Türk Göğüs Kalp Damar Cerrahisi Dergisi 2012;20(3):584-589
doi: 10.5606/tgkdc.dergisi.2012.111

The effects of clopidogrel, acetyl salicylic acid and tirofiban on acetylcholine-induced dilation in rat thoracic aorta segments

Klopidogrel, asetilsalisilik asit ve tirofibanın sıçan torasik aort segmentlerinde asetilkolin ile uyarılan gevşeme üzerine etkisi

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Background: In this article, we aimed to investigate the effects of antithrombotic agents, including acetyl salicylic acid (ASA), clopidogrel, and tirofiban on the acetylcholine (ACh)-induced dilation responses in rat thoracic aorta segments.

Methods: Twenty-eight Wistar albino male rats weighing between 250-300 g were included in the study. The rats were randomly divided into four groups: the ASA group (n=7), the clopidogrel group (n=7), the tirofiban group (n=7), and the control group (n=7). Intraperitoneal (IP) injection of ASA (1.5 mg/kg) or clopidogrel (1 mg/kg) was administered 12 hours before the experiment. In the tirofiban group, 150 μgr/kg tirofiban IP was given twice at one and 12 hours before the experiment. The rats in the control group did not receive any medication. After the rats were decapitated, the segments of the thoracic aorta were removed and suspended in an oxygenated Krebs solution in a tissue bath, and the dose dependency in the responses to the ACh-induced dilation in the aortic rings were studied.

Results: The dilation responses of the aortic rings to ACh in the lowest molar concentration (10-9) were found to be reduced in the ASA (94.3%±3.3) and clopidogrel (94.6%±3.1) groups when compared with the control group (86.7%±8.1) ($p<0.05$). The responses to higher concentrations of ACh were observed to be similar in all of the groups.

Conclusion: Acetyl salicylic acid and clopidogrel treatments resulted in a slight delay of endothelium-mediated relaxation while tirofiban had no significant effect. These findings suggest that ASA and clopidogrel may have a limited effect on endothelium-mediated vasodilation.

Key words: Acetylsalicylic acid; clopidogrel; endothelium; tirofiban.

Amaç: Bu çalışmada, asetilsalisilik asit (ASA), klopidogrel ve tirofiban gibi antitrombositer ajanların sıçan torasik aort segmentinde asetilkolin (ACh) ile uyarılan gevşeme yanıtları üzerine olan etkileri araştırıldı.

Çalışma planı: Yirmi sekiz adet ağırlıkları 250-300 g arasında olan Wistar-albino türü yetişkin erkek sıçan çalışmaya alındı. Sıçanlar randomize olarak dört gruba ayrıldı: ASA grubu (n=7), klopidogrel grubu (n=7), tirofiban grubu (n=7) ve kontrol grubu (n=7). Deneyden 12 saat önce intraperitoneal (İP) ASA (1.5 mg/kg) veya klopidogrel (1 mg/kg) enjeksiyonları yapıldı. Tirofiban grubunda, 150 μgr/kg tirofiban İP deneyden 1 ve 12 saat önce olmak üzere, iki kez verildi. Kontrol grubundaki sıçanlara herhangi bir ilaç verilmedi. Sıçanlar dekapite edildikten sonra torasik aort segmentleri çıkartıldı ve oksijenize Krebs çözeltisi içinde organ banyosuna asıldı ve aort halkalarında asetilkolin ile uyarılan gevşeme yanıtının doza bağımlı değişimleri incelendi.

Bulgular: Asetilkolinin en düşük molar konsantrasyonu (10-9) ile elde edilen gevşeme yanıtının ASA (%94.3±3.3) ve klopidogrel (%94.6±3.1) grubunda kontrol (%86.7±8.1) grubuna göre azalmış olduğu bulundu ($p<0.05$). Daha yüksek asetilkolin konsantrasyonlarında ise, tüm gruplarda gevşeme yanıtlarının benzer olduğu gözlemlendi.

Sonuç: Asetilsalisilik asit ve klopidogrel tedavilerinin sonucunda endotel aracılı gevşeme yanıtında hafif bir gecikme ortaya çıkmış ve tirofibanın belirgin bir etkisi olmamıştır. Bu bulgular ASA ve klopidogrelin endotel aracılı vazodilatasyon üzerinde sınırlı bir etkisi olabileceğini düşündürmektedir.

Anahtar sözcükler: Asetilsalisilik asit; klopidogrel; endotel; tirofiban.

Available online at
www.tgkdc.dergisi.org
doi: 10.5606/tgkdc.dergisi.2012.111
QR (Quick Response) Code

Received: February 10, 2012 *Accepted:* March 1, 2012

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Thrombocytes play a key role in the pathogenesis of atherothrombotic diseases such as myocardial infarction, stroke, and peripheral vascular occlusions. Acetyl salicylic acid (ASA) and clopidogrel are widely administered antiplatelet agents that play an essential part in therapy for these diseases. These antiaggregant drugs prevent vascular disorders with well-known indications, which have also been implicated in the guidelines.[1-4] Tirofiban is a parenteral antiplatelet drug which is frequently used in patients undergoing percutaneous coronary intervention (PCI) in the setting of acute coronary syndrome.[5]

The endothelium is a selective, permeable barrier between the circulating blood and the vessel wall. Physiologically, it controls the capillary transportation of water and water-soluble compounds along with the levels of plasma lipids. Endothelial cells are sensitive to hemodynamic and humoral alterations and are activated to release endothelium-derived factors and biologically-active substances.

Beyond these functions, the endothelium bears anticoagulant, antiaggregant and fibrinolytic properties and regulates the production of vascular reactive molecules. Endothelial cells also interact with various types of other cells, including platelets, through numerous biochemical pathways which display complex and diverse actions. As an example, adenosine diphosphate (ADP), which stimulates platelet aggregation, leads to the release of vasodilator prostacyclin from the endothelial cells.[6,7] Therefore, the endothelium plays an important role in maintaining homeostasis by providing regular blood flow and adjusting the diameter of the vessel lumen.

The aim of this experimental study was to investigate the effects of antiaggregant pretreatment (ASA, clopidogrel, and tirofiban) on the acetylcholine-induced relaxation response of the endothelium in rat thoracic aorta segments in vitro.

MATERIALS AND METHODS

The animal ethics and research committee of Adnan Menderes University approved all protocols prior to the commencement of the study. The experiments were carried out on 28 adult male Wistar albino rats (250-300 g) obtained from the experimental animal laboratory of the Medical Faculty of Adnan Menderes University. The rats were fed a standard laboratory diet and water ad libitum and housed in cages in a temperature-controlled (22±2 °C) room with a 12-hour dark-light cycle before the experiments. The study was conducted at the Electrophysiology and Cardiovascular Research Laboratory of Adnan Menderes University. The rats were randomly assigned into four experimental groups.

Tablets of ASA (Coraspin® 100 mg tb-Bayer, Turkey) and clopidogrel (Plavix® 75mg tb-Sanofi Aventis, Turkey) were crushed in sterile containers, and each tablet was dissolved in distilled water to obtain a homogenous mixture. Tirofiban (Aggrastat® 0.25 mg/ml-Merck Sharp Dohme, Turkey) was obtained in sterile vials.

ASA group (n=7): Acetyl salicilic acid (1.5 mg/kg) was administered intraperitoneally 12 hours before the experiment.

Clopidogrel group (n=7): Clopidogrel (1 mg/kg) was administered intraperitoneally 12 hours before the experiment.

Tirofiban group (n=7): Tirofiban (150 μgr/kg) was administered intraperitoneally twice, once at the first hour and again 12 hours before the experiment.

Control group (n=7): No medication was administered before the experiment.

Under high-dose ether anesthesia, a median sternotomy was performed, the heart and lungs were retracted, and the arcus and thoracic aortic segments, adjacent to the thoracic vertebrae, were removed. The rats were then sacrificed by decapitation. The aorta was carefully dissected free from the adipose and connective tissue remnants with the help of surgical binocular loupes (x3.5) by paying meticulous attention to avoid damage to the endothelium, and segments of the aortic rings, each 3-4 mm in length, were quickly prepared. The rings were then suspended in four parallel bath organ chamber systems (May IOBS 99, COMMAT Pharmacolgy and Physiology Instruments, Ankara, Turkey) each containing 25 ml Krebs solution [118.3 mM sodium chloride (NaCl), 4.7 mM potassium chloride (KCl), 1.2 mM magnesium sulfate ($MgSO_4$), 1.22 mM monopotassium phosphate ($KH2PO_4$), 2.5 mM calcium chloride ($CaCl_2$), 25.0 mM sodium bicarbonate ($NaHCO_3$), and 11.1.mM glucose (Sigma-Aldrich, St. Louis, Missouri, USA) maintained at 37 °C and bubbled with 95% oxygen (O_2), 5% carbon dioxide (CO_2). Two stainless steel clips were passed through the lumen of each ring. One was anchored to the bottom of the chamber while the other was suspended in order to connect with a transducer system (May GTA030, and Biopac Systems Inc. Model MP 100, COMMAT, Ankara, Turkey).[8,9] The data was obtained by means of the AcqKnowledge 3.8.2 hardware system (COMMAT, Ankara, Turkey).

After maintaining a state of equilibrium, the aortic rings were distended under a force of 1 gram (gr) and

progressively stretched by increasing the force by 1 gr every 10 minutes until a tension of 3 gr was reached. At the end of this cycle, a period of 10 minutes had passed, and the rings were contracted by the addition of norepinephrine (Sigma-Aldrich) (0.1 mlx10^{-4}M) to the baths. The responses were recorded, and a plateau level was then achieved. The baths were washed with Krebs solution, and the rings were distended with a final force of 4 g. Provided a stable state was reached, contraction was stimulated again by adding norepinephrine (0.1 mlx10^{-4}M) into the baths, which was subsequently followed by acetylcholine (ACh) (Sigma-Aldrich) (0.1 mlx10^{-4}M) (Figure 1). The relaxation responses of the aortic rings to the ACh were recorded, and then the baths were washed twice and allowed to equilibrate in Krebs solution for 45 minutes before starting the experiment. The baths in which an absence of contraction or relaxation was observed were excluded from the study, suggesting that the endothelium of the aortic rings in these baths may have been damaged during the harvest, causing them to fail to respond properly.[10]

The experiment was started by stimulating the contractile response with the addition of an equal amount of norepinephrine (0.1 mlx10^{-4}M) to each bath. When a stable plateau level was achieved, an initial dose of 0.1 mlx10^{-9}M ACh was given. After each given concentration of ACh, the relaxation response was recorded. When a plateau level was reached, subsequent doses (each 0.1ml. in volume) were administered. In this manner, the concentration of ACh was gradually increased, and a final dose of 0.1mlx10^{-4}M was achieved as depicted in Table 1.

The change in the tonus of the ring with each relaxation from the maximum tonus as a response to given doses of acetylcholine was calculated and expressed in percentages. The data was analyzed statistically by analysis of variance (ANOVA) and by Student's t-test. The differences were considered to be statistically significant when $p<0.05$.

RESULTS

The relationship between the percentage of relaxation and concentration of ACh in the baths for each group is shown in Figure 2. The relaxation response to the lowest concentration of Ach (10^{-9}) in the ASA (94.3%±3.3) and clopidogrel (94.6%±3.1) groups was found to be significantly decreased when compared with the control (86.7%±8.1) group ($p<0.05$). However, the relaxation responses for all the remaining concentrations of Ach were similar between the groups, and no statistical significance was present ($p>0.05$).

DISCUSSION

Antiplatelet therapy has a pivotal role in the treatment of ischemic vascular diseases; however, the mechanisms by which the antiplatelet agents inhibit thrombocytes may

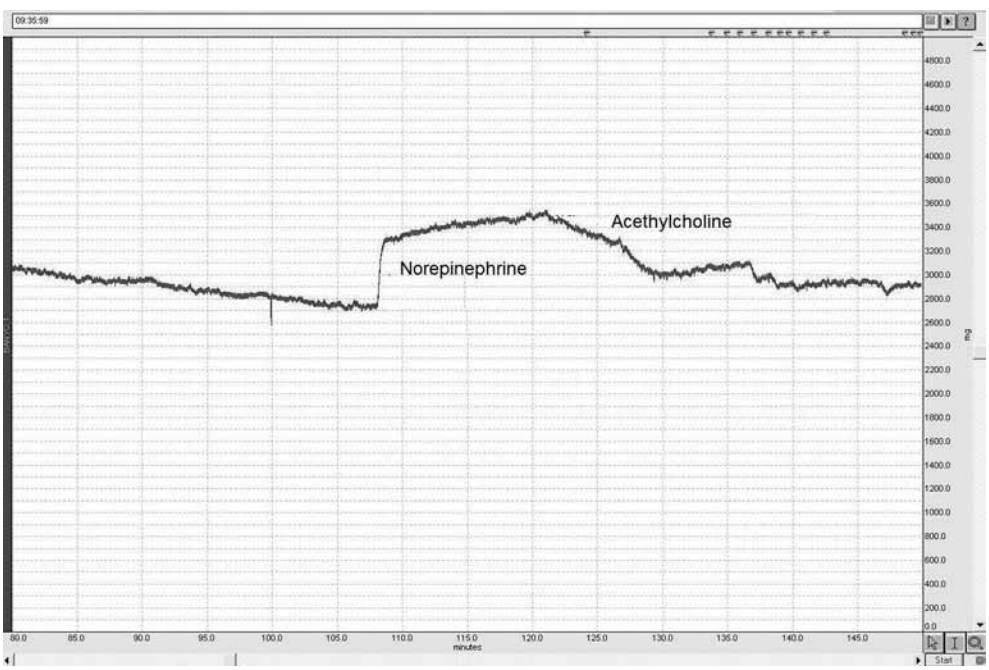

Figure 1. An example of the contraction response to norepinephrine, followed by acetylcholine induced relaxation of the aortic ring.

Table 1. Concentrations of acetylcholine doses

Dose	Acetylcholine Molar concentrations
1	10-9
2	3 x 10-9
3	10-8
4	3 x 10-8
5	10-7
6	3 x 10-7
7	10-6
8	3 x 10-6
9	10-5
10	3 x 10-5
11	10-4

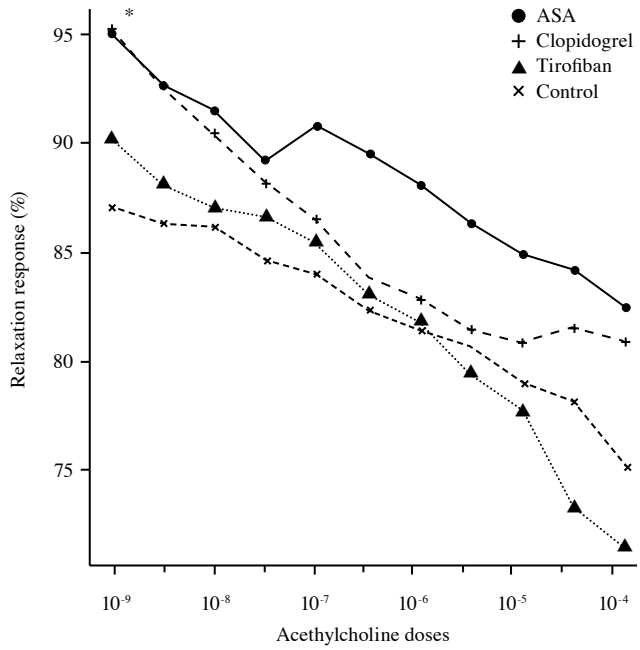

Figure 2. The relationship between the acetylcholine concentration and the percentage of decrement in the tonus from the maximum tonus is shown. The vertical axis represents the relaxation response, and the horizontal axis represents the subsequent acetylcholine doses. * p<0.5; ASA: Acetyl salicylic acid.

also interfere with physiological processes elsewhere and cause adverse effects. The protection and preservation of normal endothelial functions are important issues to be considered during the management of thrombotic disorders. Therefore, we aimed to assess the effects of aspirin, clopidogrel and tirofiban, commonly employed antiplatelet drugs, on endothelial functions by examining the Ach-induced dilatation responses of the aortic rings, obtained from pretreated rats, in a tissue bath.

The inhibition of cyclooxygenase by ASA results in an irreversible blockage of the thrombocyte aggregation, which is the principal therapeutic action. Although ASA inhibits both the thromboxane and prostacyclin pathways, the effect on the former is more evident in low doses.[11] The members of prostaglandin family may exhibit vasodilator or vasoconstrictor properties and, therefore, counteract to form a balance, which is an important factor in the determination of the vascular tonus. Aspirin, when applied in antiaggregant doses, strongly inhibits cyclooxygenase-1 (Cox-1) and blocks the synthesis of thromboxane A2 (TXA2), thereby preventing the aggregation of thrombocytes and vasoconstriction.[12] However, the inhibitory effect of the antiaggregant doses of ASA on cyclooxygenase-2 (Cox-2), which is associated with the synthesis of the vasodilator prostacyclin, is less evident.[13] In experimental studies, ASA has been reported to decrease the dilation response to ACh in rat aorta segments.[14,15] On the other hand, in vivo studies have demonstrated that ASA induces a reduction in vascular resistance and augments coronary blood flow.[16] In this study, the dilation responses of the aortic rings to minimum ACh concentration (10^{-9}), harvested from the rats pretreated with ASA, were observed to be significantly reduced when compared with the responses in the control group. Nevertheless, we did not observe a blunting of the dilation response in the ASA group with higher concentrations of ACh used in this study. This suggests that this state reflects a limited latency of the endothelium to respond to ACh. In addition, we did not note any remarkable augmentation of the Ach-induced dilation response with the ASA treatment in comparison with the control group.

Clopidogrel is a thienopyridine derivative and a selective inhibitor of the P2Y12 type of ADP receptors that is expressed by platelets which cause the secretion of thromboxane A2 (TXA2).[17,18] These receptors have also been found to exist in cells other than platelets, including the rat and human vascular smooth muscle cells.[19] Adenosine diphosphate has been shown to induce vasoconstriction via P2Y12 receptors; however, it is also believed to cause arterial relaxation by increasing the synthesis of endothelium-derived nitric oxide (NO).[19-21] Thus, the effect of ADP-mediated pathways on the endothelium is currently unclear.[22] Clopidogrel has been reported to exhibit vasomodulatory activity[23] and direct endothelial effects independent from antiplatelet actions,[24] which are still not fully understood. Recently, Froldi et al.[25] reported that the clopidogrel pretreatment of rats resulted in only a slight decrement of the constriction response of the aortic tissues to phenylephrine in vitro. In the present study,

although the dilation response to the lowest ACh (10^{-9}) concentration in the clopidogrel group was observed to be reduced in comparison with the response in the control group, there was no significant difference in the responses between the two groups for increased ACh concentrations.

Glycoprotein IIb/IIIa (GPIIb/IIIa) receptors are integrins expressed on platelets, and the activation of these receptors leads to firm binding to fibrinogen and platelet aggregation.[26] Vascular injury is a trigger for vascular cells for the expression of such integrins, which results in the platelet-endothelium adhesion among other hazardous effects.[27] Tirofiban is a competitive inhibitor of GPIIb/IIIa receptors,[28] and it has been suggested that it has protective effects on the endothelium, acute coronary syndrome, and symptomatic coronary artery disease.[29,30] Warnholtz et al.[31] reported improvement in the flow-mediated dilation of the brachial artery after PCI with tirofiban treatment. However, these studies were all performed in clinical settings where there was obvious endothelial damage, and the vascular effects of tirofiban on an intact endothelium is uncertain. Kintscher et al.[32] found that tirofiban had no direct effect on cultured human vascular endothelial cells and suggested that it acts on the endothelium via indirect mechanisms. In this study, we observed no statistically significant difference in the dilation response between the control and tirofiban groups at any given concentration of Ach.

In conclusion, ASA and clopidogrel pretreatments resulted in a slight delay of the dilation response to ACh in rat thoracic aorta segments while the tirofiban pretreatment had no significant effect. These findings suggest that ASA and clopidogrel may have a limited effect on endothelium-induced vasodilation, but further investigations are necessary to understand the influence of these agents on endothelial functions and the underlying mechanisms.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding

The authors received no financial support for the research and/or authorship of this article.

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