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The Similarity Between Aspirin Treatment and the Lack of Response to Epinephrine and the Frequency of Aspirin Resistance in Healthy Males

Engin Sagdilek,[1,2] Ahmet Korkmaz,[2] Sukru Oter,[2] Ferit Avcu,[3] Turgut Topal,[2] Mehmet Ozler,[2] Bulent Uysal,[2] Oral Nevruz,[3] Cengiz Beyan,[3] and Kasim Ozluk[4]

Abstract

Objectives: The lack of response of platelets against epinephrine has been discovered with a frequency of 14% to 40% in previous studies. There are studies that have demonstrated the effect of aspirin on platelets may resemble the lack of response to epinephrine. In this study, the extent of the effects of aspirin treatment on aggregation and secretion in healthy males with a lack of response to epinephrine and the frequency of aspirin resistance were investigated. **Methods:** Blood samples were collected at the beginning and at the end of a 10-day aspirin usage in 52 healthy males. Epinephrine, adenosine diphosphate (ADP), collagen, arachidonic acid (AA) and thrombin aggregations, and adenosine triphosphate (ATP) secretion were studied. Participants were assigned to nonresponder (<20%), semiresponder (20%-60%), and responder (>60%) groups, depending on their maximum aggregation responses to epinephrine. Participants who displayed an aggregation to AA at the end of the aspirin treatment were accepted to be aspirin resistant. **Results:** Of the 52 participants, 4 were found to be nonresponders and 3 of 52 of the participants were found to be semiresponders. Although the lack of response to epinephrine and aspirin treatment displayed similarities in aggregations using epinephrine, ADP, collagen, and thrombin, they differed in aggregations using AA and for ATP secretion. The ratio of aspirin resistance was determined to be 4:52. **Conclusions:** The observation of AA aggregation in the participants with a lack of response to epinephrine demonstrates that epinephrine nonresponse cannot substitute aspirin treatment. The fact that aspirin resistance is observed in healthy males supports the view that aspirin resistance exists even before the first usage.

Keywords

platelets, optical aggregometer, epinephrine, aspirin resistance

Introduction

Epinephrine (Epi) is a weak inducing agent that activates platelet aggregation. It activates the $Gi_{\alpha z}$ protein via α_{2A} adrenergic receptors, and thus inhibiting adenylate cyclase (AC). Although the decrease in cyclic adenosine monophosphate (cAMP) concentration is not exactly sufficient for platelet activation, it aids other inducing agents in initiating aggregation at lower concentrations. Apart from inhibiting AC, it is known that Epi also activates Rap1, stimulates the synthesis of thromboxane A_2 (TxA_2), indirectly activates phospholipase C, and causes secretion. Additionally, it has been demonstrated that the $Gi_{\alpha z}$ protein intrinsically conducts a slow guanosine triphosphate (GTP) hydrolyses and it activates protein kinase C and p21-activated kinase.[1]

The situation when platelets do not form aggregates after being introduced to a dose of Epi in optical aggregometry is known as "The Lack of Response to Epinephrine." It is suggested that the lack of response to Epi, which may be observed with high rates such as 14% to 40% for various populations, may be from the result of a defect in the intracellular activation mechanisms independent of the AC/cAMP pathway. In participants with a lack of response to Epi, it is demonstrated that the response to adenosine diphosphate (ADP) and collagen (Col) is decreased, the lag times with Col are prolonged, aggregations

[1] Department of Biophysics, Ege University, Faculty of Medicine, Izmir, Turkey
[2] Department of Physiology, Gulhane Military Medical Academy, School of Medicine, Ankara, Turkey
[3] Department of Haematology, Gulhane Military Medical Academy, School of Medicine, Ankara, Turkey
[4] Department of Physiology, Uludag University, Faculty of Medicine, Bursa, Turkey

Corresponding Author:
Engin Sagdilek, Ege University, Faculty of Medicine, Department of Biophysics, 35100 Izmir, Turkey.
Email: esagdilek@hotmail.com

induced by arachidonic acid (AA) and thrombin (Tro) are unaltered, and that the secretion stimulated by Col is unaffected. Tendency to bleed was not reported in participants with a lack of response to Epi. There are studies that demonstrate the resemblance of aspirin effects on platelets with the lack of response to Epi.[2-9]

In the thrombus model generated using Col-Epi, the mortality rate was found to be significantly lower and thrombus formation in the lung sections was found to be significantly less in the $G_{\alpha z}$ knockout mice compared to the wild type. No difference was found for the Col-ADP model.[10] Because $G_{\alpha z}$ is the active subunit of the G protein interacting with the Epi receptor, it can be thought that the lack of response to Epi may likewise decrease arterial thrombus formation and may have a protective effect in patients with cardiovascular diseases.

It is reported in the last meta-analysis of Antithrombotic Trialists' Collaboration that patients under a high-risk benefit from aspirin treatment despite the increased risk of major extra cranial bleeding.[11] Considering that not all patients are in a high-risk group, and that there may be cranial bleedings, whether it is necessary for a cardiovascular patient with a lack of response to Epi should use an antiplatelet drug and whether using such a antiplatelet drug would lead to an increase in the tendency to bleed are important questions.

Aspirin is an antiplatelet drug most frequently used in cardiovascular diseases. The basic mechanism underlying the effect of aspirin on platelets is the suppression of TxA_2 synthesis. In many recent studies, it is demonstrated that aspirin does not exhibit the same effect on all patients and that there existed patients shown to have aspirin resistance.[12-15] Whether aspirin resistance appears in time, in connection with usage, or whether it exists even before the first usage is a question to be investigated.

The aim of this study is to reveal how much aspirin treatment affects aggregation and secretion of platelets in healthy males who exhibit a lack of response to Epi and to investigate whether aspirin resistance is present at the end of a 10-day aspirin treatment in this group of participants who have no cardiovascular problems and who have not previously taken an aspirin treatment for antiplatelet purposes.

Methods

Participants

Fifty-four healthy male volunteers with ages ranging from 19 to 42 years took part in the experiments. All participants were lecturers or students of Gulhane Military Medical Faculty. The study has been carried out in accordance with the protocols and the approval of "Local Ethical Committee of Drug Investigations of Gulhane Military Medical Faculty." The experiments were conducted in Gulhane Military Medical Faculty Hematology Laboratory through January to May 2005. The participants were screened for any hemorrhagic condition in their personal or family history, smoking, alcohol consumption, and the use of drugs in the last 10 days known to affect platelets. The participants who were allergic to aspirin or who had hemorrhage or dyspeptic complaints due to nonsteroid anti-inflammatory drug use were excluded from the study. Two participants were excluded from the study after the collection of first blood samples because they had taken another drug in the 10 days during aspirin was administered. Our experiment was initiated with 54 participants and was completed with 52 participants.

Collection of Blood Samples

After informing the volunteers, the first blood samples were taken around 08.00 to 08.30 in the morning. The same day after dinner, the participants started using a 300-mg loading dose of aspirin (Dispril 300-mg effervescent tablet). During the next 9 days, aspirin (Aspirin 100-mg tablet) was administrated with a dose of 100 mg/d. In the morning of the 11th day, the second blood samples were taken, again on fasting, and the study was completed. Fifteen milliliters blood samples taken from the antecubital vein into 0.129 mol/L sodium citrated tubes after the application of a mild venous stasis to the upper limb were used in the aggregation studies; 5 mL blood samples taken into EDTA tubes were used in hemogram tests.

Optical Aggregometer

Optical aggregometers are modified spectrophotometric devices. These devices work on the principle of measuring the change in the amount of light transmitted through platelet-rich plasma (PRP), where aggregates form by the addition of a stimulating agent. The blood samples were centrifuged at 150*g* for 10 minutes and the PRP was obtained, centrifuging at 2000*g* for another 15 minutes provided the platelet-poor plasma (PPP). The platelet content of PRP was evaluated using the Cell-Dyn 4000 (Abbott, Illinois, USA) hemogram device. The platelet count was kept between 300 000 $\pm$ 50 000/μL. For values above 350 000/μL, the PRP was diluted with autologous PPP.

As previously explained,[16] platelet aggregation and secretion were measured using a platelet lumiaggregometer (Model 560CA; Chrono-log Corporation, Havertown, Pennsylvania) according to the manufacturer's instructions. As inducing agents, Epi, ADP, Col, AA, and Tro (Chrono-log Corporation) were used. To evaluate secretion, ATP Standard and luciferase (Chrono-lume reagents; Chrono-log Corporation) were used. Final concentrations used in the test were Epi 100 μmol/L, ADP 10 μmol/L, Col 6 μg/mL, AA 1 μmol/L, and Tro 0.4 U/mL.

First, by making calibration with PPP, maximum light permeability was set as 100%. This was followed by putting PRP into a second aggregation tube, setting light permeability as 0%, and then adding the inducing agent. The aggregation process that was initiated with inducing agents was monitored for 10 minutes. Experiment results were expressed in percentages of maximum aggregation.

Secretion of ATP was measured by bioluminescence, and collagen (6 μg/mL final concentrations) was used to induce the secretion of ATP. Fifty microliters luciferase was added to 450

Table 1. The Effects of Aspirin Treatment on Platelet Aggregation and Secretion in the Epinephrine Responders Group[a]

Epi Responder (n = 45)		Preaspirin	Postaspirin	*P*
Maximum aggregation %	Epi	78.93 ± 12.98 60-108	24.96 ± 12.58 4-77	<.001
	ADP	79.78 ± 10.81 60-102	70.93 ± 10.99 50-99	<.01
	Col	76.24 ± 12.90 50-101	65.36 ± 12.53 43-92	<.001
	AA	78.31 ± 9.29 58-96	41:45 aspirin sensitive 3.37 ± 2.84 (0–9) 4:45 aspirin resistant 80.50 ± 15.07 (62-98)	<.001 >.05
	Tro	Total aggregation	Total aggregation	>.05
ATP secretion		2.46 ± 1.51 0.41-8.00	1.39 ± 1.02 0.12-5.10	<.001
Disagregasyon	ADP	None	38:45	<.001
	Col	None	38:45	<.001
	Epi	None	13:45	<.001
	AA	None	1:45	>.05

NOTES: AA = arachidonic acid; ADP = adenosine diphosphate; Col = collagen; Epi = epinephrine; Tro = thrombin.
[a] The values presented as means ± standard deviations, ranges and ratios.

μL PRP in the 2 aggregation tubes, which were then immediately placed into the aggregometer wells and stirred for 2 minutes. After 2 minutes, a standard amount of ATP (2 nmol) was added to the first aggregation tube and the luminescence was calibrated. Collagen was added to the second aggregation tube and the luminescence was measured. ATP secretion results were expressed in nmol (10^{-9} mol).

Aggregation and secretion tests started at most 1 hour after the collection of blood samples and were completed within 3 hours.

For the first blood samples, for 40 of 52 participants, the aggregation response to Epi was evaluated 2 times. In samples with a lack of response to Epi, a third test was conducted with a final Epi concentration of 400 μmol/L.

Hemogram tests were made using the Cell-Dyn 4000 (Abbot) hemogram device.

Statistical Analysis

For evaluating the difference between preaspirin and postaspirin states, the Wilcoxon signed-rank test and McNemar tests were used. For the evaluation of the mean platelet volume before and after centrifuging, the paired *t* test was used. *P* values smaller than .05 were accepted to be statistically significant.

Results

The hemogram results of 52 participants (average age: 22.3 ± 5.1 years) were found to be within normal limits.

In a total of 104 evaluations before and after aspirin usage, it was observed that the mean platelet volume (MPV) in PRP was significantly lower than the MPV in the hemogram (7.19 ± 1.17 [4.9-10.4] and 7.55 ± 1.27 [5.1-11.3], respectively, $P <$ .001). Mean platelet volume decreased after the centrifuging procedure in 92 of 104 measurements (88.5%); MPV was constant in 1 measurement and in 11 measurements a maximum of 0.5 fL increase was seen in the MPV.

The participants were assigned to 3 groups according to percentages of maximum aggregation response to Epi in the optical aggregometer using the first blood samples.

1. Participants with a maximum aggregation response of 20% or lower (nonresponder, NR),
2. Participants with a maximum aggregation response between 20% and 60% (semiresponder, SR),
3. Participants with a maximum aggregation response of 60% or higher (responder, R).

Epi nonresponse was detected in 4 of 52 (7.7%) participants whose response to Epi were analyzed; in 3 (5.8%) participants, Epi semiresponse was detected.

When the effects of aspirin on platelets in 45 participants with normal Epi response were analyzed (Table 1), it was observed that aspirin significantly suppressed aggregation induced by Epi or AA and reduced ATP secretion by half. It was found that aspirin had a limited effect on ADP- or Col-induced aggregations and had no effect on aggregation induced using Tro. In addition, it was observed that aspirin caused a significant disaggregation especially in aggregations induced by ADP or Col. Although no disaggregation was observed in the

Table 2. The Effects of Aspirin Treatment on Platelet Aggregation and Secretion in the Epinephrine Nonresponders and Semiresponders Groups[a]

		Epi Nonresponder (n = 4)		Epi Semi-Responder (n=3)		Epi Non/Semi-Responder (n=7)		*P*
		Preaspirin	Postaspirin	Preaspirin	Postaspirin	Preaspirin	Postaspirin	
Maximum aggregation %	Epi	3-9-9-19	4-6-11-19	40-44-54	9-31-10	25.43 ± 20.24	12.86 ± 9.30	>.05
	ADP	46-62-65-70	47-84-59-67	61-69-70	73-65-41	63.29 ± 8.48	62.29 ± 14.80	>.05
	Col	55-62-64-65	40-42-74-37	58-64-67	53-79-50	62.14 ± 4.22	53.57 ± 16.68	>.05
	AA	69-74-81-88	4-0-1-7	62-66-70	3-4-1	72.86 ± 8.99	2.86 ± 2.41	<.001
	Tro	Total aggregation	Total aggregation	Total aggregation	Total aggregation	Total aggregation	Total aggregation	>.05
ATP secretion (nmol)		1.5-2.1-2.2-8.6	1.3-0.2-0.7-0.1	0.8-1.7-1.9	0.9-0.3-1.8	2.68 ± 2.64	0.75 ± 0.61	<.05
Disaggregation	ADP	None	4:4	None	3:3	None	7:7	<.05
	Col	None	3:4	None	3:3	None	6:7	<.05
	Epi	None	1:4	None	2:3	None	3:7	>.05
	AA	None	None	None	None	None	None	>.05

NOTES: AA = arachidonic acid; ADP = adenosine diphosphate; Epi = epinephrine; Col = collagen; Tro = thrombin.
[a] The values presented as means ± standard deviations and ratios. The preaspirin values are presented in an increasing order and the postaspirin values are ordered, respectively. The *P* values presented are given for Epi non-/semiresponder group (n = 7) in pre- to postaspirin comparisons.

participants before aspirin usage, after aspirin usage disaggregation was observed with ADP or Col aggregations, with a ratio of 38:45; with Epi aggregations the ratio was 13:45.

Because the number of participants with nonresponse and semiresponse to Epi was low, the results of these participants were given in Table 2 in detail. Epinephrine nonresponse and semiresponse groups were combined for evaluation and preaspirin, postaspirin comparisons were made. In the group with a defective Epi response (nonresponse + semiresponse), although aggregation induced by Epi was reduced to half, this was not statistically significant. Aggregation induced by AA was significantly suppressed and a significant decrease was found in ATP secretion. A significant disaggregation was observed when ADP or Col was used as the inducing agent.

After 10 days of aspirin usage, the participants who had an aggregation response of 10% or lower to AA were considered aspirin resistant. Aspirin resistance was observed with a ratio of 4:52 (7.7%). It was observed that the participants with aspirin resistance had normal responses to Epi. Epinephrine-, ADP-, and Col-induced aggregations and ATP secretion in aspirin-resistant participants were similar with the postaspirin group values.

Discussion

In our study with healthy males who did not have a hemorrhagic condition or any cardiovascular problems, it was found that a maximum aggregation response of below 60% to Epi was observed with a ratio of 7:52 (13.5%), and this group displayed aspirin resistance with a ratio of 4:52 (7.7%).

The aggregation responses of healthy participants who had no hemorrhagic tendency in personal or family history to Epi were spread over a wide range (3%-108%). The fact that no hemorrhage was observed in nonresponders to Epi makes us think that this defect in platelets is not involved in one of the principal mechanisms stopping hemorrhage.

In various studies carried out on the lack of response to Epi, the frequency of participants with a defective response to Epi was found to be in the wide range of 14% to 40%. However, in these studies, the criteria used in the definition of the lack of response to Epi showed some variation. The monitoring of the aggregation procedure initiated with Epi varied between 5 and 10 minutes. In some studies, participants with a maximum aggregation response to Epi below 20% were considered Epi nonresponders; participants with a maximum aggregation response between 20% and 60% were considered Epi semiresponders. In some studies, participants with a maximum aggregation response to Epi below 40% were considered Epi nonresponders or similarly in other studies participants who gave primary aggregation but did not give secondary aggregation were considered Epi nonresponders. In addition, final Epi concentrations varied between 10 and 300 μmol/L.[2-9]

The duration of the transition from primary aggregation to secondary aggregation for the Epi-induced aggregation response may show some interpersonal variations. It was indicated that in pregnant women the lack of response to Epi was not observed; this may be due to a late-rising response to Epi.[17] Monitoring the aggregation process for 5 to 6 minutes may be insufficient to detect a late-rising response to Epi.

Ten micromoles per liter and higher final Epi concentrations are called supraphysiologic concentrations.[18] The fact that no response is seen at very high concentrations in Epi nonresponders indicates that the defect is not concentration dependent. Therefore, working with very high concentrations is not necessary. Because at lower concentrations, such as 0.4 and 1.5 μmol/L, concentration-dependent differences can be seen,

evaluating lack of response to Epi at very low concentrations is not preferable, either.

In a study carried out with 3 different concentrations of Epi, which were 0.4, 1.5, and 10 μmol/L, responses varying between 1% and 100% were reported; however, for all 3 concentrations, the number of participants with a maximum aggregation response between 40% and 60% was low.[19]

Based on these findings, monitoring the aggregation with a minimum final Epi concentration of 10 μmol/L for 10 minutes, it would be more accurate to define Epi nonresponders as the participants who display a primary aggregation below 40% and do not display a secondary aggregation.

In our study, the percentage of defective responses to Epi was found to be 13.5%, and this is less frequent than the rates reported in other studies. Previous studies reported no relationship between the lack of response to Epi and age or gender.[2,6,8,9] Considering the distinguished military institution from which the participants were selected and the health controls routinely conducted in the institution, the fact that the lack of response to Epi was low make us think that there may be a relationship between a health parameter that we did not notice and the lack of response to Epi.

Nakamura et al,[3] using the particle counting technique, reported that Epi nonresponders and platelets from Epi responders incubated with aspirin formed only small aggregates and their aggregation curves were similar. In previous studies, it was observed that aspirin reduced the maximum aggregation response to Epi, ADP, and Col; completely suppressed AA aggregation.[20] The Epi, ADP, Col, and Tro aggregation values were found to be similar for Epi nonresponders and aspirin users, in this study. In the group with a defective Epi response, the fact that AA aggregation and ATP secretion change with aspirin usage is the most significant finding pointing out to the distinction between the lack of response to Epi and aspirin usage. The main indication of antiplatelet aspirin usage is the suppression of TxA_2 synthesis and AA aggregation. When an Epi nonresponder person needs to use an antiplatelet drug due to a cardiovascular disease, it is clear that aspirin is necessary. Still, whether aspirin usage for antiplatelet purposes would increase the risk of hemorrhage in individuals with a defective Epi response should be clinically analyzed in prospective studies.

Aspirin resistance is a favorite subject in the recent years. Emergence of obstructive vascular cases despite aspirin usage and the indication of insufficient inhibition of platelet functions through laboratory studies were defined as ''aspirin resistance.''[21] It was reported that genetic heritage was one of the reasons for aspirin resistance and that aspirin resistance was more common in old age.[12-14] In our study, with young and middle-aged male group who had no cardiovascular problems and never used aspirin for antiplatelet purposes previously, after 10 days antiplatelet aspirin treatment, aspirin resistance rate was found to be 4 of 52. The fact that 3 participants with aspirin resistance were 20 years old, the fourth one was 31 years old, clearly indicated that resistance may also be seen in young ages without an existing cardiovascular problem. It also indicates that resistance could exist at the moment when aspirin was first taken.

The weakest aspect of our study is the low number of the participants with a defective response to Epi (7 of 52). The need for a higher number of participants was one of the limitations of our study that prevented the use of parametrical tests to assess the data.

The fact that MPV in PRP was lower than that in the hemogram, although anticoagulant substances are different, indicates that during the centrifuge procedure the larger platelets sediment. The removal of larger and thus younger platelets, which contain more granules, from the material that will be studied in vitro, is a cause of the divergence from in vivo conditions in our evaluation.

In summary, the lack of response to Epi is observed in the wide range of 14% to 40% and is caused by a defect in a pathway after the receptor or the secondary messenger. The fact that the lack of response to Epi does not effect TxA_2 synthesis or AA aggregation, which are the main effect mechanisms of aspirin, make us think that, although the lack of response to Epi and the other effects of aspirin are similar, a lack of response to Epi does not create a sufficient antiplatelet effect. Still, whether aspirin usage of an Epi nonresponder individual causes hemorrhage in the long term should be clinically evaluated with prospective studies. Monitoring aspirin resistance in healthy, young, and middle-aged males with no cardiovascular problems, who did not previously receive aspirin treatment for antiplatelet purposes, support the view that aspirin resistance exists even prior to the first usage.

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Declaration of Conflicting Interests

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

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