Platelet Glycoprotein IIb/IIIa Inhibitor Tirofiban Ameliorates Cardiac Reperfusion Injury

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Summary

There are many published articles on the effects of the antithrombolytic function of platelet glycoprotein IIb/IIIa inhibitors (GP IIb/IIIa inhibitors) in myocardial infarction. However, few studies have explored the effects and optimal concentration of tirofiban in diminishing the extent of myocardial reperfusion injury (RI).

Rats received 120 minutes of coronary ligation and 180 minutes of reperfusion. The rats were then divided into 7 groups based on the concentration of tirofiban administered intravenously 30 minutes prior to coronary reperfusion to the end of reperfusion. The ratio of myocardial necrotic area to area at risk (AAR), and myocardial malondialdehyde (MDA) and plasma myeloperoxidase (MPO) activities were measured. The apoptotic index (AI) was the percentage of myocytes positive for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) out of all myocytes stained by 4’, 6-diamidino-2-phenylindole (DAPI).

The ratio of myocardial necrotic area to AAR significantly decreased in all tirofiban subgroups. The MDA activity for tirofiban concentrations of 2 and 5 ug/kg/minute showed a slight reduction. MPO activity was significantly decreased at a tirofiban concentration of 2 ug/kg/minute. The AI was significantly decreased at a tirofiban concentration of ≥ 0.4 ug/kg/minute.

The results indicate that a tirofiban can significantly ameliorate the cardiac RI and myocyte apoptosis in rats. (Int Heart J 2015; 56: 335-340)

Key words: IIb/IIIa platelet inhibitor, Coronary artery disease, Apoptosis

Traditionally, coronary flow reperfusion is the only reliable and reasonable method for salvaging the ischemic myocardium and reducing infarct size in patients suffering from coronary occlusion. However, paradoxically, coronary reperfusion itself can cause myocardial injury, which means reperfusion injury (RI). A number of studies have demonstrated that effective therapies to reduce RI have proven elusive, and most of the clinical trials that attempted to prevent or diminish the extent of RI had disappointing outcomes. In addition, different experimental results and conclusions occurred even in the same pharmacological trials in previous studies. Platelet glycoprotein IIb/IIIa inhibitors (GP IIb/IIIa inhibitors) act as a potential inhibitor of platelet aggregation by binding to the glycoprotein IIb/IIIa platelet receptor on the surface of activated human platelets. The antiplatelet function and mechanism have proven to be effective against acute coronary syndrome. The administration of a GP IIb/IIIa inhibitor during primary percutaneous coronary intervention improved myocardial reperfusion and clinical outcomes in ST-elevation myocardial infarct patients. Recently, experimental studies have suggested that GP IIb/IIIa inhibitors exert additional antiplatelet, antithrombotic, and anti-inflammatory effects while local medication concentrations are high.

However, few studies have investigated the effects of GP IIb/IIIa inhibitors in myocardial RI. Furthermore, the dose-dependent effects of GP IIb/IIIa inhibitors on myocardial RI have not been well-defined. The goals of this study were to determine whether exposure of the heart to a tirofiban would increase resistance to subsequent ischemia and reduce myocardial RI. This study also explored what tirofiban concentration would provide optimal protection for the heart.

Methods

Animal preparation: Male Sprague-Dawley rats (8 weeks old, body weight about 250-300 g) were fed a standard diet and acclimated in a quiet quarantine room for 7 to 10 days before the experiments were conducted. Rats used in this study received humane care. The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Chang
rats were given 100% O2 before and after the LAD ligation. The occurrence. Because of the high prevalence of lethal arrhythmias and dyskinesis of the myocardium, the blood of which was supplied via the right femoral artery catheter every 30 minutes to keep the rats adequately hydrated. Additionally, 1 mL of normal saline was injected directly into the heart just before ligation and after reperfusion. In addition, one drop of 0.5% xylocaine was injected into the left atrium just to the left of the midline of the chest wall. For the purpose of reducing the mortality of rats due to significant arrhythmia during the experimental period, the position of the left anterior descending coronary artery (LAD) ligation was not higher than the bottom of the left atrium. Regional ischemia was achieved by snaring the LAD with a 6-0 silk suture. Ischemia was confirmed by a visual assessment of cyanosis and dyskinesis of the myocardium, the blood of which was supplied by the LAD, a fall in blood pressure, or arrhythmia occurrence. Because of the high prevalence of lethal arrhythmia leading to mortality, LAD reperfusion was performed. The rats were given 100% O2 before and after the LAD ligation and reperfusion. In addition, one drop of 0.5% xylocaine was injected directly into the heart just before ligation and after reperfusion. During the period of ischemia and reperfusion, the open wound was kept wet and covered with parafilm to prevent dehydration. Additionally, 1 mL of normal saline was supplied via the right femoral artery catheter every 30 minutes to keep the rats adequately hydrated.

**Grouping of rats:** The animals were divided into 7 groups (n = 6/group) based on the different tirofiban infusion concentrations: sham, 0 (as the control group), 0.1, 0.4, 1, 2, and 5 ug/kg/minute. All animals received 120 minutes of LAD ligation and 180 minutes of coronary blood reperfusion. The tirofiban was administered continuously into the rats through the femoral vein. The drug was infused 30 minutes prior to LAD reperfusion and until the end of LAD reperfusion.

**Assessment of infarction size and area at risk (AAR) of the left ventricle:** At the end of the ischemia and reperfusion study protocol, the ligature around the LAD was repositioned. Evans blue dye (1 mL of 2% solution) was injected into the left atrium to stain the area of the myocardium perfused by the patent coronary arteries, and the area unstained by the dye was defined as myocardial area at risk (AAR). The atria, right ventricle, and major blood vessels were subsequently removed from the heart. The left ventricle was then sliced into 5 parallel sections (myocardial slice) of 1-mm thickness along the atroventricular groove. The unstained portion of myocardium (the AAR) was separated from the stained portion. These unstained myocardial slices were incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 5 minutes and fixed in 10% formalin. The unstained portion was defined as the myocardial necrotic area. All these serial slices were scanned using an Epson AL-CX11 flatbed scanner (Epson, Long Beach, CA). The total left ventricular area, necrotic area, and the AAR of the left ventricle of each slice were measured using NIH ImageJ software (computer-assisted planimetry with ImageJ-1.37 software). The percentages of the necrotic area to AAR were then averaged to calculate an overall value for each heart.

**Determination of tissue malondialdehyde (MDA) and myeloperoxidase (MPO) activities:** Following the 180-minute reperfusion period, tissue samples were taken from the AAR zone for the MDA and MPO activity analysis. The MDA, which was detected by Abcam (UK), reacted with thioarbituric acid (TBA) to generate the MDA-TBA adduct. This adduct can be quantified by an ELISA reader using an optical density of 532 nm. The MPO was detected with a rat MPO ELISA kit (Sunred, China). Rat plasma samples were added to a monoclonal antibody enzyme well that was pre-coated with rat MPO monoclonal antibody. MPO antibodies labeled with biotin were then added and combined with streptavidin-horseradish peroxidase (HRP) to form an immune complex. After a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and the color developed in proportion to the amount of MPO bound in the initial step. The color development was stopped, and the intensity of the color was measured with colorimetry. The MDA activity is expressed as nmol/mg of tissue sample and the MPO activity as ng/mg of tissue sample.

**Statistical analysis:** Statistical Package for Social Sciences (SPSS) 14.0 for Windows was used to conduct the statistical analysis, and all values are expressed as the mean ± SE. Statistical analysis was performed using the general linear model equation (GEE). The 95% confidence intervals were used to identify which groups were significantly different in multiple comparisons. A P value < 0.05 was considered significant.

**Results**

**Effects of tirofiban in the ratio of necrotic area to AAR:** As shown in Figure 1, the ratio of necrotic area to AAR showed a significant reduction at tirofiban concentrations of 0.1 (46.28 ± 1.46%, P = 0.02), 0.4 (43.02 ± 2.19%, P = 0.002), 1 (39.90 ± 0.58%, P < 0.001), 2 (36.86 ± 0.85%, P < 0.001), and 5 (36.63 ± 2.65%, P < 0.001) ug/kg/minute in comparison with the control group (54.05 ± 3.26%). Among different tirofiban concentrations, the concentrations of 2 and 5 ug/kg/minute yielded a more significant RI reduction effect in comparison with that of the control group.

**Effects of tirofiban on MDA and MPO activities:** As shown in Figure 2, the MDA activities in all tirofiban groups were lower
than those in the control group, however, no significant differences were found. However, a slight reduction was found at tirofiban concentrations of 2 (2.41 ± 0.27 nmol/mg, \(P = 0.056\)) and 5 (2.47 ± 0.42 nmol/mg, \(P = 0.072\)) ug/kg/minute in comparison with the control group (3.43 ± 0.28 nmol/mg).

As shown in Figure 3, the MPO activity decreased significantly at a tirofiban concentration of 2 ug/kg/minute (0.24 ± 0.02 ng/mg, \(P = 0.029\)) in comparison with that of the control group (0.34 ± 0.01 ng/mg).

**Effects of tirofiban in apoptosis of myocytes:** The DAPI stained all cell nuclei and the TUNEL-positive myocytes at different tirofiban concentrations, as illustrated in Figure 4. As shown in the Table, the AI was 1.38 ± 0.20, 13.96 ± 2.04, 10.70 ± 0.28, 8.98 ± 1.07, 4.80 ± 0.55, 5.20 ± 0.26, and 4.59 ± 0.40 % in the sham, control, and tirofiban concentration groups of 0.1, 0.4, 1, 2, and 5 ug/kg/minute, respectively, with the values for the various groups being significantly increased in comparison with those of the sham group (\(P < 0.05\)). Compared to the AI in the control group, AI was markedly decreased at tirofiban concentrations of 0.4 (\(P = 0.04\)), 1 (\(P < 0.001\)), 2 (\(P = 0.001\)), and 5 (\(P < 0.001\)) ug/kg/minute.

**Discussion**

As confirmed by a previous study, platelet activation causes microvascular injury and RI in acute myocardial infarction. In an animal study, Xu, et al reported that circulating platelets are activated early in reperfusion. Platelet activation depends on the duration of the preceding coronary occlusion (more than 45 minutes) and is proportional to the extent of myocardial injury. Furthermore, platelets were found in the myocardium immediately after coronary reperfusion. Serebruany, et al also reported that myocardial stunning after coronary ligation is associated with substantial dynamic changes in platelet aggregation and other haemostatic factors. Moreover, many studies have illustrated that the addition of platelets to the perfusate impairs coronary blood flow, decreases ventricular and postischemic contractile recovery, and promotes the occurrence of life-threatening cardiac arrhythmia in numerous models of myocardial RI.

GP IIb/IIIa inhibitors have been reported to be potential inhibitors of platelet activity that improves outcomes in acute myocardial infarction. They have also been clinically used in patients with acute coronary syndrome or unstable angina. In addition, they have been shown to improve the recovery of microvascular function in patients treated with primary stenting for acute myocardial infarction. However, it is still not known if some of the benefits of GP IIb/IIIa inhibitors in myocardial ischemia and infarction are due to a reduction in RI. In the present study, we confirmed that the GP IIb/IIIa in-
hibilities. Although our study found that the beneficial effect of the GP IIb/IIIa inhibitor was a reduction of RI, the mechanisms by which it protects against myocardial RI are still unclear. Several mechanisms may account for the benefits of GP IIb/IIIa inhibitors in myocardial RI. First, the antithrombolytic effect may directly prevent microvascular obstruction caused by the formation of platelet emboli or distal microthrombi and then yields an early restoration of epicardial blood flow. These discordant experimental outcomes sometimes confuse the therapeutic strategies in RI. A possible reason for these differing treatment results is that various animal models had been developed in previous studies. This variation will affect the presentation and identification of results and may result in a contrary conclusion. According to the report of Xu, circulating platelets become activated early in reperfusion, and their activation effect depends on the duration of the preceding coronary occlusion. Consequently, in our opinions, whether the medications for RI therapy experimented in the most RI extent period will interfere with the results of pharmacological effects in RI therapy. In an unpublished study, we established and confirmed the time sequence of coronary ligation (120 minutes) and reperfusion (180 minutes) to obtain the maximal RI in a rat animal model. We think that medications or interventional therapies implemented in this time sequence will produce the largest RI, and the therapeutic effects will perhaps be well-illustrated and conclusive. Therefore, we conducted the rat model protocol with coronary ligation of 120 minutes and reperfusion of 180 minutes in this study.

The occurrence of cardiomyocyte apoptosis in myocardial infarction reperfusion therapy may lead to the development of heart failure, and blocking or abating this process could slow or even prevent the heart failure process. Therefore, to prevent or diminish cardiac apoptosis is an important and reasonable indicator in RI therapy. The extent of necrotic and apoptotic cell death after GP IIb/IIIa inhibitor administration was examined in this study. The results showed that GP IIb/IIIa inhibitor administration significantly decreased TUNEL-positive cardiomyocytes at concentrations greater than 0.4 ug/kg/minute. At concentrations of 1, 2, and 5 ug/kg/minute, a larger AI reduction effect was observed.

Leukocytes and platelets have been found to contribute to RI by interacting with endothelial cells to promote neutrophil-induced RI. Within minutes after coronary reperfusion, platelets are among the first line cells to be recruited and are colonized with leukocytes in areas of the infarct. Extra-cellular MPO can be defined as an index of polymorphonuclear leukocyte infiltration in response to inflammation. This enzyme is an index of neutrophil accumulation in the heart. In our study, the MPO activity showed a significant decrease at a tirofiban concentration of 2 ug/kg/minute.

Reactive oxygen species degrade polyunsaturated lipids, forming MDA, which is used as a biomarker to measure the level of oxidative stress in an organism. In our study, the MDA activity in all GP IIb/IIIa inhibitor groups showed no significant reduction in comparison with the control group. However, the MDA in the 2 and 5 ug/kg/minute GP IIb/IIIa inhibitor groups was slightly reduced. A possible reason could be that each group in this study had a relatively small number of animals.

Disclosure

Competing interests: The authors declare that they have no competing interests.

References

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