Expression of Genes Encoding Kinin B_1 and B_2 Receptors in Pripheral Blood Mononuclear Cells (PBMC) from Patient with Slow Coronary Flow

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ABSTRACT

Objective: Slow coronary flow (SCF) is specified by the delayed passage of contrast in the absence of obstructive epicardial coronary disease. Its etiopathology remains unclear. Kinins are mediators of vasodilatation as well as inflammation in human body. This study aimed to investigate the gene expression of kinins B_1 and B_2 receptors (B_1R and B_2R) in peripheral blood mononuclear cells (PBMC) from patients with SCF.

Methods: Thirty patients with SCF (22 male/ 8 female; age: 53 ± 11.83 years) and 30 healthy controls (22 male/ 8 female; age: 51.37 ± 11.89 years) were enrolled for the study. Patients and controls were matched with age, gender and body mass index. Peripheral blood mononuclear cells (PBMC) were obtained for mRNA extraction. To analyze the gene expression levels of B_1R and B_2R, mRNA extraction, cDNA synthesis and quantitive reverse transcriptase polymerase chain reaction (QRT-PCR) were carried out for control and SCF groups.

Results: B_1R expression showed statistically significant difference between SCF patients and controls (0.15 ± 0.03 vs. 0.98 ± 0.15 fold, respectively; $p < 0.0001$), SCF patients with 3 slow flow coronary arteries and controls (0.15 ± 0.04 vs. 0.98 ± 0.15 fold, respectively; $P = 0.001$) and SCF patients with <3 slow flow coronary arteries and controls (0.14 ± 0.04 vs. 0.98 ± 0.15 fold, respectively; $p = 0.001$). There was no found statistically significant difference between SCF (0.58 ± 0.11 fold) and control groups (1.22 ± 0.28 fold) in analysis of B_2R gene expression ($p = 0.143$).

Conclusion: It can be concluded that decreased B_1R gene expression and its signaling pathway may provide a structural basis of the important role of kinins in SCF pathogenesis.

Keywords: Kinin receptors, peripheral blood mononuclear cell, slow coronary flow

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INTRODUCTION

Slow coronary flow (SCF), which was first demonstrated by Tambe in 1972, is an angiographic finding described by the delayed passage of contrast in the absence of obstructive epicardial coronary disease that may affect one or all coronary arteries (1). The incidence of SCF is 1%-7% in patients undergoing diagnostic coronary angiography (2) and has more frequently been reported in young men and smokers (3). Histological researchs have demonstrated myofiber hypertrophy, hyperplastic fibromuscular thickening of small arteries, swelling and degeneration of endothelial cells with constriction of the vascular lumina in SCF patients (4). However pathophysiological mechanism of SCF remains unclear (5). Microvascular dysfunction including elevated resting coronary microvascular tone (5), endothelial thickening in small vessels (6), patchy fibrosis (4), impaired endothelial release of nitric oxide (NO) (7), increased microvascular resistance at rest (8) and the other hypotheses including an earlier form of atherosclerosis, platelet aggregability, an imbalance between vasoconstricting and vasodilating factors (9) and inflammation (10) have been known to be implicated in the pathogenesis of SCF.

Kinins are peptides contributing in several physiological processes such as vasodilation, vascular permeability, pain and inflammatory reactions (11). Their various actions are mediated by kinin B_1 and B_2 receptors (B_1R and B_2R respectively). The B_1R is activated specifically by des[Arg9]-Bradykinin (des[Arg9]-BK) and Lys-des[Arg9]-BK (12). The B_2R is responsible for the main physiological functions of kinins and agonist of witch is BK (12, 13). B_1R is expressed in inflammation, sepsis and tissue injury (14) and its expression is stimulated by Lys-des[Arg9]-BK (12), IL-1β (12) TNF-α (11) and infectious stimuli (11). Kinins intensively affect the activity of inflammatory cells by stimulating the synthesis of cytokines (15), eicosanoids (15), NO (16) and chemotactic factors (15). Kinins have an important role in the cardiovascular system (17). As kinins have half-life < 1 min and

their function are largely dependent on the expression rates of B_1R and B_2R (18), the aim of the present study was to assess the expression of genes encoding B_1R and B_2R in peripheral blood mononuclear cells (PBMC) in patient with SCF.

SUBJECTS AND METHODS

Study groups

The patient group was consisted of 30 patients (22 male/8 female) aged 35-76 years (53±11.83 years) with SCF. Entry criteria included; exertional chest pain, positive treadmill test, normal angiogram and the Thrombolysis In Myocardial Infarction (TIMI) frame count (TFC) (quantitative way of assessing coronary artery flow) greater than 23 frames (19) for SCF patients group. Patients with known coronary or peripheral vascular disease, renal and hepatic dysfunction, ectatic coronary arteries, evidence of ongoing infection or inflammation, known malignancy, hematological disorders and diabetes mellitus were excluded from the study. Thirty healthy subjects (22 male/8 female; 51.37±11.89 years) matched with sex, age and body mass index (BMI) were included as control group.

The study was approved by Medical Ethics Committee (Ethical approval code. IR.umsu.rec.1393.49) at Urmia University of Medical Sciences, Urmia, Iran; and all subjects were given written informed consent.

Applied methods

Blood samples (10 ml) were collected from the femoral artery of SCF patient after angiography and basilic vein of control group into tubes containing ethylenediaminetetraacetic acid (EDTA-K_3) and PBMC was separated by Ficoll density gradient centrifugation. Twenty ml of peripheral blood and phosphate buffere saline (PBS)

mixture was stratified above 5 ml of Ficoll–Hypaque (Baharafshan, Iran). Then the sample was centrifuged 20 min at 800g at room temperature. PBMC were isolated from buffy coat layer and were washed two times with PBS. Total RNA was extracted from PBMC, using the guanidine/ phenol solution (RNX-Plus, CinaGen Co., Tehran, Iran). Purity of RNA extracts were evaluated by measuring the ratio of the absorbance at 260 and 280 nm (A260/ A280 greater than 1.8) by Biophotometer (Ependrof AG, Germany). Primer sequences were shown in Table 1 (18) (TAG Copenhagen A/S). The β-actin gene was used as the endogenous control gene.

BioRT cDNA first strand synthesis kit (Bioflux-Bioer, Hangzho, China) was used to synthesize complementary DNA (cDNA) from mRNA according to manufacturer's instructions. In order to determine gene expression of B_1R and B_2R, quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) was performed using SYBR Green RT-PCR kit (Bioneer, Accu Power 2X Green Star^{TM} qPCR Master mix, Deajeon, Korea). Cycling conditions were as follows: polymerase activation: 95ºC for 5 min; 45 cycles: 95ºC for 15 s and 68ºC for 30s for B_2R, 60 ºC for 30s for B_1R, and 72º for 30s. PCR products were run for electrophoresis in 2% agarose gel and Tris-borate-EDTA (TBE) buffer pool then visualized and imaged under UV light. All reactions were carried out in duplicate. Relative quantification Real-time PCR of expression for B_1R and B_2R with internal control of β-actin were determined by the ΔΔCT method in SCF and control groups, as described previously. Data are presented as the fold change in gene expression normalized to β-actin as endogenous reference along with standard error.

Also, the Complete Blood Count (CBC) including red blood cell (RBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular valume (MCV), mean corpuscular homoglobin concentration (MCHC), mean corpuscular homoglobin (MCH), white blood count (WBC) and platelet (Plt), prothrombin time (PT), partial thromboplastin time (PTT) and international

normalized ratio (INR) were performed for all patient and control groups. Statistical Package for Social Sciences (SPSS) 22 software was used for data analysis. The Kolmogorove-Smirnov test was performed to detect normal distributions. In normal distribution, the *t*-student test was used; otherwise the Mann-Whitney *U* test was used. The values for gene expression were represented as means ± standard error of mean (mean ± SEM) and others were expressed as means ± standard deviation (mean ± SD). If the p-value was less than 0.05, Differences were considered to be significant.

RESULTS

Demographic and clinical characteristics including age, sex, BMI, heart rate, systolic and diastolic blood pressure, smoking, familly history of coronary heart disease (CHD) and medicines taken by SCF patient (Aspirin, Statins, β-blockers) are summarized in table 2.

Both mean systolic blood pressure (128.03 ±15.85 vs. 137.48±13.79 mmHg, p=0.005; respectively) and diastolic blood pressure (81.86±22.08 vs. 88.72±10.31mmHg, p=0.007; respectively) were significantly lower in SCF group than control group.

The SCF patient group was divided into subgroups according to the number of slow flow coronary artery (TIMI frame count (TFC)>23) and smoking status. Then the TIMI frame count of 3 coronary artery (right coronary artery (RCA) TFC: 36.33±8.03, left circumflex artery (LCX) TFC: 33.20±5.42, left coronary artery (LAD) TFC: 43.51±8.52, total TFC: 40.01±7.58 Frame) and ejection fraction (EF) (52.50±7.51%) were compared in smoker and nonsmoker SCF patients and SCF patient with <3 and 3 slow flow coronary artery. The results were represented in table 3.

Results of gene expression study were expressed as mRNA fold. We observed significantly decreased gene expression in B_1R comparing SCF group versus control group

(P< 0.0001), SCF patient with three slow flow coronary versus control group (P=0.001) and SCF patient with 3> slow flow coronary versus control group (P=0.001). There was no significant difference in the comparison mRNA fold of B_2R between groups (Table 4). In this survey, spearman analysis between B_1R and B_2R expression revealed that there was positive correlation at the 0.001 level (r=0.373, p=0.003).

Complete blood count (CBC) was compared between SCF and control groups. There was no significant difference between the parameters of CBC except MCV and PT. Table 5 shows the results of comparation between SCF vs. control, SCF with 3 and <3 slow flow coronary artery (together and with control), smoker and nonsmoker SCF.

DISCUSSION

The results of several studies suggest that inflammation plays an important role in the pathogenesis of SCF (10). The inflammatory cytokines in inflammation leads to endothelial activation as well as reduced coronary blood flow (9). The renin-angiotensin system (RAS) is associated with inflammatory conditions (20). Angiotensin converting enzyme (ACE) or kininase II, one of the RAS members, converts angiotensin I (Ang I) to angiotensin II (Ang II), a powerful vasoconstrictor, that acts on vascular smooth muscles and inactivate BK (21). Serum kininase II activity is higher in subjects with deletion/deletion (D/D) alleles than in subjects with insertion(I) and D alleles and is related to cardiovascular disease (22) that the DD genotype and D allele was reported to be higher in SCF patients (23). On the other hand, Ang II has two receptor subtypes, Ang II receptor I and II (AT_1 and AT_2), which the AT_1 is present in several tissues, including the cardiovascular system (24) and Ang II is found in

large quantities in mononuclear cells as well as levels of AT_1 receptors and kininase II are increased in monocytes isolated from atherosclerotic plaque (25).

The kinins mediate their effects via the selective activation of kinin B_1R and B_2R. The B_2R is constitutively expressed and regulate the majority of the acute vascular actions of BK (21). In addition, B_1R can be up-regulated by a different inflammatory stimuli that include cytokines (13). Following an inflammatory insult, receptor expression is induced in variety of cell types including vascular smooth muscle cells, endothelial cells (26) and circulating inflammatory cells, including monocytes (27).

A cross-talk between AT_1 and B_1R occur, since AT_1 blocker increases protein and mRNA level of B_1R, while AT_1 over expression decreases B_1R expression in the rat myocardial infarction model (28). Finally, The beneficial effects of ACE inhibitors are attributed to decreased Ang II generation and BK degradation (16). ACE inhibitors increase B_1R and B_2R signaling and promote NO production. Greater B_2R signaling activates endothelial cell nitric oxide synthase (eNOS), produce a short yield of NO; while activation of B_1R results in prolonged NO output by inducible NOS (iNOS) (16) so reduced expression of kinin B_1R and B_2R lead to decreased NO, as; *Sezgin et al* have shown that reduced NO support the presence of endothelial damage in the pathogenesis of SCF (7). So, we expect the reduced gene expression of kinin B_1R and B_2R in patients with slow coronary syndrome.

We have shown that (i) B_1R-gene expression tend to be decreased in SCF patients; SCF patients with three slow flow coronary artery; SCF patients with <3 slow flow coronary arteryversus healthy individuals and the difference was significant statistically (ii) B_2R-gene expression was not significantly lower in SCF patient group nor in SCF patients with three slow flow coronary artery and SCF patients with <3 slow flow coronary artery versus control group; however, the difference did not reach statistical significance (iii) Smoking is not

effective factor on B_1R and B_2R expression (iv) there are the positive correlation between B_1R and B_2R expression.

Several studies evaluated kinin B_1R and B_2R expression in cardiovasculare disease. *Raidoo et al.* have shown an intense immunostaining for the B_1R and a low expression of B_2R on human foamy cells in atherosclerotic plaques (29). Our results demonstrated insignificant expression of B_2R. *Dabek et al* demonstrated that the B_1 receptor / B_2 receptor ratio was inversed in patients with acute coronary syndrome versus control group and the low expression of B_2R was significant (18). In another study, they evaluated expression of kinin receptors in PBMC in cardiac syndrome x patients and reported that expression of B_1R and B_2R were 7 and, 2.5 fold higher than control group respectively (30).

As well as checking blood cells characteristics showed no significant difference in the parameters between all groups. Only MCV in SCF patients, SCF patient with three slow flow coronary and SCF patients with <3 slow flow coronary compared to control group significantly was decreased and PT in SCF patients with <3 slow flow coronary compared to the control group showed a significant decrease but due to difference blood specimen type obtained from SCF patients (arterial specimen) and control group (venous specimen), cannot be say conclusive opinion about this difference.

CONCLUSION

We concluded that according to significant reduced expression of kinin B_1R and decreased expression of B_2R and its contrast with RAS, endothelial dysfunction, imbalance between vasoconstricting and vasodilating factors and impaired endothelial release of NO due to disturbance in kinin signaling is the major pathogenesis of SCF.

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Table 1: Primer sequences used for QRT – PCR

Primer	Forward	Reverse
B_1R	5′-ctgcacagagtgctgccgacatt-3′	5′-acaccagatcagaggctgccagg-3′
B_2R	5′-cacggtgctagtcctggttgtgct-3′	5′-aggtccgcagtgtgcccatg-3′
β-actin	5′-tcacccacatgtgcccatctacga-3′	5′-cagcggacccgctcattgccaatgg-3′

Table 2: Demographic and clinical characterstics of participants

Parameter	Control n=30	SCF n=30	P-value
Age (years)	51.37 ± 11.89	53 ± 11.83	0.596
Sex (Female/Male)	8/22	8/22	1
Body mass index (kg/m2)	27.44 ± 3.60	26.93 ± 4.46	0.626
Heart rate (n)	78.12 ± 10.03	74.16 ± 7.69	0.104
Systolic BP (mmHg)	137.48 ± 13.79	128.03 ± 15.85	0.005
Diastolic BP (mmHg)	88.72 ± 10.31	81.86 ± 22.08	0.007
Smoking (%)	-	60.71	-
Family history of CHD (%)	-	26.66	-
Aspirin (%)	-	70	-
Statins (%)	-	63.33	-
β- blockers (%)	-	66.66	-

CHD : coronary heart disease, BP: blood pressure

Table 3. TIMI frame count and ejection fraction in SCF patient

Parameter		Smoking		P-value	Number slow flow coronary artery		P-value
		Smoker SCF	nonsmoker SCF		<3 artery	3 artery	
EF (%)		51.66±8.74	53.63±5.51	0.693	52.35±8.85	52.69±5.63	0.713
RCA (frame)	**TFC**	33.55±6.96	38.25±8.59	0.287	38.40±8.20	35.53±8.16	0.552
LCX (frame)	**TFC**	32.14±6.41	33.28±4.30	0.702	28.50±2.12	33.92±5.45	0.199
LAD (frame)	**TFC**	41.47±8.90	45.90±7.50	0.183	42.50±8.16	44.76±9.13	0.486
Total (frame)	**TFC**	38.89±7.77	41.42±7.51	0.439	41.50±8.26	38.07±6.36	0.226

EF : ejection fraction, RCA : right coronary artery, LCX : left circumflex artery, LAD left anterior descending

Table 4: Gene expression of B_1R and B_2R as mRNA fold

Parameter	Control	SCF	Smoker	Nonsmoker	3 SCF artery	<3 SCF artery	p^1	p^2	p^3	p^4	p^5
B_1R (fold)	0.98±0.15	0.15±0.03	0.15±0.04	0.15±0.03	0.15±0.04	0.14±0.04	<0.0001	0.498	0.721	0.001	0.001
B_2R (fold)	1.22±0.28	0.58±0.11	0.59±0.15	0.62±0.15	0.52±0.11	0.63±0.17	0.143	0.652	0.950	0.234	0.231

[p1] Comparison between control and SCF groups
[p2] Comparison between smoker and nonsmoker SCF groups
[p3] Comparison between SCF with 3 and <3 slow flow coronary artery
[p4] Comparison between control and SCF with 3 slow flow coronary artery
[p5] Comparison between control and SCF with <3 slow flow coronary artery

Table 5: Complete Blood Count, PT and PTT in participants

Parameter	Control	SCF	Smoker	Nonsmoker	3 SCF artery	<3 SCF artery	p[1]	p[2]	p[3]	p[4]	p[5]
Red Blood Cell (RBC, Mill/mm^3)	4.93±0.42	5.38±2.06	5.57±2.60	5.12±0.47	5.12±0.47	5.56±2.69	0.467	0.829	0.308	0.212	0.877
Hemoglubin (Hb, g/dl)	14.31±1.21	14.08±1.32	13.89±1.31	14.48±1.33	14.14±1.40	14.02±1.29	0.473	0.255	0.815	0.689	0.450
Hematocrit (Hct, %)	43.07±2.99	41.92±3.12	41.55±3.37	42.70±2.77	42.36±3.23	41.56±3.08	0.155	0.352	0.499	0.493	0.114
Mean Corpuscular Volume (MCV, fL)	87.50±3.17	84.12±3.70	83.36±3.09	84.95±4.42	84.06±3.86	84.17±3.68	<0.0001	0.265	0.938	0.004	0.002
Mean Corpuscular Hemoglobin (MCH, Pgm)	29.06±1.29	28.27±1.81	27.79±1.51	28.97±2.13	28.21±1.60	28.31±2.0005	0.056	0.094	0.888	0.075	0.126
Mean Corpuscular Hemoglobin Concentration (MCHC, %)	33.19±1.03	33.59±1.15	33.31±1.03	34.18±1.24	33.58±0.83	33.60±1.36	0.168	0.060	0.960	0.258	0.254
platelet (Plt, x1000/mm^3)	223.16±58.66	212.56±42.82	224.50±41.84	197.18±40.21	195.23±39.11	225.82±4.78	0.427	0.095	0.051	0.125	0.870
White Blood Cell (WBC, x1000/mm^3)	6.75±1.62	7.52±2.68	7.49±2.74	7.80±2.72	7.80±3.49	7.30±1.94	0.433	0.637	0.867	0.822	0.324
Lymphocyte (%)	34.63±6.58	32.53±9.28	34±9.59	31.22±9.94	30±12.13	34.22±7.12	0.691	0.256	0.441	0.537	0.873
Neutrophil (%)	56.50±7.23	59.06±10.26	57.40±10.87	60.22±11.02	62.33±12.92	56.88±8.16	0.754	0.501	0.440	0.126	0.891
Mixed[6] (%)	8.86±2.54	8.33±2.16	8.60±2.88	8.55±1.58	7.50±2.07	8.88±2.14	0.407	0.970	0.236	0.149	0.987
Prothrombin time (PT, Sec)	12.23±0.66	11.87±0.93	11.63±0.86	12.17±0.98	12.18±0.88	11.62±0.92	0.110	0.135	0.109	0.657	0.042
Partial thromboplastin time (PTT, Sec)	27.82±2.85	26.76±5.001	25.71±562	28.36±3.72	26.61±7.07	26.87±2.78	0.469	0.230	0.423	0.902	0.324
International normalized ratio (INR)	0.94±0.09	0.90±0.11	0.88±0.08	0.93±0.14	0.92±0.13	0.89±0.09	0.109	0.217	0.399	0.355	0.102

[1]Comparison between control and SCF groups
[2] Comparison between smoker and nonsmoker SCF groups
[3] Comparison between SCF with 3 and <3 slow flow coronary artery
[4] Comparison between control and SCF with 3 slow flow coronary artery
[5] Comparison between control and SCF with <3 slow flow coronary artery
[6] Monocyte and eosinophil