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Monocyte/macrophage β2-AR as a target of antisympathetic excitation-induced atherosclerotic progression

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ABSTRACT. The aim of this study was to determine whether monocyte/macrophage β2-AR could act as the therapeutic target of antisympathetic excitation-induced atherosclerotic progression. Cultivated human THP-1 cells were divided into different groups and incubated with isoprenaline, metoprolol, propranolol or β2-AR blocker for 24 h, together with oxidized low-density lipoprotein (ox-LDL). Afterwards, each group was analyzed for C-C chemokine receptor type 2 (CCR2) expression, monocyte chemotactic protein 1 (MCP-1) release into medium and cell migration ability. In the isoprenaline group, CCR2 protein level was increased, as well as the secretion of MCP-1, and cell motility was enhanced, in a concentration-dependent manner. Propranolol and ICI 118,551 significantly reversed the stimulatory effect of isoprenaline on THP-1 cells induced by ox-LDL, but only high concentrations of metoprolol interfered significantly with the action of isoprenaline ($P < 0.05$). Isoprenaline or a β-AR blocker could mediate through β2-AR, affecting MCP-1 secretion, CCR2 protein expression and cell migration capacity of THP-1 cells. Therefore, monocyte-macrophage β2-AR may act as a target of antisympathetic excitation-

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induced atherosclerotic progression.

Key words: β2-AR; Mononuclear cell; Atherosclerosis; Stress

INTRODUCTION

A number of studies have discovered that atherosclerosis has a non-linear relation with hyperlipidemia: approximately 40% patients with atherosclerosis do not have risk factors such as smoking, hyperlipidemia, hypertension, diabetes, and others. However, atherosclerosis is related to psychological stress (Stansfeld et al., 2002; Madden, 2003; Yusuf et al., 2004). Pathological studies have found that the fibrous cap of unstable atherosclerotic plaque contains a macrophage-rich inflammatory infiltrate. Monocytes/macrophages play an important role in the formation and stability of atherosclerotic plaques (Palermo-Neto et al., 2003; Vucević et al., 2012; Woollard, 2013). Neuroimmunology studies have found that the surface of immune cells has a high density of β2 receptors (Black and Garbutt, 2002). These receptors are the molecular basis of sympathetic transmitter regulation of immune system. In various situations, with the activity of sympathetic nerve-adrenal medulla increasing, the catecholamine neurotransmitters released by the body would directly or indirectly act on the receptors of immune cells, causing changes in related immune functions of the body, or would even be a significant factor in accelerating the occurrence and development of certain diseases (Cruickshank, 1990; Yla-Herttuala et al., 1991; Sanders et al., 2003; Saederup et al., 2008). Various studies of relationships between the occurrence of non-acute coronary syndrome and the polymorphism of β2-AR genes have found that β2-AR is closely related to the occurrence of acute coronary syndrome (Lanfear et al., 2005; Jaillon and Simon, 2007). Internationally, there have been a large number of studies suggesting that the treatment with β receptor blockers could slow down the progression of atherosclerosis (Cruickshank, 1990), but none of them has confirmed that the "the slower the heart rate, the less effects of blood flow dynamics" is the only mechanism for β receptor blockers controlling atherosclerosis (Tsuchiya et al., 1980; Fleury et al., 2000; Orth-Gomér et al., 2000; Stansfeld et al., 2002). Still, there has not been any study on the specific mechanism.

In view of the fact that there is a large amount of mononuclear cell-rich infiltration in unstable atherosclerotic plaques, we concluded that β2-AR, which exists on the surface of monocytes/macrophages may act as the target of antisympathetic excitation-induced atherosclerotic progress. We used the THP-1 cell line as the research model (Qi, 2012) to determine the influence of β2-AR agonists and blockers on CCR2 protein expression, release of MCP-1 factor and cell motility.

MATERIAL AND METHODS

Main material and reagents

Human mononuclear cell strain THP-1 was purchased from the Chinese Classic Culture Preservation Center of Wuhan University; RPMI 1640 powder from Sigma Chemical Company (Sigma-Aldrich Shanghai Trading Co. Ltd. Shanghai, China); calf serum from Hangzhou Sijiqing Biological Engineering Materials Co.; ox-LDL from Guangzhou Yiyuan Biotech Co. rabbit anti-human CCR2 monoclonal antibody from Abcame UK Company; horseradish peroxide tagged anti-rabbit/mouse both from Shenzhen Jingmei Biotechnology Co.; ELISA reagent from Americould R & D Company; Transwells from Sigma Chemical Company (USA),

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and isoprenaline, adrenaline, metoprolol, propranolol, and ICI 118,551 original medicine all from Sigma Chemical Company.

Cell culture

Cells were cultivated in RPMI1640 culture medium containing 10% calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, in 5% CO_2 incubator at 37°C. The cell concentration was then adjusted to 1 x 10^9/L for the follow-up tests when the cells grew well and the trypan blue exclusion test showed a cell vitality >95%.

Experimental groups

This experiment was divided into different treatment groups: β2-AR agonist (isoprenaline), metoprolol, propranolol, and β2-AR blocker (ICI 118,551). The concentrations used were: isoprenaline at 0.1, 1, 10, and 50 μM; metoprolol at 0.1, 1, 10, and 50 μM; propranolol at 0.01, 0.1, 1, and 10 μM; and ICI 118,551 at 0.1, 1, 10, and 50 μM. Each concentration group had a control group, with 3 duplicate wells on a 6-well culture plate, and every well was inoculated with 1 x 10^6 cells.

Western blotting detection of cell chemokine receptor CCR2 protein

After washing the cells collected of each group with precooled PBS, protein was extracted according to the instructions on the nuclear and cytoplasmic protein extraction kit and quantitated by the BCA method, and the protein concentration adjusted to the same level in each group. The samples were prepared for storage after putting in boiling water for 5 min. A 20-μg protein sample was taken from each group and separated by SDS-PAGE, where β-actin was used as the internal reference. After membrane transfer, the blots were blocked with PBS containing 5% skim milk powder for 2 h. Afterwards, appropriate amounts of rabbit anti-human CCR2 antibody (1:3000) and mouse anti-human β-actin antibody (1:5000) were added, respectively, which had already been diluted with PBS containing 2% skim milk powder, and the blots were incubated overnight at 4°C. The membranes were rinsed 3 times with PBS, 10 min each time. According to the sources of primary antibodies, appropriate amounts of HRP-tagged goat anti-rabbit IgG (1:500) and HRP-tagged goat anti-mouse IgG (1:5000) diluted with PBS containing 2% skim milk powder were added, respectively; after 2 h at room temperature, the membranes were washed three times with PBS, 10 min each time. They were identified with enhanced chemical luminescence agent (ECL reagents). A semi-quantitative measurement of the band intensity was performed by Quantity One. The Ge-1 Pro Analyzer (Ver. 3.0) software was used to detect the gray-scale value of the protein bands; protein expressions was calculated using the ratio between the gray-scale value of CCR2 band and that of the internal reference β-actin band. 1.2.4 ELISA was used for detecting the MCP-1 level in the cell culture medium. We followed the steps of the instructions of the ELISA kit to detect the MCP-1 level in the cell culture medium of each group.

Transwell chemotaxis experiment

The THP-1 cells of each group were centrifuged, washed 3 times with PBS and then

resuspended. The chemotaxis experiment was carried out in Transwell chambers with the lower chamber containing the chemotactic factor MCP-1 at 500 µg/mL and the upper chamber the cell suspension. The barrier between the upper and lower chamber was a Millipore nitrocellulose filter with 5-µm pore size. Each group was equipped with three chemotactic chambers. These chambers were put into an incubator filled with 5% CO_2 at 37°C and incubated for 60 min. The filter membrane was then taken out and washed, and the membrane surface was scraped to recover the cells in suspension. Finally, the cells were counted using a hemocytometer in five fields under a microscope at 200X.

Statistical analysis

Experimental data are reported as means ± standard deviation (means ± SD), where the comparison between two groups was performed by analysis of variance and *t*-tests. Statistical analysis was performed with the SPSS 12.0 statistical software. $P < 0.05$ meant that the difference was considered to be statistically significant.

RESULTS

Changes in CCR2 protein levels in isoprenaline group

In comparison with the control group, the different concentrations of isoprenaline increased CCR2 protein expression in cells under the stimulus of ox-LDL, in a concentration-dependent manner ($P < 0.05$), as shown in Figure 1.

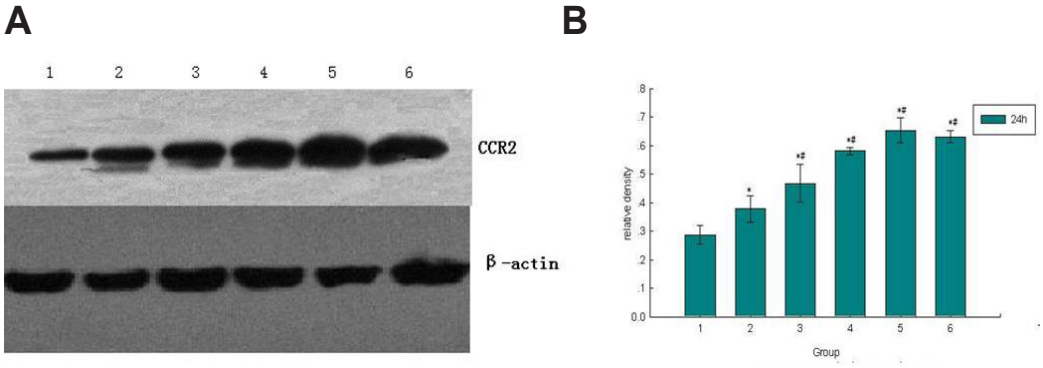

Figure 1. Influence of isoprenaline on the CCR2 protein expressions of THP-1 cells. **A.** Results of Western-blotting. **B.** Statistical graph of Western-blotting. *Lane 1* = control group; *lane 2* = ox-LDL group; *lane 3* = 0.1 µM isoprenaline; *lane 4* = 1 µM isoprenaline; *lane 5* = 10 µM isoprenaline; *lane 6* = 50 µM isoprenaline; *P < 0.05, in comparison with control group, P < 0.05, # in comparison with ox-LDL group, N = 3.

Changes in CCR2 protein levels in β1-AR blocker (metoprolol) group

In comparison with the control group, metoprolol inhibited the stimulatory effect of isoprenaline on CCR2 protein expression in THP-1 cells but only at the higher concentrations of 10 and 50 µM, as shown in Figure 2.

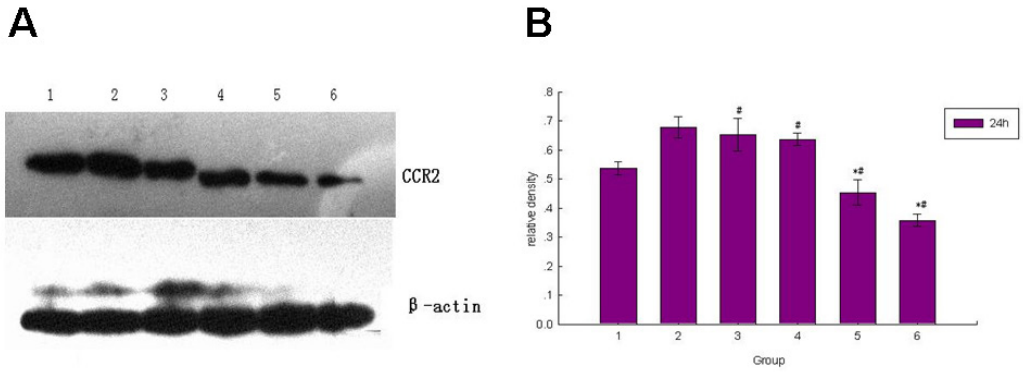

Figure 2. Influence of metoprolol on the CCR2 protein expression of THP-1 cells. **A.** Results of Western-blotting. **B.** Statistical graph of Western-blotting. *Lane 1* = ox-LDL group; *lane 2* = Isoprenaline; *lane 3* = 0.1 μM metoprolol; *lane 4* = 1 μM metoprolol; *lane 5* = 10 μM metoprolol; *lane 6* = 50 μM metoprolol; *P < 0.05, in comparison with isoprenaline group, P < 0.05, # in comparison with ox-LDL group, N = 3.

Changes in CCR2 protein levels in β-AR blocking agent (propranolol) group

Compared with the control group, propranolol suppressed the upregulation of CCR2 protein expression in THP-1 cells by isoproterenol in a concentration-dependent manner. The results are shown in Figure 3.

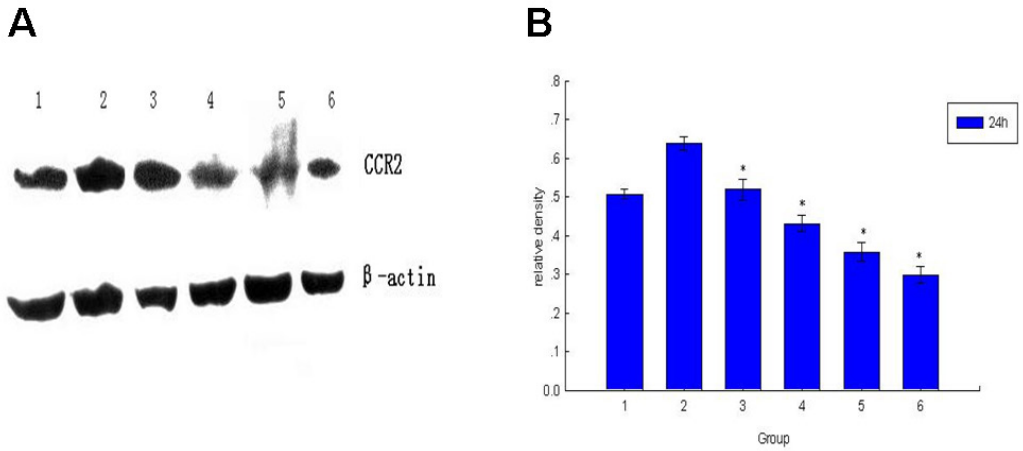

Figure 3. Influence of propranolol on CCR2 protein expression of THP-1 cell. **A.** Results of Western-blotting. **B.** Statistical graph of Western-blotting. *Lane 1* = ox-LDL group; propranolol group; *lane 3* = 0.01 μM propranolol; *lane 4* = 0.1 μM propranolol; *lane 5* = 1 μM propranolol; *lane 6* = 10 μM propranolol; (compared with the propranolol group, P < 0.05, N = 3).

Changes in CCR2 protein levels in β 2-AR blocking agent (ICI 118,551) group

β2-AR blocking agent (ICI 118,551) suppressed the upregulation of CCR2 protein expression in THP-1 cells by isoproterenol induced by ox-LDL, in a concentration-dependent

manner, compared with the control group, where the difference was considered to be statistically significant ($P < 0.05$). The results are shown in Figure 4.

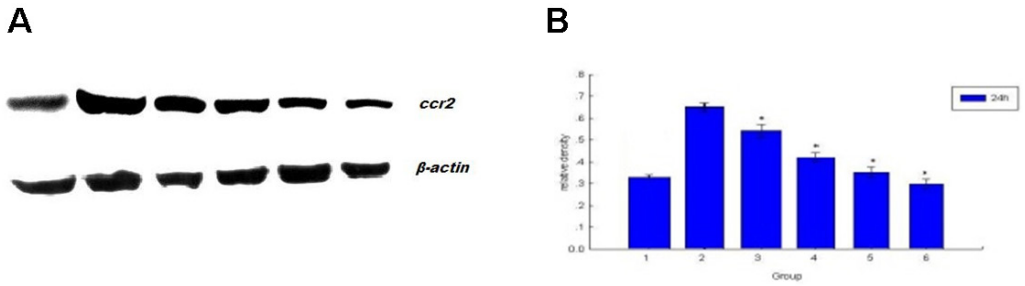

Figure 4. Influence of ICI118551 of different concentrations on CCR2 protein expression of THP-1 cell. **A.** Results of Western-blotting. **B.** Statistical graph of Western-blotting. *Lane 1* = ox-LDL group; isoprenaline group; *lane 3* = 0.01 μM ICI118551; *lane 4* = 0.1 μM ICI118551; *lane 5* = 1 μM ICI118551; *lane 6* = 10 μM ICI118551; *P < 0.05, compared with the isoprenaline group; #P < 0.05, compared with the ox-LDL group, N = 3.

Changes in MCP-1 concentration and number of cells migrating in culture medium of β-AR excitomotor (isoprenaline) group

In comparison with the control group, isoprenaline promoted THP-1 cells induced by ox-LDL to secret MCP-1 cytokine, in a concentration-dependent manner ($P < 0.05$); isoprenaline at different concentrations enhanced migration of THP-1 cells induced by ox-LDL, compared with the control group ($P < 0.05$). Compared with the ox-LDL group, isoprenaline at concentrations of 1, 10, and 50 μM enhanced the migration of THP-1 cells, in a concentration-dependent manner ($P < 0.05$). The results are shown in Table 1.

Table 1. MCP-1 concentrations and number of cell migration of β-AR excitomotor (isoprenaline) group (means ± SD).

Group	MCP-1 (pg/mL)	No. of cell migration
Control group	310 ± 10.5	17 ± 1.3
ox-LDL group	380 ± 14.5*	28 ± 2.3$^{\Delta}$
0.1 μM isoprenaline	400 ± 17.3*#	32 ± 3.4$^{\Delta\&}$
1 μM isoprenaline	413 ± 13.7*#	45 ± 1.6$^{\Delta\&}$
10 μM isoprenaline	439 ± 16.1*#	56 ± 1.7$^{\Delta\&}$
50 μM isoprenaline	450 ± 15.8*#	63 ± 2.4$^{\Delta\&}$

MCP-1 monocyte chemotactic protein 1; ox-LDL = oxidized low-density lipoprotein. Compared with the control group, *P < 0.05. Compared with the ox-LDL group, #P < 0.05. Compared with the control group, $^{\Delta}$P < 0.05. Compared with the ox-LDL group, $^{\&}$P < 0.05.

Changes in MCP-1 concentration and number of cells migrating in culture medium of β-AR blocking agent (metoprolol) group

In comparison with the control group, metoprolol reversed the action of isoprenaline in increasing the MCP-1 level in culture medium of THP-1 cells induced by ox-LDL ($P > 0.05$). Stimulus of metoprolol at concentrations of 0.1 and 1 μM could not inhibit the stimulatory effect of isoprenaline on THP-1 cell migration, compared with 50 μM isoprenaline ($P > 0.05$), while metoprolol at 10 and 50 μM promoted the migration of THP cells ($P < 0.05$) (Table 2).

Table 2. MCP-1 concentrations and number of cell migration of β1-AR blocking agent (metoprolol) group (means ± SD).

Group	MCP-1 (pg/mL)	No. of cell migration
ox-LDL group	308 ± 9.3	30 ± 1.5
50 μM isoprenaline group	454 ± 11.2*	70 ± 2.1
0.1 μM metoprolol	450 ± 15.8*#	68 ± 3.4Δ
1 μM metoprolol	453 ± 12.7*#	69 ± 2.6Δ
10 μM metoprolol	451 ± 13.9*#	62 ± 1.4&
50 μM metoprolol	449 ± 12.8*#	60 ± 2.2&

In comparison with ox-LDL group, *P < 0.05; In comparison with 50 μM isoprenaline group, #P > 0.1. In comparison with 50 μM isoprenaline group, ΔP > 0.05; In comparison with 50 μM isoprenaline group, &P < 0.05. For other abbreviations, see legend to Table 1.

Changes in MCP-1 concentration and number of cells migrating in culture medium of β-AR blocking agent (propranolol) group

In comparison with the control group, propranolol interfered with the stimulatory effect of isoprenaline on MCP-1 level in culture medium of THP-1 cells in a concentration-dependent manner (P < 0.05). In comparison with isoprenaline, propranolol R different concentrations (0.01, 0.1, 1, and 10 μM) inhibited the stimulatory effect of isoprenaline on THP-1 cell migration, in a concentration- dependent manner (P < 0.05) (Table 3).

Table 3. Changes of MCP-1 concentrations and number of cell migration of β1-AR blocking agent (propranolol) group (means ± SD).

Group	MCP-1 (pg/mL)	No. of cell migration
ox-LDL group	385 ± 9.7	32 ± 1.5
50 μM isoprenaline group	458 ± 11.2	68 ± 2.1
0.01 μM propranolol	451 ± 13.8*	63 ± 1.4Δ
0.1 μM propranolol	440 ± 8.7*	55 ± 2.3Δ
1 μM propranolol	420 ± 10.4*	45 ± 3.4Δ
10 μM propranolol	402 ± 15.8*	36 ± 1.2Δ

In comparison with 50 μM isoprenaline group, *P < 0.05. In comparison with 50 μM isoprenaline group, ΔP < 0.05. For other abbreviations, see legend to Table 1.

Changes in MCP-1 concentrations and number of cells migrating in culture medium of β2-AR blocking agent (ICI118551) group

In comparison with the control group, β2-AR blocking agent ICI 118,551 lowered the stimulatory effect of isoprenaline on MCP-1 level in culture medium of THP-1 cells induced by ox-LDL, in a concentration-dependent manner (P < 0.05) (Table 4).

Table 4. Changes of MCP-1 concentrations and number of cell migration of β2-AR blocking agent (ICI118551) group (means ± SD).

Group	MCP-1 (pg/mL)	No. of cell migration
ox-LDL group	395 ± 9.7	29 ± 1.5
50 μM isoprenaline group	460 ± 11.3	71 ± 3.1
0.1 μM ICI118551	452 ± 13.4*	63 ± 1.2Δ
1 μM ICI118551	438 ± 8.4*	54 ± 1.3Δ
10 μM ICI118551	414 ± 11.3*	39 ± 3.4Δ
50 μM ICI118551	400 ± 12.4*	32 ± 2.2Δ

In comparison with 50 μM isoprenaline group, *P < 0.05. In comparison with 50 μM isoprenaline group, ΔP < 0.05. For other abbreviations, see legend to Table 1.

DISCUSSION

Migration of peripheral blood mononuclear cells (PBMC) to blood vessel endothelium is the committed step for the occurrence of atherosclerosis. Early studies discovered that higher MCP-1 expression could be detected in atherosclerotic pathological changes in an animal model or human beings, indicating that MCP-1 plays an important role in the occurrence of atherosclerosis (Yla-Herttuala et al., 1991). By means of gene knock-out techniques, Saederup et al. (2008) proved that CCR2 had an independent function in the accumulation of mononuclear cells at blood vessel endothelium and the formation of atherosclerotic plaque. As an ideal model for the study of atherosclerosis (Qi, 2012), the THP-1 cell line was selected as the object of this study, where the changes in delivery of MCP-1 factor, CCR2 protein expression and migration ability of mononuclear cells were the experimental observation indices.

The results of this study indicated that in THP-1 cells the β-AR agonist isoprenaline, in the presence of ox-LDL, stimulated CCR2 protein expression, delivery of MCP-1 factor and cell migration ability, in a concentration-dependent manner. We found that 50 μM isoprenaline produced the strongest activity, which suggested that under stringent conditions, the body releases catecholamine hormones into the blood circulation, enhancing the expression of CCR2 protein on the surface of mononuclear cells, delivery of MCP-1 factor and migration ability of mononuclear cells by acting on β2-AR on the surface of PBMC. This would result in the accumulation of monocytes-macrophages at the atherosclerotic plaque, presenting a possible biological manifestation for promoting the progression of the atherosclerotic plaque.

This study also found that a nonselective β-AR blocker and selective β2-AR blocker ICI 118,551 could, even at a low concentration, inhibit CCR2 protein expression in THP-1 cells, reduce the release of MCP-1, and lower the migration ability of the mononuclear cells, in a concentration-dependent manner. β1-AR blocker metoprolol, only at high concentrations, suppressed CCR2 protein expression in THP-1 cells, reduced the release of MCP-1 and lowered the migration ability of the mononuclear cells, which would hamper the aggregation of mononuclear cells at atherosclerotic plaques, showing a possible biological manifestation of slowing the progression of atherosclerosis. Metoprolol, only at high concentrations, could regulate the immune function and foster some changes in favor of hampering atherosclerosis progression. This fact was in accordance with the evidence-based medical research report, where we emphasized the use of metoprolol in the clinic at the most tolerated dose for fully exploiting its effects. After acute myocardial infarction, using appropriate amounts of β-AR blocker early and permanently could reduce the risks of getting myocardial infarction again for coronary heart disease patients and also could reduce the mortality rate of patients with previous myocardial infarction (Cruickshank, 1990; Camejo et al., 1991). The aim is to reduce the blood's harmful mechanical effects on atherosclerotic plaques, as well as to stabilize the plaques, mostly considering the relation to the β1-AR blocker blocking β receptors, slowing down the heart rate, weakening the myocardial contractility and reducing myocardial oxygen consumption. Since the fundamental cause of acute coronary heart disease is the decrease in atherosclerotic plaque stability and thrombogenesis caused by plaque ulceration, the very key to coronary heart disease treatment is to stabilize the atherosclerotic plaque in veins. Through this experimental research, we discovered that the use of a β1-AR blocker as a long-term functional mechanism in the clinic could involve mechanisms other than the β1-AR blocker blocking β1-AR. It meant that when using appropriate amounts of β1-AR blocker early and permanently to block the β1-AR in veins and in cardiac muscle, we could not rule out the

possibility that β1-AR combined with the mononuclear cell β2-AR outside the veins and cardiac muscle. By modifying the functions of numerous mononuclear cells in the atherosclerotic plaque, the immune response in the atherosclerotic plaque and the blebbing of the cells could be reduced, hampering atherosclerotic progression, as well as to stabilize atherosclerotic plaques.

In conclusion, this study suggested that β2-AR on the surface of monocytes/macrophages could act as a molecular target for β-AR to stabilize atherosclerotic plaque, especially when a coronary heart disease patient has combined sympathetic excitation quality, or his/her extensive mononuclear cell infiltrate in atherosclerotic plaque shows instability. Meanwhile, this study provided some ideas for new research to define the biggest beneficiaries of β-AR blocker treatment and the best treatment course (Bangalore et al., 2012; Thompson, 2013).

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