Original Article

Effect of olmesartan medoxomil on number and survival of circulating endothelial progenitor cells and calcitonin gene related peptide in hypertensive patients

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Objective: Injury of vascular endothelium, crucial in vascular disease, is repaired via circulating endothelial progenitor cells (cEPCs). In hypertension, cEPCs number is reduced and function impaired adding further risk for cardiovascular (CV) events. Angiotensin II (Ang II)-induced oxidative stress (OxSt), accelerates cEPCs senescence. Calcitonin gene-related peptide (CGRP), able to prevent and reverse Ang II-induced cEPCs senescence, is reduced in hypertension and stimulated by the antioxidant and anti-inflammatory heme oxygenase (HO)-1. In essential hypertensive patients olmesartan reduced OxSt and markers of CV remodeling and increased HO-1. This study reports in essential hypertensive patients the effect of 6 months treatment with olmesartan on plasma level of CGRP and number and survival of cEPCs.

Methods and results: In 20 essential hypertensive patients treated with olmesartan medoxomil (20 mg per day for 6 months), cEPCs ($CD34^+KDR^+$, $CD133^+KDR^+$ and $CD34^+CD133^+KDR^+$) (direct 3-color flow cytometry analysis), apoptosis of cEPCs ($CD133^+KDR^+$ cells with Annexin V expression), CGRP determination (ELISA) and HO-1 protein level (western blot) were assessed at baseline and after 3 and 6 months of treatments. Olmesartan normalized blood pressure ($P < 0.001$), increased cEPCs from baseline ($CD34^+KDR^+$: $P < 0.003$; $CD133^+KDR^+$: $P < 0.0002$; $CD34^+CD133^+KDR^+$: $P = 0.0008$), reduced cEPCs apoptosis ($P < 0.001$) and increased CGRP ($P < 0.013$) and HO-1 ($P = 0.039$).

Conclusion: These results provide a mechanistic rationale for the olmesartan's antioxidant and anti-inflammatory potential translation toward antiatherosclerotic and antiremodeling effects reported on clinical ground.

Keywords: CGRP, endothelial progenitor cells, heme oxygenase-1, hypertension, Olmesartan

Abbreviations: BS/GS, Bartter's/Gitelman's syndromes; CGRP, calcitonin gene-related peptide; EPCs, endothelial progenitor cells; HO-1, heme oxygenase-1

INTRODUCTION

Impaired vascular repair mechanisms resulting from a reduced number and/or impaired function of endothelial progenitor cells (EPCs) are considered essential for cardiovascular outcomes [1]. Injury of vasculature is normally repaired in part via migration and proliferation of endothelial cells from the border zone or from adjacent branching blood vessels and in part via bone marrow-derived EPCs [2,3]. In hypertension, circulating EPCs number is reduced and their function impaired [4] and this represents an additional risk for cardiovascular events [5].

Angiotensin II (Ang II) and Ang II-induced oxidative stress play a pivotal role in EPCs status by accelerating the onset of their senescence, which, in turn, leads to impairment of their proliferative activity [6], while Ang II type 1 receptor blockers correct EPCs dysfunction [7–9]. The relationships between endothelial function and EPCs number and function are well known [1,10], all of their alterations in hypertension [4,11,12] mainly due to oxidative stress [13,14].

Among antihypertensive drugs, olmesartan, an Ang II type 1 receptor blocker, widely used in the treatment of hypertension, has been shown, to possess antioxidant and activating nitric oxide (NO) system-related effects. Consistent results with olmesartan and oxidative stress-related processes have been, in fact, obtained both in in-vitro and in animal model studies [15–18]. Moreover, although indirectly, the vasoprotective, anti-inflammatory and anti-atherosclerotic effects of olmesartan shown in humans in the EUTOPIA, VIOS, MORE and OLIVUS clinical trials [19–22] can be linked with an inhibitory effect on oxidative

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stress and oxidative stress signaling as a mechanism involved in the effects of olmesartan observed in these studies.

In addition, we recently reported in essential hypertensive patients that treatment with olmesartan, reduced oxidative stress [23], as shown by the reduced expression of $p22^{phox}$, a subunit of NADPH oxidase essential for the induction of oxidative stress [24], reduced the phosphorylation state of ERK1/2, the effector of oxidative stress mediated Ang II signaling for cardiovascular remodeling [25], and reduced the plasma level of oxLDL, a marker of oxidative stress. In addition, the protein expression of heme oxygenase (OH)-1 was increased.

HO-1 is a potent antioxidant and anti-inflammatory protein [26,27], whose induction effect on re-endothelialization has been linked to its ability to increase the number of circulating EPCs [28]. Furthermore, HO-1 has been reported to reduce Ang II-induced oxidative stress [29].

Calcitonin gene-related peptide (CGRP) is a vasorelaxant, which prevents circulating EPCs senescence, reverses Ang II-induced senescence of EPCs and reduces blood pressure in spontaneously hypertensive rats [30]. Moreover, CGRP is stimulated by HO-1 [31] while its level is reduced in hypertensive patients [30].

The present study reports in never before treated essential hypertensive patients the effect of a relatively short-term treatment with olmesartan on the plasma level of CGRP, number and survival of circulating EPCs and HO-1 protein level.

PATIENTS AND METHODS

Patients

The study was carried out in 20 uncomplicated, nonsmoking and never before treated essential hypertensive patients (15 men, five women, aged 36–60 years) attending our Hypertension Clinic, Department of Medicine, Clinica Medica 4 at the University of Padova. Their blood pressure ranged from 144 to 155 mmHg SBP and 93 to 99 mmHg DBP. Diabetes was ruled out by fasting serum glucose test less than 126 mg/dl and impaired renal function by serum creatinine less than1.0 mg/dl and urinary albumin/creatinine excretion less than 30 mg/g. Secondary hypertension was excluded by the evaluation of plasma renin activity and plasma aldosterone before and after 50 mg of captopril (captopril test). Lipid profile was normal and patients were not taking lipid-lowering drugs or aspirin. BMI was also normal ($<25\,kg/m^2$). None of the patients had cardiac failure, evidence for coronary heart disease and established left ventricular hypertrophy by conventional M-mode echocardiography. Informed consent was obtained from all the study participants.

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After enrollment (baseline), patients were treated with olmesartan medoxomil (20 mg per day) and were seen after 1, 3 and 6 months. Blood pressure was measured in the sitting position with a mercury sphygmomanometer, to the nearest 2 mmHg by a trained observer. The mean of three consecutive measurements, 2 min apart, was used for the analysis of the results. At baseline and at 3 and 6 months, 35 ml of peripheral blood was withdrawn from the antecubital vein after overnight fast for ex-vivo molecular biology and biochemical determinations.

All the patients were under a diet containing approximately 150 mmol sodium per day and no substantial difference in the usual diet of the patients was revealed by a dietary questionnaire administered at 3 and 6-months particularly regarding salt and alcohol consumption.

Evaluation of circulating endothelial progenitor cells number

Peripheral blood progenitor cells were analysed for the expression of cell surface antigens with direct three-color analysis using fluorescein isothiocyanate-conjugated, phycoerythrin-conjugated and allophycocyanin-conjugated monoclonal antibodies (mAbs) by flow-cytometry analysis (FACSCalibur; Becton Dickinson, Franklin Lakes, New Jersey, USA, http://www.bd.com), as previously reported [32]. Briefly, 150 μl of peripheral blood were incubated with 10 μl of fluorescein isothiocyanate-conjugated antihuman CD34 mAb (Becton Dickinson), with 5 μl of allophycocyanin-conjugated antihuman CD133 mAb (Miltenyi Biotec, Bergisch Gladbach, Germany) and 10 μl of phycoerythrin-conjugated antihuman kinase-insert domain receptor (KDR) mAb (R&D Systems, Minneapolis, Minnesota, USA), followed by incubation at 4 °C for 30 min. Unlabeled cells or antiisotype antibody served as control. The frequency of peripheral blood cells positive for the above reagents was determined by a two-dimensional side-scatter fluorescence dot-plot analysis of the samples, after appropriate gating. After morphological gating to exclude granulocyte and cell debris, we gated $CD34^+$ peripheral blood cells and then examined the resulting population for dual and triple expression of KDR and CD133. EPCs were defined as $CD34^+KDR^+$ or $CD133^+KDR^+$ and $CD34^+CD133^+KDR^+$ cells. For fluorescence-activated cell sorting analysis, 500 000 cells were acquired and scored using a FACSCalibur analyzer (Becton Dickinson, Franklin Lakes, New Jersey, USA).

Quantification of circulating endothelial progenitor cells apoptosis

Apoptosis of cEPCs was analysed after gating on the previous cells events based on Annexin V (Becton Dickinson) binding to externalized phosphatidylserine.

In particular, apoptotic subpopulation was analyzed using 150 μl of peripheral blood incubated at 4°C for 30 min with 5 μl of fluorescein isothiocyanate-conjugated antihuman Annexin V mAb (Becton Dickinson), 5 μl of allophycocyanin-conjugated antihuman CD133 mAb (Miltenyi Biotec, Bergisch Gladbach, Germany) and 10 μl of phycoerythrin-conjugated antihuman kinase-insert domain receptor (KDR) mAb (R&D Systems, Minneapolis, Minnesota, USA). Apoptosis was analysed after gating on $CD133^+KDR^+$ cells events based on Annexin V expression. Data were processed using the Macintosh CELLQuest software program (Becton Dickinson).

Measurement of plasma calcitonin gene-related peptide

Fasting venous blood was collected in a sterile EDTA vacutainer and plasma stored at −70°C until further use.

CGRP assay was performed according to the manufacturer's specifications (Human CGRP ELISA kit; Uscn, Wuhan, China) as previously reported [33]. Absorbance was measured at 450 nm. Resultant readings were plotted against the standard curve to find out the concentration of CGRP in each sample and expressed as pg/ml.

Mononuclear cell HO-1 protein expression (western blot)

Peripheral blood mononuclear cells were isolated by Ficoll-Paque PLUS gradient (Amersham Biosciences, Uppsala, Sweden) from 35 ml of EDTA anticoagulated blood.

Western blot

HO-1 protein expression was assessed using Western blot analysis, as previously reported [34]. In brief, total protein extracts was obtained by cell lysis with an ice-cold buffer (Tris-HCl 20 mmol/l, NaCl 150 mmol/l, EDTA 5.0 mmol/l, Niaproof 1.5%, Na_3VO_4 1.0 mmol/l, sodium dodecyl sulfate 0.1%) added with protease inhibitors (Roche Diagnostics, Mannheim, Germany). Protein concentration was evaluated by BCA protein assay (Pierce, Rockford, Illinois, USA). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences), and blocked overnight with no-fat milk (5% in Tween-PBS). Membranes were probed with primary polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, California, USA) and then horse radish peroxidase (HRP) conjugated secondary antibody (Amersham Biosciences) was added, and immunoreactive proteins were visualized with chemiluminescence using Super-Signal WestPico Chemiluminescent Substrate (Pierce). Protein expression on Western blots was quantified using a PC-based densitometric semiquantitative analysis using NIH image software (Research Services Branch, National Institutes of Health, Bethesda, Maryland, USA) and were normalized to GADPH, a housekeeping gene.

Statistical analysis

Data are expressed as the mean ± SD and were analysed using JMP (vers 9.0) (SAS, Cary, North Carolina, USA) statistical package running on a Mac Pro (Apple, Cupertino, California) and evaluated using ANOVA for paired data and Student's *t*-test for paired data. Values at less than 5% level ($P < 0.05$) were considered significant.

RESULTS

In hypertensive patients the treatment with olmesartan normalized blood pressure since the third month of treatment (BP $\leq$ 140/90 mmHg) in all 20 patients (ANOVA: $P < 0.001$): 148 ± 5.1 mmHg at baseline vs. 136.79 ± 2.00 at 3 months, $P < 0.001$ with a further reduction to 134.2 ± 2.3 at 6 months $P < 0.001$ and $P < 0.01$ vs. 3 months, for SBP and 96.2 ± 2.0 at baseline vs. 87.35 ± 2.6 at 3 months, $P < 0.001$ and 84.25 ± 1.2 at 6 months, $P < 0.001$ and $P < 0.01$ vs. 3 months for DBP.

Effect of the treatment with olmesartan on calcitonin gene-related peptide

Figure 1 shows that in hypertensive patients significantly CGRP increased upon olmesartan treatment (ANOVA: $P < 0.013$) from 198.81 ± 51.98 pg/ml at baseline to 218.97 ± 41.13 at 3 months and to 263.91 ± 43.08 at 6 months ($P = 0.0001$ for 6 months vs. baseline and $P = 0.03$ for 6 months vs. 3 months).

Effect of the treatment with olmesartan on circulating endothelial progenitor cells

Table 1 shows that in hypertensive patients upon olmesartan treatment, the three circulating EPC populations, $CD34^+KDR^+$, $CD133^+KDR^+$, $CD34^+CD133^+KDR^+$, as defined by cell surface antigens, significantly increased at 3 and 6 months from baseline.

Effect of the treatment with olmesartan on apoptosis of circulating endothelial progenitor cells

Apoptosis of circulating EPCs, as assessed by Annexin V binding to cell surface of $CD133^+KDR^+$ cells expressing the early apoptosis marker phosphatidylserine, was reduced (ANOVA: $P < 0.001$) from $44.28 \pm 12.38\%$ $CD133^+KDR^+$/Annexin at baseline to 27.24 ± 9.64 at 3 months ($P < 0.01$) and further decreased at 6 months to 16.83 ± 15.68 ($P < 0.001$ for 6 months vs. baseline and $P < 0.004$ for 6 months vs. 3 months) (Fig. 2).

Effect of the treatment with olmesartan on HO-1 protein level

As expected and in keeping with our previous study [23] olmesartan treatment increased HO-1 protein level (ANOVA: $P = 0.039$) from 0.81 ± 0.21 densitometric units at baseline to 0.95 ± 0.21 at 3 months ($P = 0.031$) and further increased at 6 months to 1.1 ± 0.26 ($P = 0.0001$ vs. baseline and $P = 0.01$ vs. 3 months) (Fig. 3).

DISCUSSION

Oxidative stress and oxidative stress induced endothelial dysfunction, although may not be the sole cause, play a

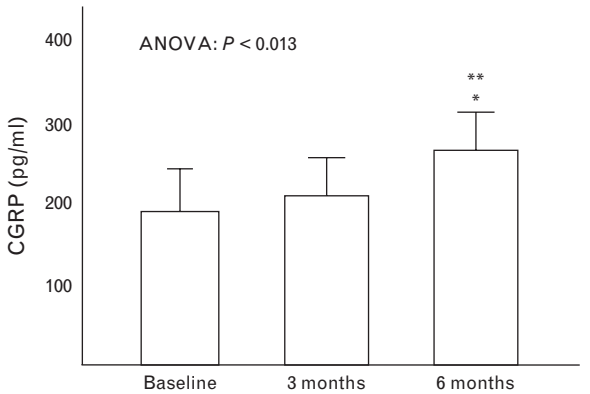

FIGURE 1 Plasma calcitonin gene-related peptide (CGRP) concentration in hypertensive patients at baseline, at 3 and 6 months of olmesartan treatment. **: $P = 0.0001$ vs. baseline; *: $P = 0.03$ vs. 3 months.

TABLE 1. Changes of circulating endothelial progenitor cell populations from baseline in hypertensive patients upon Olmesartan treatment

	Baseline	3 months	6 months	
$CD34^+KDR^+$	35.11 ± 25.98	59.11 ± 35.30	112.89 ± 53.44*§	ANOVA: $P < 0.003$
				*: $P = 0.005$ vs. baseline
				§: $P = 0.002$ vs. 3 months
$CD133^+KDR^+$	20.90.11 ± 14.58	49.50 ± 45.20	107.60 ± 37.09*§	ANOVA: $P < 0.0002$
				*: $P = 0.0001$ vs. baseline
				§: $P = 0.003$ vs. 3 months
$CD34^+CD133^+KDR^+$	3.67 ± 3.61	15.78 ± 18.59	38.11 ± 19.64*§	ANOVA: $P < 0.0008$
				*: $P = 0.0007$ vs. baseline
				§: $P = 0.0028$ vs. 3 months

major role in the pathophysiology of hypertension. Evidence suggests, in fact, that they have a causal role in the molecular processes leading to hypertension [35], are consistently observed in hypertensive patients and oxidative damage is important in the molecular mechanisms associated with cardiovascular and renal injury in hypertension [36].

Endothelial dysfunction defines a complex molecular and biochemical picture of inflammatory, proliferative, structural and functional abnormalities of the vasculature and EPCs play an important role for the protection from these abnormalities, which led to the hypertension-induced cardiovascular and renal organ damage [1]. The role of Ang II in the induction of oxidative stress via activation of NADPH oxidase, which contributes to the induction of hypertension and its long-term sequelae through the induction of endothelial dysfunction, is recognized [24,37]. In fact, endothelial function, including the endothelium-mediated vasodilation, and EPCs number and function are strictly related [1,10], all of these relationships are altered in hypertension [4,11,12] and in these alterations oxidative stress plays a pivotal role [13,14].

The re-endothelization of vascular injured areas is physiologically achieved through proliferation of endothelial cells as well as via circulating EPCs [2,3]. Of concern is that the number and function of circulating EPCs is reduced in hypertension and this reduction is considered to have a role in cardiovascular disease and to be predictive of cardiovascular events [5]. Understanding the factors that determine both the number and function of EPCs and, in particular, their reduction in hypertension, is likely to provide insight into potential targets for therapies designed to promote normalization of vascular function via stimulation of EPCs.

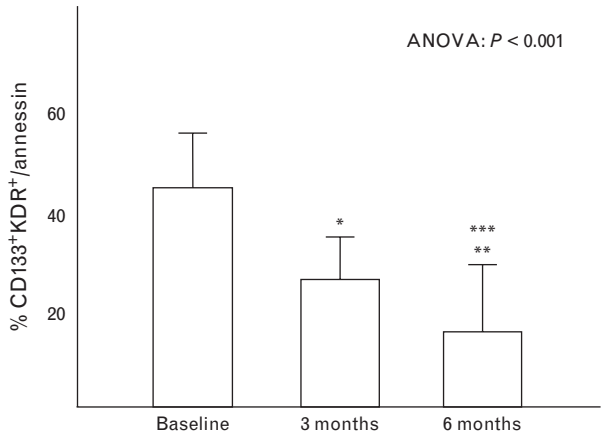

FIGURE 2 Evaluation of apoptosis of circulating $CD133^+KDR^+$ cell population in hypertensive patients at baseline, at 3 and 6 months of olmesartan treatment. ***: $P < 0.001$ vs. baseline; **: $P < 0.004$ vs. 3 months; *: $P < 0.01$ vs. baseline.

Imanishi *et al*. [6] reported that Ang II-induced oxidative stress accelerates EPCs senescence and impairs EPC function, while the treatment with Ang II type 1 receptor blockers corrects EPCs dysfunction thus pointing to a role for Ang II and Ang II-mediated oxidative stress in the reduction of EPCs number and function [7,8] as well as endothelial dysfunction.

Using a molecular biology approach, we have recently reported 'ex-vivo' in mononuclear cells of essential hypertensive patients that the treatment for 6 months with olmesartan, an Ang II type 1 receptor blocker, which has been shown to possess antioxidant and activating NO system-related effects [15–18], reduced the protein expression of $p22^{phox}$, a subunit of NADPH oxidase essential for the induction of oxidative stress, reduced the phosphorylation state of ERK1/2, effector of oxidative stress mediated Ang II signaling for cardiovascular remodeling and reduced the plasma level of oxLDL, a marker of oxidative stress [23]. In addition, treatment of these patients with olmesartan increased the protein expression of HO-1 [23], potent antioxidant and anti-inflammatory protein [26,27], whose induction effect on re-endothelialization has been linked to its ability to increase the number of circulating EPCs [28].

The present study demonstrates that in essential hypertensive patients treated for 6 months with olmesartan, circulating EPCs number is increased and EPCs apoptosis is reduced, which fits with the previous documented reduction of oxidative stress [23]. Furthermore, the plasma level of CGRP, a vasorelaxant which prevents circulating EPCs senescence, reverses Ang II-induced senescence of EPCs and reduces blood pressure in spontaneously hypertensive rats [30], was increased by olmesartan and, confirming the results of our previous study [23], HO-1 protein expression was also increased.

EPCs are typically identified either by surface antigens and flow cytometry or by in-vitro colony-forming assays. The precise criteria for identifying EPCs by flow cytometry remains a contentious issue. The combined surface expression, that is, the expression of at least one immaturity/stemness marker such as CD34 or CD133 and at least one endothelial antigen, usually KDR (VEGFR-2), is, however,

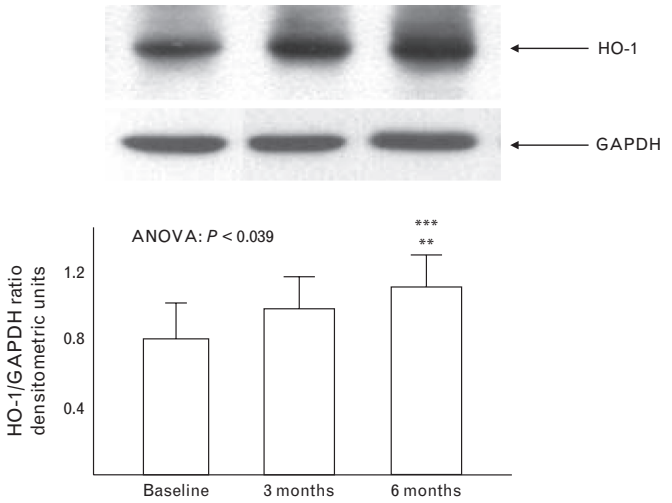

FIGURE 3 Densitometric analysis of the ratio of HO-1 to GAPDH in essential hypertensive patients treated with olmesartan at baseline, 3 and 6 months. The top of the figure shows HO-1 western blot products of 1 representative essential hypertensive patient treated with olmesartan. ***: $P = 0.0001$ vs. baseline; **: $P = 0.01$ vs. 3 months; *: $P = 0.031$ vs. baseline.

generally accepted as the most stringent criteria for EPCs [38]. On the contrary, it is difficult to establish the likely different biological meaning of EPCs phenotype populations as no exact surface phenotype of EPCs and no single antigenic combination can unequivocally identify the biological meaning of a particular EPC phenotype. Therefore clinical correlation/association data may be useful to attribute more biological relevance to putative EPC phenotypes. The number of $CD34^+KDR^+$ phenotype cell count has been, in fact, shown to correlate with pathological vascular damage and increased cardiovascular risk [39], whereas $CD133^+KDR^+$ and $CD34^+CD133^+KDR^+$ phenotype cell count are likely related to oxidative status and endothelial function [32]. This is also suggested by the increased number of all these EPCs phenotypes as well as survival in hypertensive patients treated with olmesartan of this study, which fits with the biochemical and molecular picture of good endothelial status, in terms of reduced oxidative stress and reduced phosphorylation state of ERK1/2, effector of oxidative stress mediated Ang II signaling for cardiovascular remodeling, shown in the hypertensive patients treated with olmesartan of our previous study [23], and further supported by the association of the increased number of the EPCs phenotypes with the increased HO-1 and CGRP level found in the hypertensive patients treated with olmesartan of this study.

Upregulation of HO-1, a potent antioxidant, antiapoptotic and antiinflammatory protein was reported to protect against vascular diseases, including atherosclerosis, by promoting re-endothelialization, inducing anti-inflammatory activities, inhibiting smooth muscle cell proliferation, regulating vascular tone, and by increasing cellular antioxidant activities [26,27]. As noted earlier, Wu *et al.* [28] linked the HO-1 induction effect on re-endothelialization to HO-1's ability to increase the number of circulating EPCs. In addition, HO-1 was demonstrated to reduce Ang II induced oxidative stress [29]. The link between HO-1 level and EPCs appears to be mediated in part via HO-1 effects on CGRP. Zhou *et al.* showed, in fact, that induction of the vasorelaxant CGRP, which is reduced in hypertensive patients, prevents circulating EPCs senescence, reverses Ang II-induced senescence of EPCs and leads to reduced blood pressure in spontaneously hypertensive rats. [30]. CGRP synthesis is stimulated by HO-1/CO pathway as well as related to stimulation of NO production [31]. Given the essential role played by HO-1 both in the reduction of Ang II-induced oxidative stress [29], a major determinant of circulating EPCs senescence, and in the induction of CGRP [31], the effector of HO-1 for the prevention of Ang II-induced circulating EPCs senescence, and given that the treatment with olmesartan is able both to induce HO-1 [23] and to increase EPCs number in diabetic patients [9], it comes as no surprise that olmesartan in essential hypertensive patients may be able to improve EPCs number and survival via CGRP. This is exactly what our results demonstrate. Further support for a role of olmesartan and in general for Ang II type 1 receptor blockers in the CGRP induction and EPCs increased number and survival is given by our data in patients with Bartter's and Gitelman's syndromes, a human model of endogenous Ang II type 1 receptor antagonism.

Bartter's and Gitelman's syndromes despite increased plasma levels of Ang II and activation of the renin–angiotensin system, present with normo/hypotension, hyporesponsiveness to pressors, absence of cardiovascular remodeling [40–43], blunted Ang II signaling and related pathways [40,41,44–46], reduced oxidative stress alongside increased HO-1 gene expression [41], upregulation of NO system, and increased NO-dependent vasodilation [40–42], all of which producing a mirror image of the alterations found in hypertension. In these patients we recently showed that the number of circulating EPCs and HO-1 protein expression are increased and strongly related and that the plasma level of CGRP is increased and strongly related with both HO-1 plasma level and circulating EPCs [32,33].

The results obtained in the present study reproduce those provided by a human model of endogenous Ang II type 1 receptor antagonism and strengthen the importance of the oxidative stress/HO-1/CGRP/EPCs relationships in hypertension and its long-term complications providing a mechanistic rationale for the vasoprotective effect of olmesartan achieved by increasing HO-1, CGRP and cEPCs number and improving cEPCs vitality/function, likely via the reduction of Ang II-induced oxidative stress. In addition, together with our previous data [23], they provide a mechanistic rationale on a molecular and biochemical ground for the vasoprotective, anti-inflammatory and antiatherosclerotic effects of olmesartan reported on a clinical ground in the EUTOPIA, VIOS, MORE and OLIVUS clinical trials [19–22].

Limitations of this study are the relatively small number of patients and the use of the patients as their own control. Although this could lessen the likelihood of observing statistically significant effects, the picture depicted for the oxidative stress/HO-1/CGRP/EPCs relationships in hypertensive patients treated with olmesartan, which reproduces the picture provided by a human model of endogenous Ang II type 1 receptor antagonism, suggests that the results are up to be robust.

In conclusion, this study demonstrates in essential hypertensive patients a vasoprotective effect of olmesartan via reduction of Ang II-mediated oxidative stress and increased CGRP-mediated improvement of endothelial dysfunction, likely due also to the increased number of EPCs and their improved survival/function. In addition, it provides a mechanistic rationale for the omelsartan's antioxidant and anti-inflammatory potential translation, in the long term, toward antiatherosclerotic and antiremodeling effects reported on the clinical ground.

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Conflicts of interest

There are no conflicts of interest.

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Reviewers' Summary Evaluations

Reviewer 1

The role of endothelial progenitor cells (EPCs) in endothelial dysfunction, essential hypertension and related co-morbidities is under active investigation. Although the molecular mechanisms are not clear an increase in number of EPCs associated with a decrease in oxidative stress is now being viewed as a possible marker of vascular effects of antihypertensive treatment. The results presented in this manuscript show the angiotensin receptor blocker olmesartan can reduce oxidative stress via induction of heme-oxigenase-1 and thus maintain the number of EPCs. The manuscript suggests an anti-inflammatory and antioxidant role of olmesartan independent of angiotensin receptor blockade.

Reviewer 2

The aim of the study was to extend a previous observation of FH Bahlmann *et al.* related to an increase in the number of circulating endothelial progenitor cells (EPC) during treatment with olmesartan. The authors provide a mechanistic rationale for this finding, demonstrating a possible role of calcitonin gene-related peptide and heme-oxygenase-1 in explaining olmesartan's antioxidant and anti-inflammatory properties.

The issue is interesting, and the observation relevant; however, the characterization of EPC by flow cytometry represents a controversial issue, since different patterns of surface antigens may identify different populations, with different properties. This could represent a limitation of the study.