Platelet Glycoprotein IIb/IIIa Inhibitor Tirofiban Ameliorates Cardiac Reperfusion Injury

Shih-Tai Chang,1,2 MD, Chang-Min Chung,1,2 MD, Chi-Ming Chu,3 PhD, Teng-Yao Yang,1,2 MD, Ku-Li Pan,1,2 MD, Jen-Te Hsu,1,2 MD, and Ju-Feng Hsiao,1,2 MD

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: 000-000)

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).1 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.2,3 In addition, different experimental results and conclusions occurred even in the same pharmacological trials in previous studies.4-11 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.12-14 Recently, experimental studies have suggested that GP IIb/IIIa inhibitors exert additional antiplatelet, antithrombotic, and anti-inflammatory effects while local medication concentrations are high.15

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
Gung Memorial Hospital. All animals were anesthetized with a mixture of 80-100 mg/kg ketamine and 5-10 mg/kg xylazine by intraperitoneal injection (in same syringe), and the dose was repeated every 30 minutes if needed. The rats were then placed in a supine position with their paws taped to the operating table. Airway maintenance was conducted with an endotracheal tube (PE-50) and ventilation was conducted at a rate of 70-120 beats/minute with a tidal volume of 0.6-1.25 mL/minute using a rodent ventilator (SAR-830/AP, CWE Inc., Ardmore, USA). The blood oxygen saturation of the rats was maintained at greater than 95% during the experimental procedure. Body temperature was monitored by a rectal probe connected to a digital thermometer to maintain a constant core temperature of 37°C.

**Induction of ischemia:** A loading dose of heparin 200 u/kg was administered to all rats before surgery. A heparinized catheter was inserted into the right femoral artery for blood pressure monitoring. The heart was exposed through a left vertical thoracotomy and pericardiectomy. The thoracotomy was performed 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 border 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 and dyskinesis of the myocardium, the blood of which was supplied via the right femoral artery catheter every 30 minutes for the experimental period, control of blood pressure was conducted. The blood pressure monitoring was determined according to the different LAD ligation concentrations, the concentrations of 2 and 5 ug/kg/minute yielded a more significant RI reduction effect in comparison with that of other concentrations. The AAR size was measured with colorimetry. The MDA activity was expressed as nmol/mg of tissue sample and the MPO activity as ng/mg of tissue sample.

**Determination of myocardial apoptosis:** Myocardial apoptosis was qualified using a commercially available terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) kit. TUNEL staining was performed with fluorescein-dUTP for apoptotic cell nuclei, and all cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The apoptotic index (AI) was measured as the ratio of TUNEL-positive myocytes to the total number of myocytes stained by DAPI from a total of 40 fields per heart in a blind manner by confocal microscopy at 400 times magnification. Data from 6 animals were averaged.

**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 generalized estimating 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 = 0.025), 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.

### 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 ($P < 0.05$) and drug-free group.

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-
hibitor tirofiban can limit the cardiac infarct size in a rat model of RI when administered after a period of ischemia. This beneficial effect began even at the low concentration of 0.1 ug/kg/minute. In addition, our results showed a reducing effect on myocardial infarction size that depended on the concentration of tirofiban.

In RI therapy, paradoxical therapeutic results occurred in many trials in previous studies. According to the Viehman, et al study, the degree of myocardial necrosis was only 18 ± 4% of the AAR. Nevertheless, other animal data suggest that up to 50% of an infarct size may be attributable to RI. 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 intervention 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 infarction. 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.

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. The TIMI 14 study showed that the GP IIb/IIIa inhibitor abciximab enhanced the speed as well as the degree of epicardial reperfusion. In addition, many previous studies showed that GP IIb/IIIa inhibitors may improve the clinical outcomes, including the diminished infarct size in myocardial infarct patients receiving primary stenting and GP IIb/IIIa inhibitor administration. The better clinical manifestations for GP IIb/IIIa inhibitor administration may be due to not only their antiplatelet aggregation effect, but also their benefits in RI alleviation, which could reduce further tissue injury and necrosis. Second, Hohlfeld, et al reported that blocking the platelet activity may improve the effect of myocardial contractile recovery from myocardial stunning. The direct contribution of activated platelets to myocardial injury after a prolonged myocardial ischemic period was well-documented by Xu, et al. They found that activated platelets play an important role in the myocardial RI process, and that platelet-derived P-selectin is a critical mediator in this process. Therefore, GP IIb/IIIa inhibitors exert their anti-inflammatory effect during a state of myocardial ischemia and reperfusion to reduce the extent of RI. Finally, it is possible that the interaction of a GP IIb/IIIa inhibitor with receptors other than the GP IIb/IIIa inhibitor receptor, such as the SAFE and/or RISK pathways, may prevent RI at the time of coronary reperfusion. Although no study has examined this yet, further research should be conducted to explore the exact components of signal pathways for the role of GP IIb/IIIa inhibitors in RI.

Disclosure

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

References

5. Gonon AT, Gourine AV, Middelveld RJ, Alving K, Pernow J. Limitation of infarct size and attenuation of myeloperoxidase activity


