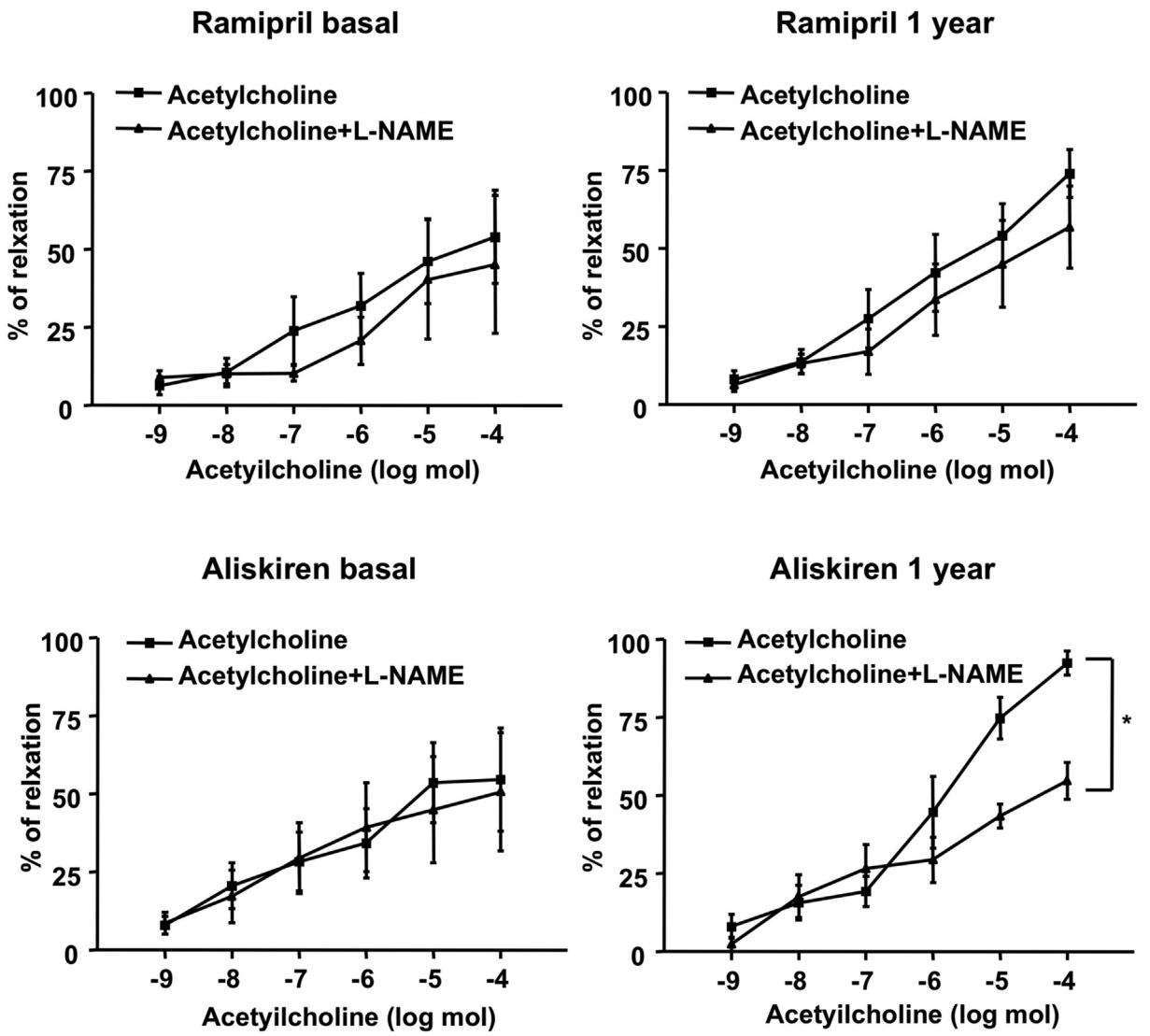

FIGURE 2 Dose–response curves to acetylcholine ± *N* omega-nitro-L-arginine methyl ester hydrochloride in resistance arteries precontracted with norepinephrine before and after aliskiren and ramipril treatment. $^{*}P < 0.05$.

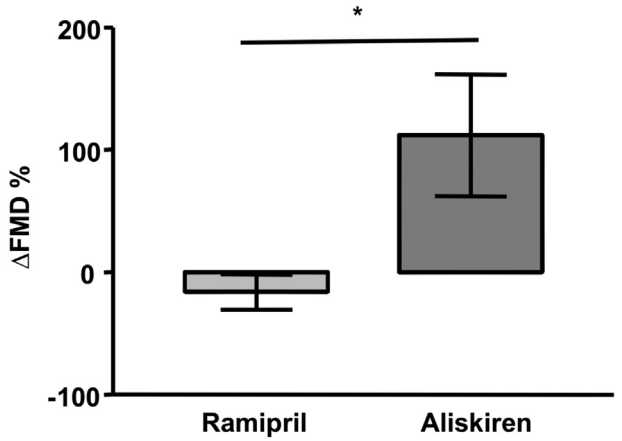

FIGURE 3 Flow-mediated dilation in patients treated with ramipril and aliskiren. The results are expressed as the magnitude of flow-mediated dilation variation at the end of the treatment with respect to the values before the treatment for each group. ΔFMD = magnitude of flow-mediated dilation variation at the end of treatment with respect to the basal values. $^{*}P < 0.05$.

the enhancement of nitric oxide production possibly through the increased expression of S1177-p-eNOS in the endothelium of hypertensive and diabetic patients.

DISCUSSION

In spite of the evidence on the beneficial effects of aliskiren in monotherapy on BP control and vascular protection, several concerns have been raised about the use of aliskiren in diabetic patients alone or in combination with other RAAS blockers [13,30]. The precise mechanisms of the protective/adverse effects in the vascular system of diabetic and hypertensive patients remain not fully elucidated. Therefore, we sought to expand further on the putative vascular protective mechanisms of aliskiren treatment in hypertensive and diabetic patients. We previously reported that aliskiren improves vascular remodeling in hypertensive and diabetic patients [21], here we wanted to focus on the vascular functional effects of aliskiren treatment. The major

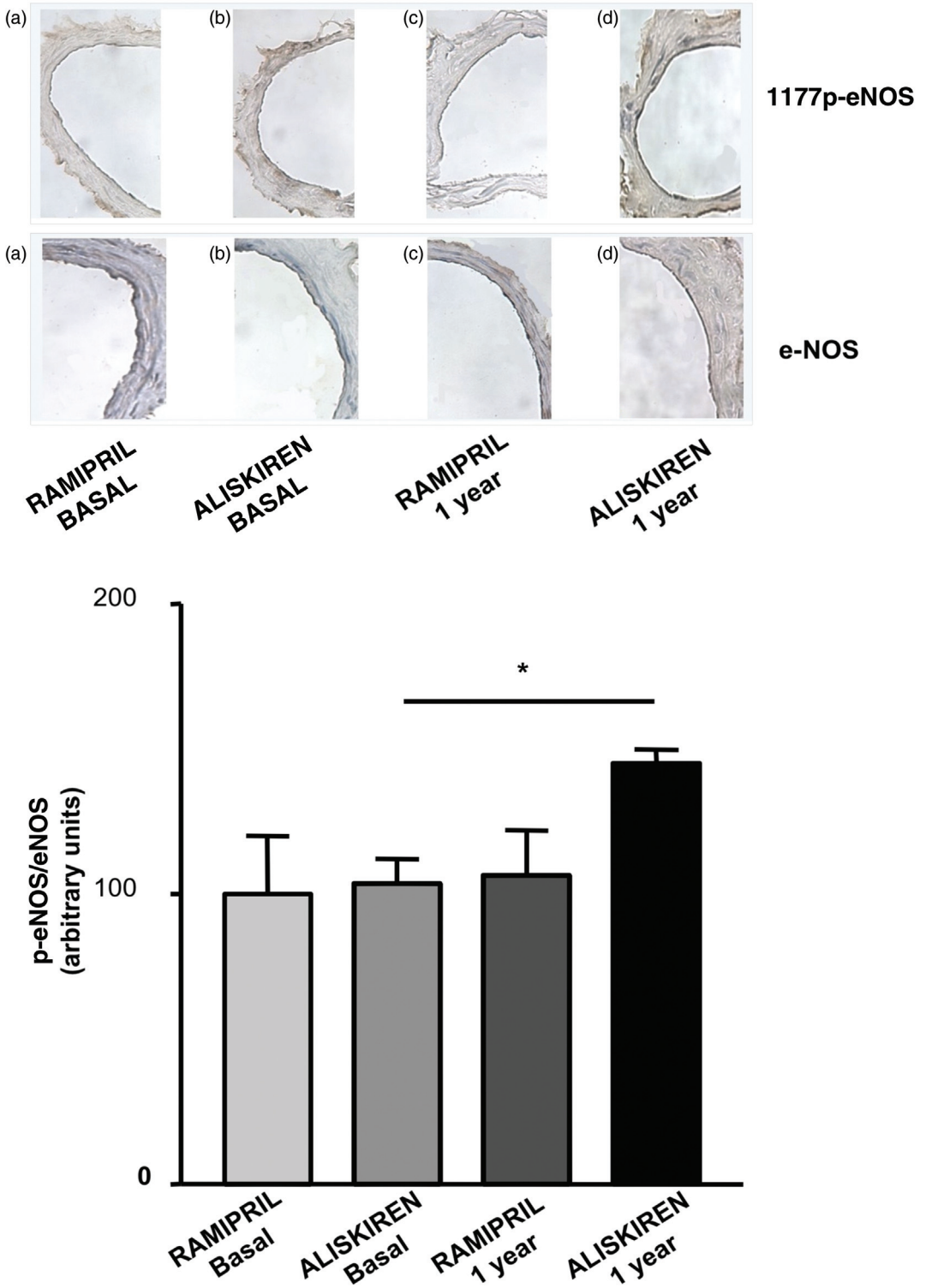

FIGURE 4 p-Endothelial nitric oxide synthase/endothelial nitric oxide synthase expression in resistance arteries of hypertensive and diabetic patients treated with aliskiren and ramipril. $^{*}P < 0.05$.

findings from this study are the following: equieffective doses of aliskiren and ramipril had similar hemodynamic effects, as both drugs induced similar BP control after 1-year treatment in hypertensive and diabetic patients; only aliskiren improved endothelial function of resistance as well as conduit arteries of hypertensive and diabetic patients; consistently only aliskiren increased the nitric oxide bioavailability and the expression of the phosphorylated active form of eNOS in the endothelium, and it had NS effect on oxidative stress production in the vasculature of diabetic and hypertensive patients who are at higher cardiovascular risk.

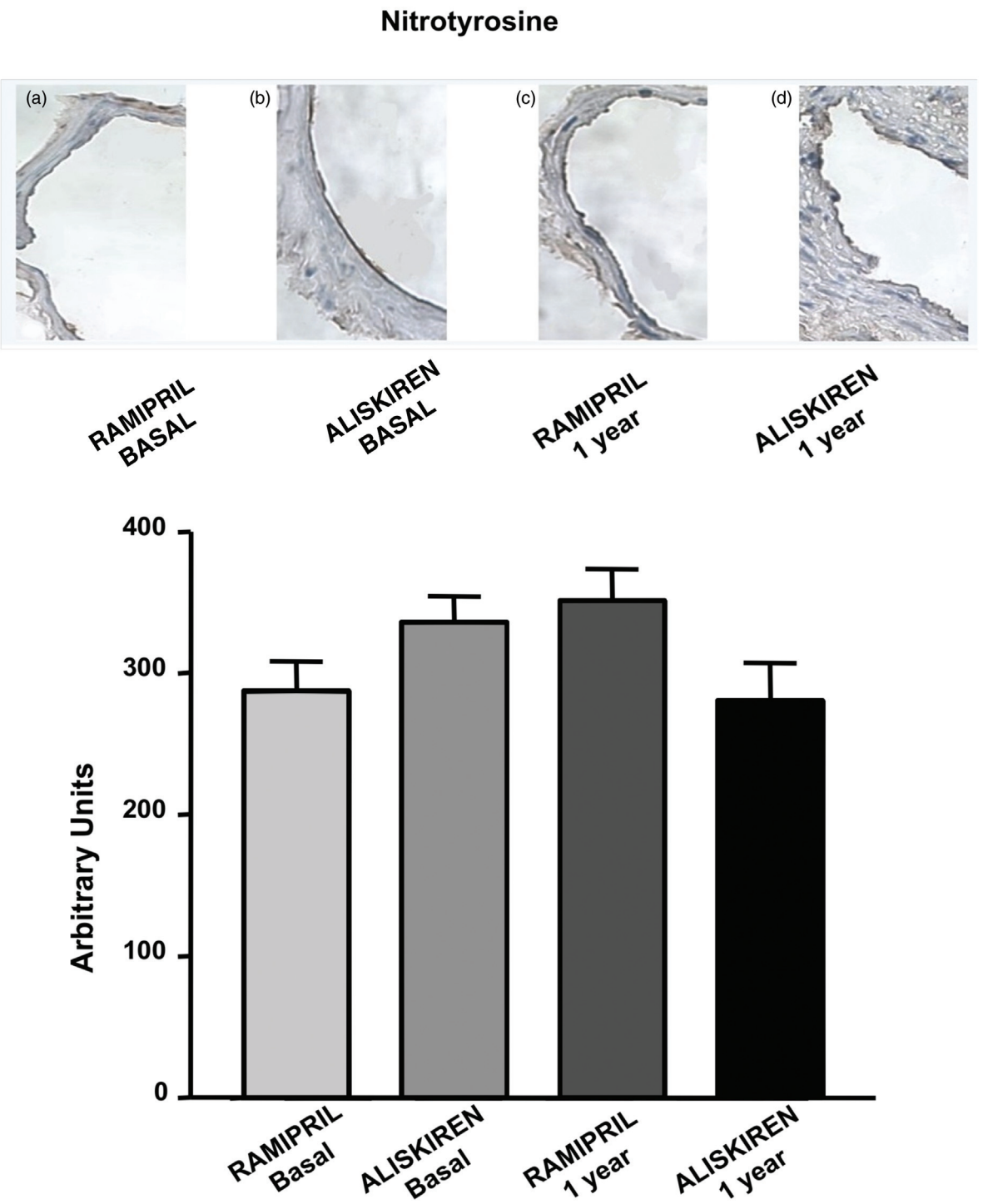

FIGURE 5 Nitrotyrosine expression in resistance arteries of hypertensive and diabetic patients treated with aliskiren and ramipril.

This is the first prospective study aimed to compare the effect of long-term treatment with a DRI vs. an ACE inhibitor on endothelial function and vascular protection of resistance arteries in high cardiovascular risk patients such as the hypertensive patients with NIDDM. Resistance arteries represent the key element in BP control, since resistance arteries increase peripheral vascular resistance to blood flow, and functional and structural changes in the microcirculation may directly affect BP values. In hypertension as well as diabetes, resistance arteries undergo functional alterations and vascular remodeling (reduced vascular lumen with increased media thickness) that may be functional, mechanical, and structural, which also play an important role in increasing vascular resistance [5,31,32]. Endothelial dysfunction, reduced eNOS activity and vascular remodeling [1,32] are the first vascular alterations of the cardiovascular system that may occur in hypertensive patients and may have prognostic significance [31,32]. This

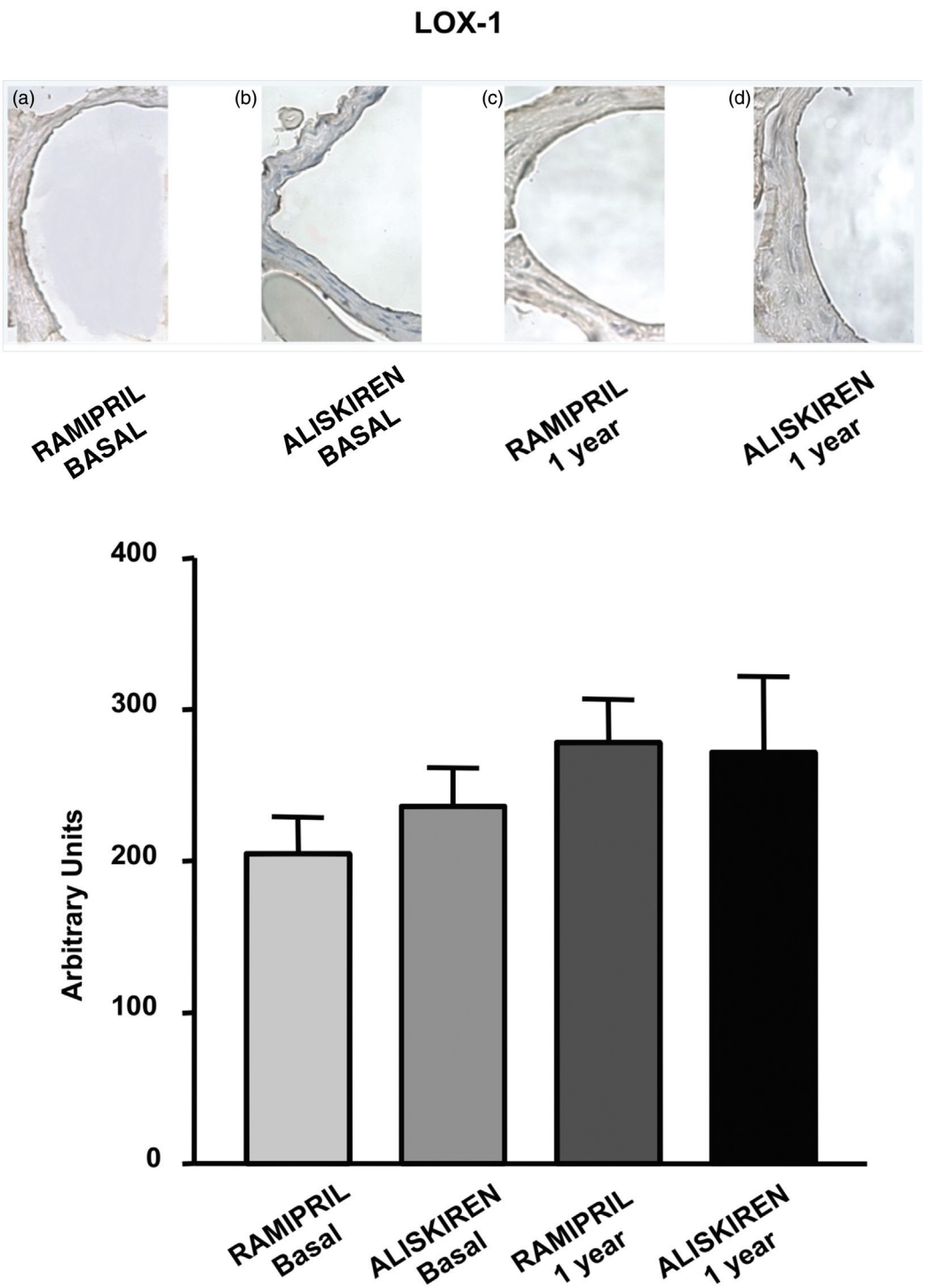

FIGURE 6 LOX-1 expression in resistance arteries of hypertensive and diabetic patients treated with aliskiren and ramipril. LOX-1, lectin-like oxidized LDL receptor 1.

concept has been also supported by our data, since, as reported here and in our previous publication [21], at basal the hypertensive and diabetic patients presented impaired endothelial function and vascular remodeling and had preserved cardiac structure and function [21]. Data from clinical and experimental studies suggest that drugs that enhance eNOS activity may play a protective role on endothelium through the increased nitric oxide production and bioavailability [33]. The improvement of endothelial function is also correlated to the positive modulation of vascular inflammation and remodeling and ultimately to the protection of the cardiovascular system [1,32]. Our findings in high cardiovascular risk patients are consistent with previous experimental and clinical evidence in patients at lower cardiovascular risk [14–16,34–36], although in these studies a shorter duration of aliskiren treatment was used. In hypertensive patients without diabetes, aliskiren treatment improved endothelial function of conduit

arteries as assessed by FMD. Further evidence has shown that aliskiren in monotherapy or in combination with ACE inhibitors or calcium channels blockers improved endothelial function in different categories of patients [14–16,34–37]. Nevertheless, in patients at higher cardiovascular risk, such as the hypertensive and diabetic patients, little evidence on the role of aliskiren on vascular protection is reported [13,38], particularly in resistance arteries after long-term treatment.

In this study, peripheral BP was similarly reduced by both aliskiren and ramipril although changes in peripheral DBP were statistically significant only in the aliskiren group. This may imply that aliskiren is able to induce BP reduction possibly by inducing a more prompt peripheral vasodilation [13]. In a meta-analysis aimed to investigate the antihypertensive effects and tolerability of aliskiren in comparison with other antihypertensive drugs and placebo in patients with hypertension, more patients achieved BP control with aliskiren, and in particular aliskiren was superior to ACE inhibitors in lowering DBP, while it had similar effects to ACE inhibitors on SBP reduction [39]. Moreover, in a large real-life registry in hypertensive patients with diabetes it has been reported that an aliskiren-containing regimen showed better BP reductions than patients without RAS-blockade, or an ACE-inhibitor/ARB-containing regimen [40]. Furthermore, it has been reported that aliskiren-based therapy both as monotherapy and with optional addition of amlodipine provided superior BP reductions to hydrochlorothiazide-based therapy with good tolerability in patients with hypertension and obesity [41]. Thus, aliskiren is more effective to induce peripheral vasodilation as compared with other class of drugs including ACE inhibitors, suggesting a better protection on peripheral vascular function [13]. Only aliskiren selectively increased the endothelium-dependent vasodilation in both resistance and conduit arteries, suggesting a restoration of nitric oxide bioavailability. This was further supported by the evidence that only aliskiren increased the expression of the active form of eNOS in the endothelium of resistance arteries in hypertensive and diabetic patients. These data are also supported by our previous report in double-transgenic rats (rats with increased expression of human renin) in which aliskiren was able to improve vascular function and increase nitric oxide bioavailability and eNOS expression and function in the vascular system as compared with ramipril [42]. Also in other experimental models such as the Watanabe heritable hyperlipidemic rabbits, a model of atherosclerosis, aliskiren improved the impaired nitric oxide bioavailability by increasing plasma nitric oxide concentrations [34]. Significantly, ramipril failed to improve endothelium-dependent vasodilation in both resistance and larger conduit arteries of hypertensive and diabetic patients, as also shown in previous reports [9,14,41]. Available evidence indicates that ACE inhibitors and ARBs do not improve always endothelium-dependent vasodilation particularly in the peripheral resistance arteries although both are able to reverse vascular structural alterations in essential hypertension and diabetes [8,9,11,14]. A possible explanation of this discrepancy in the action of both aliskiren and ramipril on endothelial function could be related to the fact that endothelial function is also regulated by the hyperpolarizing factor, which is modulated by circulating vasoactive substances such as bradykinin that can modulate vascular tone and BP in condition of high renin plasma levels in experimental models of hypertension as well as in humans [9,10,43–45]. Thus, bradykinin may exert a countervailing beneficial effect on endothelial function, and ACE inhibitors can selectively increase only vasodilation to bradykinin [9,10,43–45].

RAAS activation promotes oxidative stress, inflammation, cell growth, fibrosis [1,8], and the inhibition of eNOS activity in the vascular wall of hypertensive patients [1,2,8], contributing to endothelial dysfunction and vascular remodeling. These deleterious effects are further enhanced in patients with diabetes [2,5,8]. On the other hand, the increased nitric oxide concentration plays an important anti-inflammatory role by counteracting oxidative stress [46] and in turn it may further contribute to the improvement of vascular remodeling. Aliskiren has been proven to reduce fibrosis in several organs [47]. Moreover, aliskiren induced favorable effects similar to that induced by ACE inhibition in terms of improving vascular remodeling of resistance arteries in hypertensive rats with high plasma renin levels, independently of BP control [42]. We further extended this evidence in hypertensive and diabetic patients and showed that aliskiren reduced remodeling and fibrosis of resistance arteries [21]. Nevertheless, direct renin inhibition did not improve central indexes of arterial stiffness (PWV), as we have shown in the current study. The aforementioned evidence was associated to the enhancement of nitric oxide bioavailability in the vascular system of hypertensive and diabetic patients, thus it is conceivable that increased nitric oxide production may contribute further to the improvement of vascular inflammation and remodeling particularly in resistance arteries [21,35,42].

Surprisingly, aliskiren and ramipril were not able to exert any significant further effect on markers of oxidative stress at least in resistance arteries of hypertensive and diabetic patients. Although cannot be excluded an influence derived by the limited numerosity of the population enrolled, it can be speculated that, in conditions of increased production of oxidative stress as may occur in the vascular system of hypertensive and diabetic patients, a more complete RAAS blockade (in both upstream and downstream sites) could be required. It has been shown that antihypertensive effects of aliskiren alone do not prevent hypertension-induced vascular oxidative stress and endothelial dysfunction in 2-kidneys-1-clip rat model, unless a combination therapy with an ARB was used [48]. Moreover, the combination of aliskiren and valsartan exerts a synergistic organ-protective effect through attenuation of oxidative stress in eNOS-deficient mice [49]. Furthermore, aliskiren, significantly enhanced the protective effects of valsartan against diabetic nephropathy in db/db mice by different mechanisms, suggesting that aliskiren might protect against type 2 diabetic nephropathy, through pleiotropic effects [50]. Taken together, this evidence supports the concept that aliskiren alone could not prevent hypertension-induced vascular oxidative stress, and that renin inhibition is not enough to prevent hypertension-induced impaired redox biology, particularly in high cardiovascular risk conditions.

In the past years, the efficacy and safety of aliskiren has been the focus of attention, especially when used in patients on therapy with ACE inhibitors or ARBs, although the efficacy of aliskiren has been challenged particularly in diabetic patients [13,30]. In particular, in a subgroup analysis of The Aliskiren Trial to Minimize Outcomes in Patients with Heart Failure in patients with congestive heart failure and diabetes no harmful effect was found, rather a trend towards benefit was observed when aliskiren monotherapy was compared with an ACE inhibitor. On the other hand, combined aliskiren and enalapril treatment led to more adverse events with no improvement in outcomes [19]. Taken into account the data of our study and other available evidence [35,42] it is conceivable to argue that the increased nitric oxide bioavailability induced by aliskiren in the peripheral arteries may contribute to the beneficial effects of aliskiren when used in monotherapy. Nevertheless, this could play also a role in the harmful effect of aliskiren when associated with other RAAS blockers in diabetic patients particularly in high risk clinical conditions such as congestive heart failure, in which a remarkable reduction of BP and peripheral vasodilatation could outcome the benefit of RAAS blockade.

In conclusion, aliskiren is an antihypertensive agent with a mechanism based on the inhibition of the catalytic activity of the renin enzyme, which prevents the activation of the RAAS cascade, and has antihypertensive effect similar to that induced by ACE inhibition. It seems to have some advantages compared with ramipril in terms of enhanced nitric oxide production and improvement of endothelial function particularly in resistance arteries of hypertensive and diabetic patients. These findings may have important clinical repercussion, since the improvement of endothelial dysfunction may have good prognostic significance particularly in high cardiovascular risk patients. However, in clinical studies, despite the positive effect on BP reduction, aliskiren did not reduce total mortality or cardiovascular death particularly in patients with diabetes, mainly when aliskiren add-on therapy was used [13]. Thus, the role of the use of aliskiren monotherapy remains to be further investigated particularly in high cardiovascular risk patients with associated metabolic alterations.

ACKNOWLEDGEMENTS

Conflicts of interest

There are no conflicts of interest.

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