



INFLUENCE OF PHOSPHATIDIC ACID ON RESPIRATORY RATE AND SERUM CREATININE LEVELS WITH GLYCOPROTEIN IIB/IIIA RECEPTOR ANTAGONIST ADMINISTRATION

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ABSTRACT

Phosphatidic acid (PA) is a putative second messenger released after the breakdown of phosphatidylcholine by phospholipase D and may have important relationship with glycoprotein (GP) IIb/IIIa receptor. The effect of Eptifibatide and Tirofiban, two widely used GP IIb/IIIa receptor antagonists in the cardiac medicine was evaluated on respiratory rate and serum creatinine *per se* and after pretreatment with phosphatidic acid. Respiratory rate was ascertained in the Wistar rat groups (n=6). Eptifibatide and tirofiban significantly changed the respiratory *per se*. Serum creatinine was estimated using the Jaffe's reaction. The results of our study suggest no major changes in respiratory rate after PA pretreatment and subsequent treatment with Tirofiban and Eptifibatide but significant changes in serum creatinine levels with similar treatments.

Keywords: Phosphatidic acid, Eptifibatide, Tirofiban.

INTRODUCTION

In patients undergoing GP IIb/IIIa inhibitor therapy, it is necessary to measure the creatinine levels. As creatinine levels serve as the important indicators for adjusting the dosage levels for GP IIb/IIIa inhibitors. Increase in doses may lead to other adverse outcomes especially in patients with renal insufficiency and in females. Renal impairment (RI) is also known to be an independent risk factor for the progression of cardiovascular disease.¹ Both, Eptifibatide and Tirofiban are widely used in the field of cardiac medicine. They are used in treating patients with acute coronary syndrome (ACS), percutaneous transluminal coronary angioplasty (PTCA), unstable angina, in patients undergoing percutaneous coronary intervention (PCI), and their role in transient ischemic attacks (TIA) is still under study.

On the other hand, catalytic activity of phospholipase D (PLD) generates choline, phosphatidic acid, and lysophosphatidic acid, the latter two representing potent platelet activators²⁻⁴. Phospholipase D is also involved in platelet activation by collagen and thrombin,⁵ monocyte and macrophage activation by oxidized LDL,⁶ matrix-metalloproteinase secretion,⁷ and endothelial cell dysfunction⁸. Phospholipase D and choline have important interrelationships with the GP IIb/IIIa receptor representing the final common pathway to platelet aggregation⁹. Phosphatidic acid dose-dependently promotes fibrinogen binding to the GP IIb/IIIa receptor¹⁰ and, after conversion to diacylglycerol, regulates activation of the GP IIb/IIIa receptor via protein kinase C¹¹. Eptifibatide, a selective high-affinity inhibitor of the platelet glycoprotein IIb/IIIa receptor, produces a dose-dependent inhibition of platelet aggregation and has been shown to reduce the frequency of acute ischemic complications in percutaneous coronary revascularization. The GP IIb-IIIa belongs to the integrin family of receptors for adhesive proteins (integrin α IIb β 3) and plays an essential role in platelet physiology¹².

Platelet stimulation with thrombin results in the activation of GP IIb-IIIa, which then binds released fibrinogen and triggers platelet aggregation¹³. Recently, it has been demonstrated that GP IIb-IIIa may also act as a transducer of signals into the platelets that can influence platelet function. In our study, these antagonists were tried with a combination of Phosphatidic acid to see their effects on respiratory rate and serum creatinine levels. Respiratory rates were measured as certain medications may have serious effects on respiration. Thus, it becomes necessary to study the effects of GP IIb/IIIa receptor antagonist alone and also in combination with phosphatidic acid on the serum creatinine levels. This study was conducted to ascertain the effect of GP IIb/IIIa receptor antagonists alone and in combination with phosphatidic acid on respiratory rate and serum creatinine levels. The results of our study suggest that after combination of phosphatidic acid with GP receptor antagonists there was no significant change in respiratory rate however the serum creatinine levels were affected.

MATERIALS AND METHODS

Animals and Housing Conditions

Experiments were performed on female Swiss albino mice weighing 25 - 35g and Wistar rats weighing 150 - 200g obtained from experiment animal centre of Christian Medical College, Vellore, India. Animals were housed in groups of 5 - 6 animals in polypropylene plastic cages under hygienic conditions, lined with paddy-husk bedding. Animals were housed in a colony room under controlled temperature (25 ± 0.5 °C), relative humidity of ($60 \pm 2\%$) and were exposed to a 12-hour light: 12 hour dark cycle (light on at 6.00am), with food and water available *ad libitum*. All experiments were conducted during the light phase, between 8.00 and 13.00 hrs. Experimental protocol was approved by the Institutional animal ethics committee (IAEC).

Drugs and chemicals used

Eptifibatide injection (Integrilin injection) (M/s. Sicoor Pharmaceuticals Inc, California, USA), Tirofiban hydrochloride i.v injection (Gland Pharma Limited, Hyderabad, India), Phosphatidic acid (Sigma chemical company), 0.9% Saline (Nirlife health care, India), 10% Sodium tungstate (Forbes Chemicals, India), 2/3 N H₂SO₄ (Qualigens, India), Standard Creatinine (Glaxo chemicals, India), Picric acid 0.04 M (Qualigens, India), NaOH 0.75M (Fisher, India) were used for this study.

Estimation of serum creatinine by alkaline picrate method

The normal serum creatinine ranges from 0.7 to 1.4 mg %. This estimation was performed in Wistar rats. Measuring serum creatinine is a useful and inexpensive method of evaluating renal dysfunction. Creatinine is a non-protein waste product of creatine phosphate metabolism by skeletal muscle tissue. Creatinine production is continuous and is proportional to muscle mass. Creatinine in serum was determined by the Jaffe's reaction¹⁴. Here a protein-free filtrate of the sample is treated with alkaline picrate to yield a yellow-red (orange) colour and the intensity of the colour is a measure of the concentration of creatinine present in the sample.

Procedure for preparation of serum

Estimation of serum creatinine was determined in Wistar rats. Blood was collected from the ocular retro orbital sinus region (by glass capillary method) or from the sub-mandibular region of the animals. 2.5-5ml of blood was collected (not anticoagulated) according to standard procedures in glass tubes. The whole blood was fractioned by centrifuging at 1500-2000 X g for 10-15 min at room temperature. This would separate the blood into an upper serum layer, a lower red blood cell (RBC) layer, and a thin interface containing the WBCs. A sterile plastic or glass pipette to transfer the serum into a clean and dry boiling tube was used. 4 ml of serum was pipette out into the boiling tube. Into the boiling tube, addition of 6 ml

water, which is to be followed by the addition of 2 ml of 10% sodium tungstate and 4 ml of 2/3 N H₂SO₄. The contents were mixed in the boiling tube thoroughly and let stand for 10 minutes. The contents of the tube filtered through a WHATMAN No.1 filter paper and preserve the filtrate for creatinine estimation by spectrophotometric technique.

Estimation of respiratory rate

Respiratory rate was counted visually by observing the thoracic movements. The rat was held upside down by gently holding into one's hand. The other hand was placed on the upper chest to feel it rise and fall. Each rise/fall cycle counts as one respiratory breath. Respiratory rate was determined by counting the number of times the chest rises or falls per minute. In this study, an average of about 6 minutes for each rat to get a more accurate value was ascertained.

RESULTS

Respiratory rate measured in Wistar rats

The effect of tirofiban and an eptifibatide on respiratory rate in Wistar rats was evaluated. Tirofiban [62.5 µg/kg, (Dose: 0.5 ml/rat; concentration: 25µg/ml)] injected intraperitoneally (i.p) significantly altered the respiratory rates (Average no. of breaths per minute) and changed the control values [86.8833 ± 1.78128 (Mean ± SEM) to 72.4167 ± 5.97263 (Mean ± SEM) [Figure 1]

Similarly, Eptifibatide [200 µg/kg, (Dose: 0.2 ml/rat; concentration: 200µg/ml)] injected intraperitoneally (i.p) produced a significant decrease in the respiratory rates (Average number of breaths per minute) and changed the control values from [86.8833 ± 1.78128 (Mean ± SEM) to 72.9833 ± 2.69820 (Mean ± SEM) [Figure 2]

Phosphatidic acid (P.A) [50 µg/kg, (Dose: 0.1 ml/rat; concentration: 100µg/ml)] injected intramuscularly (i.m) produced a significant decrease in the respiratory rates (Average number of breaths per minute) and changed the control values [86.8833 ± 1.78128 (Mean ± SEM) to 74.0333 ± 2.72270 (Mean ± SEM) [Figure 1 & 2].

Eptifibatide [200 µg/kg, (Dose: 0.2 ml/rat; concentration: 200µg/ml)] in combination with Phosphatidic acid (P.A) [50 µg/kg, (Dose: 0.1 ml/rat; concentration: 100µg/ml)] did not significantly alter the respiratory rates (Average number of breaths per minute) [86.8833 ± 1.78128 (Mean ± SEM) to 78.0333 ± 2.97833 (Mean ± SEM) [Figure 2].

Tirofiban [62.5 µg/kg, (Dose: 0.5 ml/rat; concentration: 25µg/ml)] in combination with Phosphatidic acid (P.A) [50 µg/kg, (Dose: 0.1

ml/rat; concentration: 100µg/ml)] did not significantly alter the respiratory rates (Average no. of breaths per minute) [86.8833 ± 1.78128 (Mean ± SEM) to 78.4500 ± 3.40076 (Mean ± SEM, [Figure 1].

Serum Creatinine levels estimated in Wistar rats

The effect of various drugs was ascertained on serum creatinine levels. Eptifibatide [200 µg/kg, (Dose: 0.2 ml/rat; concentration: 200µg/ml)] injected intraperitoneally (i.p) did not significantly alter the [0.8333 ± 0.06839 (Mean ± SEM) to 0.8725 ± 0.14767 (Mean ± SEM), [Table 1].

On the other hand, Tirofiban [62.5 µg/kg, (Dose: 0.5 ml/rat; concentration: 25µg/ml)] injected intraperitoneally (i.p) significantly altered [0.8333 ± 0.06839 (Mean ± SEM) to 2.4067 ± 0.08751 (Mean ± SEM), [Table 1].

Eptifibatide [200 µg/kg, (Dose: 0.2 ml/rat; concentration: 200µg/ml)] in combination with Phosphatidic acid (P.A) [50 µg/kg, (Dose: 0.1 ml/rat; concentration: 100µg/ml)] significantly altered the [0.8333 ± 0.06839 (Mean ± SEM) to 1.1533 ± 0.04529 (Mean ± SEM), [Table 1].

Tirofiban [62.5 µg/kg, (Dose: 0.5 ml/rat; concentration: 25µg/ml)] in combination with Phosphatidic acid (P.A) [50 µg/kg, (Dose: 0.1 ml/rat; concentration: 100µg/ml)] significantly altered the [0.8333 ± 0.06839 (Mean ± SEM) to 1.2417 ± 0.05250 (Mean ± SEM), [Table 1].

DISCUSSION

The results of this study show that glycoprotein IIb/IIIa receptor antagonists, eptifibatide and tirofiban decrease respiratory rate in the rats although this was not significantly altered by phosphatidic acid pretreatment. Both eptifibatide and tirofiban bind to the platelet surface glycoprotein IIb/IIIa complexes and thus inhibits binding to these receptors, and this indirectly blocks the final mechanism of platelet aggregation. Clinically used GPIIb/IIIa blockers are ligand mimetics, and thereby their binding can induce conformational changes of the platelet integrin GPIIb/IIIa. A fast reversibility of the conformational change of GPIIb/IIIa after dissociation of GPIIb/IIIa blockers could be demonstrated as an intrinsic property of the GPIIb/IIIa receptor¹⁵. This mechanism prevents general platelet aggregation after dissociation of ligand mimetic GPIIb/IIIa blockers¹⁶. The two GP IIb/IIIa antagonists eptifibatide and tirofiban when used in rats produced a significant decrease in respiratory rates *per se*. The changes seen in the respiratory rate of rats may be attributed to modification of pulmonary vascular permeability.

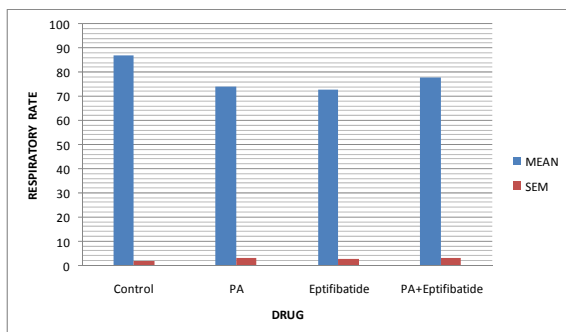


Fig. 1: Effect of PA (50 µg/kg) and tirofiban alone on respiratory rate (no. of breaths/minute) and combination of PA and tirofiban (62.5µg/kg) in Wistar rats (n=6)

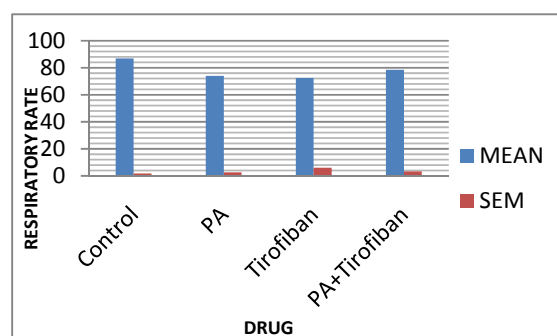


Fig. 2: Effect of PA (50 µg/kg) and eptifibatide alone on respiratory rate (no. of breaths/minute) and combination of PA and eptifibatide (200 µg/kg) in Wistar rats (n=6)

Table 1: Depicts mean creatinine values in rat serum after treatment with tirofiban and eptifibatide alone and in combination with PA and the level of statistical significance as compared with control

Treatment	Mean Serum creatinine (mg %)	Mean±SEM	'P' value
1. Saline	0.8333	0.8333 ± 0.0683	-
2. Tirofiban	2.4067	2.4067 ± 0.0875	P<0.05
3. Eptifibatide	0.8725	0.8725 ± 0.1476	P>0.05
4. PA+Tirofiban	1.2417	1.2417 ± 0.5250	P<0.05
5. PA+Eptifibatide	1.1533	1.1533 ± 0.0452	P<0.05

The reason for using phosphatidic acid in this study was because it is a second messenger derived from the action of phospholipase D enzyme. Phosphatidic acid (PA) is a putative second messenger that causes actin polymerization, calcium²⁺ mobilization and mitogenesis in fibroblasts. Phosphatidic acid after conversion to diacylglycerol regulates activation of the GP IIb/IIIa receptor via protein kinase C. They also enhance the binding of integrin alpha IIb beta 3 to the fibrinogen necessary for platelet aggregation. PA increases the proportion of fibrinogen binding-competent GP IIb/IIIa complexes without altering their affinity for fibrinogen.¹⁷

Several studies suggest that serum creatinine levels could help determine and titrate the dosage of GP IIb/IIIa receptor antagonists. In this study, PA pretreatment was able to modify the levels of serum creatinine after treatment with eptifibatid or tirofiban. According to James A. Shaw et al,¹⁸ 2008, serum creatinine levels serve as the major indicators in adjusting the dosage levels especially in patients with renal insufficiency and also in women. The results of our study on serum creatinine clearly show the interaction of PA with GP IIb/IIIa receptor antagonists. PA pretreatment was able to significantly alter the serum creatinine levels in conjunction with GP IIb/IIIa receptor antagonists. Previous studies support the interpretation that the overall effect of tirofiban on PLD activation and choline release is evident after tirofiban infusion and platelet inhibition. Blocking of the GP IIb/IIIa receptor inhibits platelet aggregation, which also reduces sustained stimulation of platelet activation pathways like PLD, leading to a significant reduction of generated choline; however, the pathophysiology of fibrinogen binding and PLD is complex and it is controversial whether changes of whole blood choline are directly related to platelet activation and/or inhibition. Studies by Martinson¹⁶ indicate that platelet PLD is activated by protein kinase C via an integrin alpha IIb beta 3-independent mechanism. Martinson also suggested that PLD is involved in signal transduction events occurring upstream of integrin alpha IIb beta 3 activation and fibrinogen binding. Inhibition of fibrinogen binding (i.e. with tirofiban) could therefore inhibit signal transduction events upstream of fibrinogen binding including PLD, leading to a decrease of choline levels. Platelet phosphatidylcholine-specific phospholipase C activity and phosphocholine-specific phosphatase activity converting phosphocholine to choline are other important mechanisms affecting choline concentrations in this setting.

The importance of serum creatinine in relation to phosphatidic acid could have tremendous implications for cardiac pathophysiology. According to Naranjan S. Dhalla¹⁹, 1997, Most of the positive inotropic agents which are known to stimulate cardiac hypertrophy, have been shown to increase the level of PA in cardiac sarcolemma. By using single cardiomyocytes, it is seen that PA increased the basal [Ca²⁺] level without significant effect on the amplitude of Ca²⁺-transients. PA has also been shown to stimulate protein synthesis in cardiomyocytes, which is inhibited by a PKC inhibitor as well as a Ca²⁺-chelator. PA stimulates the activity of PLC in cardiac sarcolemma which was attenuated by a PLC inhibitor. Since DAG, formed due to the activation of PLC, is considered to play a crucial role in regulating the activity of protein kinase C (PKC), the positive feedback effect of PA on this pathway may be essential for maintaining the sustained elevation in the activity of PKC during the development of cardiac hypertrophy. Thus PA may be a potential signal transducer for the development of cardiac hypertrophy.

Thus in conclusion it can be summarized based on the results our study, that GP IIb/IIIa receptor antagonists *per se* modified the respiratory rate in experimental rats and in combination with PA were able to alter the serum creatinine levels.

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