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In vitro effect of intermedin/adrenomedullin 2 on platelet aggregation in human

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ÖZET

Intermedin/adrenomedullin 2'nin insan platelet agregasyonu üzerine in vitro etkileri

İntermedin/Adrenomedullin (IMD/AM2) kalsitonin genle ilişkili peptid ailesinin yeni bir üyesidir. Homeostaz için bir vasküler düzenleyici ve vasküler hastalıklara karşı önemli bir endojen koruyucu faktördür. Bu in vitro çalışma IMD/AM2'nin sağlık gönüllüler üzerinde çeşitli pletelet agregan ajanlar ile trombosit agregasyonu üzerinde etkisini araştırmak için tasarlanmıştır. Bu çalışma 7 sağlıklı gönüllü üzerinde gerçekleştirilmiştir. Kan örnekleri santrifüje edilerek trombositten zegin ve trombositten fakir plazma elde edildi. Trombositten zengin plazma, trombositten fakir plazma ile dilüe edilerek son trombosit sayısının 300,000±25,000 platelets L–9 olduğu trombositten zengin plazma elde edildi. IMD/AM2' nin 10-8, 10-7, and 10-6 M olacak şekilde 3 konsantrasyonu hazırlandı. IMD/AM2' nin her bir konsantrasyonu bir saat süreyle 37 C'de trombositten zengin plazma ile inkübe edildi. Trombosit agregasyon çalışmalara trombositten zengin plazma ile 3µg ml–1 kollajen, 10µM ADP ve 10µM epinephrine kullanılarak yapıldı. Ayrıca IMD/AM2' nin her bir konsantrasyonu için spontan agregasyon trombositten zengin plazma inkübasyonu ile çalışıldı. Kontrol grubu ile karşılaştırıldığında kollajen, ADP, epinefrin indüksiyonu ile IMD/AM2' nin tüm dilüsyonlarda yapılan pre-inkübasyonunun trombosit agregasyonu üzerine saptanabilir cevabı ile spontan agregasyon etkisi yoktur. Agregasyon testinde trombosit agregasyon amplitütleri ve eğrileri tüm gruplarda istatistiksel olarak birbirine benzerdir. Bu in vitro çalışma doz bağımlı bir şekilde IMD/AM2' nin trombosit agregasyonu üzerine etkisi olmadığını göstermiştir.

Anahtar Kelimeler: İntermedin (17-47)/Adrenomedüllin 2 (17-47); trombosit kümelenmesi; canlı dışında

SUMMARY

Intermedin/Adrenomedullin 2 (IMD/AM2) is a novel peptide related to the calcitonin gene-related peptide family. It is a vascular regulatory factor of homeostasis and a vital endogenous protective factor against vascular diseases. This in vitro study was designed to the effect of IMD/AM2 on platelet aggregation with several platelet aggregation agents in the healthy volunteers. The study was carried out on 7 healthy volunteers. Blood samples were centrifuged to prepare platelet-rich plasma and platelet-poor plasma. The platelet-rich plasma was diluted with the platelet poor plasma to yield test platelet-rich plasma with a final platelet count of 300,000±25,000 platelets L–9. Three concentration of IMD/AM2 solutions were prepared that would result in 10-8, 10-7, and 10-6 M. Each concentration of IMD/AM2 was incubated with platelet-rich plasma at 37 C during one hour. Platelet aggregation studies were carried out in platelet-rich plasma using 3µg ml–1 collagen, 10µM ADP, and 10µM epinephrine. In addition, spontaneous aggregation was performed for each concentration of IMD/AM2 incubated platelet-rich plasma. Compared to control, pre-incubation with all dilutions of IMD/AM2 had no detectable effect on platelet aggregation response induced collagen, ADP, epinephrine, and no detected spontaneous aggregation. The platelet aggregation amplitudes and slopes were statistically similar among all groups by the aggregation test. This in vitro study suggested that IMD/AM2 had no effect on platelet aggregation in a dose-dependent manner.

Key words: *Intermedin (17-47)/Adrenomedullin 2 (17-47); platelet aggregation; in vitro techniques*

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Introduction

Intermedin (IMD/AM2) also called, as adrenomedullin (AM) 2 is a novel member of the calcitonin/calcitonin gene-related peptide family (1). IMD/AM2 and its receptors are present in the cardiovascular system, and IMD/AM2 is present at low levels in plasma. In the cardiovascular system, IMD/AM2 has multiple functions such as regulation of blood pressure and cardiac function, pro-angiogenesis, endothelial barrier function protection, anti-oxidative stress, and anti-endoplasmic reticulum stress. IMD/AM2 participates widely in the pathogenesis of atherosclerosis, hypertension and vascular calcification. Its encoding genes are highly homologous and it plays an important role in vascular regulatory factor of body homeostasis and a vital endogenous protective factor against vascular diseases (2). The endothelium performs a crucial role in maintaining vascular integrity leading to whole organ metabolic homeostasis and IMD/AM2 is a vital bioactive peptide maintaining vascular homeostasis (1). Adrenomedullin has multifunctional biological activities such as increasing platelet cyclic AMP (cAMP) (3,4). Pharmacological analysis showed that IMD/AM2 binds to and acts on both CGRP (CRLR/RAMP1) and AM (CLRL/RAMP2, CLRL/RAMP3) receptors in a non-specific manner and suggested to activate intracellular cAMP pathway in the majority of tissues (5). Platelets are indispensable for primary haemostasis, but their function needs to be tightly regulated to prevent excessive platelet activity, possibly leading to atherothrombotic events. An important mediator of the platelet activity is cAMP, which inhibits platelet aggregation (6). In hemostasis and thrombosis; platelets, vascular vessel wall, and cAMP plays an important role and IMD/AM2 may be an important mediator of this. Although Schiller et al. (7) studied the effect of adrenomedullin on platelet functions, there is no study available so far demonstrating the effect of intermedin on platelet functions. Therefore this study was designed to test the effect of IMD/AM2 in vitro on platelet aggregation with several platelet aggregation agents in the healthy volunteers.

Materials and methods

This study was performed in accordance with the Helsinki declaration. Local Ethical committee of Gulhane Military Medical Academy approved the study (23 Jul 2014-GATA (KA-14009)). Following the approval of the informed consents, blood samples were collected from seven healthy volunteers. Venous blood was collected under light tourniquet through 19 gauge needles into vacutainers from antecubital vein. Four tubes with 4.5 ml including tri-sodium citrate (0.109 M) blood samples were collected (blood to anticoagulant ratio: 9/1). The collection was performed early in the morning following a light breakfast. All subjects were required to have not ingested as-

Table 1: Effects of intermedin on human platelet aggregation, in vitro. The platelet aggregation was induced by addition of collagen (3 μg ml−1), ADP (10 μM), epinephrine (10 μM), respectively.

		IMD/AM2 groups		
	Control Group	10-8 M	10-7 M	10-6 M
Collagen (3 μg l−1)	57,85±10,74	56,33±11,14	54,33±8,50	56,71±6,94
ADP (10 μM)	53,28±6,51	54,66±8,99	51,66±6,80	55,42±8,15
Epinephrine (10 μM)	59,85±9,38	60,66±8,49	56,66±8,08	58,14±8,44

IMD/AM2: Intermedin/Adrenomedullin 2, ADP; Adenosine diphosphate

pirin or other nonsteroidal anti-inflammatory agents for at least 10 days, no garlic or Chinese food for 2 days, and no alcohol for 24 h. Furthermore, patients with an abnormal platelet count, a history of thrombosis or abnormal bleeding, active neoplasia or active inflammatory disease were also excluded. Testing on all samples was completed within 2–4 h of collection. We counted platelets using an automated cell counter device (ABX Pentra DX 120, Horiba, France). Platelet aggregation was measured by using a platelet lumiaggregometer (Model 560CA, Chrono-log Corporation, Havertown, PA, USA) according to the manufacturer's instructions. Platelet-rich plasma (PRP) was separated from 3.2 % citrate-anticoagulated blood as previously described. (8-10) The remaining blood was centrifuged for further 10 min at 1250 g to prepare platelet-poor plasma (PPP). Calibration was made with PPP when using PRP. Aggregation studies were carried out in PRP containing 300,000±25,000 platelets L-9. A Teflon-coated stir bar was inserted into the silicon-coated cuvette with PRP then into the heater block to a temperature of 37 C, and the aggregation procedure was done in computerized aggregometer. (8)

Platelet-rich plasma was incubated with increasing doses (10-8, 10-7, 10-6 M) of IMD/AM2 (Bachem, Torrance, CA, USA) for 1 hour at 37 °C, and then the stimulating agents (Chrono-par reagents, Havertown, PA, USA) including collagen (3 μg ml−1), adenosine diphosphate (ADP) (10 μM), and epinephrine (10 μM) were added to the cuvette, and changes in light transmission were observed for 10 minutes. The maximal amplitudes of the aggregation curves, expressed as percentage, were used for quantitative analysis. Spontaneous aggregation for increasing concentrations of IMD/AM2 was performed after incubation with PRP and non-incubated PRP control.

Statistical analysis

We analyzed data using SPSS 15.0 (SPSS Inc. Software, Chicago, Illinois, USA) statistical software. Aggregation data presented as mean ± SD. Friedman repeated measures analysis of variance was used to compare platelet aggregation response to ADP, collagen, epinephrine between control and each IMD/AM2 solution. A wilcoxon test was used to determine differences between paired data. $P<0.05$ was considered statistically significant.

Results

All volunteers had normal haemogram parameters. When IMD/AM2 was added to normal PRP in vitro, the effect on platelet aggregation was dependent upon the agonist and upon the concentration of IMD/AM2. The response to 3 μg ml−1 collagen, 10 μM ADP, 10 μM epinephrine were not noticeably suppressed by increasing concentrations of IMD/AM2 compared with control group. Amplitude percentages of platelet aggregation of each group were shown in Table1. There was no relationship between IMD/AM2 concentration (10-8, 10-7, 10-6 M) and platelet aggregation responses in dose-dependent manner. Spontaneous aggregation was not also detected on PRP incubation with increasing concentrations of IMD/AM2 and non-incubated control PRP.

Discussion

Intermedin is a 47 amino acid peptide formed by enzymatic degradation of preprointermedin. Initially isolated from the puffer fish, the IMD/AM2 sequence is conserved across species including human, rat and mouse. Intermedin has been reported to be expressed in the kidney, lung, thymus, gastrointestinal tract, submaxillary gland and brain by using real time-polymerase chain reaction (RT-PCR). Intracerebrovascular administration of IMD/AM2 promotes anorexia, water restriction, and release of prolactin, oxytocin, vasopressin and adrenocorticotropin as well as inhibition of growth hormone release. Peripheral administration, similar to intracerebrovascular administration, promotes anorexia. However it also promotes gastoparesis, oliguria, diuresis and antinatriuresis. (1)

Systemic and regional vascular responses to IMD/AM2 have been reported in the conscious and anesthetized mouse and rat. Intracerebrovascular administration of IMD/AM2 increases systemic arterial pressure (SAP), whereas intravenous (i.v) administration of IMD/AM2 decreases SAP. Intrarenal infusion of IMD/AM2 increases renal blood flow and decreases renal vascular resistance in vivo whereas IMD/AM2 does not relax porcine renal arterial conductance vessels. Moreover, IMD/AM2 reduces myocardial injury in the ischemia-reperfused rat heart in vitro and relaxes isolated porcine coronary arterial rings. Furthermore IMD/AM2 possesses marked vasodilatory activity in pig coronary vascular bed. (1)

IMD/AM2 shows its biological activities mainly through the G protein coupled calcitonin receptor-like receptor (CRLR), which is shared by CGRP and AM. Three different subtypes of the receptor activity modifying protein (RAMP) determine ligand selectivity of CRLR. The complex of CRLR and RAMP1 shows a higher affinity with CGRP; the complex of CRLR and RAMP2 or RAMP3 has a higher interaction with AM. However, IMD/AM2 can interact with the three complexes non-selectively. These receptor components for IMD/AM2 are abundant in both central and peripheral tissues involved in the cardiovascular regulation. (11)

IMD/AM2 stimulates cAMP production like AM and CGRP and cAMP-dependent signaling may be the primary molecular

mechanism subsequent to activation of CRLR receptor. IMD/AM2 binds to and acts on receptor then activates intracellular cAMP pathway in the majority of tissues. (11)

It is well known that an increase in intracellular cAMP inhibits platelet activation and aggregation. Therefore, Lang-Rollin et al. (12) examined the possible influence of adrenomedullin on the function of human platelets. According to their study, there was no direct effect of adrenomedullin on thrombin-induced platelet aggregation, even when high adrenomedullin concentrations were used. It was also similar to the results of Schiller et al. which reported that adrenomedullin does not alter the aggregatory response of human platelets to ADP, due probably to the lack of functional adrenomedullin receptor.

Similar to the findings of AM studies, in the current study we found that IMD/AM2 also does not have any effect on human platelet aggregation. Different from the study of Schiller et al. (7) we studied not only ADP-induced platelet aggregation, but also we studied the collagen and epinephrine induced aggregation.

According to the Schiller et al. (7) the reason for the absence of an effect on human platelet function is uncertain but may suggest that functional AM receptors coupled with adenylate cyclase are not present on the membrane of human platelets. Although it is not proven, similar to AM, the reason for absence of an effect on human platelet may be related to inexistence of IMD/AM2 receptors coupled with adenylate cyclase on the membrane of human platelets.

Conclusion

Increasing evidence suggests that IMD/AM2 is a vital regulatory peptide for cardiovascular homeostasis and involved in occurrence and development of various vascular diseases. Furthermore it also has protective effect on atherosclerosis, vascular calcification and hypertension. Therefore it could be a novel therapeutic agent for cardiovascular system diseases. Since it has no effect on platelet aggregation, this may increase its safety profile when it is used for cardiovascular disorders.

Conflicts of Interest

The authors declare no conflicts of interest

References

1. Doganci S, Yildirim V, Bolcal C, Demirkilic U, Ozal E, Akdag E, Arslan M, Gumusel B, Lippton H. Intermedin (IMD/AM2) dilates the pig coronary vascular bed through release of nitric oxide. Turkish J Thorac Cardiovasc Surg 2011; 19: 242-246.
2. Ni X, Zhang J, Tang C, Qi Y. Intermedin/adrenomedullin2: an autocrine/paracrine factor in vascular homeostasis and disease. Sci China Life Sci 2014; 57: 781-9.
3. Marutsuka K, Hatakeyama K, Yamashita A, Sato Y, Sumiyoshi A, Asada Y. Adrenomedullin augments the release and production of tissue factor pathway inhibitor in human aortic endothelial cells. Cardiovasc Res 2003; 57: 232-237.
4. Parlapiano C, Paoletti V, Campana E, Labbadia G, Giovanniello T, Califano F, Pantone P, Clemente G, Donnarumma L, Musca A. Adrenomedulline assay and its clinical significance. Eur Rev Med Pharmacol Sci 1999; 3:53-61.
5. Kandilci HB, Gumusel B, Lippton H. Intermedin/adrenomedullin-2 (IMD/AM2) relaxes rat main pulmonary arterial rings via cGMP-dependent pathway: role of nitric oxide and large conductance calcium-activated potassium channels (BK(Ca)). Peptides 2008; 29: 1321-8.
6. Noé L, Peeters K, Izzi B, Van Geet C, Freson K. Regulators of platelet cAMP levels: clinical and therapeutic implications. Curr Med Chem 2010; 17: 2897-2905.
7. Schiller NK, Champion HC, Hugghins SY, Timothy AM, Murphy WA, Coy DH, Peter JR, Kadowitz PJ, McNamara DB. Adrenomedullin Does Not Inhibit Human Platelet Aggregation. J Cardiovasc Pharmacol Ther 1998; 3: 223-228.
8. Ural AU, Yilmaz MI, Avcu F, Yalcin A. Treatment with cerivastatin in primary mixed hyperlipidemia induces changes in platelet aggregation and coagulation system components. Int J Hematol 2002; 76: 279-283.
9. Kaptan K, Erinç K, Ifran A, Yildirim V, Uzun M, Beyan C, Işik E. Levosimendan has an inhibitory effect on platelet function. Am J Hematol 2008; 83: 46-49.
10. Kose EA, Nevruz O, Honca M, Yildirim V. In vitro effect of dexmedetomidine on platelet aggregation. Braz J Anesthesiol 2013; 63: 415-418.
11. Li P, Sun HJ, Han Y, Wang JJ, Zhang F, Tang CS, Zhou YB. Intermedin enhances sympathetic outflow via receptor-mediated cAMP/PKA signaling pathway in nucleus tractus solitarii of rats. Peptides 2013; 47: 1-6.
12. Lang-Rollin I, Schurek JO, Lang RE. Platelet-induced inhibition of adrenomedullin secretion. Eur J Pharmacol 2001 14; 427: 115-118.