

Tirofiban Preserves Endothelial Junctions and Decreases Endothelin-1

**Jing-Lin ZHAO, Yue-Jin YANG *, Wei-Dong PEI, Yu-Hua SUN,
Mei ZHAI, Ya-Xin LIU, Run-Lin GAO**

Department of Cardiology, Cardiovascular Institute and Fu-Wai Heart Hospital,
Chinese Academy of Medical Sciences and Peking Union Medical College,
Beijing, China.

Abstract

It has been verified that glycoprotein IIb/IIIa inhibitor tirofiban can attenuate myocardial no-reflow. Myocardial no-reflow has been associated with alterations in endothelial junctions. In addition, ET-1 is an important mechanism for myocardial no-reflow. However, the effect of tirofiban on endothelial junctions and Endothelin-1 (ET-1) is unknown. Methods: Twenty-eight mini-swines were randomized into 3 study groups: 10 in control, 10 given an intravenous infusion of tirofiban and 8 in sham-operated. Acute myocardial infarction and reperfusion model was created with three-hour occlusion of the left anterior descending coronary artery followed by one-hour reperfusion. Results: In control group, plasma ET-1 significantly increased, ET-1 or VE-cadherin level in the reflow and no-reflow myocardium was significantly higher or lower than that in normal myocardium. Compared with the control group, tirofiban significantly decreased plasma ET-1 and myocardial tissue ET-1, maintained VE-cadherin level. Conclusions: Tirofiban is effective in preserving endothelial junction. This beneficial effect of tirofiban could be partly due to its reduction of ET-1.

Keywords

Acute myocardial infarction • Endothelin-1 • Reperfusion • Tirofiban • No-reflow

* Corresponding author: E-mail: realplayone@yahoo.com.cn (Y.-J. Yang).

Introduction

The main goal of reperfusion therapy for acute myocardial infarction (AMI) is to restore both epicardial and microvascular blood flow to the ischemic myocardium. Primary percutaneous coronary intervention (PCI), the preferred treatment for AMI, can achieve normal epicardial coronary flow. However, studies have shown that despite complete restoration of epicardial vessel blood flow, myocardial tissue perfusion evaluated with myocardial contrast echocardiography (MCE) remains incomplete, even no perfusion, known as slow flow or no-reflow phenomenon [1, 2], which accounts for 37% [3] of the patients with a first anterior AMI after receiving coronary reflow. No-reflow has been associated with severe myocardial injury, progressive left ventricular remodeling, congestive heart failure, and poor prognosis [4–6]. Therefore, myocardial tissue perfusion is now accepted as a target of reperfusion therapy for AMI [7]. Our previous study has already shown that specific platelet glycoprotein IIb/IIIa receptor inhibitor with powerful antiplatelet aggregation property attenuates no-reflow [8]. However, the exact cause of this beneficial effect has remained unclear. As demonstrated in animal models of coronary artery occlusion and reperfusion, localized endothelial swelling and protrusions, are predominantly confined to the capillary bed [9], indicating that endothelium plays an important role in tissue-level perfusion. Endothelin-1 (ET-1) is considered to be indices of endothelial dysfunction that is associated with arterial injury [10, 11]. However, it is unknown whether the mechanism of glycoprotein (GP) IIb/IIIa inhibitor for myocardial no-reflow is partly due to its effect on ET-1. In addition, myocardial no-reflow was always associated with microvascular structural disintegrity (structural no-reflow), which caused vascular leakiness. Vascular endothelial (VE)-cadherin, a specific endothelial cadherin, is an important determinant of vascular structural integrity [12]. Therefore, in this study, we used the same mini-swines in the previous study to further assess the effect of specific glycoprotein IIb/IIIa inhibitor tirofiban on vascular structural integrity and ET-1.

Experimental

Animal Preparation

Animal Preparation has been described in our previous study [8]. The mini-swines (30.3 ± 3.0 kg) were fasted overnight, sedated with 10 mg/kg of azaperone intramuscularly, anesthetized with 10 mg/kg of thiopental intravenously and ventilated with a respirator (Siemens elema sv 900). Tidal volumes of 12 ml/kg with a set respiratory rate of 16 breaths per minute were used. Anesthesia was maintained with a continuous infusion of thiopental. A middle thoracotomy was performed, and the heart was suspended in a pericardial cradle. The middle and distal portion of left anterior descending coronary artery (LAD) was dissected free from surrounding tissue, and was encircled by a suture. The two ends of the suture were threaded through a length of plastic tubing, forming a snare, which could be tightened to achieve coronary artery occlusion.

Experimental Protocol

Twenty-eight animals were randomized into three study groups: 10 in control , 10 in tirofiban–treated and 8 in sham-operated groups. In the tirofiban–treated animals, the dose of tirofiban employed was 1.5-fold higher than that in the RESTORE and TARGET studies [13, 14] based on body surface area [15]. Thus, tirofiban (Wuhan Yuanda Pharma Co, Ltd) was given as a 15 µg/kg iv. bolus, followed by an infusion administered at a rate of 0.3 µg/kg/min from 30 min before occlusion to the end of protocol. Control animals received the same amount of saline intravenously. AMI and reperfusion model were created with 3 hours of LAD occlusion followed by 1 h of reperfusion. In the sham-operated animals, LAD was only encircled by a suture, but not occluded. After reperfusion 1 h, no-reflow region was delineated by intra-atrial injection of 1 mL/kg of the fluorescent dye thioflavin S (Sigma Chemical Co). Then the LAD was reoccluded, and Evans blue dye was injected into the left atrium to determine ligation area. Under an ultraviolet light in a dark room, the areas not perfused by thioflavin S were identified. Normal region was defined as the region stained by Evans blue, while no-reflow region was

defined as the non-fluorescent area within ligation area. Samples were then taken from the myocardium in the normal, reflow and no-reflow regions, washed thoroughly with saline and snap-frozen in liquid nitrogen.

Assay of plasma ET-1

Blood samples (5 mL) were collected in tubes containing 30 μ L of 7.5% EDTA and 40 μ L aprotinin and were centrifuged immediately at 1500 g at 4°C for 10 min. The plasma was decanted and kept at -70°C until analysis. Plasma ET-1 concentrations were measured with a commercially available [125 I]-endothelin radioimmunoassay kit (Beijing Dong-ya Biotechnology Institute, China), as described previously [16]. Briefly, a 6 h incubation period at 37°C with ET-1 antiserum was followed by the addition of [125 I]-labelled ET-1 tracer and a second 24 h incubation period at 4°C. Separation of the bound from unbound ligands was achieved by a precipitating reagent and samples were incubated at room temperature for 15 min. The mixture was centrifuged at 2000 g at room temperature for 20 min. The supernatant was aspirated off and radioactivity in the pellet was determined with a gamma counter. The assay was performed in duplicate. The plasma ET-1 concentration is given in pg/mL.

Radio-immuno assay of ET-1 in myocardial tissue

Frozen tissue samples were pulverized under 1mol/L HAC 1 mL using a mortar and pestle. The resultant powder was boiled for 10 min in water and the homogenized on ice. Homogenates were centrifuged at 3000 g at 4°C for 15 min and the supernatant was collected and stored at -70°C before analysis. Protein concentration of the final supernatant was determined by Bradford method [17]. ET-1 concentration was measured with an endothelin radioimmunoassay kit from Beijing Dong-ya Biotechnology Institute following the manufacturer's instructions and expressed as pg ET-1/100 mg protein.

Western blot analysis for VE-cadherin in myocardial tissue

Myocardial tissue samples were separately suspended in 5 ml of ice-cooled lysis buffer containing (mM)-Tris-HCl 20 (pH 7.4), EDTA 1, NaCl 150, DTT 1,

2-mercaptoethanol 10, freshly added proteinase inhibitor and disrupted by using a tissue homogenisator. The particulate material was discarded by centrifugation at 100,000g at 4°C for 1 h. The clear supernatant of each tissue sample was collected and frozen at -70°C until use. Protein concentration was determined by the method of Bradford [17] using bovine serum albumin as a standard. 50µg of total protein solubilized for 10 min at 100°C was loaded per lane onto a 12% SDS – PAGE gel. Electrophoresis was performed for 1 h at 150 mA. Proteins were transferred onto Immobilon-P transfer membrane (Millipore, Bedford, MA, U.S.A.) for 1.5 h at 0.8 mA cm⁻² in a 20% methanol containing cathodes buffer. The membrane was washed three times for 20 min in PBST (0.1% Tween 20, 100 mM Tris-HCl, 150 mM NaCl, pH 7.5), blocked for 1 h in 5% nonfat milk-TTBS and incubated with VE-cadherin goat polyclonal (Santa Cruz Biotechnology, California), or mouse monoclonal beta actin (from Transduction Labs). The primary antibody was used in a 1 : 1000 dilution in PBST. After washing three times in PBST for 15 min, the membrane was incubated with a 1: 3000 dilution of the appropriated secondary antibody, either a sheep anti-mouse (Amersham Pharmacia Biotech Inc, Piscataway) or rabbit anti-goat IgG (Jackson Immunolabs) for 30 min at room temperature. To measure protein levels, the Western blots were scanned and digitized on an optical scanner (EPSON GT-8000, Seiko, Tokyo, Japan). Quantification of Western blots was done on a computer using the Gel-Pro image analysis system. All specific values of proteins were standardized to the value of beta actin used as the internal standard to ensure that equal amount of protein is loaded.

Statistical Methods

Data are expressed as mean±SEM. Comparisons of data among all stages were performed with repeated-measures ANOVA followed by Student-Newman-Keuls test for multiple comparison. Comparisons of data among groups were done with one-way ANOVA followed by Student-Newman-Keuls test for multiple comparison. A value of $P<0.05$ (2-sided) was considered statistically significant.

Results and Discussion

Four mini-swines (two receiving tirofiban and two controls) died of ventricular fibrillation during the reperfusion period and they were excluded. Therefore, 8 animals were evaluated in each group. Basic experimental parameters (such as blood pressure, heart rate and temperature) were registered. There was no significant difference in basic experimental parameters among three groups.

The effect of tirofiban on no-reflow

In control group, the area of no-reflow (ANR) was 82.3% of ligation area. Compared with those in control group, ANR was significantly decreased to 23.2% ($P<0.01$) in tirofiban-treated group.

Tab. 1. The effect of tirofiban on ANR (%).

	Histopathological evaluation	
	LA (%)	ANR (%)
control	23.5±1.98	82.3±1.90
tirofiban	23.6±3.56	23.2±1.86 [†]

[†] $P<0.01$ vs control group.

Data are expressed as the mean value±SEM; n=8 per group. LA and ANR represent ligation area and the area of no-reflow respectively.

The effect of tirofiban on plasma ET-1

In control group, plasma ET-1 significantly increased at AMI 5min, AMI 3 h, reperfusion 5min and reperfusion 1 h (all $P<0.01$) in comparison of the baseline. In the tirofiban group, plasma ET-1 levels were significantly lower than those in the control group (all $P<0.05$).

Tab. 2. The variation of plasma ET-1 (pg/ml).

Group	before AMI	AMI 5 min	AMI 3 h	reopen 5 min	reopen 1 h
sham	102±10	102±13	103±13	102±11	103±13
control	104±12	114±12	134±8 [†]	146±13 ^{†‡}	169±13 ^{†‡}
tirofiban	98±15	96±10	102±14 [▲]	109±15 [▲]	119±14 ^{*▲}

* $P<0.05$. [†] $P<0.01$ vs before AMI. [‡] $P<0.01$ vs AMI 3 h. [▲] $P<0.05$ vs control group.

Data are expressed as the mean value±SEM, n=8. AMI and ET-1 represent acute myocardial infarction and endothelin-1 respectively.

The effect of tirofiban on ET-1 in the myocardium

Compared with the control group, tirofiban significantly decreased area of no-reflow on both methods from 82.3% to 23.2% respectively ($P<0.01$). In control, as well as the tirofiban group, ET-1 in the reflow or no-reflow myocardium was significantly higher than that in normal myocardium (all $P<0.01$) with ET-1 in no-reflow myocardium further increased in comparison with that in the reflow myocardium ($P<0.01$). In the tirofiban groups, ET-1 in the reflow and no-reflow myocardium was significantly lower than that in the control group ($P<0.01$).

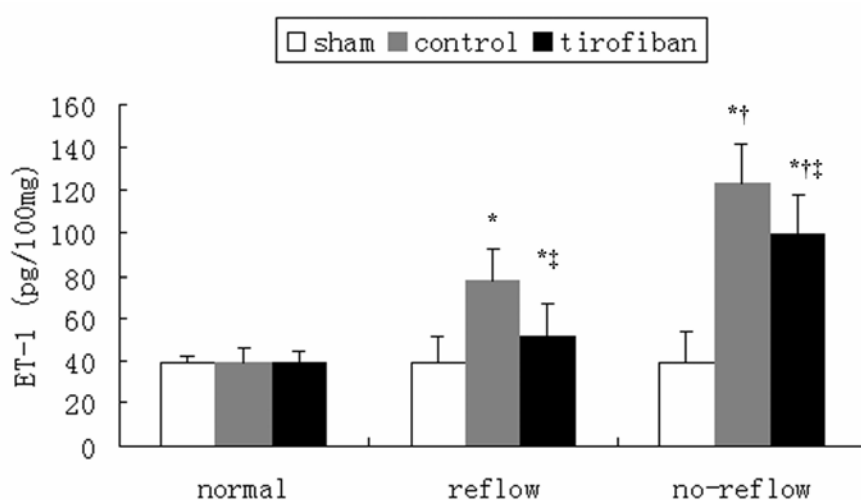


Fig. 1. The variation of ET-1 (pg/100mg) in the normal, reflow and no-reflow myocardium. * $P<0.01$ vs normal region. [†] $P<0.01$ vs reflow region. [‡] $P<0.01$ vs control group. Data are expressed as the mean value±SEM, n=8. ET-1 represents endothelin-1.

VE-cadherin in the myocardium

In control, as well as the tirofiban groups, VE-cadherin level in the reflow and no-reflow myocardium was significantly lower than that in normal myocardium ($P<0.01$). In tirofiban group, VE-cadherin level in the reflow and no-reflow myocardium was significantly higher than in the control group ($P<0.01$).

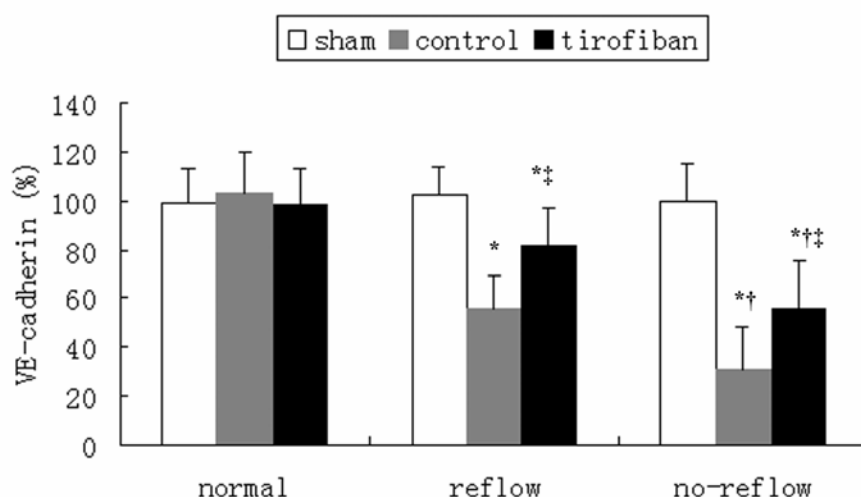


Fig. 2. The variation of VE-cadherin (%) in the normal, reflow and no-reflow myocardium. * $P<0.01$ vs normal region. † $P<0.01$ vs reflow region. ‡ $P<0.01$ vs control group. Data are expressed as the mean value \pm SEM, $n=8$.

Discussion

The proposed mechanism of the no-reflow phenomenon is multifactorial. Animal and postmortem histologic studies have demonstrated varying degrees of small-vessel vasospasm, endothelial gap and bleb formation, neutrophil plugging of capillaries as well as microvascular compression from myocytes, interstitial edema, and hemorrhage after recanalization. Although it has been suggested that plugging of capillaries by leukocytes and platelet activation contribute to no-reflow [18, 19], these blood cell elements are not necessary for the development of this phenomenon, because no-reflow has been observed in buffer-perfused hearts as well [9, 20]. In addition, our study has shown that platelet inhibition may not contribute to the beneficial effect of tirofiban on myocardial no-reflow. Thus, this

study was sought to explore the possible mechanism of tirofiban for myocardial no-reflow.

ET-1 is a peptide consisting of 21 amino acids, is one of three endothelins, and is the one produced by endothelium. Enhanced ET-1 release in AMI, therefore, might aggravate reperfusion injury by increasing microvascular vasoconstriction [21]. Of note, there is evidence that ET-1 is an important mechanism for myocardial no-reflow [22]. Niccoli et al. [22] thought that the possible mechanisms by which ET-1 favours no-reflow include 1) Enhanced release of ET-1 from ischaemia-reperfusion injured endothelium may result in intense and sustained microvascular constriction. 2) ET-1 has also relevant effects on polymorphonuclear (PMN) leukocytes which could account for its association with no-reflow. Indeed, ET-1 stimulates adhesion of PMN leukocytes to the endothelium, thus favouring PMN plugging by increasing the expression of the integrin CD11b/18 on PMN surface by inducing elastase release, which may also mediate tissue injury and oedema, and by increasing endothelium expression of ICAM-1. 3) ET-1 enhances microvascular permeability with consequent oedema resulting in microvascular compression. Our findings showed a significant increase in plasma ET-1 levels during AMI and reperfusion, which is in agreement with the report of Tønnessen [23], reflecting endothelial dysfunction caused by ischemia and reperfusion. Our findings also showed that the myocardial tissue levels of ET-1 have a significant increase in the reflow and no-reflow myocardium compared to those in the non-ischaemic myocardium, further suggesting that endothelium was damaged to some different extent in the reflow and no-reflow myocardium. The present study demonstrated that tirofiban decreased the ET-1 levels, which is in agreement with the report of Molero et al [24], implying the beneficial effect of tirofiban could be partly due to its protection of endothelial function.

Myocardial no-reflow has been classified to two different forms: structural and functional [25]. In structural no reflow, microvessels confined within necrotic myocardium exhibit irreversible structural disintegrity; in functional no reflow, patency of anatomically intact microvessels is compromised because of spasm

and/or microembolisation. VE-cadherin, a specific endothelial cadherin, is an important determinant of vascular structural integrity [12]. Our findings showed myocardial VE-cadherin level has a significant decrease in the reflow and no-reflow myocardium compared to those in the non-ischemic myocardium, reflecting microvascular structural integrity was damaged by ischemia and reperfusion. Our findings also suggested that tirofiban could maintain VE-cadherin level, indicating tirofiban could preserve endothelial junctions and attenuate structural no-reflow.

Study limitation

The results were observed in an acute experiment setting, and no long-term data are available.

In conclusion, the present study demonstrated that with preserving endothelial junctions, tirofiban also decreased the levels of ET-1 after AMI and reperfusion, suggesting this beneficial effect could be in part due to its reduction of ET-1

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