Effect of ACEI and ARB treatment on nitric oxide-dependent endothelial function

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Summary: *Background:* Angiotensin-converting-enzyme inhibitors (ACEI) and angiotensin II receptor blockers (ARB) are widely used as a first-line therapy for the treatment of cardiovascular disease. Here, ACEI modulate the bradykinin receptor (BDKRB1 and BDKRB2) system and NO-dependent endothelial function, thus determining cardiovascular health and regenerative arteriogenesis. The current study aims at evaluating nitric oxide-dependent endothelial function, and gene expression of bradykinin receptors in peripheral blood mononuclear cells (PBMC) from patients with ACEI or ARB treatment. *Patients and methods:* The WalkByLab has been established to screen cardiovascular patients for peripheral artery disease and coronary artery disease. In total 177 patients from WalkByLab with heterogenous disease and risk status were randomly selected, divided according to their medication history into the following groups: 1. ACEI group, 2. ARB group or 3. non-ACE/ARB group. Total plasma nitrite/nitrate (NO) levels were measured, endothelial function was evaluated by assessing flow mediated dilation (FMD). PBMC were isolated from peripheral whole blood, and gene expression (qRT-PCR) of bradykinin receptors and angiotensin converting enzyme were assessed. *Results:* Plasma total NO concentration in the ACEI group (24.66 ± 16.28 , $\mu\text{mol/l}$) was increased as compared to the ARB group (18.57 ± 11.58 , $\mu\text{mol/l}$, $P=0.0046$) and non-ACE/ARB group (16.83 ± 8.64 , $\mu\text{mol/l}$, $P=0.0127$) in patients between 40 to 90 years of age. However, FMD values (%) in the ACEI group (7.07 ± 2.40 , %) were similar as compared to the ARB (6.35 ± 2.13 , %) and non-ACE/ARB group (6.51 ± 2.15 , %), but significantly negatively correlated with age. Interestingly, BDKRB1 mRNA level was significantly higher and BDKRB2 mRNA level lower in the ACEI group (BDKRB1 3.88-fold ± 1.05 , BDKRB2 0.22-fold ± 0.04) as compared to the non-ACE/ARB group (BDKRB1 1.00-fold ± 0.39 , $P<0.0001$, BDKRB2 1.00-fold ± 0.45 , $P=0.0136$). *Conclusions:* ACEI treatment enhances total nitrite/nitrate concentration, furthermore, upregulates BDKRB1 in PBMC, but downregulates BDKRB2 mRNA expression. FMD is a strong determinant of vascular aging and is sensitive to underlying heterogenous cardiovascular diseases.

Keywords: flow mediated dilation, nitric oxide, kallikrein-kinin-system, angiotensin-converting-enzyme inhibitors, angiotensin receptor blockers

Introduction

Vascular aging processes impact atherosclerosis risk and arteriogenic remodelling capacity and are most commonly analyzed by measuring peripheral blood mononuclear cells (PBMCs) phenotype and endothelial function [1]. Whereas

atherosclerosis and arterial occlusion are the consequence of most vascular aging processes, regenerative arteriogenesis is the primary compensatory mechanism against ischemic vascular disease (CAD, PAD). Arteriogenesis is one of the key mechanisms of protective vascular growth, and involves the remodeling and positive outgrowth of

pre-existing collateral arteries following a stenosis or arterial occlusion [2]. For arteriogenesis it was shown as well that endothelial function and PBMCs play a critical role in endothelial proliferation, intima formation and maturation of collateral arteries [3, 4]. Thus, where vascular regeneration interacts positively with PBMC phenotypes and endothelial function, these factors are negatively influenced by pathological processes of aging, and determine the severity of diseases. Recent research has shown that in terms of vascular regeneration, PBMC activity and endothelial function are linked through bradykinin receptor signaling and NO production [1, 5]. In regard to vascular diseases, Angiotensin-converting-enzyme inhibitors (ACEI) and angiotensin II receptor blockers (ARB) are cornerstones of the pharmacological therapy for patients with coronary artery disease (CAD), peripheral artery disease (PAD), heart failure (HF) and essential hypertension [6, 7, 8, 9]. Furthermore, ACEI and ARB are discussed to be involved in arteriogenesis and vascular regeneration. Therefore, in this work, we investigate whether ACEI and ARB therapy is associated with bradykinin receptor signaling in PBMCs, NO production, and endothelial function.

Beyond the property that angiotensin-converting enzyme (ACE) inhibitors are used to treat hypertension and ischemic heart disease, many research groups are looking at the bradykinin-mediated aspects of ACE inhibition [10]. Angiotensin-converting enzyme (ACE, also known as kininase II) is the key enzyme for the degradation of bradykinin (BK). BK is one of the major endothelium-dependent vasodilators in cardiovascular homeostasis. Therefore ACEI, the inhibitor of ACE, prevents BK degradation and leads to the BK mediated and endothelium-dependent vasodilation (EDV) [11]. Furthermore, our group was the first to demonstrate that bradykinin receptor signaling is a strong modulator of therapeutic arteriogenesis [4]. Research has shown that ACEI can be regarded as the allosteric enhancer of bradykinin receptors. The bradykinin receptor family consists of two G protein-coupled receptors: bradykinin receptor 1 (BDKRB1) and bradykinin receptor 2 (BDKRB2), whereby ACEI are agonists activating BDKRB1 directly on the extracellular loop and also indirectly activating the ACE/BDKRB2 heterodimer by altering conformational change [12]. Bradykinin receptor BDKRB2 is constitutively expressed in various cell types, whereas bradykinin receptor BDKRB1 is only upregulated under the inflammatory conditions. BK and kallidin, derived from plasma and tissue kallikrein, respectively, are the corresponding ligands of BDKRB2, whereas the BK and kallidin derivatives des-Arg⁹-bradykinin and Lys-bradykinin are the corresponding ligands of BDKRB1 [13]. It was shown that BDKRB2 and BDKRB1 signaling induces nitric oxide (NO) production in endothelial cells and circulating immune cells [14]. Hence, bradykinin receptors and NO are major players in mediating arteriogenesis and endothelial function [5].

In addition to ACEI, ARB are recommended as first-line treatment options for hypertension. ARB are superior to

ACEI in regard to their more favorable (drug) safety profile ARB selectively inhibit angiotensin II type 1 receptors (AT1), thereby augmenting angiotensin II type 2 receptor (AT2) activity, whose effects antagonize AT1 [15, 16]. Interestingly, a functional heterodimerization of AT2-BDKRB2 enhances peptide ligand binding and receptor activation [17]. Animal experiments also demonstrated that angiotensin II induced upregulation of BDKRB1 is abrogated in AT1 knockout mice. BDKRB1 can be regarded as the downstream target of AT1 agonists [18].

In the current study we therefore analyze total nitrite/nitrate concentration in plasma, endothelial function by flow mediated dilation and gene expression of bradykinin receptors and angiotensin converting enzyme in PBMCs from patients with CAD, PAD, cerebrovascular disease (CVD), and hypertension treated pharmacologically with either ACEI or ARB.

Patients and methods

Study population

In total 177 patients with atherosclerotic disease (CAD, PAD, CVD) were randomly selected from the WalkByLab-Registry database. These are the patients who presented to the WalkByLab center Brandenburg/Havel (Brandenburg Clinic, Brandenburg Medical School) Germany in the period from July 2019 to February 2020 with the corresponding pre-existing cardiovascular disease. The WalkByLab aims to interdisciplinarily screen, diagnose and follow-up patients with cardiovascular disease (www.laflab.de). The WalkByLab register trial protocol was reviewed and approved by the ethical committee of the Cottbus Medical Association (Landesärztekammer Cottbus, study number of the ethics committee: AS 74(bb)/2018). The screening trial is performed in accordance with the principles of the declaration of Helsinki.

Isolation of peripheral blood mononuclear cells

15 ml blood was collected in three vacutainer EDTA tubes (Becton Dickinson) by routine venipuncture procedure. Blood was diluted 1:1 in PBS, and carefully layered onto the Ficoll-Paque density gradient media (GE Healthcare) at a ratio of 4:3 and centrifuged at 400 g for 25 min. The upper layer of plasma was collected from each sample and frozen in 2 ml aliquots at -80°C until use for determination of total nitrite/nitrate levels.

The middle layer containing PBMCs was transferred to 50 ml sterile tubes, and washed twice with 40 ml PBS. Cell counting was performed under the microscope. Aliquots including 5 million cells were centrifuged at 1500 g for 10 minutes and the pellet was frozen and stored at -80°C until use for RNA isolation.

RNA isolation and quantitative real-time PCR-based analysis

Total RNA was extracted from PBMCs by using the Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA integrity was assessed by agarose gel electrophoresis, quantitative analysis of RNA was performed using Qubit® RNA BR assay together with the Qubit® 3.0 Fluorometer (Thermo Fisher Scientific). 1 µg total RNA was used for the first strand cDNA synthesis by using QuantiTect Reverse Transcription Kit (QIAGEN). Samples were deployed in the peqSTAR thermal cycler (VWR International). The mRNA levels of the target gene were analyzed by real-time polymerase chain reaction using the LightCycler® 96 Real-Time PCR System (Roche). Each reaction system contained 1 µl cDNA, 2 µl primer working solution, 7 µl RNase/DNase-free water and 10 µl QuantiTect® SYBR® Green PCR Kit (QIAGEN). All the primers were synthesized and purchased from Eurofins Genomics Germany GmbH. All samples were run in duplicate (Table I).

Determinations of total nitrite/nitrate levels in plasma

In the cell, nitric oxide is converted into nitrite and nitrate as the stable metabolites after a series of reactions. Therefore, total nitrite/nitrate concentration is the best value for assessing the NO production in a physiological system. Plasma samples were thawed and ultrafiltered to remove hemoglobin by centrifugation in an ultra-0.5 centrifugal filter devices (Merck Millipore). Total nitrite/nitrate levels in plasma were determined according to the manufacturer's instructions (Cayman Chemical). In brief, conversion of nitrate to nitrite is achieved by utilizing nitrate reductase enzyme. During this process a deep purple azo dye is formed based on Griess reagent, and the light absorbance can be spectrophotometrically measured at 548 nm on a Spark multimode microplate reader (Tecan Group AG).

Assessment of flow mediated dilation

The AngioDefender (Everist Health) medical device was used to evaluate endothelial function by assessing flow mediated dilation (FMD) of the brachial artery. FMD measurement is regarded as the gold standard method for evaluation of endothelial function. The novel AngioDefender medical device allows for a precise, standardized, and automated measurement of FMD-value by oscillation technique, it has been proven to show less measurement errors when compared with classical FMD measurement based on ultrasound techniques. The AngioDefender measurement technique is therefore regarded superior to the classical measuring methods.

Treadmill testing

A treadmill and the "Gardner Test Protocol" are used to determine the maximum walking distance. The test is

performed under the supervision of a physician, the patient is secured with a drop stop device (safety bar with chest harness). The patient initially stands on the lateral treads and the natural walking speed is tested, while the treadmill accelerates to 3.2 km/h to allow the patient to enter the treadmill and start the test (time "zero"). Subsequently, the incline angle of the treadmill changes by 2 degrees every 2 minutes. In addition to this, the concomitant symptoms (peripheral fatigue, angina pectoris, dyspnea and others) during treadmill testing have also been recorded. However, some patients did not participate to the walking test because they suffered severe cardiovascular disease. Hence, we have results from in total 83 patients of the here analyzed patient population.

Statistical analysis

Data of demographics, plasma nitrite level and flow mediated dilation are given as mean±standard deviation (SD), data of mRNA expression are given as mean±standard error of the mean (SEM). A Kolmogorov-Smirnov test was carried out to check the distribution of quantitative variables, data following a normal distribution were analyzed by the one-way analysis of variance (ANOVA), data following a non-normal distribution were analyzed by the Kruskal-Wallis test. A p-value of $P \leq 0.05$ (two-sided) was considered to indicate statistical significance.

Results

All patients were screened for medication history, blood samples were taken, and FMD measured. Patients were divided into three groups as follows according to the medication history: 1. ACEI group, 2. ARB group, and 3. non-ACE/ARB group (neither treated with ACEI or ARB). Population characteristics are presented in Table II.

Patient age groups were 40 to 49 years (2.82%), 50 to 59 years (9.60%), 60 to 69 years (36.72%), 70 to 79 years (31.64%) and 80 to 89 years (19.21%), respectively. The mean age was 70.10 years (SD=9.35). 41.81% patients were female and 58.19% patients were male. The mean BMI of patients was 27.96 (SD=4.95) and of these patients were normal weight (BMI=18.5–23, 27.81%), overweight (BMI=23.0–27.49, 44.38%), and obese (BMI>27.5, 27.81%), respectively. Common comorbidities included: hypertension (n=167), diabetes mellitus (n=36), renal dysfunction (n=34), hyperlipidemia (n=74). 84 patients suffered from PAD, 91 patients suffered from CAD, and 12 patients suffered from CVD. For 32 patients a history of myocardial infarction was reported, heart failure for 41 patients, 74 were smokers.

Total plasma nitrite/nitrate concentration

Total plasma nitrite/nitrate level in the ACEI group (24.66 ± 16.28 , µmol/l) was significantly higher than that

Table I. qRT-PCR primer list (sense and antisense primers)

Gene	Forward (5'-3')	Reverse (5'-3')
BDKRB1	ATTCTCCACCTCAGCCTCT	CTCTGGTTGGAGGATTGGAG
BDKRB2	CTTCATGGCCTACAGCAACA	GCACACTCCCTGGTACACCT
ACE	ATGAAGACCTGTTATGGGCATGG	ATTCGCGGTAAACTGGAGGATGG
RPLPO	ACGGGTACAAACGAGTCCTG	AGCCACAAAGGCAGATGGAT

Table II. Population characteristics

	ACEI (n=73)	ARB (n=68)	Non-ACEI/ARB (n=36)	P
Demographics				
Gender, male	47	34	22	0.207
Age, y	68.45±9.40	71.85±9.09	70.11±9.38	0.097
BMI, kg/m ²	27.94±5.34	28.62±5.11	26.69±3.45	0.181
Medical history				
Hypertension	67	67	33	0.164
Diabetes mellitus	16	12	8	0.781
Renal dysfunction	12	13	9	0.566
Hyperlipidemia	32	30	12	0.513
PAD	38	30	16	0.590
CAD	38	33	20	0.784
CVD	6	4	2	0.814
Myocardial infarction	8	16	8	0.118
Heart failure	18	16	7	0.828
PCI/PPI	27	28	18	0.431
CABG/PABG	8	10	1	0.174
Smoking	28	28	18	0.506
Medication history				
Beta-blocker	42	38	23	0.725
Calcium channel blockers	22	33	7	0.007
Diuretics	31	24	6	0.028
Statins	46	45	23	0.924
Aspirin	36	42	16	0.169
Antidiabetics	10	9	4	0.928
Insulin	6	5	3	0.977

PCI: Percutaneous coronary intervention, PPI: percutaneous peripheral interventional, CABG: coronary artery bypass grafting, PABG: peripheral artery bypass grafting.

in the ARB group (18.57±11.58, $\mu\text{mol/l}$, $P=0.0046$) and non-ACE/ARB group (16.83±8.64 $\mu\text{mol/l}$, $P=0.0127$) (Figure 1A). The plasma total nitrite/nitrate level in the ARB group showed a similar concentration (18.57±11.58, $\mu\text{mol/l}$) as compared to the non-ACE/ARB group (16.83±8.64 $\mu\text{mol/l}$).

Furthermore, plasma nitrite/nitrate levels were analyzed in regard to possible sex differences in the medical treatment groups, however results showed no relation between plasma nitrite/nitrate and sex ($P=0.963$) (Figure 1B). A Spearman's rank correlation was calculated in order to investigate how plasma total nitrite/nitrate is related to the different medical treatment groups in the context with age. Nitrite/nitrate was not correlated with age in any group ($P=0.215$) (Figure 1C).

Endothelial function analyzed by flow mediated dilation (FMD)

Cardiovascular health and arteriogenic capacity are largely determined by arterial endothelial function. Endothelial function was assessed in all patients using the AngioDefender, which is a new and standardized method for determining FMD. FMD in the ACEI group (7.07±2.40, %) showed values with no significant differences as compared to the ARB group (6.35±2.13, %) and values were comparable to the non-ACEI/ARB group (6.51±2.15, %) (Figure 2A). FMD was analyzed in regard to possible sex differences and results showed no relation between FMD and gender ($P=0.229$) (Figure 2B). Correlation analysis of FMD with age showed a significant and negative correlation. FMD

Figure 1. (A) Plasma total nitrite/nitrate levels in patients of three groups (* $P \leq 0.05$). (B) Correlation analysis between plasma total nitrite/nitrate levels and gender shows no correlation. (C) Correlation analysis between plasma total nitrite/nitrate levels (log scale) and age shows no correlation. ACEI ($n=73$), ARB ($n=68$), non-ACEI/ARB ($n=36$).

decreases with increasing age in all medical treatment groups ($P < 0.001$) (Figure 2C). Furthermore, FMD does not correlate with nitrite/nitrate level in any medical treatment group ($P = 0.949$) (Figure 3A, Table III)

Treadmill testing

Treadmill testing results showed that the maximal walking distance and walking speed in the ACEI are slightly higher than in the ARB group and slight lower than in the non-ACEI/ARB but without significant differences. The results of concomitant symptoms reported during treadmill testing

Figure 2. (A) FMD of the brachial artery in patients of three groups. (B) Correlations analysis between FMD and gender shows no correlation. (C) FMD correlates with age in all treatment groups (linear model). ACEI ($n=73$), ARB ($n=68$), non-ACEI/ARB ($n=36$).

showed there is no significant differences between three groups groups (Table IV).

Gene expression analysis of markers and regulators of arteriogenesis

ACEI treatment resulted in an upregulation of BDKRB1 in PBMCs, but a downregulation of BDKRB2 mRNA expression. BDKRB1 mRNA level was significantly higher in the ACEI group (3.88-fold $\pm$ 1.05), when compared to non-ACEI/ARB group (1.00-fold $\pm$ 0.39, $P < 0.0001$). The expression level of BDKRB1 in the ARB group (2.46-fold $\pm$ 0.59)

Figure 3. Correlations analyses between FMD, plasma total nitrite/nitrate levels and gene expression of BDKRB1/BDKRB2.

was slightly lower than in the ACEI group, but significantly higher as compared to the non-ACEI/ARB group (1.00-fold $\pm$ 0.39, $P=0.0003$) (Figure 4A). Regarding BDKRB2, the mRNA expression level in the ACEI group (0.22-fold

$\pm$ 0.04) was significantly lower than in the non-ACEI/ARB group (1.00-fold $\pm$ 0.45, $P=0.0136$). Again, the ARB group (0.32-fold $\pm$ 0.14), follows the same trend as the ACEI group, was slightly higher than ACEI group, but it is significantly lower as compared to the non-ACEI/ARB group (1.00-fold $\pm$ 0.45, $P=0.0228$) (Figure 4B). Finally, the ACE mRNA level was unchanged between all groups, ACEI group (0.63-fold $\pm$ 0.06), ARB group (0.69-fold $\pm$ 0.08), and non-ACEI/ARB group (1.00-fold $\pm$ 0.30) (Figure 4C).

In the context of previously described total plasma nitrite/nitrate concentration and flow mediated dilation, we performed a correlation analysis in relation to bradykinin receptor BDKRB1 and BDKRB2 gene expression. The correlation analyses showed that FMD positive correlates with BDKRB1 in female patients ($P=0.040$), however, plasma nitrite/nitrate concentration does not correlate neither with BDKRB1 nor with BDKRB2 (Figure 3, Table III).

Discussion

This work demonstrates that ACEI, as compared to ARB treatment and non-ACEI/ARB treatment, is effective in increasing the plasma total nitrite/nitrate concentration, with concomitant BDKRB1 upregulation and BDKRB2 down-regulation in circulating PBMCs of patients with heterogeneous ischemic vascular disease. However, although NO metabolism was elevated in the ACEI treatment group, endothelial function as measured by FMD remained unchanged as compared to the ARB and Non-ACEI/ARB group, respectively. Furthermore, this study compared nitrite/nitrate levels and FMD to age and demonstrated that FMD but not nitrite/nitrate negatively correlates with age.

Nitrite/nitrates are viewed as the end metabolites of nitric oxide (NO), which is the primary vasodilator molecule and the secondary messenger of the bradykinin receptor signaling pathway. NO not only plays an important role for modulating endothelium-dependent relaxation, but also for regulating active vascular growth [19]. Previous research indicated the superiority of ACEI over ARB with regard to their effect on NO production [20, 21]. In-vivo studies have shown that the nitric oxide synthesis (NOS) inhibitor - L-NAME (N(gamma)-nitro-L-arginine methyl ester) causes an overactivation of the renin-angiotensin system (RAS), thereby leading to hypertension. However, ACE knockout mice are resistant to the hypertension induced by L-NAME [22]. Furthermore, administration of ACEI decreased serum asymmetric dimethylarginine, which is an endogenous NOS inhibitor [23, 24]. These results demonstrate that inhibition of ACE is an important and targeted molecular therapeutic option to upregulate NO levels. These publications are consistent with our finding regarding ACEI treatment elevated plasma NO levels. However, in this study we show for the first time an elevated plasma NO level in hypertensive, ACEI treated patients with different and heterogeneous ischemic vascular diseases (CAD, PAD and CVD). The elevated

Table III. Correlation analyses

	ACEI (n=73)		ARB (n=68)		Non-ACEI/ARB (n=36)		Total sample (n=177)	
	Male	Female	Male	Female	Male	Female	Male	Female
FMD_nitrite/nitrate								
r	−0.125	−0.051	−0.190	0.267	−0.087	0.358	−0.096	0.157
P	0.402	0.805	0.282	0.126	0.700	0.210	0.336	0.181
FMD_BDKRB1								
r	0.109	0.280	−0.143	0.078	0.416	0.128	0.046	0.246
P	0.476	0.175	0.442	0.672	0.076	0.678	0.657	0.040
FMD_BDKRB2								
r	−0.235	0.085	−0.198	−0.079	0.004	−0.086	−0.149	−0.032
P	0.120	0.688	0.261	0.663	0.986	0.769	0.137	0.787
Nitrite/nitrate_BDKRB1								
r	−0.042	−0.061	0.138	0.217	−0.304	0.001	0.037	0.097
P	0.782	0.774	0.461	0.233	0.206	0.996	0.724	0.425
Nitrite/nitrate_BDKRB2								
r	−0.042	−0.061	0.138	0.217	−0.304	0.001	−0.193	0.034
P	0.782	0.774	0.461	0.233	0.206	0.996	0.053	0.775

Table IV. Parameters of treadmill test

	Maximal walking distance (m)	Duration (mm:ss)	Walking speed (m/h)	Peripheral fatigue	Angina pectoris	Dyspnea	Others
ACEI (n=34)	257.97±192.16	05:13±03.55	3036.21±427.65	8	3	28	6
ARB (n=38)	250.37±175.52	05:06±03:31	2966.95±581.42	14	1	28	5
Non-ACEI/ARB (n=11)	298.73±177.90	05:44±03:28	3174.09±162.97	4	0	5	2
P	0.741	0.880	0.453	0.443	0.343	0.055	0.846

nitrite/nitrate levels found in this study have interesting scientific implications, NO is the most important molecule for the regulation of vascular tone, but also plays a crucial role (I) in modulating endothelial function, and (II) in regulating arteriogenesis [1, 5].

With regard to endothelial function, recent studies have suggested that endothelial dysfunction precedes the progression of atherosclerosis and other age-related vascular diseases. Therefore, endothelium-dependent FMD can be regarded as a prognostic indicator of major adverse cardiovascular and cerebrovascular events (MACCE) [25]. Indeed, studies measuring FMD in patients with cardiovascular disease showed that endothelial dysfunction correlates to CAD severity [26]. Furthermore, both pre-clinical studies and clinical trials have clearly illustrated that ACEI could improve FMD and prevent endothelial dysfunction [27, 28]. However, an ACEI related FMD improvement was not confirmed in this analysis. FMD is slightly increased in the ACEI treatment group as compared to the ARB treatment group, but this value is not significantly different. The reason for our deviating findings probably depends on the selection of patients with different underlying cardiovascular diseases. Endothelial function precisely maps biological age, but is also very sensitive to different forms of cardiovascular diseases. Therefore, selection of a heterogeneous group of patients with different cardiovascular

diseases could affect endothelial function differently. Nevertheless, there was a clear negative correlation of FMD with patient age. Indeed, numerous studies have shown that progressive endothelial dysfunction is one of the most important aging-associated determinants [29, 30], and we confirmed that FMD is strongly and negatively correlated with increasing age. Since, we enrolled the patients in a randomized manner, the number of males and females differed by 58.19% to 41.81%. We therefore also investigated the differences in nitrite/nitrate levels and FMD between males and females. In our analysis there were no differences in nitrite/nitrate levels and FMD related to gender, thus different gender ratios within our patient group should not bias the group differences discussed herein.

In addition, our results show for the first time that patients on ACEI medication have upregulated BDKRB1, but downregulates BDKRB2 mRNA expression in PBMCs. ACEI not only inhibit angiotensin I (Ang I) to angiotensin II (Ang II) conversion, but also increase bradykinin concentration. Bradykinin derivatives are direct ligands of BDKRB2 and BDKRB1. In fact, stimulation of BDKRB2 leads to intracellular activation of endothelial nitric oxide synthase (eNOS) in the endothelia cell layer, and stimulation of BDKRB1 results in activation of inducible nitric oxide synthase (iNOS) in immune cells, such as PBMCs

Figure 4. Gene expression of BDKRB1: ACEI (n=70), ARB (n=63), non-ACEI/ARB (n=32), gene expression of BDKRB2: ACEI (n=70), ARB (n=67), non-ACEI/ARB (n=36), and gene expression of ACE: ACEI (n=72), ARB (n=68), non-ACEI/ARB (n=36) in PBMC (* $P \leq 0.05$).

[14]. Previous research has also shown that the antagonist of BDKRB2 attenuated the augmentation of NO production induced by ACEI. While BDKRB2s are known to regulate vascular tone, they also exhibit atherogenic activity by increased reactive oxygen species generation [31]. In contrast, BDKRB1s exhibit anti-atherosclerotic activities, and mice deficient in kinin B1 receptor and apolipoprotein E showed a predisposition to atherosclerosis development [32]. Recent research shows that both of BDKRB1 and BDKRB2 can respectively bind to different heterotrimeric G proteins and thereby increase NO production by activating either iNOS or eNOS: in normal physiological conditions, BDKRB2 couples through $G_{\alpha q/11}$ and results in calmodulin-dependent activation of eNOS; whereas, in the inflammatory conditions (myocardial infarction, ischemic stroke, acute peripheral arterial occlusion), B1R

couples through $G_{\alpha i}$, $G_{\beta \gamma}$ and results in activation of iNOS through the ras-raf-mek-erk-map kinase pathway [14]. Hence, the putative ACEI-BDKRB1-iNOS and ACEI-BDKRB2-eNOS signaling pathways may be of significance as a target for anti-atherogenic and pro-arteriogenic therapies in future.

Indeed, accumulating evidence suggests that BDKRB1 are amplified and expressed constitutively in PBMC subtypes such as macrophages [33]. Our research group demonstrated, that in particular BDKRB1 signaling regulates arteriogenesis by modulation of monocyte/macrophage migration. Moreover, we tested whether bradykinin receptors play a functional role in adaptive arteriogenesis, and analyzed the role of the bradykinin receptor system for collateral artery growth by using a loss-of-function approach. We found a strong reduction in peripheral arteriogenesis for BDKRB1^{-/-} mice and only a minor reduction for BDKRB2^{-/-} mice. A transplantation of wild type into BDKRB1^{-/-} mice recovered the loss of arterial flow after an arterial occlusion surgery, as seen in untreated BDKRB1 mutant mice (gain of function). The enhanced expression of BDKRB1 on immune cells seems to be the pivotal determinant during collateral artery growth and vascular regeneration [4]. Our finding that ACEI treatment might activate BDKRB1 has a clinically perspective and evidence is growing that BDKRB1 can be activated by ACEI directly on the extracellular loop without altering conformational change [12]. Interestingly, our correlation analyses between bradykinin receptor expression and endothelial function shows that there is a positive correlation between FMD and BDKRB1 exclusively in female patients. This is indeed consistent with the findings of Wu et al. [34], who previously speculated here that sex differences in BDKRB1-induced cytokine production may occur in response to ACEI therapy. Indeed, our results here also suggest a possible sex-specific difference in BDKRB1-mediated endothelial function in response to ACEI therapy that needs to be further investigated.

Limitations

The current cross-sectional study is aimed at analyzing the gene expression of bradykinin receptors and ACE in peripheral blood mononuclear cells from patients with ACEI or ARB treatment. Here, we did not conduct any new pharmacological therapy, therefore, the evidence level grade is lower than that of a randomized clinical trial (RCT) or cohort study.

Conclusions

We firstly showed that ACEI treatment enhances total nitrite/nitrate concentration in patients with underlying heterogenous cardiovascular diseases. NO production might also enhance endothelial function in the ACEI treatment group as previously reported, however FMD

measurements showed no differences between the medication treatment groups. The selection of patients with different and concomitant cardiovascular diseases and risk status might influence FMD analysis. FMD is known to be strongly affected by different risk factor such as hypertension, diabetes or severity of atherosclerosis status [25]. Indeed, we demonstrated that FMD is strongly negatively correlated with age in patients with concomitant ischemic vascular disease (CAD, PAD and CVD) and hypertension. Hence, FMD is sensitive to underlying heterogenous cardiovascular diseases, and was again shown to be a strong determinant of vascular aging.

Secondly, ACEI treatment induces the upregulation of BDKRB1, but the downregulation of BDKRB2 mRNA expression in PBMC from patients with cardiovascular disease. The role ACEI-bradykinin receptor signaling pathway is expected to redirect away from pathologic atherosclerosis towards physiological arteriogenesis, which is worthy of further investigation.

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Conflict of interest

The authors have declared no conflict of interest.

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