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Original Paper

Blood Pressure Reduction is Associated With the Changes in Oxidative Stress and Endothelial Activation in Hypertension, Regardless of Antihypertensive Therapy

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Key Words
8-iso-prostaglandin F2-alpha • Hypertension • AT1 receptor antagonists • Calcium channel blockers • Cell adhesion molecules

Abstract
Background/Aims: Hypertensive patients present with increased oxidative stress and frequently receive angiotensin II (ANGII) receptor type I blockers (ARB) for blood pressure (BP) reduction. Recent studies revealed an important role of ANGII in maintaining vascular oxidative homeostasis, including sustaining normal sodium dismutase activity. This study aimed to investigate the effects of antihypertensive therapy and also vitamin C/E supplementation on BP, oxidative stress and endothelial activation in patients with essential hypertension. ***Methods:*** Newly discovered patients received ARB/olmesartan or the Ca^{2+}-channel blocker (CCB)/amlodipine, and additionally vitamin C/E or placebo throughout weeks 9-16. ELISA was used to determine 8-iso-prostaglendin F2-alpha (8iPGF2α) and endothelial activation markers. ***Results:*** In both groups BP was normalized during first 8 weeks of therapy. Vitamins C/E had no additional BP-lowering effect. The vitamins C/E supplementation was not effective in reducing absolute values of 8iPGF2α; however; the magnitude of 8iPGF2α reduction was significantly greater in patients taking vitamins C/E in the CCB group. Although plasma 8iPGF2α positively correlated to BP, a significant decrease occurred during an additional 8 weeks of treatment. There were no changes in endothelial activation markers related to the specific action of ARB

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or CCB. ***Conclusions:*** Present study suggests that observed oxidative stress is a consequence of hypertension. BP reduction is associated with the observed decrease in oxidative stress and changes in endothelial activation regardless of antihypertensive therapy.

Introduction

Increased oxidative stress is a hallmark of many chronic diseases, including hypertension [1, 2]. It occurs as a consequence of and altered balance between the production and elimination of reactive oxygen (ROS) and nitrogen (RNS) species via antioxidative enzymes [3]. Hypertensive patients exhibit both, increased oxidative stress and reduced antioxidative capacity [4, 5]. An oxidative stress marker, 8-iso-prostaglandin F2-alpha (8iPGF2α) is elevated in hypertensive individuals and has been associated with endothelial dysfunction in resistant hypertension [6]. That finding is in line with animal studies on the role of oxidative stress in the pathogenesis of hypertension, and the role of oxidative stress in mediating endothelial dysfunction, vascular remodeling and inflammation [3, 7, 8]. There is evidence for increases in ROS/RNS in the vascular wall of most animal models of hypertension [9, 10]. As depicted in Figure 6, the initial sources of ROS are NAD(P)H oxidases (Nox enzymes), and according to the "kindle – bonfire" theory, the process is self-amplified, leading to NOS uncoupling and mitochondrial respiratory burst chain activation resulting in escalating ROS/RNS generation [11]. NAD(P)H oxidases are activated by various stimuli, including ANGII, aldosterone, shear stress, growth factors, cytokines, endothelin 1 etc[1]. In the ANGII infusion model of hypertension NAD(P)H oxidase has a central role in the development of increased blood pressure (BP) and impaired vascular function [2, 9, 12]. Conversely, suppression of ANGII during increased dietary salt intake or pharmacological inhibition of the renin-angiotensin system by ACE and AT_1 receptor blockers have been shown to increase vascular oxidative stress [13] by affecting superoxide dismutase (SOD) activity, leading to a similar increase of ROS levels [14, 15]. In a recent study healthy normotensive volunteers subjected to a short period of high salt intake exhibited impaired endothelium dependent vasodilatation in response to reactive hyperaemia and acetylcholine infusion [16, 17]. In addition, low renin activity has been linked to higher mortality of cardiovascular events in salt sensitive individuals [18]. This is consistent with several animal studies of ANGII deprivation, which showed altered endothelium-dependent and endothelium-independent vasodilatation in different vascular beds, suggesting an important role of normal ANGII levels in maintaining vascular function [13, 19, 20].

Independent of its effects on vascular function, increased oxidative stress within the vascular wall (Figure 6) can induce intracellular redox signaling and activate transcription factors (i.e. AP-1: activated protein-1 or NFκB: nuclear factor kappa B) involved in triggering inflammation and endothelial activation (Figure 6) [1]. Increased inflammatory parameters, such as endothelial activation markers sICAM-1, sVCAM-1 and soluble P- and E-selectin have been previously reported in hypertensive patients compared to their normotensive counterparts [21].

Angiotensin converting enzyme inhibitors (ACEI) and AT_1 receptor blockers (ARBs) are widely used antihypertensive agents, directly modulating RAS and the effects of angiotensin II [22]. Calcium channel blockers (CCB) are alternatively used as antihypertensive drugs, with an equally efficient BP lowering effect [23]. In-vitro studies, as well as experiments on animals have provided evidence on their positive/direct role in reducing ROS production by attenuating NADPH oxidase expression and activity, followed also by reduced expression of inflammatory markers [24, 25]. Nevertheless, given the recent evidence on important physiological role of ANG II in maintaining antioxidative mechanisms and normal vascular function [10, 16, 26] and the lack of human studies in newly discovered hypertensive patients, the aim of the present study was to investigate the effect of AT_1 receptor blockers on oxidative stress after BP normalization in patients with essential hypertension compared to

anthypertensive therapy (CCB) that does not affect AT1 receptor - ANGII signaling pathway. The specific objective of this study was to investigate the dynamics of the oxidative stress marker 8-iso-prostaglandin F2-alpha (8iPGF2α) and vascular inflammation markers in patients with essential hypertension over the time course of 16 weeks of antihypertensive therapy. An additional aim was to test whether supplementation with antioxidants (vitamins C and E) can further decrease BP and endothelium activation by reducing the level of oxidative stress.

Patients and Methods

Subjects

Fifty seven newly discovered hypertensive subjects of both sexes (age: 53.2±9.67 yrs*)* were recruited from people who were admitted at Gesundheitszentrum Lange Reihe Dr. Tadzic und Kollegen (Hamburg, Germany) for systematic physical examination. The inclusion criterion was evident presence of essential hypertension (systolic BP≥140mmHg, and diastolic BP≥90 mmHg) without any signs or history (the exclusion criteria) of secondary forms of hypertension, autoimmune disease, angina pectoris, coronary heart disease, myocardial infarction, cerebrovascular disease, hemorrhagic stroke, ischemic stroke including transient ischemic attack, renal disease or liver disease.

All test sessions were performed in the morning. After initial clinical assessment, BP measurement and blood sampling, patients were randomly assigned to either the calcium channel blocker group (CCB; control group, N=27) or the AT_1 receptor blocker group (ARB group, N=30). Patients from control group were administered CCB amlodipine (5-10 mg/day for 16 weeks; dose to reach a BP of ≤139/89 mmHg), and the other group of patients received the ARB olmesartan (10-20 mg/day for 16 weeks; dose to reach a BP of ≤139/89 mmHg). After 8 weeks of treatment, patients of both groups were additionally subdivided into: (A) patients receiving vitamin C/E supplement (800 IU of vitamin E and 500 IU of vitamin C) or (B) placebo. Peripheral blood samples were taken prior to administration of the antihypertensive drug, and after 8 and 16 weeks of continuous antihypertensive therapy. Data on age, sex, body weight, body mass index (BMI), BP, and waist and hip circumference were collected. Basic laboratory parameters, including fasting glycaemia, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL) cholesterol, triglycerides, plasma renin activity (PRA), aldosterone, and serum sodium, potassium, creatinine and urea concentration were also determined for each time point shown in Table 3. The study was approved by the Ethical Committee of the University Josip Juraj Strossmayer Osijek, Faculty of Medicine and by the Ethical Committee of the Gesundheitszentrum Lange Reihe Dr. Tadzic und Kollegen, and was in accordance with Declaration of Helsinki. Patients as well as technicians handling sample analyses were blinded for the type of therapy and vitamin or placebo supplementation. All patients voluntary participated in the study and provided written informed consent.

Blood pressure measurement

Auscultatory readings of BP were performed using a properly calibrated sphygmomanometer (Boso LP RT) in the morning, with the patient sitting. Subjects were asked to avoid caffeine 30 minutes before the appointment and had to sit quietly for 10 minutes before beginning the measurements. A minimum of three sequential readings were taken with five minutes between readings. All BP measurements were performed according to the 2013 ESH/ESC Guidelines for the management of arterial hypertension [27].

Determination of serum/plasma 8-iso-prostaglandin F2-alpha and soluble cell adhesion molecules (sICAM-1, sVCAM-1 and sE-selectin)

All blood samples were collected at the beginning of the morning test session at the Gesundheitszentrum Lange Reihe Dr. Tadzic und Kollegen and immediately sent to the certified routine diagnostic laboratory (Labor Dres. Ennen und Gebauer, Hamburg, Germany), according to the official protocol of the clinic. Blood samples were retrieved using appropriate tubes containing either EDTA for sampling plasma or tubes without anticoagulants for collecting serum. Upon arrival to the laboratory, samples were centrifuged for 10 min at 4 ºC and 1000 rpm. Serum and plasma were collected and stored until analysis at -20 ºC. All measurements were performed using commercially available ELISA based kits, as follows: the Human sVCAM-1 Platinum Elisa (BMS 205/ BMS205TEN) for serum concentrations of soluble vascular cell adhesion molecule 1 (sVCAM-1), the Human sICAM-1 Platinum Elisa (BMS201/BMS201 TEN) for soluble intercellular adhesion

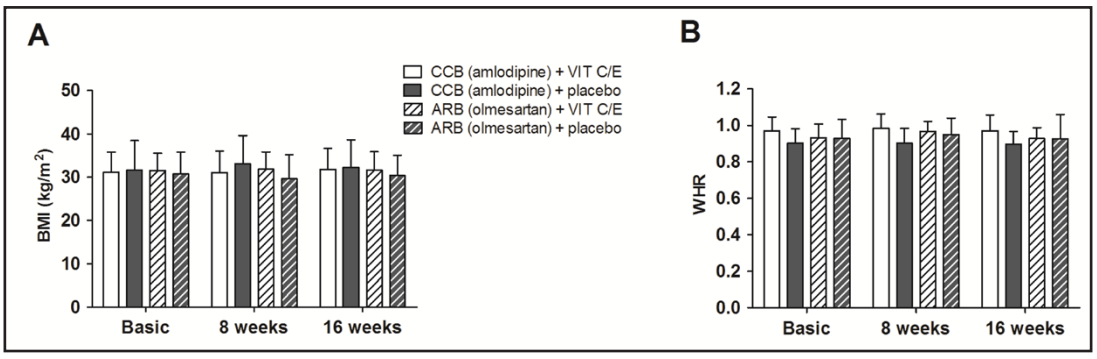


Fig. 1. Body mass index (BMI, panel A) and waist to hip ratio (WHR, panel B) in patients receiving angiotensin II receptor 1 blocker olmesatan or calcium channel blocker amlodipine with or without vitamin C/E supplementation. Data on BMI and WHR, together with data on sex and age, confirmed matched subject design of the study. Data are presented as median and interquartil range, differences were tested by two way ANOVA and Bonferrroni post hoc test and p≤0.05 was considered significant.

molecule 1 (sICAM-1) and the Human sE–selectin Platinum Elisa (BMS 205/ BMS205TEN) for soluble E-selectin. All kits were obtained from eBioscience (Vienna, Austria). Plasma levels of 8-iso-prostaglandin F2-alpha were determined using Direct EIA kit fom Enzo life Science GmbH (Lorrach, Germany).

Table 1. The effect of differences in initial diastolic blood pressure on independent variables

	Type III S um of Squares	df	Mean Square	F	*p*=
8-iso-PGF2 4P	15768.24	1	15768.24	.105	0.748
ICAM-1	12025.14	1	12025.14	3.137	0.085
E-selectin	1.37	1	1.37	.008	0.929
VCAM-1	221200.93	1	221200.93	2.592	0.116
Aldosterone	4318.54	1	4318.54	3.208	0.082

Analysis of covariance (ANCOVA) for all independent variables with diastolic blood pressure as covariate were performed, p≤0.05 was considered significant; 8iPGF2α - 8-iso-prostaglandin F2-alpha, sICAM-1 - soluble intercellular adhesion molecule 1, sVCAM-1 - soluble vascular cell adhesion molecule 1, sE-selectin - soluble E-selectin

Statistical analysis

In order to achieve a moderate effect size (0.5), power of 80% and $p \leq 0.05$ in the case of 4 study groups, the necessary total sample size was determined as 53 (G power software). Based on preliminary data for 10 patients per group for the most important independent variables (8iPGF2α, sICAM-1, sVCAM-1, sE-selectin), we performed ANOVA sample size analysis and established the adequate sample sizes to achieve the desired power (e.g. for 8-isoPGF2, alpha the test established that a sample size of 13 participants was required and appropriate). Initially, normality of data distribution was assessed by the Shapiro Wilk test. Within group differences were tested by one-way ANOVA or Kruskal-Wallis test followed by the Holm-Sidak/Tukey or Dunn's post hoc multiple comparison procedure, respectively. The Student t test and Mann-Whitney U Statistic were used to compare the differences between the CCB and ARB therapy in the case of normally distributed variables and variables that violated assumption of normality, respectively. In some cases, the differences were tested by two-way ANOVA and a Bonferroni post hoc test. Spearman's correlations were calculated where appropriate. In order to examine the moderating effect of type of the therapy and the addition of vitamins, a series of multivariate analysis of variance (MANOVA) were implemented. Analyses were performed separately for each group of parameters using the difference between the initial measurements and the measurements after 16 weeks, rather than absolute values. Types of therapy and vitamin supplementation were included as independent variables and all other measured parameters as dependent variables. Significant MANOVA main effects were further clarified by univariate analyzes of variance (ANOVA). As the size of the effect, partial eta squared (ηp2) was used. According to Cohen threshold values for this indicator were set as follows: 0.01 = small effect, .06 = medium effect size and .14 = large effect. Data are presented as mean±SD (unless otherwise stated). Except for MANOVA ($p \leq 0.1$) p values less or equal to 0.05 were considered significant.

Since a significant difference in initial diastolic BP between the CCB and ARB group was identified, we performed analysis of covariance (ANCOVA) for all independent variables with diastolic BP as covariate (Table 1). The analysis revealed that there was no significant effect of diastolic BP on the relationship of other independent variables (Table 1).

Table 2. Anthropometric measures of subjects enrolled in the study

	Mean± St.Dev.		Median, 25%, 75%		
	CCB	ARB	CCB	ARB	p=*
Sex	Male: 14 Female: 13	Male: 15 Female: 15	-	-	0.889
Age	53.4±9.79	53.0±9.71	56.0, 48.0, 61.0	54.0, 45.0, 59.0	0.875
Hight, cm	168.9±10.6	169.7±11.1	170.0, 161.4, 176.8	169.0, 161.2, 176.0	0.779
Weight, kg	90.3±19.7	89.2±15.1	89.0, 73.0, 96.0	86.5, 79.0, 99.0	0.818
BMI, kg/m^2	30.2±8.25	31.0±4.68	31.9, 26.3, 35.4	29.8, 28.0, 32.9	0.943
WHR	0.93±0.08	0.93±0.09	0.94, 0.87, 0.99	0.92, 0.87, 0.99	0.901

Distribution of sex was tested by hi square, while other variables were compared by Student t test or Wilcoxons test; *p<0.05 was considered significant; CCB – calcium channel blocker therapy (amlodipine); ARB – AT_1 receptor blocker therapy (olmesartan)

Table 3. General blood lab results of the subjects enrolled in the study

Parameter	Refe-rent values	Calcium channel blocker therapy				AT_1 receptor blocker therapy			
		Initial	8 weeks	16 weeks	p=	Initial	8 weeks	16 weeks	p=
Total Cholesterol , mg/dl	<220	212.9±34.1	213.7±43.4	210.7±37.0	0.963	222.6±38.1	229.8±39.9	237.4±42.6	0.510
HDL Cholesterol, mg/dl	>60	52.8±15.9	46.7±13.1	51.9±16.3	0.317	51.9±14.1	44.4±9.8	46.3±12.6	0.145
LDL Cholesterol, mg/dl	<100	134.2±33.7	139±39.5	135.6±36.5	0.897	132.9±34.2	157.5±39.9	154.9±51.7	0.055
Tryglicerids, mg/dl	<150	162.2±64.6	186.5±135.6	187.1±80.0	0.528	202.4±99.3	176.3±83.8	253.1±228.1	0.615
Glucose, mmol/l	3.9-5.5	6.13±2.59	6.25±1.58	7.29±2.22	0.005*	6.27±1.49	6.53±1.83	7.27±1.43	0.042*
PRA, nmol/L/h	1.68-23	17.4±32.1	39.7± 48.4	44.9±68.0	0.053	17.2± 24.8	14.9±29.7	12.2±11.7	0.312
Aldosterone, mg/l	10-160	83.1±29.8	159.9±97.5	177.6±114.0	0.002*	96.5±40.8	155.8±96.1	162.9±85.1	0.009*
Urea, mmol/L	2.5-7.5	5.33±1.47	6.25±1.87	6.18±1.63	0.121	4.74±1.44	5.52±1.33	5.15±1.33	0.110
Creatinine, mmol/L	0.55-1.1	0.78±0.16	0.79±0.16	0.79±0.17	0.955	0.74±0.19	0.80±0.15	0.76±0.19	0.454
Na^+ serum, mmol/L	137-145	138.8±1.72	140.2±2.13	141.0±1.84	<0.001*	138.8±2.37	140.25±2.07	140.5±2.07	0.064
K^+ serum, mmol/L	3.6-5.0	4.13±0.44	3.93±0.31	4.16±0.59	0.033*	4.3±0.34	4.05±0.29	4.31±0.44	0.038*

Data are presented as mean±SD, differences in the means among the measurements (initial,8 and 16 weeks) were tested by one-way ANOVA and Tuckey post hoc analysis, *p<0.05 was considered significant

Results

Blood pressure reduction

Analysis of the anthropometric measures showed that the study groups were age, sex, BMI and WHR matched (Table 2 and Figure 1). General lab blood results were not significantly different between the groups (Table 3). The BP was measured at the time of the enrollment in the study and after 8 weeks and 16 weeks of treatment. Normal BP values (<140/90) were achieved during the first 8 weeks of therapy in both groups (Figure 2A and B, p≤0.001for CCB and p≤0.001 for ABR), with no additional decrease during further 8 weeks of treatment. Although patients were randomly assigned into two study groups, later analysis revealed that at the time of first admission to the clinic (and prior to initiation of the therapy) patients receiving CCB had higher diastolic BP compared to ARB group; and this appeared to be statistically significant (98.3±3.93 vs.

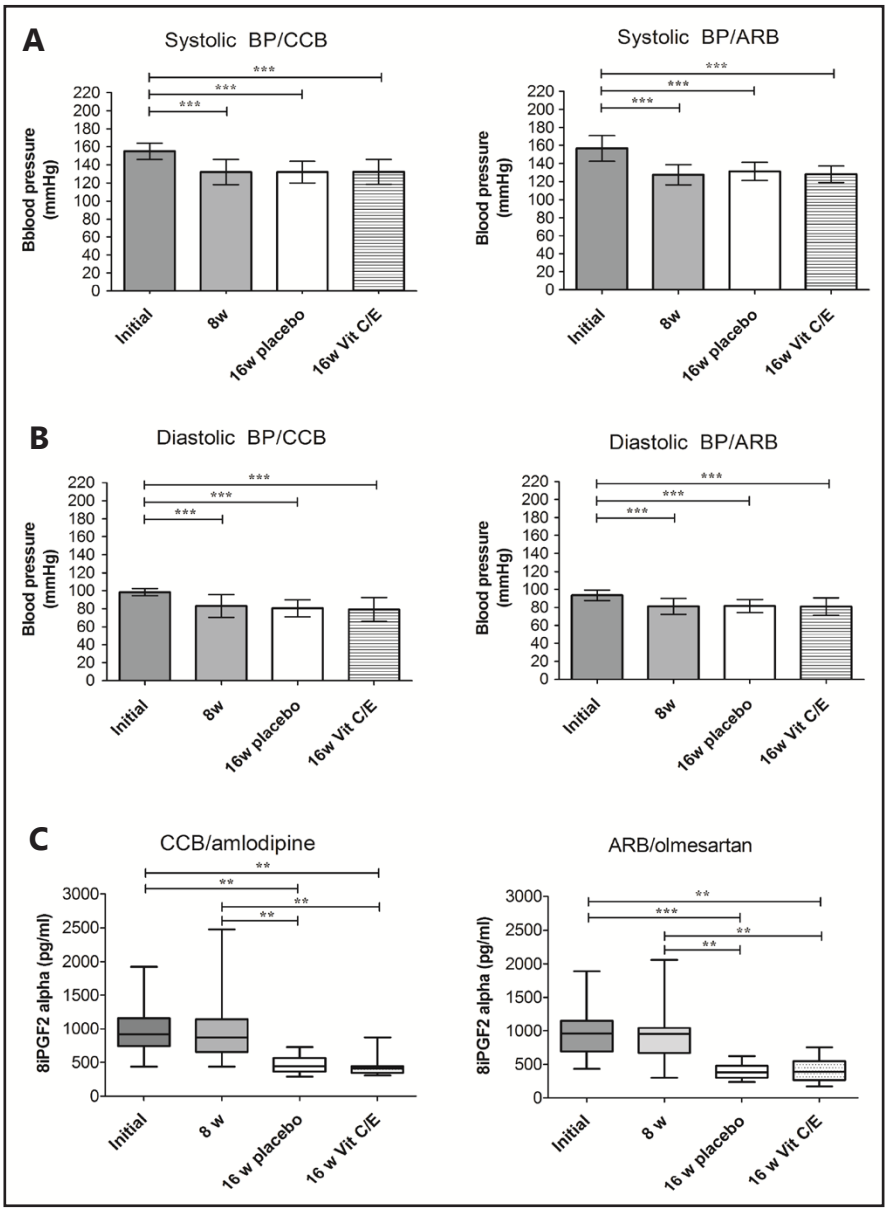


Fig. 2. Systolic and diastolic blood pressure, and changes of 8-iso-prostaglandin F2-alpha levels in patients on calcium channel blocker (CCB; amlodipine; N=27, panel A) and AT_1 receptor blocker (ARB; olmesartan; N=30, panel B) therapy with addition of vitamin C/E or placebo supplement during weeks 9th – 16th , Data are presented as mean±SD. Differences were tested by one-way ANOVA or Kruskal-Wallis One Way Analysis of Variance followed by Tukey post hoc analysis or Dunn's pairwise multiple comparison procedure, respectively; p value less or equal to 0.05 was considered significant, **p<0.01, ***p<0.001.

93.4±5.75, $p \leq 0.001$). As described in Materials and Methods section, these differences had no significant effect on any of the independent variables assessed in this study (Table 1). In contrast, there were no differences in BP levels between the groups after 8 and 16 weeks of treatment, suggesting that both therapies were equally effective in reducing BP to control values (Figure 2A and B). Supplementation of vitamin C/E during 8 weeks (9th to 16th week) had no significant effect on BP levels in either of the treatment groups.

Oxidative stress status during antihypertensive treatment

There was a significant reduction of serum 8iPGF2α levels after 16 weeks of antihypertensive treatment in both groups, irrespective of the type of therapy (Figure 2C, $p \leq 0.001$ for both groups). Conversely, levels of 8iPGF2α positively correlated to systolic (Figure 3A; r=0.369, p<0.001) and diastolic BP (Figure 3B; r=0.426, p<0.001) in CCB group, but only to diastolic BP levels in ARB group (Figure 3B; r=0.350, p=0.005). There were no differences in 8iPGF2α levels between the groups for any of the measured time points. The association between blood pressure and 8iPGF2α concentration was maintained at 8 and 16

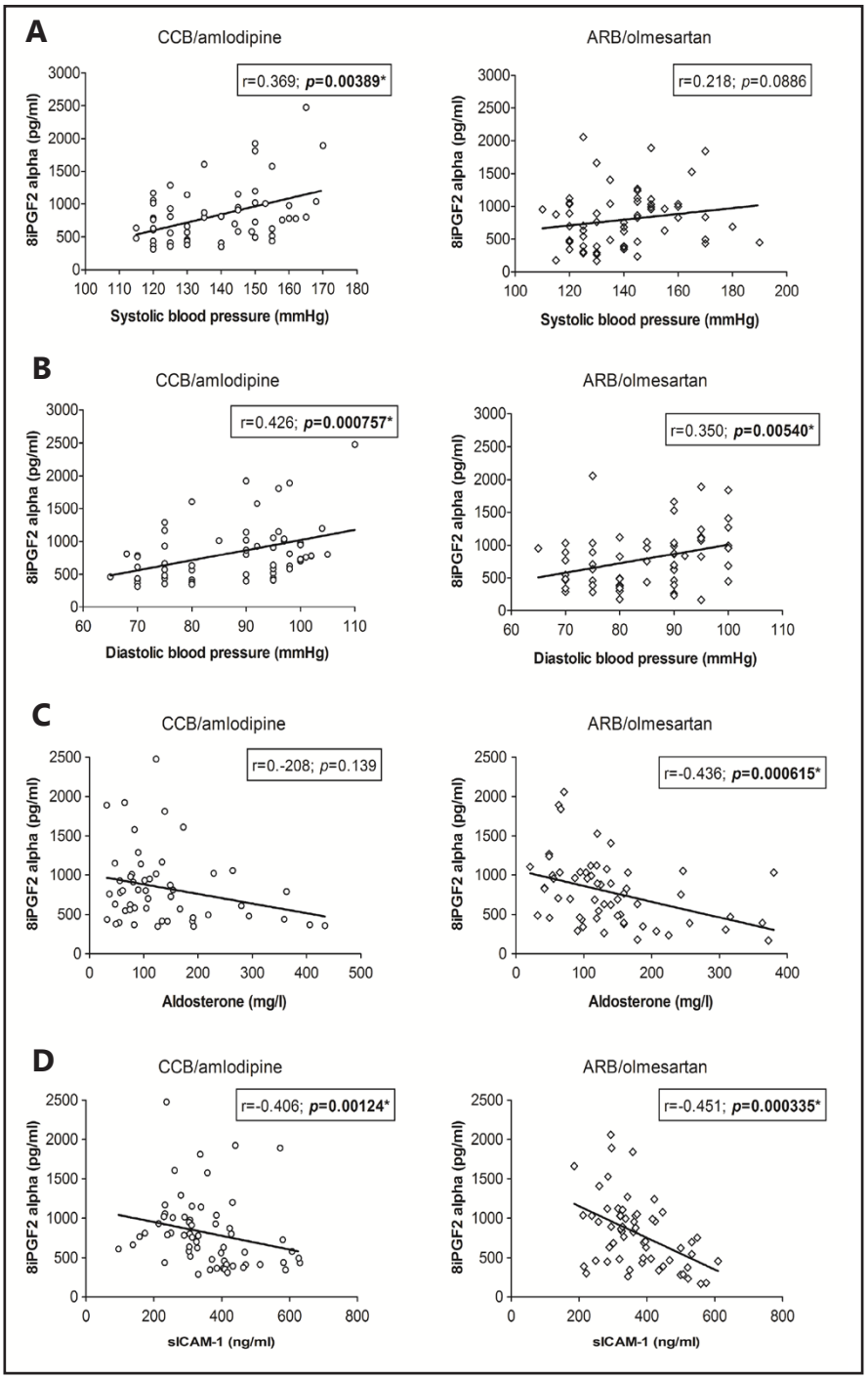


Fig. 3. Correlation between 8-iso-prostaglandin F2-alpha levels and blood pressure, aldosterone or soluble ICAM-1 levels in hypertensive patients receiving calcium channel or AT_1 receptor blockers during 16 weeks of therapy. Figure 3 shows correlation between the plasma levels of the 8-iso-prostaglandin F2-alpha (8iPGF2α) and systolic (panel A), diastolic (panel B) blood pressure (BP), aldosterone (panel C) and soluble ICAM-1 (sICAM-1, panel D) in patients receiving calcium channel blockers (CCB; amlodipine; panel A, B; N=60) or AT_1 receptor blockers (ARB; olmesartan, panel C, D; N=62). Levels of 8iPGF2α positively correlated to systolic and diastolic BP in the CCB group; but only to diastolic BP levels in the ARB group (*p* and r values are denoted at the corresponding panels), irrespective of vitamin supplementation. Aldosterone and sICAM tend to increase with the reduction of plasma 8iPGF2α levels (panel C,D). Correlations were calculated using Spearman Rank Order Correlation test and p<0.05 was considered significant.

weeks of treatments even though the blood pressure was reduced. Addition of vitamin C/E did not affect absolute values of 8iPGF2α in any of patient groups (Figure 2C). 8iPGF2α levels were negatively correlated to serum aldosterone levels in patients receiving ARB (Figure 3 panel C and Table 4; r=-0.436, p<0.001).

Table 4. Correlations between blood pressure, aldosterone, 8isoPGF2alpha and inflammatory markers

		sE-selectin		sICAM-1		sVCAM-1		Aldosteron		8isoPGF2alpha	
		CCB	ARB	CCB	ARB	CCB	ARB	CCB	ARB	CCB	ARB
SBP	r=	0.0197	-0.378	0.111	-0.0651	0.348	0.321	-0.377	-0.195	0.369	0.218
	p=	0.875	0.00236*	0.375	0.611	0.00439*	0.0104*	0.00312*	0.122	0.00389*	0.0886
DBP	r=	-0.0488	-0.291	-0.0279	-0.0888	0.206	0.402	-0.386	-0.249	0.426	0.350
	p=	0.696	0.0209*	0.824	0.488	0.0967	0.00118*	0.00247*	0.0475*	0.00076*	0.00540*
sE-selectin	r=			0.135	0.241	0.0409	-0.206	-0.0660	0.0641	-0.0428	-0.0713
	p=			0.275	0.0576	0.742	0.105	0.621	0.622	0.742	0.587
sICAM-1	r=					0.475	0.252	-0.104	0.137	-0.406	-0.451
	p=					0.00006*	0.0468	0.436	0.290	0.00124*	0.00034*
sVCAM-1	r=							-0.341	0.0646	-0.0924	-0.0833
	p=							0.00901*	0.620	0.477	0.526
Aldosteron	r=									-0.208	-0.436
	p=									0.139	0.00062*

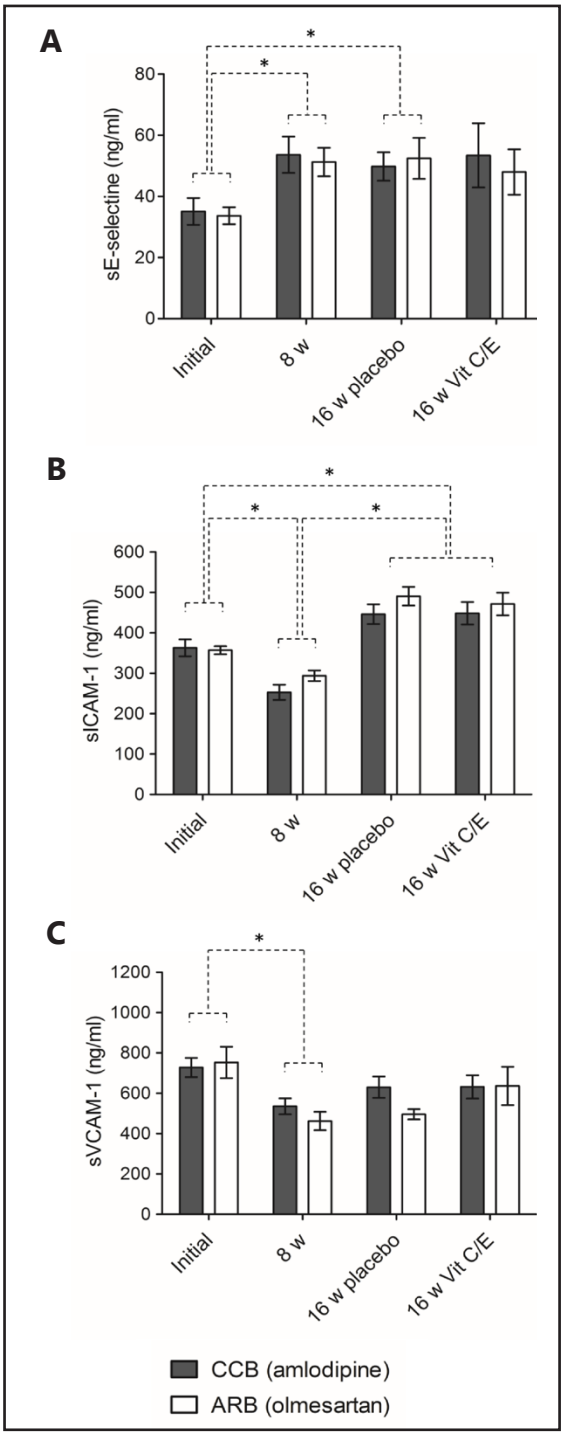
Correlations were calculated using Sperman Rank Order Correlation test; *p<0.05 was considered significant; r - Correlation Coefficient; N(amlodipine)=75; N(olmesartan)=78; CCB – calcium channel blocker therapy (amlodipine); ARB – AT_1 receptor blocker therapy (olmesartan); 8iPGF2α - 8-iso-prostaglandin F2-alpha, sICAM-1 - soluble intercellular adhesion molecule 1, sVCAM-1 - soluble vascular cell adhesion molecule 1, sE-selectin - soluble E-selectin

The effects of antihypertensive therapy on endothelial activation

Soluble E-selectin levels increased after 8 weeks of treatment and remained elevated after 16 weeks in both groups (Figure 4A; p=0.052 for amlodipine group and p=0.015 for olmesartan group), irrespective of the vitamin C/E supplementation. Serum sICAM-1 levels initially dropped significantly, but after 16 weeks of antihypertensive treatment sICAM-1 levels exceeded their initial values (Figure 4B, p≤0.001 for both groups). Serum sVCAM-1 levels decreased with the therapy and remained reduced throughout the study (Figure 4C, p=0.027 for amlodipine and p=0.008 for olmesartan group). Antioxidant vitamins supplementation during weeks 9th to 16th had no significant effect on the levels of soluble cellular adhesion molecules. In addition, there were no significant differences between the CCB and ARB groups at any of the time points. Soluble ICAM-1 levels negatively correlated to 8iPGF2α levels in both groups (Figure 4D, p values denoted at the panels), whereas there were no significant correlations between 8iPGF2α levels and serum sE-selectin (p=0.742 for CCB and p=0.587 for ARB), nor serum sVCAM-1 levels (p=0.477 for CCB and p=0.526 for ARB group). In addition, we found that sVCAM-1 levels tended to decrease together with the systolic BP in both groups (Table 4, statistically significant), while BP reduction negatively correlated to sE-selectin levels only in the ARB group and there was no correlation between sICAM-1 levels and the BP in any of the groups (Table 4).

Multivariate analysis of the effects of vitamin supplementation and antihypertensive therapy on blood pressure, oxidative stress, and endothelial activation

In order to examine the moderating effect of type of the antihypertensive therapy employed and the addition of vitamins, a series of multivariate analysis of variance (MANOVA) was implemented. MANOVA revealed a significant effect of vitamin C addition on the average change of 8iPGF2α, and this effect was moderate (Figure 5, panel B). Further univariate analyzes of variance (ANOVA) revealed that this effect was only significant in patients receiving CCB therapy (p=0.071). MANOVA failed to identify any effect of the type of the therapy or vitamin C/E supplementation on the status of endothelium activation.



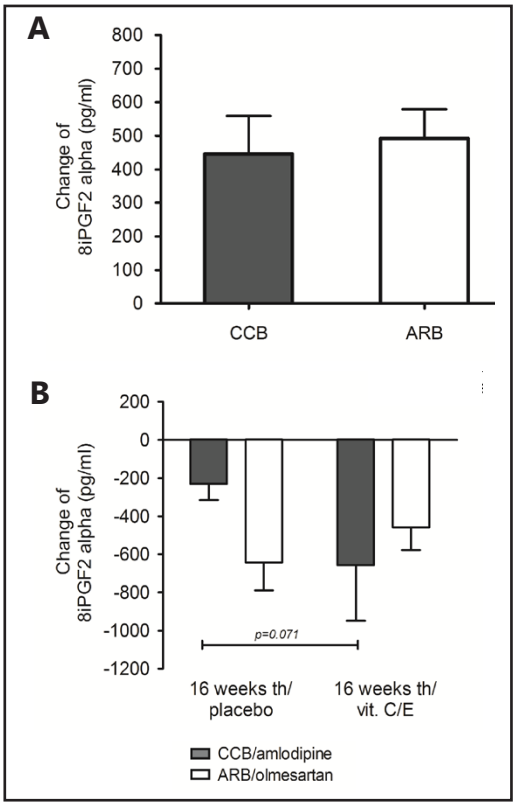


Fig. 5. The effect of vitamin C/E supplementation on the change in 8-iso-prostaglandin F2-alpha levels in patients with essential hypertension and receiving the calcium channel blocker (CCB; amlodipine; N=27) or the AT_1 receptor blocker (ARB; olmesartan; N=30), 8iPGF2α changes were calculated as the difference between the initial and last (at 16 weeks) measurement (panel A). Multivariate analysis of variance (MANOVA) revealed a significant effect of vitamin C/E addition on the average change of 8iPGF2α (panel B), and this effect was moderate. Furthermore, this effect was found to be significant only in patients receiving CCB therapy (panel B). Data are presented as mean±S.E.M, $p<0.1$ was considered significant.

Fig. 4. The effects of blood pressure reduction on the serum levels of soluble cell adhesion molecules in patients on calcium channel blocker (CCB; amlodipine; N=27) or AT_1 receptor blocker therapy (ARB; olmesartan; N=30) with addition of vitamin C/E or placebo supplement during weeks 9^{th} – 16^{th} , Endothelial (de) activation was assessed indirectly by measuring serum levels of soluble E-selectin (sE-selectin; panel A), soluble intercellular adhesion molecule 1 (sICAM-1; panel B) and soluble vascular cell adhesion molecule 1 (sVCAM-1; panel C). Data are presented as mean±SEM; differences were tested by one-way ANOVA or Kruskal-Wallis analysis of variance followed by Holm-Sidak or Dunn's post-hoc method; *p value less or equal to 0.05 was considered significant.

Discussion

The main findings of this study are: (a) 8iPGF2α levels positively correlate to systolic and diastolic BP in CCB (amlodipine) group, but only to diastolic BP levels in patients receiving ABR olmesartan; (b) the BP normalization evident after 8 weeks of therapy was not followed by an immediate reduction of oxidative stress in tested subjects; (c) 8iPGF2α levels were significantly decreased after additional 8 weeks of treatment, independently of the type of antihypertensive therapy; (d) in both groups BP levels or absolute levels of 8iPGF2α were not affected by vitamin C/E supplementation, but the magnitude of 8iPGF2α reduction was significantly greater in patients taking vitamin C/E and CCB amlodipine compared to patients receiving only CCB; and (e), changes in endothelium activation biomarkers could not be linked to the specific action of antihypertensive therapy, and, interestingly, we found a moderate negative correlation of sICAM-1 and 8iPGF2α in both groups.

Increased oxidative stress and a reduced capacity for scavenging free radicals in patients with essential hypertension is well established [5, 28]. Moreover, it has been shown that systolic and diastolic BP positively correlates to oxidative stress markers and negatively correlates to plasma antioxidant capacity in hypertensive subjects [28]. Contrary to our results, Hirooka et al. found that the CCB amlodipine was inefficient in reducing oxidative stress while the ARB valsartan reduced urine excretion of 8-isoprostane and 8-hydroxy-2'-deoxyguanosine [29]. On the other hand, Ganafa et al. established that amlodipine was efficient in preventing oxidative stress-induced hypertension in Sprague-Dawley rats [30]. Several studies demonstrated that ANGII receptor blockade by olmesartan reduces oxidative stress in human hypertensive subjects [28, 29, 31], which is supported by the mechanistic explanation that p22(phox) expression and the level of ERK1/2 phosphorylation were reduced by olmesartan treatment [31]. These mechanistic data were obtained from peripheral blood monocytes, and not from endothelium, though its relevance to vascular redox signaling in humans remains unknown. In the same study, kinetics of the observed changes varied depending on the parameter measured, which is in line with our finding of a delayed decrease of 8iPGF2α plasma levels after BP normalization (Figure 3). Restricted access to human vessel samples again limits our knowledge as to whether redox signaling pathways and antioxidant pathways are already modified by the antihypertensive therapy at 8 weeks. In our study, we identified a significant negative correlation between aldosterone and 8-iso-PGF2a in the olmesartan group, but not in the amlodipine group (Figure 4). This result indicates that ANGII has a major role in oxidative stress in essential hypertension that is independent of aldosterone. It has been shown that selective antagonism of the AT1 receptor is associated with an increase in plasma ANGII levels, which may stimulate aldosterone secretion through the AT2 receptor when the AT1 receptor is blocked - a phenomenon called “aldosterone breakthrough during AT1 receptor blockade” [32].

Several previous studies have demonstrated increased indicators of inflammation, including endothelial activation markers sICAM-1, sVCAM-1 and soluble P- and E-selectin in hypertensive patients compared to their normotensive counterparts [21, 33]. A high level of inflammation (assessed by hs-CRP) in hypertensive patients with acute ischemic stroke has been linked to an increased risk of future cardiovascular mortality [34]. A study on normotensive subjects revealed that adhesion molecules, particularly the sE-selectin, relates to obesity (waist and hip ratio), whereas the relationship between adhesion molecules and BP in hypertensive subjects is adhesion molecule-specific and varies with sex and age, which may partially explain inconsistencies found in the literature [35].

The results of similar studies investigating the effects of BP reduction on the sCAMS are contradictory, and in some cases depend on the type of the pressure reducing agent. In one study, monotherapy with the ARB irbisartan resulted in a significant decrease in sE-selectin but not in sVCAM-1 or sICAM-1 levels [36], whereas another study found that eight weeks of antihypertensive treatment with enalapril (but not losartan), significantly decreased plasma levels of all sCAMs [37]. We have previously reported a significant reduction of sICAM-1 and sVCAM-1 after 8 weeks of amlodipine treatment [38], and these results are consistent to our present findings in the CCB group, as well as in the ARB group receiving olmesartan

for 8 weeks (Figure 4). At 16 weeks of treatment, the amlodipine and olmesartan groups exhibited increased aldosterone levels; and the antihypertensive therapy increased the endothelial activation markers in most instances, suggesting that endothelial activation could be mediated by aldosterone. However, this is not supported by the Spearman Rank correlation analysis, which showed either no correlation or a weak negative correlation between sCAMs and aldosterone while at the same time there was a significant moderate positive correlation of sVCAM-1 and systolic and diastolic BP in both groups indicating that BP reduction is primarily accountable for the changes in endothelial activation.

As schematically shown in Figure 6, TNFα and IL-1 can be produced as a result of redox-signaling within the endothelium and VSMC, and also as a consequence of activation of residing monocytes-both leading to endothelium activation and expression of surface CAMs. In that respect, Fliser et al demonstrated reduced serum levels of hsCRP, TNF-a, IL-6, and MCP-1 after 6 weeks of olmesartan therapy [39]. Furthermore, combined therapy, including the ARB olmesartan and the CCB azelnidipine lowered BP and reduced plasma levels of hsCRP in patients with essential hypertension [40]. As shown in Figure 4, our present results are in line with studies demonstrating changes in endothelial activation/inflammation markers with BP reduction [37]. However, these changes remain inconsistent and do not support full endothelium deactivation in hypertensive subjects. In addition, the lack of a strong positive correlation between the oxidative stress marker 8iPGF2α and endothelium activation markers (sCAMs) makes it difficult to draw any conclusions on their relationship in this experimental setting.

Evidence of increased oxidative stress and reduced antioxidative capacity in hypertension has provided a rationale for the use of antioxidants in hypertension therapy. For example, vitamin C improved vascular function and reduced BP in spontaneously hypertensive rats (SHR) by up-regulation of eNOS and down-regulation of NOX [41]. In addition, human studies have demonstrated an inverse relation between vitamin C plasma levels and BP and oxidative stress, in normotensive and hypertensive populations [42]. Several small randomized, placebo-controlled trials have revealed short term beneficial effects of vitamin C, including significantly reduced BP and improved endothelium dependent vasodilatation [43, 44]. Conversely, a large clinical trial assessing long term effects (5-years) of vitamin C supplementation in 244 subjects has failed to prove the BP-lowering effect of vitamins C in initially normotensive subjects [45]. A few short term (8 weeks) studies examining synergistic effects of vitamin C and E have confirmed beneficial effects on BP reduction and vascular function in newly discovered hypertensive patients [46, 47]. Possible reasons for these conflicting results might be the failure to achieve functional plasma concentrations of vitamin C/E by oral intake, inability of vitamin C/E to scavenge H_2O_2 or various differences in study design [48].

In contrast to some other studies, we found ARB olmesartan and CCB amlodipine were equally efficient in reducing BP (Figures 2) [29, 49]. Vitamin C/E supplementation had no additional BP-lowering effect. The vitamin C/E supplementation was not effective in reducing absolute values of 8iPGF2α concentration; however, it had a moderate effect to reduce levels of lipid peroxidation end products in the CCB group but not in the ARB group (Figure 5). Furthermore, both antihypertensive drugs reduced the level of inflammation marker sVCAM-1, but not sICAM-1 or sE-selectin (Figure 4).

Conclusion

The results of the present study suggest that observed oxidative stress is a consequence of hypertension. BP reduction, independent of the type of antihypertensive therapy employed, is primarily accountable for the observed decrease in oxidative stress and changes in endothelial activation; Vitamin C/E supplementation has only a minor, if any, effect on markers of oxidative stress and no effect on endothelial activation.

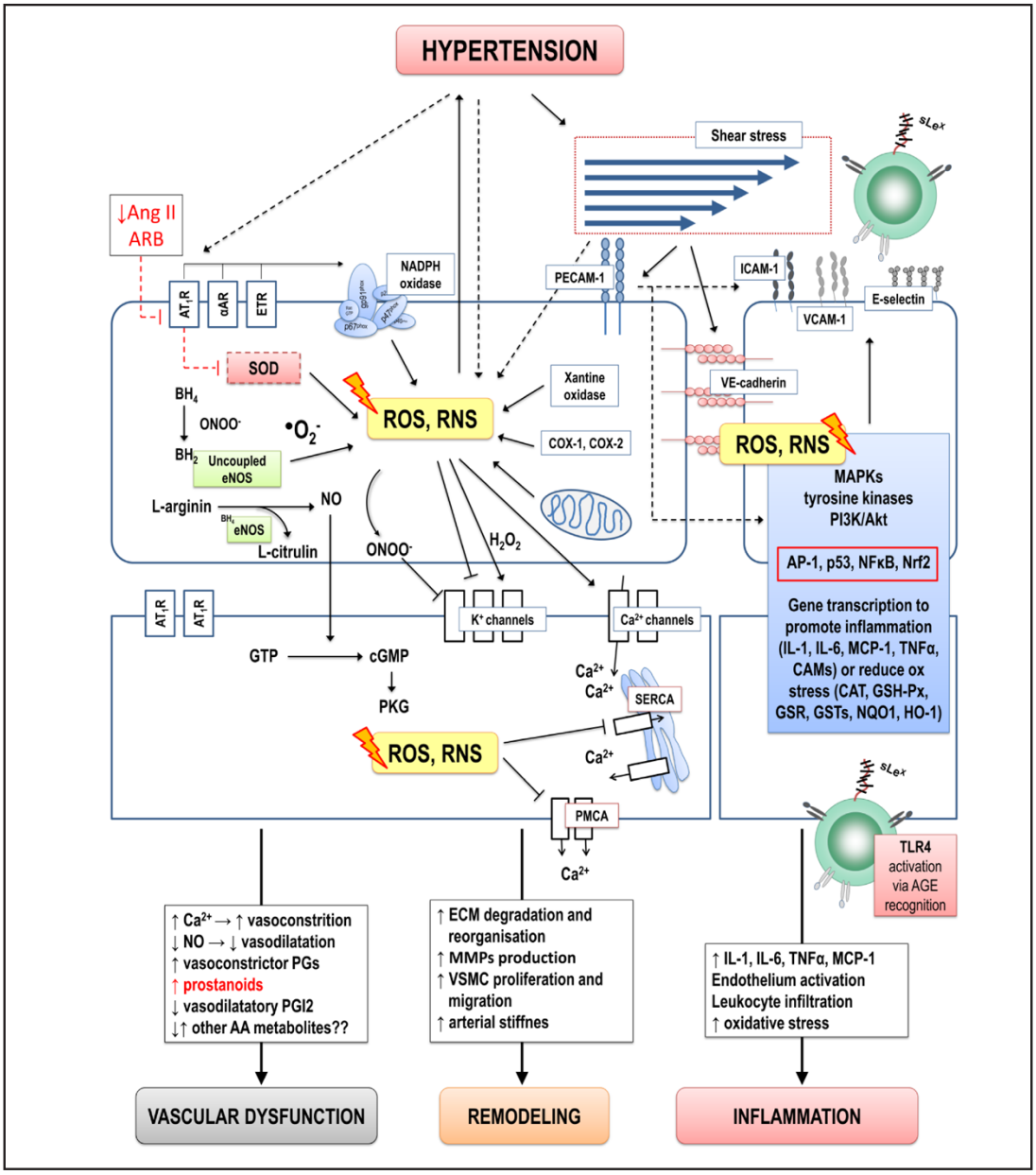


Fig. 6. ROS generation and signaling in the vascular wall during hypertension. The Figure 1 shows the sources of ROS and RNS generation in the vascular wall. A part of the stimuli for ROS generation is derived through AT_1R, αAR and ETR signaling resulting in NADPH oxidase activation. Hypertension presents with increased shear stress to the vascular wall which can also contribute to ROS production, as well as to changes in the expression of endothelial cell adhesion molecules due to endothelial activation and increased leukocyte adhesion and transmigration through the endothelium. In contrary, low angiotensin II levels, such as during high salt intake, reduce antioxidant capacity by modulating SOD expression, which can in turn lead to enhanced oxidative stress. ROS contribute to peroxynitrite formation and reduced BH_4 bioavailability leading to eNOS uncoupling, reduced NO production and further increased ROS production. ROS (except the H_2O_2) block voltage, ATP and Ca^{2+} K-channels and open Ca^{2+}-channels resulting in increased intracellular Ca^{2+} and vasoconstriction. On the other hand, ROS can activate various kinases (i.e. MAPK, PI3K/Akt) and downstream transcription factors (e.g. NFκB) leading to enhanced expression of various genes involved in antioxidant mechanisms, inflammation, and vascular remodeling. Formation of neo-antigens in the vascular wall, such as AGE and oxidized LDL can activate residing monocytes through pattern recognition receptors (e.g. TLR4) and induce endothelium activation and subsequent inflammation. Excessive ROS

and RNS production will ultimately overcome antioxidatve capacity of the vessel wall leading to vascular dysfunction, vascular remodeling and inflammation-all processes involved in the development of cardiovascular diseases. Abbreviations: AGE - Advanced glycosylation end products, ANG II – Angiotensin II, AP-1 - activated protein-1, ARB – Angiotensin II type 1 receptor blockers; AT_1R – Angiotensin II receptor Type 1 , BH_4 – Tetrahydrobiopterin , CAMs – cellular adhesion molecules, CAT – catalase, COX-1,2 - cyclooxygenase 1 and 2, ECM - extracellular matrix, eNOS - endothelial nitric oxide synthase, ETR – endothelin receptor, GSH-Px - glutathione peroxidase, GSR - glutathione reductase, GST - glutathione S-transferase, HO-1 - heme oxygenase-1, ICAM-1 – Intercellular adhesion molecule 1, MAPKs - mitogen-activated protein Kinases, MCP-1 - Monocyte chemoattractant protein-1, MMPs - matrix metalloproteinases, NFκB - nuclear factor kappa B, NQO1 - NADPH:quinone oxidoreductase 1, Nrf2 - nuclear E2-related factor 2, PECAM -1 - Platelet Endothelial Cell Adhesion Molecule-1, PI3K/Akt – phosphoinositol-3-kinase/Akt kinase, PKG G – Protein Kinase G, PMCA Plasma membrane Ca-ATPase, RNS – reactive oxygen species, RNS – reactive nitrogen species, SERCA Sarco/endoplasmatic reticulum Ca-ATPase, TLR4 – Toll like receptor 4, VCAM-1 – Vascular cell adhesion molecule 1, VSMC – vascular smooth musle cells, αAR – α adrenergic receptor.

Abbreviations

ANGII - angiotensin II, ARB - angiotensin II receptor type I blockers , CCB - Ca^{2+}-channel blocker, 8iPGF2α - 8-iso-prostaglandin F2-alpha, BP – blood pressure, AP-1: activated protein-, NFκB - nuclear factor kappa B, sICAM-1 - soluble intercellular adhesion molecule 1, sVCAM-1 - soluble vascular cell adhesion molecule 1, sE-selectin - soluble E-selectin.

Disclosure Statement

Authors declare no conflict of interest.

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